

## Rotavirus Proteins: Structure and Assembly

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<b>1</b>	<b>Introduction</b> . . . . .	190
<b>2</b>	<b>Rotavirus Proteins</b> . . . . .	192
<b>3</b>	<b>Capsid Architecture</b> . . . . .	192
3.1	VP7 Layer and VP4 Spikes . . . . .	193
3.2	Aqueous Channels . . . . .	195
3.3	VP6 Layer . . . . .	196
3.4	VP2 Layer and Transcription Enzyme Complex . . . . .	196
3.5	Genome Organization . . . . .	198
<b>4</b>	<b>Reassortants</b> . . . . .	199
<b>5</b>	<b>Protease-Enhanced Infectivity</b> . . . . .	199
5.1	Trypsin-Induced Unique Order-to-Disorder Transition in the Spike . . . . .	200
<b>6</b>	<b>Cell Entry</b> . . . . .	202
6.1	Possible Structural Alterations in VP4 During Cell Entry . . . . .	203
6.1.1	Is the VP4 Spike a Trimer? . . . . .	203
6.1.2	pH-Induced Changes of the Spike: Implication for Cell Entry and Antibody Neutralization . . . . .	203
<b>7</b>	<b>Endogenous Transcription</b> . . . . .	204
<b>8</b>	<b>Genome Replication and Packaging</b> . . . . .	205
8.1	NSP3 and Genome Translation . . . . .	206
8.2	NSP2 and NSP5 . . . . .	206
8.3	A Working Model for Genome Encapsidation in Rotavirus . . . . .	208
<b>9</b>	<b>Maturation and Release</b> . . . . .	209
<b>10</b>	<b>Conclusion</b> . . . . .	210
	<b>References</b> . . . . .	211

**Abstract** Rotavirus is a major pathogen of infantile gastroenteritis. It is a large and complex virus with a multilayered capsid organization that integrates the determinants of host specificity, cell entry, and the enzymatic functions necessary for endogenous transcription of the genome that consists of 11 dsRNA segments. These segments encode six structural and six nonstructural proteins. In the last few years, there has been substantial progress in our understanding of both the structural and functional aspects of a variety of molecular processes involved in the replication of this virus. Studies leading to this progress using a variety of structural and biochemical techniques including the recent application of RNA interference technology have uncovered several unique and intriguing features related to viral morphogenesis. This review focuses on our current understanding of the structural basis of the molecular processes that govern the replication of rotavirus.

## 1

### Introduction

Rotavirus is a major cause of gastroenteritis in young children (under age 5) worldwide. It is responsible for an estimated 600,000–870,000 annual deaths worldwide (Cohen 2001; Kapikian 2002; Midthun and Kapikian 1996; Parashar et al. 2003). Deaths from rotavirus are most prevalent in developing nations, where patients may not always receive adequate medical attention quickly enough. Rotavirus infection occurs primarily in the differentiated enterocytes of the jejunum in the small intestine, which are responsible for digestion and absorption (Moon 1994). Destruction of these cells results in the loss of nutrient and water absorption, followed by dehydration and malnutrition that ultimately can lead to death. An increasing number of reports indicate that rotavirus escapes the gastrointestinal tract resulting in antigenemia in children and viremia in animal models (Blutt et al. 2003) and the detection of rotavirus antigen or RNA in tissues of infected children and adults (Cioc and Nuovo 2002; Hongou et al. 1998; Iturriza-Gomara et al. 2002; Lynch et al. 2001, 2003; Morrison et al. 2001; Pager et al. 2000). The full clinical significance of such extraintestinal virus remains to be determined.

Rotavirus is a member of the *Reoviridae* family, which consists of 11 genera (Fields 1996). Members of this family of viruses have multilayered, nonenveloped, icosahedral capsids with a diameter ranging from approximately 600 to 1000 Å. Each member of this family encapsidates between 10–12 segments of dsRNA. In these viruses, the enzymatic machinery necessary for transcription is housed within an intact core, where the genome is transcribed. Transcriptionally active particles of these viruses are capable of repeated cycles of transcription. These viruses replicate in the cytoplasm of the cell and encode several nonstructural proteins to aid in their replication and morphogenesis inside the host cell.

Biochemical studies on rotaviruses have established much of our basic understanding of rotavirus infectivity, genome transcription, morphogenesis, and virus–cell interactions. The lack of a reverse genetics system for rotavirus, as for all members of the *Reoviridae*, has hampered a detailed understanding of the intracellular functional roles of the virally encoded proteins. In lieu of this, recombinant proteins and virus-like particles (VLPs) have been very useful, not only in rotavirus but also in other dsRNA viruses, for the understanding of both biochemical and structural properties of rotaviral structural and nonstructural proteins. All rotaviral genes of several rotavirus strains have been cloned (Estes and Cohen 1989). These genes have been successfully expressed, and co-expression of specific structural proteins has been shown to result in the spontaneous formation of virus-like particles (VLPs) and other functional complexes (Cohen et al. 1989; Crawford et al. 1994; Estes et al. 1987; Labbe et al. 1991; Mattion et al. 1991, 1992; Zeng et al. 1994). In parallel, structural studies have played an important role to help understand the virus functions in the context of the three-dimensional structures of the virus and virus-encoded individual proteins. An exciting development in the field of rotavirus biology in recent years is the application of RNA interference techniques to study the functional roles of rotaviral proteins during the process of infection (Arias et al. 2004; Campagna et al. 2005; Dector et al. 2002; Lopez et al. 2005; Silvestri et al. 2004).

Until recently, much of our understanding of the structure–function relationships in rotaviruses has come from using electron cryomicroscopy (cryo-EM) techniques (Prasad and Estes 2000). Determination of the overall low-resolution structure of rotavirus using cryo-EM techniques in 1988 (Prasad et al. 1988) began paving the way for more elaborate structural characterization of this virus (Prasad et al. 1990, 1996; Shaw et al. 1993; Yeager et al. 1990, 1994). In addition to providing a detailed description of the architectural features of this large and complex virus, including the topographical locations of all the structural proteins and their stoichiometric proportions, these structural studies using cryo-EM techniques also provided more insight into some of the biological functions of the virus such as trypsin-enhanced infectivity (Crawford et al. 2001), cell entry (Dormitzer et al. 2004, Pesavento et al. 2005), antibody interactions (Prasad et al. 1990; Tihova et al. 2001), endogenous transcription (Lawton et al. 1997a, 2000), and genome organization (Pesavento et al. 2001, 2003b).

More recently, X-ray crystallography has been successfully applied to determine the atomic structures of several of the structural and nonstructural proteins of rotavirus (Deo et al. 2002; Dormitzer et al. 2002, 2004; Groft and Burley 2002; Jayaram et al. 2002; Mathieu et al. 2001). With the lack of an X-ray structure of the rotavirus particle or any of its subassemblies, cryo-EM

reconstructions in combination with X-ray structural information have filled the void to some extent and provided more in-depth structural characterization of the particles at atomic resolution (Dormitzer et al. 2004; Mathieu et al. 2001). With the spectacular success in determining near atomic resolution structures of the bluetongue virus (BTV) core (Grimes et al. 1998) and orthoreovirus core (Reinisch et al. 2000), there is the expectation that the entire rotavirus or homologous subassemblies of rotavirus can be addressed using X-ray crystallography. The status of our current understanding of the three-dimensional structure of this important medical pathogen and some of its proteins in the context of its replication cycle is the main focus of this review.

## 2

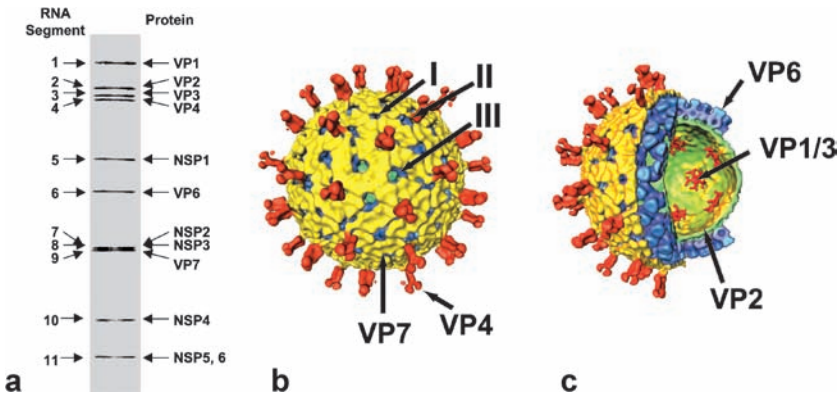
### Rotavirus Proteins

The 11 dsRNA segments of the rotavirus genome code for six structural and six nonstructural proteins (Fig. 1a). The naming of the structural proteins is based on their molecular weights, with VP1, the largest at 125 kDa, and VP8\*, one of the two proteolytic fragments of VP4, the smallest at 28 kDa. The six structural proteins form the multi-layered capsid of the mature rotavirus particle. The nonstructural proteins, except for NSP1, are essential for virus replication. NSP1 is an RNA-binding protein that directly interacts with IRF-3 (Graff et al. 2002). The loss of NSP1 does not seem to negatively affect rotavirus replication in cultured cells (Silvestri et al. 2004). However, it plays a role in pathogenesis in some animal models (reviewed in Desselberger 1997), likely by antagonizing the type I interferon response to increase viral pathogenesis (Barro and Patton 2005). In this regard, NSP1 shares some similarities with NS1 of influenza virus, although the mechanism of action appears to be unique. The function and roles that the rest of the rotaviral proteins play in the structure and replication of rotavirus are discussed below. A brief summary of the properties of the rotavirus structural and nonstructural proteins is given in Table 1.

## 3

### Capsid Architecture

The architectural features of the mature rotavirus along with the positions of various structural proteins are shown in Fig. 1b and c. The mature infectious rotavirus particle 1000 Å in diameter (including the spikes), is made of three concentric icosahedral protein layers that encapsidate the genome of



**Fig. 1a-c** **a** PAGE showing rotavirus RNA segments and gene-protein assignments. The RNA segments are numbered in order of gel migration on the *left* and their encoded protein products are indicated on the *right*. Gene segments 7, 8, and 9 are very close in length and tend to migrate nearly on top of one another. Gene 11 is alternatively processed to produce NSP5 and NSP6. (Torres-Vega et al. 2000; Welch et al. 1989). For protein molecular weights, see Table 1. **b** Surface representation of the mature rotavirus particle (TLP). Arrows indicate the three types of aqueous channels, labeled I, II, and III. The 60 VP4 spikes are colored red and the 780 copies of VP7 forming the outer capsid layer are shown in yellow. (Adapted from Pesavento et al. 2003b). **c** Cut-away of the TLP structure showing the internal structural features. The density due to genomic RNA is removed for clarity. The internal VP6 protein layer is in blue and the core VP2 layer in green. The flower-shaped VP1-VP3 transcription complex is attached to the inside of the VP2 layer at the five-fold icosahedral axes directly below the type I channels and is colored red. (Adapted from Prasad et al. 1996)

11 dsRNA segments. The complete virion is called a triple-layered particle (TLP). Like many of the members of the *Reoviridae*, the capsid architecture is predominantly based on T=13 icosahedral symmetry.

### 3.1

#### VP7 Layer and VP4 Spikes

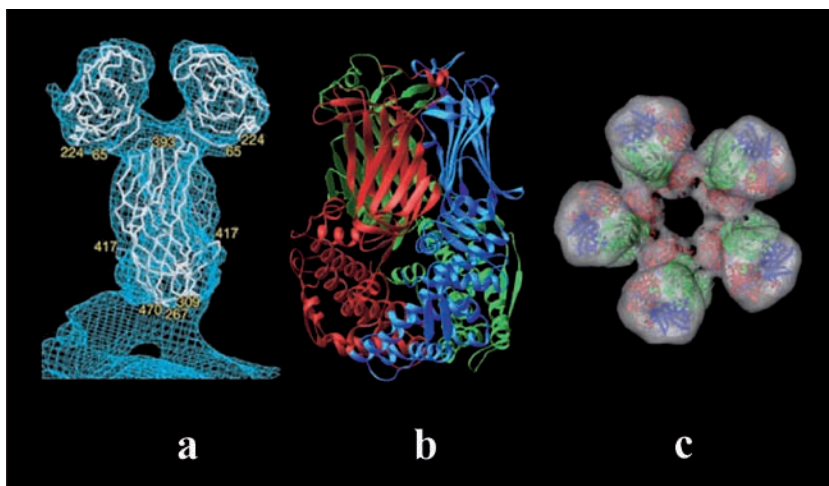
The outer layer of the TLP is composed of two structural proteins: VP7 and VP4. VP7, the major constituent of the outer layer, is a glycoprotein in most rotavirus strains although glycosylation is not required for capsid assembly (Estes 2001). Seven hundred eighty copies of VP7 are grouped as 260 trimers at all the icosahedral and local three-fold axes of a T=13 icosahedral lattice surrounding 132 channels. The outer layer is decorated by 60 spikes, each of which is formed by a dimer of VP4 (Fig. 1b). Thus each rotavirus particle has 120 copies of VP4. The composition of the spike was confirmed by cryo-EM

**Table 1** Properties of rotavirus structural and nonstructural proteins<sup>a</sup>

Gene segment	Protein	Mass (kDa) <sup>b</sup>	Post-translational modification(s)	Location (no. of copies)	Functional properties
1	VP1	125	-	SLP (12)	RNA-dependent RNA polymerase, RNA binding, interacts with VP2 and VP3
2	VP2	95	Cleaved	SLP (120)	RNA binding, interacts with VP1
3	VP3	88	-	SLP (12)	Guanylyl and methyl transferase, ssRNA binding, interacts with VP1
4	VP4 (VP5* + VP8*)	85 (58+27)	Cleaved	TLP (120)	Hemagglutinin, neutralization antigen, virulence, protease-enhanced infectivity, cell attachment, fusion region
5	NSP1	53	-	Nonstructural	RNA binding, antagonist of interferon response
6	VP6	45	-	DLP (780)	Hydrophobic trimer, group and subgroup antigen
7	NSP3	34	-	Nonstructural	Important for viral mRNA translation, PABP homologue, RNA binding, interacts with eIF4G
8	NSP2	35	-	Nonstructural	Important for genome replication/packaging, main constituent of viroplasm, NTPase, RNA binding, interacts with NSP5
9	VP7	34	Cleaved signal sequence, high mannose glycosylation and trimming	TLP (780)	RER integral membrane glycoprotein, neutralization antigen, Ca <sup>++</sup> binding
10	NSP4	20	Uncleaved signal sequence, high mannose glycosylation and trimming	Nonstructural	RER transmembrane glycoprotein, role in morphogenesis, viral enterotoxin
11	NSP5	26	Phosphorylated, O-glycosylated	Nonstructural	Constituent of viroplasm, interacts with NSP2, RNA binding, Protein kinase
11	NSP6	11	-	Nonstructural	Constituent of the viroplasm, interacts with NSP5

<sup>a</sup> A number of known functional properties were added, many taken from Estes 2001

<sup>b</sup> Molecular weights based on apparent molecular weights by SDS-PAGE analysis



**Fig. 2a–c** X-ray structure of the VP8\*, VP5\*, and VP6. **a** X-ray structures of VP5\* and VP8\* (shown in the backbone representation) fitted into the cryo-EM envelope of the VP4 spike derived from a 12-Å resolution map. (Adapted from Dormitzer et al. 2004). **b** X-ray structure of the VP6 trimer (monomers in red, green, and blue) shown in ribbon representation. (Mathieu et al. 2001). **c** Fitting of the X-ray structure of the VP6 trimers into the trimers around the type I channel in the cryo-EM map of the DLP

studies of the rotavirus complexed with VP4-specific monoclonal antibodies (Prasad et al. 1990; Tihova et al. 2001).

The VP4 spike exhibits a distinct structure with two distal globular domains, a central body, and an internal globular domain that is tucked inside the VP7 layer in the peripentonal channel of the T=13 icosahedral lattice (Shaw et al. 1993; Yeager et al. 1994). X-ray structures of proteolytic fragments of VP4, VP8\*, and VP5\* have been determined (Dormitzer et al. 2002, 2004), and provide strong evidence that the distal globular domain of the VP4 spike is composed of VP8\* with the remaining body of the spike consisting of VP5\* (Fig. 2a). The crystallographic studies on VP5\*, as discussed in connection with cell entry below, have also indicated the possibility of an alternate oligomerization state of VP4 (Dormitzer et al. 2004).

### 3.2

#### Aqueous Channels

One of the distinctive features of the rotavirus architecture is the presence of large channels that penetrate through the VP7 and VP6 layers. These channels allow for the passage of aqueous materials and biochemical substrates into

and out of the capsid. The 132 channels at the five-fold and quasi six-fold positions of the T=13 lattice are grouped into three distinct types. Twelve type I channels are located at the five-fold vertices of the capsid (arrows, Fig. 1b). There are 60 type II channels at each of the pentavalent locations surrounding the type I channels, near which VP4 is attached to VP7 and VP6 (Fig. 1b). The 60 type III channels are located at the remaining hexavalent positions on the capsid surrounding the icosahedral three-fold axes (Fig. 1b).

### 3.3

#### VP6 Layer

The intermediate layer is formed by the VP6 protein, and is in direct contact with the VP7 layer. Particles carrying VP6 on the outside are called double-layered particles (DLPs). The VP6 layer maintains the same icosahedral symmetry as the VP7 layer with 780 copies of VP6 arranged as 260 trimers on a T=13 icosahedral lattice (Fig. 1c). These trimers are located right below the VP7 trimers such that the channels in the VP7 and VP6 layers are in register. The DLP is the transcriptionally competent form of the virus during the replication cycle. VP6 is the major protein of the rotavirus particle by weight. It plays a key role in the overall organization of the rotavirus architecture by interacting with the outer layer proteins, VP7 and VP4, and the inner most layer protein VP2. Thus, it may integrate two principal functions of the virus: cell entry (outer layer) and endogenous transcription (inner layer). The X-ray structure of VP6 has been determined and it shows that VP6 has two domains (Fig. 2b, c) (Mathieu et al. 2001). In its overall structure, VP6 is similar to the VP7 of BTV (Grimes et al. 1997, 1998) and to the  $\mu 1$  protein of orthoreovirus (Liemann et al. 2002). The distal domain with an eight-stranded antiparallel  $\beta$ -sandwich fold makes contact with the VP7 layer, and the lower domain, consisting of a cluster of  $\alpha$ -helices, makes contact with the inner VP2 layer. Fitting of the X-ray structure of VP6 into the cryo-EM structure of the DLP shows that the VP6 trimers interact laterally to form the T=13 layer (Mathieu et al. 2001). There appear to be two types of contacts between the trimers. The contacts, across the quasi two-fold axes and closer to the icosahedral three-fold axis are similar, whereas the contacts are varied as the trimers approach the icosahedral five-fold axis. In contrast to VP7 of BTV (Grimes et al. 1998), the VP6 trimer exhibits extensive lateral interactions involving charged residues.

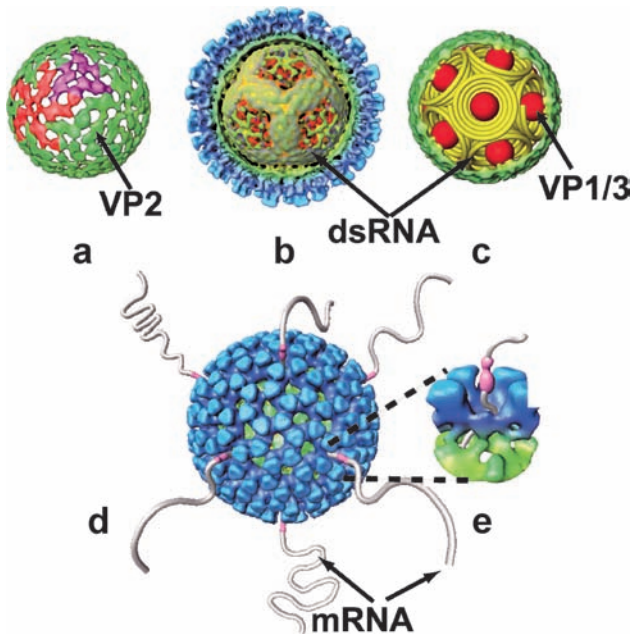
### 3.4

#### VP2 Layer and Transcription Enzyme Complex

Underneath the VP6 layer is the innermost protein layer of the rotavirus structure. The particle structure at this level is referred to as the single-layer



particle (SLP). The SLP houses the dsRNA genome within a protein layer composed of 120 copies of VP2 (Fig. 3a) arranged in an unusual T=1 icosahedral lattice with two molecules in the icosahedral asymmetric unit (Lawton et al. 1997b). All the structurally characterized members of the *Reoviridae* and of other dsRNA viruses such as phi6 and LA viruses exhibit this unique



**Fig. 3a–e** Structural organization of the VP2 layer, genomic dsRNA, and transcription by the rotavirus DLP. **a** Surface representation of the outer portion of VP2. In one of the 60 dimers that constitute this layer, the VP2 subunits are colored in *red and purple* to indicate their orientations and connections to one another. (Adapted from Lawton et al. 1997b). **b** Cut-away view of the DLP. The VP6 and VP2 layers were peeled halfway to expose the outermost layer of genomic organization. The outer layer of RNA has a dodecahedral appearance and surrounds each of the VP1–VP3 star-shaped complexes at the five-fold vertices. (Adapted from Prasad et al. 1996). **c** Model for genome organization around the VP1/3 transcription enzyme complex. The outer *green* portions represent a cut-away view of the VP2 layer. The *yellow spirals* indicate dsRNA gene segments and the *red spheres* represent the VP1/3 transcription complexes. (Adapted from Pesavento et al. 2003). **d** A DLP is shown with mRNA transcripts exiting out by the proposed pathway through the type I channel at a five-fold vertex. The transcripts are colored as *gray strands*. **e** Close-up view of a transcribing DLP. The *pink bowling-pin-shaped density* is the result of the exiting transcript seen in the reconstructions of actively transcribing DLPs. (Lawton et al. 1997a)

organization of the core protein (reviewed in Prasad and Prevelige 2003). The structural organization of the corresponding layers in three of the *Reoviridae* members—BTV (Grimes et al. 1998), orthoreovirus (Reinisch et al. 2000), and rice dwarf virus (Nakagawa et al. 2003)—have been visualized at the atomic level. From the X-ray structure of the BTV core particle, which closely resembles the rotavirus DLP, Grimes et al. (1998) have argued that the pentameric caps of VP3 (equivalent of rotavirus VP2) dimers are building blocks in the assembly of this layer. VP2 expressed using the baculovirus expression system, forms helix-like structures that can form spherical particles at lower concentrations (Zeng et al. 1994) and co-expression of VP2 with VP1 and/or VP3 results in the self-assembly of these proteins into VP1/2, VP2/3, and VP1/2/3 virus-like particles (VLPs) (Wentz et al. 1996). Comparative cryo-EM analysis of these particles showed that 12 copies of the VP1/VP3 transcription enzyme complexes are attached to the inner surface of the VP2 layer at each of the five-fold vertices of the SLP and surrounding each of the transcription complexes is genomic dsRNA (Fig. 1c). Similar structural localization of the enzymes, particularly the polymerase, required for endogenous transcription is found in other members of the *Reoviridae* such as BTV (Gouet et al. 1999; Nason et al. 2004), rice dwarf virus (Nakagawa et al. 2003; Zhou et al. 2001), aquareovirus (Nason et al. 2000), orthoreoviruses (Zhang et al. 2003), and cypovirus (Zhang et al. 1999). Such structural conservation is not surprising given that in all these viruses endogenous transcription of multiple segments is a common and necessary phenomenon. However, a contrasting feature is in regard to the location of the capping enzyme. In viruses such as rotavirus, BTV, and rice dwarf virus, the capping enzyme is suggested to be inside the core layer, whereas in viruses such as the orthoreovirus, aquareovirus, and cypovirus, the capping enzyme forms a distinctive turret structure with a central hole localized at the virion five-fold axis (Hill et al. 1999).

### 3.5

#### Genome Organization

The question of how the dsRNA segments are arranged inside the capsid is particularly interesting considering that they are transcribed simultaneously and repeatedly within the confines of the capsid. By analyzing the structural differences between empty virus-like particles (VLPs) and native rotavirus particles, Prasad et al. (1996) showed that a significant portion of the genome is statistically ordered and manifests as concentric layers of density inside the icosahedrally averaged reconstructions of the rotavirus particles (Fig. 3b). Similar structural manifestation of the genome is indeed seen in the X-ray structure of the BTV core and cryo-EM reconstructions of several

other dsRNA viruses. However, because of the implicit use of icosahedral symmetry averaging in the structure determination of these viruses, either by crystallography and or cryo-EM, the precise organization of the individual genome segments is lost. Interestingly, in rotavirus, using a combination of biochemical and cryo-EM techniques, Pesavento et al. (2001) showed that the rotavirus genome can undergo reversible condensation and expansion without affecting the integrity of the surrounding capsid layers. A plausible model that emerges from the available biochemical and structural data for rotaviruses and other dsRNA viruses, is that each genome segment is spooled around a transcription complex (consisting of VP1 and VP3) that is anchored to the inner surface of the VP2 layer at the five-fold axis (Gouet et al. 1999; Pesavento et al. 2003b). Such a model (Fig. 3c) allows for up to 12 independent transcription complexes, each associated with an individual dsRNA segment for concurrent transcription.

## 4

### Reassortants

Although most of the cryo-EM structural studies have been performed on a few selected strains of rotavirus, these studies clearly indicate that the general architectural features are generalizable and independent of the strains. Cryo-EM structural studies have been reported on several rotavirus reassortants. These structural studies indicate that the capsid structure remains unaltered except for the VP4 spikes. Rotavirus reassortment occurs widely in nature and represents a major force for genetic diversity along with point mutations and gene rearrangements (Desselberger 1996; Iturriza-Gomara et al. 2001). The structures of reassortants show that while VP4 generally maintains the parental structure, when moved to a heterologous protein background, in certain reassortants there are subtle alterations in the conformation of VP4 (Pesavento et al. 2003a). The alterations in the VP4 conformation correlated with the observation of unexpected VP4-associated phenotypes. Interactions between heterologous VP4 and VP7 in reassortants expressing unexpected phenotypes appear to induce the conformational alterations seen in VP4.

## 5

### Protease-Enhanced Infectivity

From their locations in the structure of rotavirus, VP7 and VP4 are obvious candidates to be implicated in the cell entry processes. Although early studies

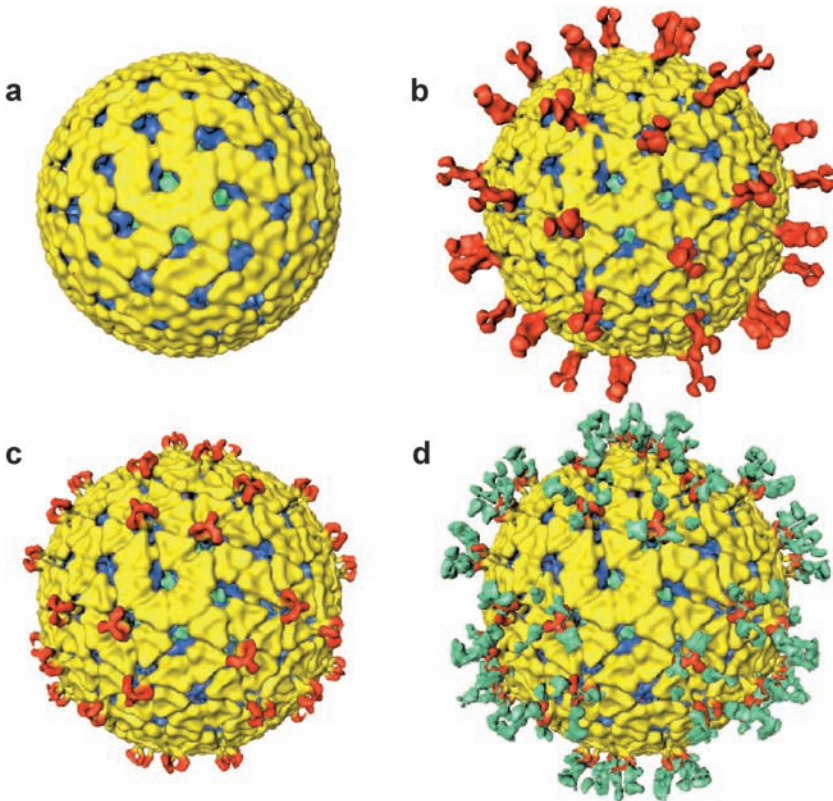
implicated VP7 in the cell entry process (Fukuhara et al. 1988; Sabara et al. 1985), subsequent studies have increasingly indicated the involvement of VP4 not only in cell attachment and cell penetration, but also in hemagglutination, neutralization, virulence, and host range (Burns et al. 1988; Fiore et al. 1991; Kirkwood et al. 1998; Lopez et al. 1985; Ludert et al. 1996, 1998; Mackow et al. 1988). Prior to its interaction with the host cell, VP4 is proteolytically cleaved for efficient internalization of rotaviruses into cells. This is particularly relevant considering that rotavirus replication takes place in enterocytes in the small intestine, an environment rich in proteases. Proteolytic cleavage of VP4 enhances viral infectivity by several fold (Arias et al. 1996; Estes et al. 1981) and facilitates virus entry into cells (Kaljot et al. 1988). Proteolysis of VP4 generates two fragments, VP8\* (aa 1–247) and VP5\* (248–776) and these fragments remain associated with the virion (Fiore et al. 1991; Lopez et al. 1985). Trypsinized viruses enter cells more efficiently without using the endosomal pathway, compared to particles that are not trypsinized (Kaljot et al. 1988; Keljo et al. 1988). In vitro experiments have shown that proteolytically activated particles, as well as recombinant VP5\*, possess lipophilic activity (Dowling et al. 2000; Nandi et al. 1992; Ruiz et al. 1994). Although rotavirus is a nonenveloped virus, it is interesting to note some parallels between rotavirus VP4 and cell attachment proteins in enveloped viruses such as influenza viruses. Proteolytic cleavage is as essential for infection in influenza virus as it is for rotavirus, because it primes the HA (hemagglutinin) protein for an ensuing irreversible conformational change, which occurs in the low-pH environment of endosomes prior to membrane fusion. The rotavirus VP5\* and VP8\* trypsin cleavage products are analogous to the proteolytically cleaved fragments of the influenza virus hemagglutinin, HA1 and HA2. Much like rotavirus VP8\*, the HA1 subunit plays an accessory role by providing initial binding to the cell via sialic acid containing receptors. HA2 functions more like VP5\*, as it is required and sufficient on its own for cell fusion (Wiley and Skehel 1987).

## 5.1

### **Trypsin-Induced Unique Order-to-Disorder Transition in the Spike**

The molecular mechanism of increased infectivity by proteolysis is not well understood. To understand the structural basis of trypsin-enhanced infectivity in rotaviruses, Crawford et al. (2001) examined the biochemical and structural properties of rotaviruses grown in the absence (nontrypsinized rotavirus, NTR) or presence (trypsinized rotavirus, TR) of trypsin. The infectivity of the NTR particles is drastically reduced, as anticipated. Exogenous addition of trypsin to NTR particles increased their infectivity but to nowhere

near the level of infectivity seen with TR particles. Despite clear biochemical indications for the presence of uncleaved VP4 in correct stoichiometric proportion in the NTR particles, the spikes in the cryo-EM reconstruction of these particles are not visualized in contrast to the well defined spike structure seen in the particles that are grown in the presence of trypsin (Fig. 4a , b).



**Fig. 4a–d** Effects of trypsin and pH on the spike structure. The highly flexible VP4 spike protein on rotavirus assumes altered conformations due to proteolytic cleavage or encountering high pH. **a** Rotavirus grown in the absence of trypsin (*upper panel*) has low infectivity and the VP4 spike is disordered on particles (i.e., not represented in cryo-EM reconstructions). (Crawford et al. 2001). **b** Proteolytically cleaved rotavirus has high infectivity and a well-ordered spike appearing dimeric at the top. **c** Treatment of rotavirus with ~pH 11 induces a conformational change in the spike resulting in a tri-lobed stunted spike and unmarks a cell binding domain that appears to be involved in infection of cells by a sialic acid-independent mechanism. (Pesavento et al. 2005). **d** The high-pH-altered short spikes are recognized by VP5\*-specific 2G4-Fab fragments, and three Fab fragments are seen binding to each altered spike (Pesavento et al. 2005)

These results thus indicate that trypsin cleavage imparts structural order to the VP4 spikes on *de novo* synthesized virus particles and that these ordered spikes make virus entry into cells more efficient (Crawford et al. 2001).

The idea of a trypsin-induced disorder-to-order transition is indeed unique and has not been documented with any other virus thus far. Does trypsin act from within or outside of cells? One possibility is that during virus infection, trypsin acts outside cells on the newly formed VP4 and that this trypsinized VP4 is able to assemble properly onto the rotavirus particles. This hypothesis is consistent with the finding, using confocal microscopy of virus-infected MA104 cells, that high amounts of VP4 are present at the plasma membrane approximately 3 h after infection and that the N-terminal region, i.e., VP8\*, is accessible to antibodies (Nejmeddine et al. 2000). Similar results were obtained with cells transfected with a VP4 plasmid, suggesting that VP4 targeting depends on signals in the protein rather than on the presence of virus particles. Targeting of VP4 to the plasma membrane appears to be a general phenomenon as it is seen in both polarized and nonpolarized cells (Sapin et al. 2002). Further structural and biochemical studies are needed to provide a better understanding of how and where trypsin affects spike assembly.

## 6 Cell Entry

The consensus opinion that has emerged from several recent studies is that rotavirus cell entry is a coordinated multistep process involving sequential interactions with sialic acid (SA) -containing receptors in the initial cell attachment step. Next, interactions are thought to occur with hsp70, and integrins such as  $\alpha v\beta 3$ ,  $\alpha 4\beta 1$ ,  $\alpha 2\beta 1$  during the subsequent postattachment steps (reviewed in Lopez and Arias 2004). In the entry process, the VP8\* domain is involved in the interactions with SA, whereas VP5\* is implicated in the interactions with integrins. Involvement of VP8\* in cell attachment is further supported by studies that show that several VP8\*-specific neutralizing mAbs block cell attachment. The X-ray structure of the VP8\*-SA complex has shown that VP8\* has a beta-sandwich fold similar to that of galectins, whose natural ligands are carbohydrates (Dormitzer et al. 2002). The SA binds to a shallow pocket between the two  $\beta$ -sheets, a region that is distinct from the carbohydrate binding pocket in the galectins, which is blocked in the VP8\*. Involvement of SA during rotavirus infections is not an essential step in all rotavirus strains. For many of the rotavirus strains, including human rotaviruses, cell entry is SA-independent (Ciarlet et al. 2001). In these viruses, the majority of neutralizing mAbs select mutations in VP5\* (Kirkwood et al.



1996, 1998; Padilla-Noriega et al. 1995), suggesting that cell entry is mediated mainly by the VP5\*. An interesting question is what the role of VP8\* might be in these SA-independent viruses.

## 6.1

### Possible Structural Alterations in VP4 During Cell Entry

How does VP4 facilitate such multistep entry processes in rotavirus? It is possible that VP4 undergoes distinct conformational changes at various stages during cell entry to mask certain epitopes and reveal others in order to optimally interact with different receptors and the cellular membrane. Such distinct conformational states during cell entry processes have been observed in viruses such as influenza virus (Bullough et al. 1994), flavivirus (Modis et al. 2004; Mukhopadhyay et al. 2003), alphavirus (Gibbons et al. 2004) and picornaviruses (Belnap et al. 2000). Recent studies on rotavirus clearly point to conformational changes of VP4 during cell entry. In addition to the drastic conformational change from a flexible to a rigid-bilobed spike structure upon trypsinization, as discussed above (Crawford et al. 2001), recent X-ray crystallographic studies of VP5\* (Dormitzer et al. 2004) and cryo-EM studies in high-pH-treated rotaviruses suggest the possibility of further structural changes in the spike structure that may be relevant during rotavirus cell entry.

#### 6.1.1

##### Is the VP4 Spike a Trimer?

In the crystal structure, VP5\* is a trimer with substantial intersubunit interactions (Dormitzer et al. 2004). That is, by itself, VP5\* has a propensity to form strong trimers. Why, then, in the cryo-EM structures is the spike a dimeric structure? Two individual monomers of VP5\* clearly fit into the main body of the spike in the cryo-EM structure (Fig. 2a). A proposed possibility is that each spike is indeed a trimer of VP4, and upon trypsinization, two of them form the visible spike, as seen in the cryo-EM reconstruction of the trypsinized rotavirus particles, with the other monomer being floppy and not visible in the reconstruction (Dormitzer et al. 2004). During cell entry, by a yet unknown entry-associated event, the floppy VP4 monomer together with the other two molecules, trimerizes as seen in the VP5\* crystal structure.

#### 6.1.2

##### pH-Induced Changes of the Spike:

##### Implication for Cell Entry and Antibody Neutralization

Recent studies on high-pH-treated rotavirus have uncovered an interesting phenomenon that appears to substantiate the above proposal (Pesavento et al.

2005). At elevated pH, the spike undergoes a drastic irreversible conformational change and becomes stunted with a pronounced tri-lobed appearance (Fig. 4c). Biochemical analysis of pH-treated particles indicates that VP4 is present in the same amount as in native particles. Three Fab fragments of the VP5\*-specific neutralizing monoclonal antibody, 2G4, are seen to bind to the altered spike structure (Fig. 4d). One strong possibility from these observations is that VP4 has undergone a dimer to trimer transition. Despite the loss of infectivity and the ability to hemagglutinate, the high-pH-treated particles surprisingly exhibit SA-independent cell binding, in contrast to native virions, which exhibit SA-dependent cell binding. These studies have also shown that the binding of 2G4-Fab to native particles completely protects the spikes from undergoing pH-induced conformational changes and preserves the SA-dependent cell binding and hemagglutinating functions of the virion. However, when 2G4 is bound to the pH-altered particles, cell binding is completely lost. A hypothesis that emerges from this study is that high-pH treatment triggers a conformational change that mimics a post-SA attachment step to expose an epitope recognized by one of the downstream receptors in the rotavirus cell entry process, and the mechanism by which the 2G4 antibody neutralizes infectivity is by preventing this conformational change.

In their cell attachment, the pH-treated particles appear to resemble the nar3 mutant of rhesus rotavirus (RRV) (Graham et al. 2003; Zarate et al. 2000a). This mutant exhibits SA-independent cell binding in contrast to its parental strain and has been shown to attach to the cell surface by interacting with integrin  $\alpha 2 \beta 1$  through the DGE motif in VP5\*. As in the high-pH-treated particles, 2G4 antibody binding to the nar3 mutant inhibits cell binding (Zarate et al. 2000b). A distinct possibility is that the DGE motif (residues 308–310) becomes exposed in the pH-treated particles, and the 2G4-Fab inhibits cell binding of the pH-treated particles by sterically hindering the accessibility of this motif. In the studies by Pesavento et al. (2005), pH was used to trigger the conformational changes. During a natural infection process, it is not known what triggers the conformational changes necessary to interact with downstream receptors. As yet there are no structural studies reported of rotavirus complexed with any of the multiple, proposed receptors molecules.

## 7

### Endogenous Transcription

The next stage in the replication cycle of the virus is the transcription of dsRNA segments into viable mRNA molecules that can be processed for



template generation and viral protein production. During the process of cell entry, the outer layer is removed and the resulting DLPs in the cytoplasm become transcriptionally competent (Estes et al. 2001). The dsRNA segments are transcribed within the structural confines of the DLP. Cryo-EM structural studies have shown that DLPs remain structurally intact during the process of transcription, and the nascent transcripts exit through the type I channels that penetrate the inner VP2 and outer VP6 capsid layers of the DLP at the five-fold vertices (Fig. 3d) (Lawton et al. 1997a). The DLP possesses the complete enzymatic activities needed to synthesize not only mRNA transcripts but also to properly guanylate and methylate the cap structure at the 5' end of each mRNA to facilitate translation by the cellular translation machinery. These enzymatic functions are carried out by VP1, the RNA-dependent-RNA polymerase (Valenzuela et al. 1991), and VP3, a guanylyltransferase and methyltransferase (Chen et al. 1999). While DLPs are transcriptionally competent both *in vitro* and *in vivo*, the TLPs are transcriptionally incompetent. Certain monoclonal antibodies, which bind to the distal end of VP6, almost 140 Å away from the site of transcription initiation, inhibit transcription (Ginn et al. 1992; Kohli et al. 1993; Thouvenin et al. 2001). From cryo-EM studies of DLPs complexed with these antibodies, it has been proposed that binding of ligand, such as an antibody or VP7, induces a conformation change at the interface of the VP2 and VP6 layers to inhibit sustained elongation and translocation of the transcripts (Lawton et al. 1999). Further higher-resolution structural analysis of TLPs and DLPs is necessary to understand the structural basis of transcriptional activation and inhibition.

## 8

### Genome Replication and Packaging

Following endogenous transcription and release of the transcripts, the rotavirus replication cycle may be viewed as having three subsequent major stages: (1) translation and synthesis of the viral proteins; (2) replication, genome packaging, and DLP assembly; (3) budding of the newly formed DLPs into the ER and assembly of the outer layer to form mature TLPs (reviewed in Estes 2001). The positive-stranded RNA transcripts encode the rotaviral proteins and function as templates for production of negative strands to make the progeny dsRNA. Recent studies with siRNA have indicated that there are likely to be two separate pools of mRNA for these distinct functions (Silvestri et al. 2004).

## 8.1

### NSP3 and Genome Translation

The nonstructural protein NSP3 is implicated in the specific recognition of the rotaviral mRNAs and in facilitating their translation using the cellular machinery (Piron et al. 1998, 1999; Vende et al. 2000). NSP3 is a functional homologue of cellular poly(A) binding protein (PABP). While the N-terminal domain of NSP3 interacts with the 3'-consensus sequence of the rotaviral viral mRNAs, the C-terminal domain interacts with eIF4G to enable circularization of viral mRNA and its delivery to the ribosomes for viral protein synthesis. The X-ray structures of both the N-terminal domain complexed with the consensus rotaviral mRNA sequence, and that of the C-terminal domain bound to a peptide that corresponds to the binding site on eIF4G have been determined (Deo et al. 2002; Groot and Burley 2002). These studies clearly indicate that NSP3 functions as a homodimer. Both the domains have novel folds. While the RNA binding domain forms a heart-shaped asymmetric dimer, the C-terminal domain forms a rod-shaped symmetric dimer. The dimeric N-terminal domain tightly binds to the consensus 3'-end of the mRNA inside a tunnel formed at the dimeric interface. The binding of NSP3 to the mRNA had also been proposed as a possible mechanism to transport newly made mRNAs to viroplasms for subsequent replication.

## 8.2

### NSP2 and NSP5

Replication, genome packaging and assembly of the DLP occur in perinuclear, nonmembrane-bound, electron dense inclusions called viroplasms, which appear 2–3 h after infection. Several *in vivo* and *in vitro* studies have strongly implicated two of the nonstructural proteins NSP2 and NSP5, not only in the formation of the viroplasm, but also in genome replication and packaging (Afrikanova 1998; Aponte et al. 1996; Gallegos and Patton 1989; Kattoura et al. 1994; Petrie et al. 1984). Co-expression of NSP2 and NSP5 in uninfected cells form viroplasm-like structures (Fabbretti et al. 1999). NSP5 is a dimeric phosphoprotein rich in Ser and Thr residues that undergoes O-linked glycosylation (Afrikanova et al. 1996; Poncet et al. 1997). In co-transfection experiments with NSP5 and NSP2, NSP2 has been shown to upregulate phosphorylation of NSP5 (Afrikanova 1998). *In vivo* studies have shown that these two proteins along with VP1, the viral RNA polymerase, are co-localized in the viroplasms and that they are the main constituents of the replication intermediates (reviewed in Taraporewala and Patton 2004). Further evidence for the involvement of NSP2 and NSP5 in the formation of viroplasms, genome replication, and virion assembly is provided by recent studies using siRNA

techniques, which showed that suppression of either NSP2 or NSP5 expression inhibits the formation of viroplasms, genome replication, and viral assembly (Campagna et al. 2005; Silvestri et al. 2004). Noting that the viral mRNA located outside the viroplasms that are involved in translation are susceptible to siRNA-induced degradation, while the mRNA in the viroplasms that undergo replication are not, Silvestri et al. (2004) have suggested that the transcriptionally active progeny DLPs form foci for the formation of the viroplasms, thus eliminating the necessity for two spatially distinct locations for transcription and replication. This model eliminates the necessity of having to transport viral mRNAs and viral proteins, as per an earlier model, to the viroplasms for negative strand synthesis and subsequent DLP assembly and genome packaging.

Biochemical studies on recombinant NSP2 have shown that it readily forms an octamer and has NTPase (nucleotide triphosphatase), ssRNA-binding, and helix destabilizing activities (Taraporewala et al. 1999, 2001; Taraporewala and Patton 2001). Based on these properties, it has been suggested that NSP2 may function as a molecular motor using the energy derived from NTP hydrolysis to facilitate genome packaging. The X-ray structure of NSP2 has provided some insights into the locations of NTP and RNA binding sites (Jayaram et al. 2002). NSP2 is a two-domain  $\alpha/\beta$  protein. The two domains are separated by a deep cleft. The N-terminal domain is predominantly  $\alpha$ -helical with only a few  $\beta$ -strands, whereas the C-terminal domain has a twisted antiparallel  $\beta$ -sheet with flanking  $\alpha$ -helices. Despite any detectable sequence similarity, the polypeptide fold in this domain is highly similar to that observed in the HIT (histidine triad) family of nucleotidyl hydrolases (Lima 1997). Based on this similarity, it was suggested that this domain contains the NTP binding pocket. Recent mutational analysis based on the structural observations is consistent with such a prediction (Carpio et al. 2004). NSP2 forms a doughnut-shaped octamer with a 35-Å-wide central hole, and four grooves related by a four-fold axis on the sides of the octamer. These grooves, lined with basic residues, are suggested to be the sites for RNA binding. Thus while the NTPase activity is localized in the monomeric subunit, the ability to bind RNA and other proteins such as NSP5 and VP1 may require the formation of the octamer.

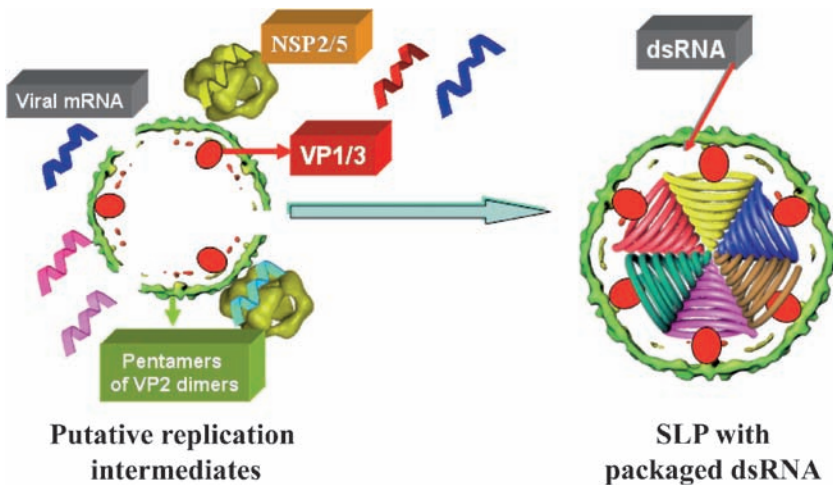
Based on the structure of NSP2 and its functional properties, it is tempting to speculate that the replication complex is organized around the NSP2 octamer providing a platform or a scaffold (Jayaram et al. 2004). It is possible that the hydrophobic side of the octamer, around the four-fold axis, may bind to VP1; given that NSP5 is an acidic protein, the basic grooves of the NSP2 octamer may be the binding sites for NSP5. Although the role of NSP5 in the overall replication process remains to be elucidated, it is plausible that by having its binding site on NSP2 overlap with that of the RNA binding site, the

function of NSP5 is to regulate the binding of RNA by NSP2 during replication and packaging. It is still unclear whether NSP6, which is encoded by an alternating open reading frame in the gene segment 11 along with NSP5 and is also present in the viroplasm, has any role in genome replication and/or packaging. NSP6 interacts with NSP5 and it is suggested that it might have a regulatory role in the self-association of NSP5 (Torres-Vega et al. 2000).

### 8.3

#### A Working Model for Genome Encapsidation in Rotavirus

How the correct set of 11 segments of dsRNA get encapsidated into each virion remains entirely unclear. Given that multiple segments of varied length have to be encapsidated, and that each one has to occupy different vertices to associate with a transcription enzyme complex, as per the current model of genome organization, it is unlikely that the dsRNA genome segments are encapsidated into preformed empty capsids as in some of the bacteriophages. Instead, the encapsidation could be concurrent with the capsid assembly as proposed by Pesavento et al. (2003). In this model (Fig. 5), the capsid assembly begins with the association of 12 units, each unit consisting of pentamers of VP2 dimers in complex with a transcription enzyme complex (VP1/VP3) and a genome segment, to form the SLP and provide a scaffold for the subsequent assembly



**Fig. 5** A working model for genome encapsidation in rotavirus. Based on the available biochemical and structural data, one possible model for genome encapsidation is shown. All the components that are likely to be involved in this process are indicated. See Sect. 8.3 in the text for details

of the VP6 trimers leading to the assembly of a DLP. The proteinaceous parts of each of these units may represent the replicase complex in which mRNA, brought in with the aid of nonstructural proteins (NSP2/NSP5), is fed into the enzyme complex for the synthesis of the negative strand and the formation of the duplex RNA, which gets spooled around the enzyme complex. In such a process, NSP5 may function as an adapter, with its ability to interact with VP2 and to facilitate interactions between NSP2 and the VP1–VP3–VP2 complex (Berois et al. 2003). This model raises an important question as to how a correct set of 11 (as in rotavirus) distinct segments is brought together. It is possible that specific RNA–RNA interactions coordinate this process.

## 9

### Maturation and Release

Maturation and release represent the final steps of the rotavirus replication cycle. Once formed, DLPs bud from the viroplasms into the proximally located ER (Estes 2001), and by a mechanism that is not clear DLPs acquire the outer layer consisting of VP7 and VP4. This budding process is facilitated by the nonstructural proteins NSP4, which has a binding site for VP6 (Au et al. 1989, 1993; Meyer et al. 1989; Tian et al. 1996). Both NSP4 and the outer layer protein VP7 are synthesized on the ER-associated ribosomes and co-translationally inserted into the ER membrane. NSP4 is a predominantly  $\alpha$ -helical glycoprotein that forms a tetramer with its C-terminal 131 residues on the cytoplasmic side of the ER. The C-terminal residues form a binding site for VP6 (O'Brien et al. 2000; Taylor et al. 1992, 1993). As yet there is no structural information on NSP4, except that of a small region that is responsible for tetramerization (Bowman et al. 2000). Recent studies using RNA interference have shown that accumulation of rotaviral proteins and indeed, DLPs and TLPs, are blocked by silencing the expression of the NSP4 gene (Lopez et al. 2005). This result indicates that NSP4 may have previously unexpected functions related to virus maturation. Aside from its role in viral morphogenesis, NSP4 is a viral enterotoxin capable of inducing diarrhea on its own in mice (Ball et al. 1996; Estes 2001, 2003; Sasaki et al. 2001).

During the budding process, DLPs get enveloped transiently in the ER. This may be an intermediate stage during acquisition of the VP7 layer. Silencing the expression of VP7 does not affect the assembly of DLPs but leads to the accumulation of enveloped DLPs in the ER, thereby suggesting that VP7 is required for removal of the lipid envelope (Lopez et al. 2005). Although the assembly of the VP7 layer onto the DLPs, as generally agreed, takes place in the ER, where and how the spike protein VP4, which is synthesized on free

cytosolic ribosomes, is assembled onto the particles is unclear. The cryo-EM structure of the particles produced by silencing the VP4 gene during virus infection clearly shows all the features of the native TLP structure except for the VP4 spikes (Arias et al. 2004; Dector et al. 2002). These results suggest that neither the proper assembly of VP7 nor the budding of the DLPs into the ER require VP4. Based on the results that indicate VP4 alone can traffic to the plasma membrane of the infected cells (Nejmeddine et al. 2000; Sapin et al. 2002), a likely possibility is that assembly of VP4 onto viral particles may take place at the plasma membrane shortly before particle release and that VP4 may be involved in the early stages of virus release. The presence of trypsin or a protease outside of cells may access the VP4 and bring about appropriate structural alterations for its proper assembly on the particles with the VP7 layer already assembled.

## 10 Conclusion

In last few years, there has been tremendous progress in our understanding of the structural and biochemical aspects of a variety of the molecular processes involved in rotavirus morphogenesis, including protease enhanced infectivity, cell entry, antibody neutralization, genome replication, and maturation. This has been made possible by the appropriate use of structural techniques such as cryo-EM and X-ray crystallography either independently or in combination. Particularly noteworthy are the insights provided by the atomic structures of several of the rotaviral proteins, including VP4, VP6, NSP3, NSP4, and NSP2. In parallel, this progress was facilitated by equally important advances in the molecular biology of rotaviruses, resulting in recombinant proteins and virus-like particles, along with the successful application of RNA interference techniques. These studies have uncovered several unique aspects of rotavirus morphogenesis and as always raise several intriguing new questions about these viruses such as:

1. How and where does the assembly of VP4 take place in infected cells?
2. How does trypsin facilitate proper assembly of the VP4 spike?
3. How does VP4 facilitate interactions with the variety of proposed receptors?
4. How is the endogenous transcription controlled by the addition or the removal of the outer capsid layer?

5. How is the process of genome replication, packaging and assembly orchestrated and controlled by the interplay between structural and non-structural proteins?
6. What is the structural and molecular basis of NSP4 function both in relation to viral pathogenesis and morphogenesis?

Dissecting the rotavirus functions in terms of its individual proteins would have been much easier if a reverse genetics system was available. Given the complexity of this virus, or any other member of the *Reoviridae* for that matter, establishing such a system is indeed a daunting task. A major achievement in the near future, as a result of continued and better understanding of the processes that control rotavirus morphogenesis, could be the establishment of a reverse genetics system.

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