

Chapter 2

Rotavirus Biology

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1 Introduction

Acute, infectious diarrhea is one of the most common causes of morbidity and mortality among children living in developing countries. In 2010, 1.7 billion cases of diarrheal events were estimated to have occurred worldwide in children under 5 years of age [123]. Diarrheal diseases are the third cause of death in this age range, after perinatal problems and respiratory infections [1]; however, it is complicated to calculate the number of deaths associated with a particular enteric pathogen [39, 115]. Updated global estimates of rotavirus mortality in children less than 5 years of age indicate a decline from 296,000 deaths in 2008 to 215,000 in 2013, with a slight decrease in the proportion of diarrheal deaths caused by rotavirus, from 39% to 37% in this same period [115]. Developing countries bear the major burden of mortality from rotavirus, with about 85% of these cases occurring in six countries in Africa and Asia and very few in industrialized nations [39, 115].

Rotaviruses continue to be the leading etiological agent of severe diarrheal disease, even though two live attenuated vaccines have been licensed in more than 100 countries since 2006, [115]. These live oral vaccines have shown a lower efficacy in countries with a high burden of diarrheal disease [17, 39, 114], and the majority of those currently using rotavirus vaccines are low-mortality countries, so the impact of vaccine use on global estimates of rotavirus mortality has been limited [115]. Furthermore, the recent Global Enteric Multicenter Study showed that rotavirus was the leading cause of infant diarrhea among more than 20,000 children studied in seven sites across Asia and Africa [60]; this study also reported that each episode of severe diarrhea in children increased the risk of delayed physical

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and intellectual development as well as increased mortality by 8.5 fold [60, 114]. Thus, the development of improved vaccines and therapeutic strategies is needed to efficiently control rotavirus infection, and in fact new rotavirus vaccines are under investigation or have recently been licensed in various parts of the world [121]. Fundamental to these developments is a basic understanding of the molecular mechanisms by which rotaviruses interact with their host cell.

Although rotavirus can infect older children and adults, severe diarrheal disease is primarily observed in children less than 2 years of age [37]. Rotavirus infection is primarily restricted to mature enterocytes located at the tip of intestinal villi. However, additional extraintestinal spread of rotavirus during infection of animals indicates a wider host tissue range than previously appreciated [88, 99]. In vitro, rotaviruses bind to a wide variety of cell lines, although only a subset of these, including cells of renal or intestinal origin and transformed cell lines derived from breast, stomach, bone, and lung, are productively infected [23]. The initial stages of rotavirus interactions with the host cell are complex and are the focus of intense current research. Most of these studies have been performed using model cell culture lines, the monkey kidney epithelial cell line MA104 and the human colon carcinoma cell line Caco-2, both of which are highly permissive to rotavirus infection and are the most commonly employed.

The mature rotavirus infectious particles are formed by a triple-layered protein capsid that encloses the genome, composed of 11 segments of double-stranded RNA (dsRNA). The innermost layer, formed by 120 dimers of VP2, contains the viral genome and 12 copies each of VP1, the virus RNA-dependent RNA polymerase (RdRP), and VP3, a protein with guanylyltransferase, methylase, and phosphodiesterase enzymatic activities; these viral elements constitute the core of the virus. The addition of 260 trimers of VP6 on top of the VP2 layer produces double-layered particles (DLPs). The outermost layer is made by 780 copies of the glycoprotein VP7 arranged in trimers, which form a smooth surface layer from which 60 spikes composed of trimers of VP4 protrude to form the characteristic, infectious, triple-layered particles (TLPs) [37].

During or shortly after cell entry, the infecting TLP loses the external protein layer and is converted to a DLP. Once in the cytoplasm, the DLP, which is transcriptionally active, begins the synthesis of viral mRNAs that direct the synthesis of six structural proteins (VP1 to VP4, VP6, VP7) and six nonstructural proteins (NSP1 to NSP6). In addition to their function as mRNAs, the viral transcripts also serve as RNA templates for the synthesis of negative-strand RNAs to form the dsRNA genomic segments. The newly synthesized viral proteins are recruited to viroplasms, electrodense cytoplasmic structures, where the viral genome replicates and double-layered replication intermediate (RI) particles assemble. The DLPs newly formed in the viroplasms mature by budding into the lumen of the endoplasmic reticulum (ER) through the ER membrane, which is modified by the viral glycoproteins VP7 and NSP4. During this process, mediated by the interaction of VP6 with NSP4, the DLPs acquire a transient lipid envelope that is subsequently lost to yield mature infectious TLPs. Finally, in MA104 cells, the virus is released into the medium by cell lysis, whereas in Caco-2 cells, the virus exits through a non-lytic mechanism that is not well characterized [37] (Fig. 2.1).

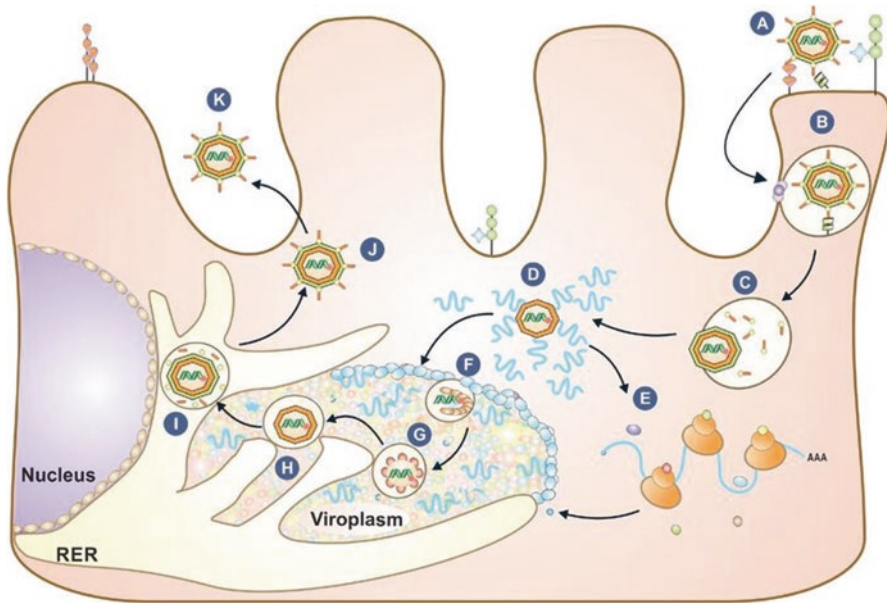


Fig. 2.1 Rotavirus replicative cycle. The virus replication cycle starts with the binding of the virus to the cell surface **A** and its internalization by endocytosis **B**. Inside the cell, the outer protein layer is shed **C**, and the double-layered particle becomes transcriptionally active **D**, giving rise to 11 RNA transcripts that encode 12 viral proteins. **E** Once a critical mass of viral protein is accumulated, the mRNA transcripts also serve as templates for the synthesis of the genomic double-stranded RNA (dsRNA), which occurs in replication intermediate particles within electrodense structures called viroplasms that are composed of viral proteins, viral RNA, and some cellular proteins **F–H**. Newly synthesized single- and double-layered particles assemble concurrently with genome replication, and **I** the double-layered particles then bud through a NSP4- and VP7-modified endoplasmic reticulum membrane into the lumen of the Rough Endoplasmic Reticulum (RER), where the final maturation of the virus particle takes place. **J, K**. Triple-layered, infectious particles exit the cell either by lysis or through a non-lytic process, depending on the cell line

Considering the purpose of this book, we describe in this chapter aspects of rotavirus biology where significant contributions by researchers working in Latin America have been made, with emphasis in our own work. This manuscript does not pretend to be a comprehensive review of the area, and we apologize to the colleagues we do not cite because of length restrictions.

2 Rotavirus Cell Entry

Among our principal contributions to the field of rotavirus is the characterization of the early events of virus–cell infection. Our research group has described the existence of at least four distinct interactions between the virus and host cell-surface molecules that mediate the attachment of the virus particle to the cell membrane and its subsequent entry into the cell. We have identified cell receptors and co-receptors,

as well as the viral proteins that interact with these cell-surface molecules. The characterization of these interactions allowed us to propose a model for rotavirus cell entry based on the concept of multiple virus–cell-surface molecule interactions, at least some of which occur in a sequential and well-coordinated manner, from the initial contact of the virus with the cell surface to penetration of the virus particle into the cell cytoplasm. This model is now the paradigm followed by researchers in the field, conceptually different from the “one viral protein–one viral receptor” prevalent at that time. Our group has also described that the interactions described here induce the endocytosis of the virus particle to initiate an intracellular vesicular trafficking that ends with the uncoating of the viral particle in distinct endosomal compartments, which, in some cases, involves the participation of the acidic proteases, cathepsins. In this section, we summarize our advances in this area.

2.1 *Virus Attachment*

The first step in the virus infectious cycle is the attachment of the virus particle to the cell surface, which is mediated by VP4 that has essential functions in the early interactions of the virus with the cell, including receptor binding and cell penetration [27, 64, 65, 79, 80, 84, 133]. The properties of this protein are therefore important determinants of host range, virulence, and induction of protective immunity. To be infectious, the virus depends on the specific trypsin cleavage of VP4, of 776 amino acids, to yield polypeptides VP8 (aa 1–231) and VP5 (aa 248–776), both of which remain associated to the virion [11, 25, 35, 36, 64]. The cleavage of VP4 does not affect cell binding, but rather it seems to be required for virus entry. The VP8 domain of VP4 mediates the attachment of the virus to the cell, whereas VP5 and the surface glycoprotein VP7 interact with downstream post-attachment molecules [72].

Rotavirus strains were initially classified as neuraminidase (NA) sensitive or NA resistant, depending on their ability to infect cells that had been previously treated with NA. Most human rotaviruses are NA resistant, whereas animal rotaviruses can be either NA sensitive or NA resistant [24, 53, 72]. Rotaviruses whose infectivity is decreased by NA treatment bind to the cell surface through terminal sialic acids (SAs), which are susceptible to NA cleavage. On the other hand, some NA-resistant viruses bind to internal SAs, which are not cleaved by NA [49], while yet others bind to human blood group antigens (HBGAs) [51, 52].

In the case of NA-sensitive rotavirus strains, gangliosides have been associated with rotavirus cell attachment for some time [14, 31, 49, 55]. However, knocking down the expression of two key enzymes involved in ganglioside synthesis decreased ganglioside levels as well as the infectivity of both NA-resistant and NA-sensitive rotavirus strains, but did not affect their binding to the cells, suggesting that gangliosides are not essential for cell-surface binding but rather they are needed during a later step of the entry process, regardless of the NA sensitivity of the virus [81].

2.2 *Post-attachment Interactions*

After the initial attachment to glycans on the cell surface, rotaviruses interact with additional surface molecules to gain access into the cell. Among these molecules are some integrins ($\alpha 2\beta 1$, $\alpha X\beta 2$, $\alpha V\beta 3$) and the heat shock cognate protein 70 (hsc70) [71, 72, 83, 85, 132, 134, 135]. Whether all these molecules are used by all rotavirus strains and whether the interactions of the virus with them are sequential or alternative is not known; however, in the particular case of the rhesus rotavirus strain (RRV), we showed that some of these interactions occur sequentially [71, 72, 83, 85, 132, 134, 135]. Interestingly, not all rotavirus strains interact with integrins, although all the strains tested require hsc70 for efficient cell infection [42, 45, 46].

The interaction of rotavirus with integrin $\alpha 2\beta 1$ is mediated by a DGE motif located toward the amino-terminal end of the VP5 domain of VP4 and the domain I of the integrin subunit $\alpha 2$ [42, 134]. On the other hand, integrin $\alpha V\beta 3$ interacts with rotavirus through a linear sequence in VP7 [135]. The interaction between the viral particle and hsc70 is mediated by VP5 (amino acids 642 and 659) and the peptide-binding domain of hsc70, and it has been suggested that the ATPase domain of hsc70 could be involved in promoting conformational changes in the viral particle to facilitate virus entry or uncoating [96, 134]. Furthermore, it has been shown that gangliosides, as well as integrins $\alpha 2\beta 1$, $\alpha V\beta 3$, and hsc70, are associated with detergent-resistant membrane microdomains, where infectious viral particles are also present during cell entry [54], and we showed that the integrity of these microdomains is fundamental for viral infection [44, 46].

Integrins have a polarized distribution in epithelial cells, localizing primarily at the basolateral face of the plasma membrane. Therefore, rotaviruses reaching the intestinal epithelium would find the integrin receptors hidden beneath the tight junctions (TJs). How might then rotavirus, with putative basolateral ligands, infect polarized epithelia? A possible explanation was offered when it was shown that a recombinant VP8 protein was able to decrease the trans-epithelial electrical resistance of polarized Madin–Darby canine kidney (MDCK) cells [89]. The ability of VP8 to generate a leaky TJ could allow integrins to diffuse to the apical surface, so that the virus could bind and infect from the apical side. The ability of virus particles to disrupt TJs during their early interaction with polarized epithelia, however, remains to be shown. Furthermore, we have shown that rotavirus infects polarized cells more efficiently through the basolateral face in comparison to the apical surface [22, 100]. In addition, we recently reported that the TJ protein JAM-A is important for the entry of some rotavirus strains at a post-attachment step, and we also found that occludin and ZO-1 are relevant for virus entry [116, 117].

It is of note that the assays used to block the interaction of rotaviruses with each of these proposed receptors and co-receptors using different approaches, such as proteases, antibodies, peptides, sugar analogues, or siRNAs, only decrease viral infectivity by less than tenfold, suggesting that either a more relevant entry factor for rotavirus has yet to be found, the virus can use more than one route of entry, or the cellular factors that allow the entry of rotavirus are redundant.

2.3 *Virus Internalization*

The cell entry of rotavirus by endocytosis is supported by several experimental approaches, including pharmacological inhibitors, overexpression of dominant-negative mutant proteins, and knocking down the expression of proteins implicated in different endocytic routes. In addition, actinin 4 and the activation of the small GTPase RhoA and Cdc42, as well as its activator CDGAP, which are involved in different types of endocytic processes, have been implicated in the entry of rotavirus [32, 46, 126]. Of interest, all tested rotavirus strains, with the exception of the RRV strain, enter cells through clathrin-mediated endocytosis [32, 46], whereas RRV uses an atypical endocytic pathway that is clathrin- and caveolin independent but depends on dynamin 2 and on the presence of cholesterol [107, 111]. The requirement for cholesterol and dynamin is also shared by those rotaviruses that are internalized by clathrin-dependent endocytosis [46], although contradictory results were recently reported in MDCK cells [126].

It is interesting to note that the interactions of the virus with the putative receptor and co-receptor molecules characterized so far do not seem to determine the endocytic pathway used, because both NA-resistant and NA-sensitive strains, as well as rotaviruses that interact with HBGAs, can enter cells using a clathrin-dependent mechanism [32]. In addition, using reassortant viruses, our group recently reported that the outer layer protein VP4 determines the endocytic pathway used, and a single amino acid substitution in the VP8 domain of RRV can change its entry pathway from a clathrin-independent to a clathrin-dependent mechanism [32]. We also showed that the infectivity of rotavirus is enhanced by calcium and that internalization of the virus induces an early permeabilization of cells [28, 92].

2.4 *Intracellular Vesicular Traffic and the ESCRT Machinery*

After internalization, rotavirus travels along the intracellular vesicular traffic moving from the cell periphery to the perinuclear space. During this traffic, the virus is transported by endocytic primary vesicles to early endosomes (EEs), then to maturing endosomes (MEs) that contain intraluminal vesicles (ILVs), and finally to late endosomes (LEs) [33, 111] (Fig. 2.2). The formation of the characteristic ILVs present in the ME is generated by the endosomal sorting complex required for transport (ESCRT) machinery [128]. Independent of the nature of the cell-surface receptor and the endocytic pathway used for cell internalization [32, 33, 46, 107, 111], all rotavirus strains tested converge in EEs during entry [32, 33, 46, 127] and depend on a functional ESCRT machinery, as knocking down the expression of components of the ESCRT complex by RNAi reduces virus infectivity [13, 111]. Why the entry of rotaviruses depends on the ESCRT machinery and what is the role of ILVs in this process has not been elucidated (discussed in [111]).

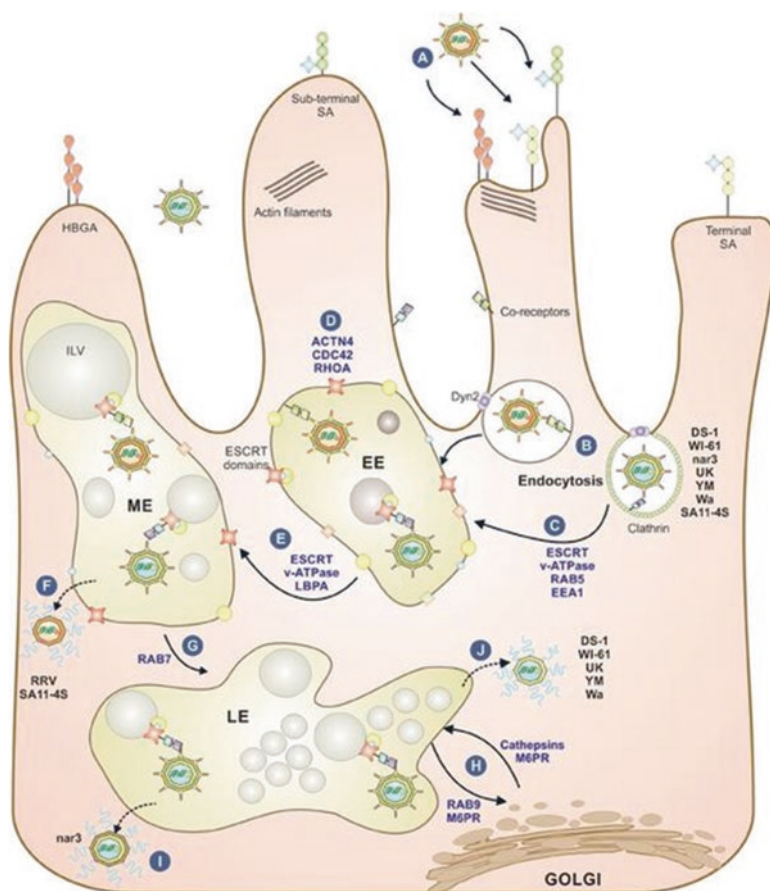


Fig. 2.2 Working model for rotavirus (RV) cell entry pathway in MA104 cells. **A** RVs attach to the cell surface through different glycans, depending on the virus strain. After initial binding, the virus interacts with several co-receptors concentrated at lipid rafts. **B** RVs are internalized into cells by clathrin-dependent or clathrin-independent endocytic pathways, depending on the virus strain. **C** Regardless of the endocytic pathway used, all RV strains reach early endosomes (EEs) in a process that depends on RAB5 and EEA1 and probably on HRS and the v-ATPase. **D** At the EE, the virus probably begins to be internalized into the endosomal lumen through the action of VPS4A. **E** EEs progress to mature endosomes (MEs), with a progressive decrease in pH and intraendosomal calcium concentration through the function of the v-ATPase; during this process, the formation of intraluminal vesicles (ILVs) increases. **F** E-P rotaviruses RRV and SA11-4S reach the cytoplasm from MEs. **G** GTPase Rab7 participates in the formation of late endosome (LE) compartments; ILVs increase in number. **H** The stability and function of LEs depend on the arrival of cellular factors (e.g., cathepsins) from the trans-Golgi network, traffic that is mediated by mannose-6-phosphate receptors (M6PRs) and the GTPase Rab9, among other factors. **I** L-P RV strains reach late endosomes; RV nar3 exit from LEs requires the function of Rab9. **J** RV strains UK, Wa, WI61, DS-1, and YM require, in addition to Rab9, the function of the CD-M6PR and the activity of cathepsins to productively infect cells. **F, I, J** The cytosolic double-layered particles begin transcribing the RV genome to continue the replication cycle of the virus

How far rotaviruses go into the different vesicular compartments depends on the virus strain. For rotaviruses RRV and SA11, the intracellular traffic comes to an end at MEs, and, based on this observation, these strains have been considered as early-penetrating viruses (Fig. 2.2). In contrast, all other rotavirus strains tested depend on the expression of Rab7 [33, 111], suggesting that these viruses continue their travel through the endosomal network to reach LEs [33]. In this regard, Rab7-dependent rotaviruses behave as late-penetrating viruses. Whether the virus travel to EE or reach LE is also determined by the spike protein VP4 [33].

2.5 *M6PR and Cathepsins*

The small GTPase Rab9 is a key component of LEs and orchestrates the transport of mannose-6-phosphate receptors (M6PRs) from LEs to the trans-Golgi network. Rotavirus strains that reach LEs depend on a functional Rab9 to infect the cell, and most of them also require the activity of the cation-dependent (CD) M6PR. Lysosomal acid hydrolases, such as cathepsins, are delivered from the trans-Golgi network to endosomes by M6PRs, and the recycling of these receptors to the Golgi depends on Rab9 [18]. We recently showed that the infectivity of rotavirus strains whose infectivity depends on Rab9 and CDM6PR is inhibited by pharmacological inhibitors of cathepsins B and L or when the expression of cathepsins B, L, or S is knocked down by RNAi [33], suggesting that these rotavirus strains require the activity of these hydrolases for cell entry (Fig. 2.2).

3 **Structural and Functional Characterization of Viral Genes and Cellular Proteins Required for Rotavirus Genome Replication and Virus Morphogenesis**

Our group was involved from the dawn of rotavirus research in the characterization of the proteins coded by each of the 11 segments of the viral genome [5, 6] and in determining the primary structure of the genes and their encoded protein products [2, 7, 64–69, 104]. This involvement allowed us to identify structural domains and predict potential antigenic and functional regions of the viral polypeptides [2, 7–10, 29, 38, 63–65, 67, 68, 70, 90]; however, the characterization of the role of the different proteins in the rotavirus life cycle was more difficult as it was limited by the technological tools available in the late 1990s. At the beginning of the past decade, a breakthrough for the analysis of gene function of mammalian cells occurred with the adaptation of the RNA interference (RNAi) system to efficiently and specifically knock down the expression of cellular genes [34]. In 2002, we reported that it was possible to inhibit the expression of rotaviral genes using this system: this represented one of the first reports in virology and the first

in the rotavirus field that demonstrated the feasibility of inhibiting the expression of animal virus genes by RNAi [12, 30]. Using this technology, we knocked down the expression of all rotavirus genes and characterized the function of the encoded proteins during different stages of the life cycle of the virus, including the replication of the viral genome [16, 76], the formation of viroplasm [75, 76], the assembly of double-layered RI particles [16, 75, 76], and the morphogenesis of mature, infectious viruses [30, 75, 82]. We also characterized the role of viral proteins in the control of the unfolded protein response [119], the mechanism of inhibition of cellular protein synthesis [87, 102, 103, 108], and the control of formation of stress granules [87]. Others have used this technology to prove the role of NSP4 in altering the Ca^{2+} homeostasis in rotavirus-infected cells [130]. Some of these contributions are briefly described following and in the next section.

Regarding the replication of the viral genome, it has been proposed that the synthesis of the negative strand of each genome segment occurs in viroplasms, concurrently with packaging of the positive-stranded RNAs (equivalent to the mRNAs) into core RI particles [93]. The analysis of the kinetics of transcription and replication of the viral genome throughout the replication cycle of the virus allowed us to provide evidence for the existence of a second round of transcription originated from newly assembled, transcriptionally active, double-layered RI particles, resulting in a second wave of assembly of DLPs [16]. In agreement with earlier studies in rotavirus genome transcription and replication by Eugenio Spencer and colleagues [94], this analysis also showed that all the proteins that form the DLPs (VP1, VP2, VP3, VP6) are essential for replication of the dsRNA genome, because in their absence there was little synthesis of viral mRNA and dsRNA [16]. In a parallel study, we also showed that the efficient replication of the viral genome depends on the ubiquitin-proteasome system (see following).

Once DLPs assemble in viroplasms, they mature by budding into the adjacent ER membrane, which is modified by the viral glycoproteins VP7 and NSP4. During this process, mediated by the interaction of DLPs with NSP4, the particles acquire a transient membrane envelope that contains VP4, NSP4, and VP7, which is later removed to yield the mature TLPs [37]. The mechanism of removal of the transient lipid envelope is largely unknown, although we demonstrated that VP4 is not involved and VP7 is important for this step [30], suggesting that rather than the membrane-piercing activity of VP4, as had been previously suggested, the assembly of the VP7 trimers into DLPs is responsible to exclude the lipid membrane from the viral particles. We also showed that the correct assembly of mature, infectious rotavirus particles is influenced by the two folding systems involved in the ER quality control. Grp78, protein disulfide isomerase (PDI), calnexin, and calreticulin were found to promote the timely trimming of the carbohydrate chains of VP7 and NSP4, the correct formation of VP7 disulfide bonds, and the incorporation of properly folded VP7 into TLPs to yield infectious virus, indicating that these chaperones are involved in the quality control of rotavirus morphogenesis [82]. On the other hand, Grp94 and Erp57 do not seem to be required for rotavirus morphogenesis [82]. PDI has also been suggested to be involved in rotavirus cell entry [20].

To better understand the replication of the viral RNA and virus morphogenesis, we have also analyzed the composition, dynamics, and gene function requirements for viroplasm formation, underscoring the highly organized nature and complex regulation of this structure [21, 40, 41, 75–77, 118]. We are currently studying the highly organized nature of viroplasms by super-resolution confocal microscopy.

The approach of knocking down the expression of one-by-one cellular proteins to search for those relevant for virus replication was not practical, but the scenario changed when the RNAi system became amenable for genome-wide screening of cellular functions. Using this system, we identified more than 500 cellular genes involved in rotavirus replication [78, 111]. These genes clustered functionally into several biological processes potentially involved in various steps of the rotavirus life cycle. Among these functional clusters were endocytic processes, the tight junction protein network, and the ubiquitin-proteasome protein degradation system.

The endocytic process of the virus and the role of tight junction proteins in virus infection were described in the previous section. With regard to the proteasome-ubiquitin components, *in silico* proteomics showed a strong cluster of positive hits in our data set that included E3 ligases regulated by deubiquitinase PAN2, heat shock proteins, and components of the 26S proteasome subunits [111]. These findings were supported by our demonstration, and that of a different group, of the requirement of the proteasome-ubiquitin pathway for rotavirus replication [26, 77]. We showed that both the proteolytic and ubiquitination activities of the ubiquitin-proteasome system were needed for the correct incorporation into viroplasms of the viral polymerase VP1 and the capsid proteins VP2 and VP6, as well as for the efficient replication of the viral genome [77], suggesting that this system has a very complex interaction with the rotavirus life cycle.

4 Rotavirus Strategies to Control the Antiviral Response of the Host Cell

Stress and innate immune cell responses are closely linked and overlap at many levels. The outcomes of these responses serve to reprogram host expression patterns to prevent viral invasions. In turn, viruses fight back against these responses to ensure their replication through various mechanisms, depending on the virus. Interestingly, the first step to control the antiviral response of the cell, and a solution seen in several virus families, is to take over the translation machinery of the host, such that the translation of viral proteins is ensured while the expression of the stress and antiviral responses of the cell is blocked. In addition, immediately upon infection, the cellular RNA decay pathways and the innate immune responses are triggered. To guarantee their successful replication, viruses have evolved different tools to subvert these pathways. Our group has been interested in characterizing the interactions between rotavirus and its host cell to understand the mechanisms by which this virus is able to establish a productive infection based on controlling the antiviral response of the cell [73, 74] (Fig. 2.3).

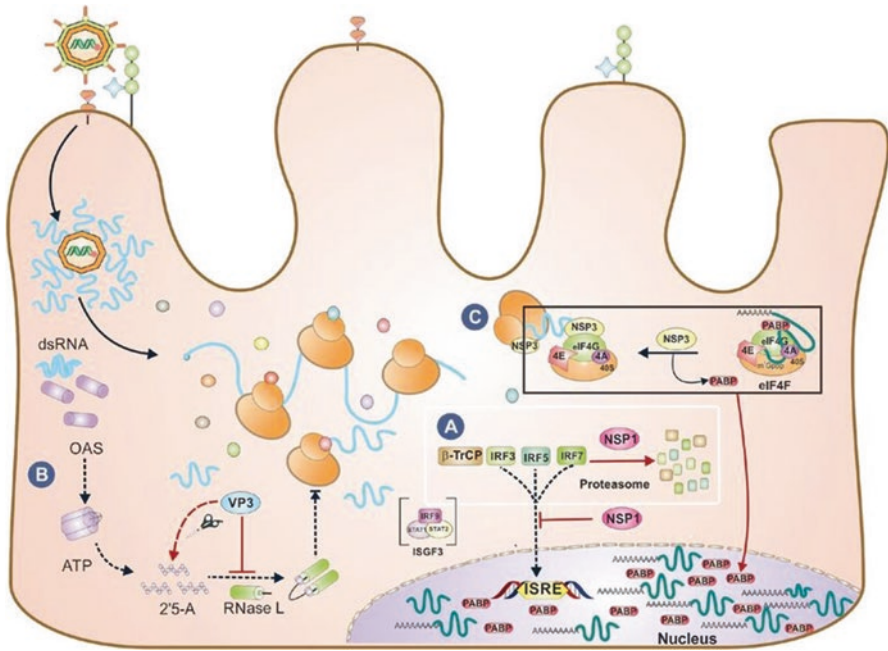


Fig. 2.3 Rotavirus measures to counteract the host response. **A** During the infection, NSP1 interacts with and causes the proteasome-dependent degradation of IRF3, IRF5, IRF7, and beta-TrCP, and also prevents the activation and translocation of ISGF3 in a proteasome-independent manner. **B** In the presence of dsRNA, 2'-5'-oligoadenylate synthetase (OAS) oligomerizes and synthesizes 2'-5'-oligoadenylates (2-5A), which in turn interact with RNase L, causing its dimerization and activation. The phosphodiesterase activity of VP3 degrades the 2-5As, preventing RNase L activation. **C** NSP3 interacts specifically with eIF4G, displacing poly(A)-binding protein (PABP) and preventing the translation of poly(A)-containing mRNAs. Also in the infection, PABP and poly(A)-containing mRNAs accumulate in the cell nucleus. 2-5A 2'-5'-Oligoadenylate, *IRF* interferon regulatory factor, *ISRE* interferon-stimulated response element, *OAS* 2'-5'-oligoadenylate synthetase, *dsRNA* double-stranded RNA

4.1 Protein Synthesis in Rotavirus-Infected Cells

As obligate intracellular parasites, viruses depend on the cell translation machinery for the production of their proteins. Although every step of the translation process is amenable to regulation, in general, mRNA translation is regulated mainly at the level of initiation [113], a process mediated by the eukaryotic initiation factors (eIFs). The main checkpoints for the control of polypeptide chain initiation are the formation of the eIF4F complex and the activity of eIF2, both of which are targets of control by viruses (reviewed in [124]). In eukaryotic cells, mRNA translation initiation begins with the recruitment of mRNAs by the eIF4F complex and the subsequent assembly of the 40S and 60S ribosomal subunits. The eIF4F complex is formed by several canonical eIFs; the cap-binding protein eIF4E recognizes the cap structure present at the 5'-end of mRNAs; eIF4A is an ATP-dependent RNA helicase that unfolds secondary structures of mRNAs and eIF4G, which functions as a

scaffolding protein where several eIFs bind; and eIF4F complex favors the interaction of the mRNA with the 40S ribosomal subunit [56]. Once the 40S ribosomal subunit is bound to the mRNA, it is scanned in the 5′–3′ direction, until the first AUG codon is found, and it is selected for translation initiation [50]. A ternary complex composed of eIF2-GTP-Met-tRNA charges the initiator Met-tRNA to begin translation, and the 60S ribosomal subunit is then joined to form an 80S initiation complex. The released binary complex formed by GDP-eIF2 is recycled by eIF2B, which exchanges GDP for GTP, and a new tRNA-Met is loaded to form a ternary complex, ensuing new rounds of initiation [50, 56].

Early in the infection, rotaviruses take over the host translation machinery, causing a severe shutoff of cell protein synthesis, whereas the synthesis of viral proteins proceeds very robustly. At least three different mechanisms have been found to be involved in the control of the host protein synthesis machinery (Fig. 2.3).

(i) *The poly(A)-binding protein (PABP) is displaced from its binding site in eIF4G.*

In general, all eukaryotic mRNAs contain a poly(A) tail at their 5′-end, which is recognized by PABP, which in turn binds to eIF4G, favoring the circularization of the mRNAs that are also bound to eIF4G through the cap-binding protein. Rotavirus mRNAs contain 5′-methylated cap structures, and, instead of the poly(A) tails characteristic of most cellular mRNAs, they have at their 3′-end a consensus sequence (GACC) that is conserved in all 11 viral genes [95, 98]. The nonstructural protein NSP3 binds through its amino-terminal domain to this consensus sequence, and it also binds through its carboxy-terminal domain to eIF4G, at the same site where PABP binds. Thus, it was proposed that during infection, NSP3 evicts PABP from eIF4G, impairing the translation of cellular mRNAs while leading to an enhanced translation of rotaviral mRNAs [97, 98]. However, despite the essential role proposed for NSP3 in infected cells, we found that silencing the expression of this protein by RNAi indeed blocks the translation of cellular mRNAs but the viral mRNAs were still efficiently translated. We also found that the knockdown of NSP3 results in an increased production of viral progeny [86]. These findings were questioned with the argument that even small undetected amounts of NSP3 could be able to initiate the synthesis of viral proteins, at a time in the infection where there is little viral mRNA to compete with the cellular mRNAs [43]. Differences in the viral strains used and on the cell lines or experimental paradigms used may also account for these discrepancies.

(ii) *PABP is accumulated in the nucleus of the cell, and there is a block in the nucleocytoplasmic transport of polyadenylated cellular mRNAs.* Interestingly, we and others have recently found that NSP3 has an additional mechanism to prevent the translation of cellular mRNAs: PABP is a protein that assists the transport of mRNAs from the nucleus to the cytoplasm, where they are available to the translation machinery; during rotavirus infection, PABP becomes accumulated in the nucleus of infected cells [15, 47, 87, 103], and it was shown that the eIF4G-binding domain of NSP3 is important for the nuclear localization of PABP [47, 87], although the precise mechanism through which this occurs has not yet been determined [103].

Furthermore, we found that the accumulation of PABP in the nucleus of rotavirus-infected cells also resulted in the accumulation and hyper-polyadenylation of poly(A)-containing mRNAs [97], suggesting that the shutoff of cell protein synthesis during the infection might be caused by a blocking of the nucleocytoplasmic transport of polyadenylated mRNAs [103].

(iii) *Phosphorylation of the translation initiation factor eIF2*. In rotavirus-infected cells, the inhibition of cell protein synthesis is also regulated by a third mechanism because the alpha-subunit of eIF2 becomes phosphorylated early in the infection and it is maintained in this state throughout the virus replication cycle [87]. When eIF2 α is phosphorylated, the eIF2-GDP complex binds with higher affinity to eIF2B, preventing the exchange of GDP to GTP catalyzed by eIF2B, which reduces the formation of pre-initiation translation complexes and causes a severe reduction in global translation [56]. The phosphorylated status of eIF2 α is beneficial for the virus, because under these conditions the viral mRNAs are efficiently translated but the synthesis of most cellular proteins is prevented. We and others have found that the dsRNA-dependent protein kinase, PKR, is the enzyme responsible for the phosphorylation of this translation initiation factor in rotavirus-infected MA104 [102] and intestinal epithelial cells [122].

The precise mechanism involved in viral protein synthesis has not been identified. However, we have found that during the infection, the amount of viral transcripts produced is in the range of tens of thousands of molecules per cell [103]. The huge number of viral mRNAs in a cell where the translation of poly(A)-containing mRNAs is inhibited by at least three different mechanisms [eIF2 α , poly(A)-containing mRNAs sequestered in the nucleus, and eviction of PABP from eIF4G] leaves the translation of viral mRNAs with little competition for the protein synthesis machinery and explains the severe shutoff host translation caused by rotaviruses.

4.2 Stress Response of the Cell

Two of the most common stress responses of the cell are the formation of stress granules [3] and an integrated stress response known as the unfolded protein response [129]. These responses have been characterized in rotavirus-infected cells (Fig. 2.3).

(i) *Stress granules* (SGs) are cytoplasmic aggregates of stalled translational pre-initiation complexes that accumulate during stress [59]. In addition to its direct effect on protein synthesis, the phosphorylation of eIF2 α is one of the signals that induces the formation of SGs. It has been proposed that SGs are sites in which the integrity and composition of mRNAs are triaged and then mRNAs are sent either to translation, degradation, or storage (reviewed in [91]). Because the main function of SGs is to arrest protein synthesis until the stressful conditions are

resolved, viruses have to interact with these structures to ensure the translation of their mRNAs, and several different viral strategies have been developed to cope with their deleterious effect (reviewed in [125]).

Interestingly, we have found that even though eIF2 α is phosphorylated in rotavirus-infected cells, SGs are not formed [87]. Furthermore, we found that rotaviruses prevent the formation of SGs, because these structures are not formed in cells infected with rotavirus when induced to form SGs by treatment with arsenite, a well-characterized SG inducer. The mechanism by which the formation of these structures is prevented during the infection has not been determined.

(ii) *Unfolded protein response (UPR)*. The accumulation of misfolded proteins in the ER causes stress and leads to activation of a coordinated adaptive program called UPR (reviewed in [48, 57, 129]). The function of the UPR is to handle unfolded proteins by upregulating the expression of chaperone proteins and degradation factors to refold or eliminate misfolded proteins and to reduce the incoming protein traffic into the ER by attenuation of translation [106] (Fig. 2.4). Failure to alleviate ER stress leads to activation of apoptotic pathways and cell death [58]. Rotavirus infection induces the UPR; however, this response is modulated by the virus [119, 131]. At least two of the three arms of the UPR appear to be activated in rotavirus-infected cells; the mRNA of Xbp1 was spliced by IRE1, and the transcription of GRP78 and CHOP is induced, indicating that the ATF6 pathway was activated. The UPR, however, is suppressed at the translational level by NSP3 [119]. The consequences of suppressing this response during rotavirus infection have not been addressed.

4.3 Antiviral Response of the Cell

Double-Stranded RNA Double-stranded RNA (dsRNA) is considered a key mediator of interferon (IFN) induction in response to virus infection. When the cell sensors detect dsRNA, a cascade of events is activated that promote the shutoff of cell protein synthesis, the induction of transcription of genes encoding IFN and other cytokines, and finally cell death [101]. Several findings indicate that rotaviral dsRNA is exposed to cell sensors at some point during virus replication: (a) the kinase that phosphorylates eIF2 α in rotavirus-infected cells is PKR, which is activated by dsRNA [25]; (b) RIG-I and MDA5 are active and mediate the IFN response in rotavirus-infected cells [19, 110]; and (c) viral dsRNA can be detected in the cytoplasm (outside viroplasm) of rotavirus-infected cells [102]. These observations suggest that during rotavirus infection, either naked viral dsRNA, or highly structured viral mRNA [62], or both are present in the cytoplasm where they are detected by RIG-I and MDA5 with the consequent activation of the IFN response and the PKR activity that leads to the phosphorylation of eIF2 α and the modification of the cellular translation machinery. Another pathway that is activated by dsRNA is the 2'-5'-oligoadenylate synthetase (OAS)/RNase L pathway. OAS is activated by

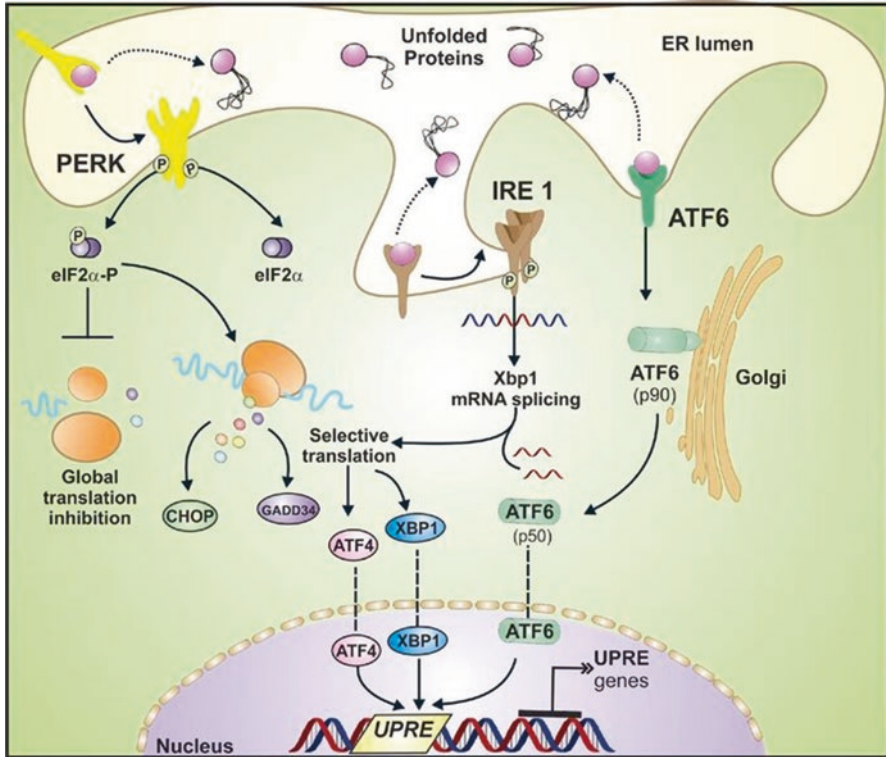


Fig. 2.4 The unfolded protein response. Accumulation of misfolded proteins in the endoplasmic reticulum (ER) causes stress and leads to activation of a coordinated adaptive program called the unfolded protein response (UPR). Three ER-resident transmembrane proteins are activated in response to ER stress: the PKR-like ER kinase (PERK), the activating transcription factor 6 (ATF6), and the inositol-requiring enzyme 1 (IRE1). Under normal conditions, the ER chaperone GRP78 is bound to the luminal domain of each sensor. When misfolded proteins accumulate in the ER, GRP78 binds these proteins and releases the sensors. Upon release, PERK and IRE1 homodimerize, causing autophosphorylation and activation, and released ATF6 relocates to the Golgi apparatus where it is cleaved and activated. Once activated, PERK phosphorylates the alpha-subunit of IF2 at Ser51. Phosphorylated eIF2a inhibits global translation and stimulates the translation of ATF4, which in turn transcriptionally activates UPR-responsive genes encoding proteins that ameliorate the ER stress. The gene encoding the CCAT/enhancer-binding protein (CHOP) is a target of ATF4, and this protein can function as a proapoptotic or prosurvival transcription factor, depending on the strength or duration of the stress. Both transcription factors, ATF4 and CHOP, can induce the transcription of the *GADD34* gene, encoding a protein that interacts with protein phosphatase 1 (PP1) to dephosphorylate eIF2a, resulting in a negative feedback loop that recovers protein synthesis and allows the translation of stress-induced transcripts. When ATF6 is cleaved in the Golgi apparatus, one of its cleavage products becomes an active transcription factor that promotes the transcription of chaperone genes. Finally, upon dimerization of IRE1, it autophosphorylates and mediates the removal of an intron from X-box-binding protein 1 (*XBP1*) mRNA. The spliced form of XBP1 encodes a transcription factor that activates the transcription of genes encoding chaperones and proteins involved in the ER stress-associated protein degradation (ERAD) system. (Figure reproduced from *Current Opinion in Virology* 2012;2:1–10, with permission)

dsRNA to produce 2'–5'-oligoadenylates, which are the activators of RNase L; this enzyme degrades viral and cellular RNAs restricting viral infection [112]. We recently demonstrated that after rotavirus infection the OAS/RNase L complex becomes activated; however, the virus is able to control its activity using at least two distinct mechanisms: a virus–cell interaction that occurs during or previous to rotavirus endocytosis triggers a signal that prevents the early activation of RNase L, whereas later, once viral proteins are synthesized, the phosphoesterase activity of VP3 degrades the cellular 2'–5'-oligoadenylates, which are potent activators of RNase L, preventing its activation [108].

5 Rotavirus Pathogenesis and Adaptive Immunity

Rotavirus pathogenesis and immunity have not been areas of direct study by our group, but relevant work in the area regarding virus–cell interactions and the humoral and cellular immune response to natural infection or vaccination is briefly reviewed in this section.

Ionic calcium (Ca^{2+}) is a crucial second messenger that controls many intracellular processes in mammalian cells. Thus, intracellular $[\text{Ca}^{2+}]$ is finely regulated by a number of proteins that maintain Ca^{2+} intracellular homeostasis in different compartments to regulate spatiotemporal Ca^{2+} signaling. Pioneering work done in Venezuela demonstrated that rotavirus infection causes significant changes in the homeostasis of Ca^{2+} of the infected cell. These changes bring alterations in the cell cytoskeleton that may be related to pathogenesis but also help to create favorable intracellular conditions for virus maturation [105]. In addition, work from Venezuela also helped to firmly establish NSP4 as a key function in the Ca^{2+} alterations observed in infected cells.

Understanding the adaptive immune response to rotavirus infection is necessary if efficient preventive measurements are to be developed. Work carried out in Mexico and Colombia has helped greatly in understanding rotavirus immunity. A pioneering work by the Mexican Institute of Nutrition, where a cohort of more than 200 rotavirus-infected children was followed from birth to 2 years of age, answered several of the key questions necessary to launch the development of an effective rotavirus vaccine: a primordial finding of that study was that a rotavirus infection, either symptomatic or asymptomatic, would protect against subsequent infections [120]. Also, work from Mexico has helped in the identification of T-cell epitopes on the main rotavirus structural protein VP6 and its use as a potential recombinant vaccine for veterinary use [61]. Finally, work developed in Colombia has helped in the understanding of the B- and T-cell response to rotavirus infection and in the identification of correlates of protection for rotavirus vaccines [4].

6 Future Challenges

The rhythm of research on fundamental aspects of rotavirus biology has slowed down in the past decade, probably because of the successful incorporation of two rotavirus vaccines in national immunization programs for children around the world. However, it is important to keep in mind that, so far, the impact of vaccine use on global estimates of rotavirus mortality has been limited [115], and in some regions of the world rotavirus infections still place an enormous burden on societies from both health and economic perspectives. There is need for a renovated effort to better understand the life cycle of rotavirus and to improve our knowledge about its epidemiology, evolution, ecology, and pathogenesis, as well as the immune response it elicits, all of which should facilitate the development of improved vaccines and therapeutic approaches.

Our knowledge about virus biology has advanced greatly during the past years; however, most stages of virus replication are incompletely understood, such as rotavirus entry and vesicular traffic, translation of the viral polypeptides, replication of the virus genome, morphogenesis of the newly assembled viral particles, and the egress of the mature, infectious virus from cells. Most of what we know has been learned from studies using nonpolarized MA104 cells or differentiated cultures of intestinal cell lines, such as Caco-2. However, to better understand virus–cell interactions in detail, it is important to incorporate methodological advances that make possible the analysis of the host cell response at a single-cell level instead of characterizing the response of pooled and usually heterogeneous cell cultures.

Furthermore, it is of utmost importance to study the virus replication cycle and the virus–host interactions in the cells that the virus targets in a natural infection. Animal models have been very useful to characterize virus restriction factors that participate in defining host range, and virus pathogenesis, as well as the innate and acquired immune responses induced by rotavirus infection. However, these models represent a complicated system to characterize the different steps of virus replication. In this regard, the recent development of enteroids from human intestinal origin, which have been reported to mimic the complex cellular lineages and tissue architecture of the gut and to efficiently support the replication of rotavirus [109], represents an appealing alternative for these studies. This system, together with the possibility of using the CRISPR/Cas9 technology in these cells and the possibility of characterizing the interactions of the virus with the cell-surface cellular receptors/co-receptors by live cell imaging systems, and the use of novel super-resolution microscopy techniques, are important tools for advances in this field.

Acknowledgments The work in our laboratory relevant to this chapter was supported by grant # 221019 from CONACYT, Mexico, and grants # IG200114, and #IG200317 from DGAPA-UNAM, Mexico. The authors thank Dr. Liliana Sanchez Tacuba for the elaboration of the figures.

Competing Interests We declare no competing interest.

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Ludert, J.E.; Pujol, F.H.; Arbiza, J. (Eds.)

2017, XI, 474 p. 48 illus., 30 illus. in color., Hardcover

ISBN: 978-3-319-54566-0