Rotavirus Assembly: An Alternative Model That Utilizes an Atypical Trafficking Pathway

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Abstract   We review here recent advances in our knowledge on trafficking and assembly of rotavirus and rotaviral proteins in intestinal cells. Assembly of rotavirus has been extensively studied in nonpolarized kidney epithelial MA104 cells, where several data indicate that most if not all the steps of rotavirus assembly take place within the endoplasmic reticulum (ER) and that rotavirus is release upon cell lysis. We focus here on data obtained in intestinal cells that argue for another scheme of rotavirus

assembly, where the final steps seem to take place outside the ER with an apically polarized release of rotavirus without significant cell lysis. One of the key observations made by different groups is that VP4 and other structural proteins interact substantially with specialized membrane microdomains enriched in cholesterol and sphingolipids termed rafts. In addition, recent data point to the fact that VP4 does not localize within the ER or the Golgi apparatus in infected intestinal cells. The mechanisms by which VP4, a cytosolic protein, may be targeted to the apical membrane in these cells and assembles with the other structural proteins are discussed. The identification of cellular proteins such as Hsp70, flotillin, rab5, PRA1 and cytoskeletal components that interact with VP4 may help to define an atypical polarized trafficking pathway to the apical membrane of intestinal cells that will be raft-dependent and by-pass the classical exocytic route.

1
Introduction

1.1
Rotavirus Structure

Rotavirus is a relatively large (75-nm) icosahedral, nonenveloped double-stranded RNA (dsRNA)-containing virus (reviewed in Lawton et al. 2000). Its capsid encloses 11 segments of dsRNA, each segment encoding for one protein except for segment 11, which encodes two proteins. Six out of these 12 proteins are structural proteins (VP1–VP4, VP6, and VP7) and the six others are nonstructural proteins (NSP1–NSP6). The capsid is composed of three concentric protein layers (reviewed in Jarayam et al. 2004). The inner layer is mostly made of 60 dimers of VP2 to which are associated small quantities of VP1, an RNA-dependent RNA polymerase, and VP3, a guanylyl and methyl transferase required for the synthesis of capped mRNA transcripts. The intermediate layer consists exclusively of 260 trimers of VP6 that interact with VP2 and with the two remaining structural proteins of the external layer of the capsid, VP7 and VP4 (Mathieu et al. 2001).

1.2
Rotavirus Mainly Targets Enterocytes

It has been shown that rotaviruses can infect a number of cell types in vitro (Ciarlet et al. 2001), and recent work suggests that rotavirus provokes viremia, thus transgressing the epithelial barrier (Blutt et al. 2003). Several studies, however, indicate that in vivo rotaviruses mainly target enterocytes from the small intestine of young animals, infants, and young children. Rotaviruses are the major causative agent of acute infantile gastroenteritis responsible for

nearly 600,000 deaths annually worldwide (Parashar et al. 2003). Most of the clinical symptoms are restricted to the gut, suggesting that progeny virions follow a pathway that limits widespread dissemination. Indeed, it has been shown that the vast majority of progeny virions are released through an ill-defined process at the apical pole of infected intestinal cells (Jourdan et al. 1997).

1.3
The Polarized Trafficking Machinery of Intestinal Epithelial Cells

Intestinal epithelial cells display a polarized phenotype and possess an apical membrane consisting of a brush border oriented towards the lumen of the gut. The apical membrane is separated from the basolateral membrane by a sophisticated junction system containing tight and adherent junctions that strictly control and limit the dissemination through the epithelial barrier and laterally between the apical and the basolateral domain. To support this organization, epithelial cells have developed sophisticated intracellular trafficking pathways able to sort and target molecules destined to the apical or the basolateral domains (Mostov et al. 2003). Proteins synthesized on endoplasmic reticulum (ER) -associated ribosomes enter the ER and follow a common route through the Golgi apparatus to the trans-Golgi network (TGN). The polarized traffic of lipids has been less studied, but recent data indicate that similar sorting and targeting processes are also at work for several lipid species (Hoekstra and van Ijzendoorn 2000). It is now an established fact that a first sorting event takes place in the TGN, where a subset of proteins will be targeted to the apical membrane, whereas other proteins are targeted to the basolateral membrane. Several data have shown that a second sorting event takes place at the basolateral membrane where proteins containing specific, as yet unidentified targeting signals will be readdressed to the apical domain using the endosomal system through a process called transcytosis (Polishchuk et al. 2004). It is also generally accepted that proteins bear sorting and/or targeting signals that are recognized by a complex intracellular machinery and help to incorporate proteins into specific intracellular vesicles equipped to address these proteins to the correct compartment. Unequivocal basolateral targeting signals have been identified (Mostov et al. 2000), but apical targeting signals remain to be more clearly defined. It has thus been proposed that the glycosylphosphatidylinositol (GPI) anchor (Mayor and Riezman 2004), protein glycosylation (Ait Slimane et al. 2001), or some specific transmembrane domains (Ait Slimane et al. 2001) may be involved in apical targeting, although these results are challenged by other experimental data (Rajho Meerson et al. 2000; Ait Slimane et al. 2000; Lipardi et al. 2000).

2
What Is Known About Rotavirus and Rotaviral Protein Trafficking?

Although it has been clearly established that intestinal cells are the main in vivo target of rotavirus, most of the studies on virus assembly and release have been carried out in nonpolarized and nonintestinal cells, mainly MA104 cells originating from monkey embryo kidney cells (Estes 2001). The picture emerging from these studies can be summarized as follows. After rotavirus entry into cells through a process that continues to be debated (endocytosis or direct entry; for details see Lopez and Arias 2004), the external layer of the capsid (VP4 and VP7) is removed through a calcium binding process (Ruiz et al. 2000), leading to a transcriptionally competent particle (called the double-layered particle or DLP; see Jarayam et al. 2004), producing segmented mRNAs which are capped at the 5′ end but not polyadenylated at the 3′ end (Estes 2001). Most of the encoded viral proteins are then thought to be synthesized on free ribosomes, except for VP7 and NSP4, which possess signal peptides and are therefore targeted to ER-associated ribosomes (Au et al. 1993; Stirzaker et al. 1990). All the cytosolic viral proteins except VP4 have been found in an ill-defined cytoplasmic organite, the viroplasm, considered to be a viral factory from which immature particles, containing the viral genome protected by the inner and the intermediate layer of the capsid (DLPs), emerge and enter within the ER lumen through an NSP4-dependent process (Taylor et al. 1993). Electron microscopy studies have shown that these DLPs have a transient membrane envelope (Suzuki et al. 1993). It is still not clear whether VP7 is associated with the virus particles at this stage. When VP7, a glycoprotein, is incorporated into viral particles of rotavirus SA11 strain in infected MA104 cells, the protein displays a glycosylation content consisting mostly of six mannose molecules (Kabcenell et al. 1988). This would suggest that VP7 remains accessible to glycan-processing enzymes localized in a post-ER compartment. The nonstructural protein NSP4, a viral glycoprotein associated with the ER membrane, is involved in DLP entry within the ER (Taylor et al. 1993) and has also been described as part of a complex with VP4 and VP7 (Maass and Atkinson 1990). This finding, together with the fact that NSP4 never reaches the Golgi apparatus (Xu et al. 2001), has favored the hypothesis that rotavirus final assembly takes place within the ER. It is generally accepted that new rotavirus virions are released through cell lysis in MA104.

3
Rotavirus and Intestinal Cells

Few studies have been conducted on the mechanisms by which rotavirus infects intestinal cells, despite the availability, since the early 1980s, of cell systems such as HT-29 or Caco-2 cells able to reproduce a significant part of the polarization and differentiation programs of human intestinal cells in culture (Chantret et al. 1988). Interestingly, these cell lines, when grown in a very precise manner, are able to form a regular monolayer with functional tight junctions, displaying a high electrical transepithelial resistance and expressing intestinal specific markers, such as apical brush border enzymes (Trugnan et al. 1987; Darmoul et al. 1992). Harry Greenberg's group was the first to demonstrate that polarized Caco-2 cells are fully susceptible to rotavirus infection (Svensson et al. 1991). We then showed that rotavirus infection of Caco-2 cells displays three major differences as compared to nonpolarized MA104 cells: (a) the time needed for virus morphogenesis appears to be significantly longer than in MA104 since a rise in virus titer is only observed at 12–15 h postinfection (pi), suggesting a more complex assembly process and/or a delayed entry (Jourdan et al. 1997, 1998); (b) intestinal cells do not lyse upon completion of the true viral replication cycle and remained viable for at least 48 h pi; and (c) in agreement with (b), progeny virions are selectively released through the apical membrane, suggesting that the general architecture of intestinal cells was maintained and that virus was assembled using polarized sorting and targeting mechanisms (Jourdan et al. 1997). Since then, additional work has been carried out to describe the pathway used by rotavirus and rotaviral protein for assembly and release in intestinal cells (see below). The main starting point for these studies was based on the idea that apical targeting may be mediated by specialized membrane microdomains, namely rafts, known to be enriched in cholesterol and sphingolipids (Simons and Ikonnen 1997).

4
Rotavirus and Rafts

4.1
Revisiting the Fluid Mosaic Model of Biological Membrane

The classical model of Singer and Nicolson (1972) predicted that cell membranes behave like a fluid mosaic in which proteins float within a sea of

lipids. This model has proven very useful for several generations of researchers. However, using either membrane models or biological systems, several other results have shown that lipids have specific capacities to self-assemble within microdomains, and therefore membranes should be heterogeneous in composition and structure (Simons and Vaz 2004). Proteins may choose a specific lipid environment, as shown, for example, for GPI-linked proteins, and this in turn may induce lipids to reorganize (Helms and Zurzolo 2004). The concept of rafts remains, however, a matter of debate, mostly because of the lack of accurate identification methodologies (Munro 2003). Most of the studies are based on a unique biochemical property of these microdomains: they are resistant to detergent extraction (detergent resistant membranes, DRM) and can therefore be floated on density gradients, because of their high lipid content. The raft hypothesis has been largely used to explain the number of biological and pathophysiological processes, including the involvement of rafts in the interaction of enveloped viruses with their target cells (Chazal and Gerlier 2003). In contrast, until recently, there were no data available on interactions of rafts with nonenveloped viruses.

4.2
Evidence for Raft Involvement in Rotavirus Assembly

Evidence that rotavirus and rotaviral proteins become associated with rafts was demonstrated using various approaches. The group of Carlos Arias and Susana Lopez demonstrated that some particular rafts of MA104 cells may be involved in rotavirus entry (Lopez and Arias 2004; Isa et al. 2004; Sanchez-San Martin et al. 2003) (see the chapter by S. Lopez and C. Arias in this volume). Using differentiated Caco-2 cells, our group showed that VP4 associated very early after infection with detergent-resistant membranes. We also demonstrated that the other rotaviral structural proteins associated later with DRMs in a time sequence that was compatible with a role of rafts for rotavirus assembly. This was confirmed using an X-ray diffraction approach with VP4 and a lipid mixture that resemble raft composition (Sapin et al. 2002). Finally, we showed that detergent-resistant membranes extracted from infected cells were able to infect naive Caco-2 cells (Sapin et al. 2002). A direct interaction between rotavirus, rotaviral proteins, and detergent-resistant membranes was confirmed by Harry Greenberg's group, which demonstrated that in vivo rotavirus also associated with lipid rafts in infected mice (Cuadras and Greenberg 2003). Interestingly, some discrepancies between the two sets of data were noted, mainly concerning the kinetics of rotavirus protein association with rafts, which was much more rapid and simultaneous in the

latter data (Cuadras and Greenberg 2003). This may at least in part be attributable to the fact that Caco-2 cells were grown in conditions in which they do not fully differentiate (Cuadras and Greenberg 2003). In the meantime, it was shown that NSP4 also associates with microdomains (Huang et al. 2001). Other recent data seem to indicate, however, that when VP5 fragments, derived from VP4, including the putative hydrophobic domain (residues 248–274) were transfected into Cos 7 or HEK293 cells, no association with DRMs was observed (Golantsova et al. 2004), suggesting that VP4 may not interact with rafts using this hydrophobic domain and/or that VP4 interaction with DRMs is not direct, as expected for a peripheral protein that requires additional membrane factor(s) to interact. This fit very well with preliminary data from our lab suggesting that VP4 may interact with cellular raft-associated proteins (Gardet et al., Delmas et al., unpublished data; Broquet et al. 2003, unpublished data).

5
Rotavirus Assembly Needs an Extra-Reticular Step

5.1
Rational for the Extra-Reticular Hypothesis

In an attempt to understand how rafts may be involved in the final assembly of rotavirus particles, it was necessary to comprehend how these rafts interact with the ER, since DLPs enter the ER when they emerge from the viroplasm and rafts are classically known to be excluded from this compartment. In eucaryotic cells, the synthesis of sphingolipids, which are essential raft components, takes place in the Golgi apparatus (Holthuis et al. 2001) from a ceramide precursor made in the ER and transported directly to the TGN via a nonvesicular pathway, catalyzed by a recently discovered protein called CERT (Hanada et al. 2003). If rotavirus uses rafts as an assembly platform, then it can be hypothesized that immature particles (containing VP1–VP3 and VP6) emerging from the ER associate with VP7, which is already present within the ER membrane, and with VP4, which is synthesized on free ribosomes. If this is true, then VP4 must be ER-associated and therefore sensitive to drugs affecting ER exit.

5.2
The Tunicamycin Effect

Experiments have recently been conducted in our laboratory in which tunicamycin was used to perturb ER exit. Tunicamycin is known to block the

first N-glycosylation step, resulting in the accumulation of nonglycosylated proteins within the ER (Struck and Lennarz 1977). This drug has already been used to study rotavirus assembly in MA104 cells and it has been shown that virus morphogenesis is strongly perturbed (Petrie et al. 1983; Mirazimi and Svensson 1998). Our experiments revealed a similar decrease in rotavirus final assembly in Caco-2 cells in the presence of tunicamycin. In addition, these experiments demonstrated that VP4 biosynthesis and trafficking were insensitive to tunicamycin, suggesting that VP4 does not interact with the ER (Delmas et al. 2004a).

5.3
VP4 Is an Extra-ER Protein: Consequences for Rotavirus Assembly

A major consequence of these observations is that VP4 must assemble with the other structural proteins outside the ER. This conclusion is also strengthened by previous data indicating that although rotavirus and rotaviral proteins never transit through the Golgi apparatus (Jourdan et al. 1997; Xu et al. 2001), perturbation of Golgi trafficking using either brefeldin A (Mirazimi et al. 1996) or monensin (Jourdan et al. 1997) leads to an abnormal assembly or a mistargeting of the virus. Finally, recent elegant experiments using the siRNA strategy to inhibit VP4 expression have shown that rotavirus particles that contained all structural proteins except VP4 can be assembled and detected in the cell cytoplasm (Dector et al. 2002). Altogether these data suggest that an alternative model for rotavirus final assembly can be proposed that includes both ER and extra-ER steps (Delmas et al. 2004b) as shown in Fig. 1.

Fig. 1A–F   A new proposal for Rotavirus assembly in polarized Caco-2 cells. **A** Rotavirus enters Caco-2 cells through a ill-defined process. The external layer of the viral capsid (VP4 and VP7) is removed. Double layered particles are released and are competent to deliver viral dsRNA within the cytosol. **B** Viral protein synthesis starts on free ribosomes form most of structural and nonstructural proteins except NSP4 and VP7, which are synthesized on ribosomes associated with the rough endoplasmic reticulum. Several structural and nonstructural proteins are found in the viroplasm, an ill-defined cytoplasmic organelle. **C** Double-layered particles are assembled within the viroplasm in the vicinity of the ER. Early after infection, VP4 is found associated with the cytoskeleton. **D** During the endoplasmic reticulum assembly step, DLP enters the ER. Double-layered particles are surrounded with a transient lipid envelope. **E** VP7 is recruited on the external layer of the virus capsid. The viral particles lacking VP4 exit the ER. The VP4 present in the cytosol, mostly associated with the cytoskeleton, is assembled on the viral particles. **F** Virus exits the cells through a mechanism that requires lipid rafts and the cytoskeleton
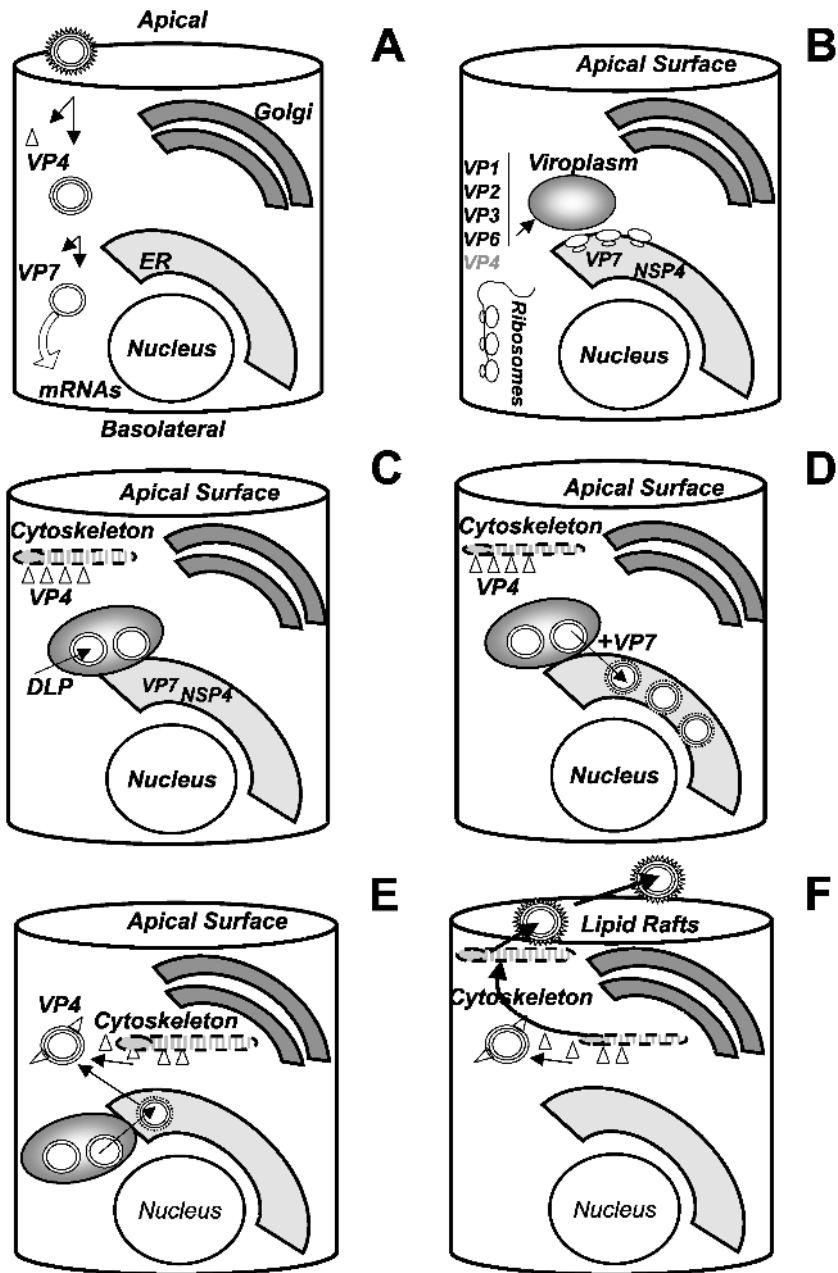
6
Unanswered Questions

6.1
How Does VP4, a Cytosolic Protein, Associate with Rafts?

As mentioned above, VP4 is synthesized on free cytosolic ribosomes, but it has been shown that this protein never displays a cytosolic pattern but rather localizes on subcellular, still unidentified structures (Petrie et al. 1982; Gonzalez et al. 2000). One of the major questions is how this protein is recruited on cell membranes. Several plasma membrane proteins have already been suggested as partners, such as the cognate heat shock protein Hsc70 (Zarate et al. 2003), which mostly localizes at the cell surface, but not on intracellular membranes. Analysis of the primary structure of VP4 (Bremont et al. 1992) provided some additional clues to this issue. Several VP4 strains display a putative caveolin-1 scaffolding-binding motif within residues 289–296 (Couet et al. 1997) that may account for interaction with lipid microdomains. However, it is important to note that Caco-2 cells do not express caveolin (Mirre et al. 1996). An integrin L-binding domain has been described and recently shown to be correctly exposed at the protein surface and to play a role in rotavirus interactions (Dormitzer et al. 2004). However, it is expected that integrins will be associated with the external leaflet of membrane bilayers within cells and thus cannot be the first partner for VP4, which is produced within the cytosol. Rotavirus VP4 protein also presents a conserved coiled-coil domain, able to mediate protein–protein interactions. The functionality of this domain has not been demonstrated and site-directed mutagenesis experiments have to be carried out in order to confirm that this domain plays a role in VP4–membrane interactions. A peroxisomal targeting signal has been predicted at the C-terminal part of VP4, and a recent paper has suggested that VP4 may interact with peroxisomes (Mohan et al. 2002). However, we were unable to confirm a peroxisomal localization of rotavirus particles and of VP4, suggesting that this signal may be not functional. We have already mentioned that a fusion domain has been described and that this domain does not seem to be involved in VP4 interactions with membrane microdomains. The identification of a galectin-like domain on the VP8 part of VP4 suggests that glycans may represent an interesting membrane target for VP4 (Dormitzer et al. 2002). However, this would suggest highly specific interactions of VP4 with sugars, which does not seem likely if one considers the large variation in sugar interactions of several rotavirus strains (Delorme et al. 2002). Cytoskeletal proteins may also be an interesting tool to mediate VP4 interactions with membrane components. It is well established that rotavirus infection induces early cytoskeleton changes (Brunet

et al. 2000a, 2000b; Obert et al. 2000). Recently, the N-terminal part of VP4 (VP8*) has been shown to directly interact with tight junctions (Nava et al. 2004). Transfected VP4 was detected on cytoskeletal elements in Cos 7 cells (Nejmeddine et al. 2000). Further studies are in progress to analyze whether these cytoskeletal components are instrumental to promoting VP4 interactions with cell membranes and microdomains (Gardet et al., unpublished data).

6.2
In Which Subcellular Compartment Does VP4 Associate with DLPs?

Our current model proposes that VP4 does not assemble with the viral particles within the ER but that this assembly takes place within rafts. It must be definitively demonstrated that ER does not participate in this final step, since it cannot be fully ruled out that an atypical subcompartment of the ER is involved. Recent data seem to indicate that some particular rafts may form in the ER vicinity (Sarnatero et al. 2004). Electron microscopy and cryo-electron microscopy studies will be required to answer these questions. We favor an alternative hypothesis that involves the presence of microdomains within the membrane of an intracellular organelle that is not the Golgi apparatus or the ER. Using two-hybrid and co-immunoprecipitation strategies, it was recently found that VP4 may interact with rab5 and PRA1, two proteins associated with the endosomal system (Enouf et al. 2003). Whether these interactions are instrumental for rotavirus assembly must be further explored. Other intracellular compartments have not received enough attention, for example, autophagosomes or exosomes that have been shown to participate in the assembly of other viruses (Prentice et al. 2004; Nguyen et al. 2003). Finally, it will be interesting to analyze the fine composition of the raft subset that specifically associates with VP4, since this composition may provide insights into their origin. Indeed, it is now recognized that there are several subtypes of cholesterol–sphingolipid-enriched membrane microdomains that may be characterized by their differential solubility in various detergents (Schuck et al. 2003). It has also been shown that some raft subsets are resistant to cholesterol removal by methyl β cyclodextrin, suggesting that they display a different molecular organization. This is particularly true for rafts extracted from the apical membrane of intestinal cells (Danielsen and Hansen 2003). Preliminary results from our laboratory indicate that VP4 containing rafts are also resistant to methyl β cyclodextrin (Delmas et al., unpublished data).

6.3

**Is There a Cellular Route Between the ER and the Plasma Membrane
That Bypasses the Golgi Apparatus and That Can Be Used by Endogenous Proteins?**

As mentioned above, rotavirus and its main structural proteins behave very differently in the final stages of morphogenesis in comparison to other viruses and do not seem to follow a classical exocytic route, although the virus is specifically delivered to the apical pole of intestinal cells (Jourdan et al. 1997). Indeed, some intermediate molecules are present within the ER and others are detected within various intracellular organelles, except the Golgi apparatus. It should be pointed out that most of the proteins of this virus are cytosolic and have no specific signals to enter the exocytic pathway. These proteins are synthesized on free ribosomes and directly released within the cytosol. Little is known on the mechanisms that control their sorting and targeting. In a recent review, Walter Nickel summarized the data on what is called the nonclassical protein secretion, a pathway that bypasses ER and Golgi compartments (Nickel 2003). Four mechanisms have been suggested for this atypical plasma membrane targeting: (1) a re-entry from the cytosol into the endosomal compartment (used for example by interleukin 1β); (2) the use of specific transporters at the cell surface (used by fibroblast growth factors 1 and 2); (3) a translocation at the membrane that probably needs a flip-flop mechanism (used by the *Leishmania* cell surface protein HASBP); and (4) exosomes that form through a membrane blebbing process (probably involved in galectin secretion). At least three such proteins that use the nonclassical protein secretion have recently been described as also being associated with rafts. One is the above-mentioned galectin family, a group of endogenous lectins that have been shown to reach the apical cell membrane through their association with particular DRMs, i.e., rafts (Braccia et al 2003; Hansen et al. 2001). The second one is annexin II, which has been proposed to be secreted through a hemi-fusion process (Danielsen et al. 2003; Faure et al. 2002). The last example is Hsp70, which has recently been shown in our laboratory to be targeted to the plasma membrane of intestinal Caco-2 cells and released in the extracellular medium through specific association with rafts, a process that is greatly increased when cells experienced heat shock (Broquet et al. 2003). Whether rotavirus and/or rotaviral proteins use one of these nonclassical protein secretion pathways remains to be demonstrated.

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