Genome-wide RNAi screen reveals a role for the ESCRT complex in rotavirus cell entry

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Rotavirus (RV) is the major cause of childhood gastroenteritis worldwide. This study presents a functional genome-scale analysis of cellular proteins and pathways relevant for RV infection using RNAi. Among the 522 proteins selected in the screen for their ability to affect viral infectivity, an enriched group that participates in endocytic processes was identified. Within these proteins, subunits of the vacuolar ATPase, small GTPases, actinin 4, and, of special interest, components of the endosomal sorting complex required for transport (ESCRT) machinery were found. Here we provide evidence for a role of the ESCRT complex in the entry of simian and human RV strains in both monkey and human epithelial cells. In addition, the ESCRT-associated ATPase VPS4A and phospholipid lysobisphosphatidic acid, both crucial for the formation of intralumenal vesicles in multivesicular bodies, were also found to be required for cell entry. Interestingly, it seems that regardless of the molecules that rhesus RV and human RV strains use for cell-surface attachment and the distinct endocytic pathway used, all these viruses converge in early endosomes and use multivesicular bodies for cell entry. Furthermore, the small GTPases RHOA and CDC42, which regulate different types of clathrin-independent endocytosis, as well as early endosomal antigen 1 (EEA1), were found to be involved in this process. This work reports the direct involvement of the ESCRT machinery in the life cycle of a nonenveloped virus and highlights the complex mechanism that these viruses use to enter cells. It also illustrates the efficiency of high-throughput RNAi screenings as genetic tools for comprehensively studying the interaction between viruses and their host cells.

Rotaviruses (RVs), members of the family Reoviridae, are the leading etiologic agents of viral gastroenteritis in infants and young children worldwide, being responsible for an estimated 453,000 deaths each year (1). The infectious particle is composed of three concentric layers of protein that enclose the viral genome formed by 11 segments of double-stranded RNA. The proteins of the outermost layer, VP4 and VP7, are involved in virus attachment and cell entry. Two domains constitute the spike protein VP4: VP5 at the base of the spike and VP8 at the head. Once inside the cell, the triple-layered particle (TLP) loses the surface proteins, leading to a double-layered particle (DLP) that is transcriptionally active. The nascent viral mRNAs can be used either for viral protein synthesis or for genome replication. Newly formed progeny DLPs assemble in cytoplasmic inclusions known as viroplasms and bud into the lumen of the ER. The outer-layer proteins then assemble on DLPs in this compartment (2). It has been recently reported, however, that RV hijacks the autophagy membrane-trafficking pathway to transport the ER-associated viral proteins required for infectious particle assembly to membranes surrounding viroplasms (3).

Even though specific steps of entry have been increasingly well characterized in recent years, the involvement of host-cell proteins in the replication life cycle of the virus has been poorly characterized. The initial interactions of the virus with the cell surface involve several molecules. Specifically, some RV strains such as rhesus RV (RRV), initially bind to sialic acid on the cell surface through the VP8 domain of the spike protein VP4, but some RVs appear to attach to subterminal sialic acid, such as that present in ganglioside GM1 (4); in addition, it was recently described that the VP8 protein of human RV strain HAL1166 and the human RV strains belonging to the most frequent VP4 genotypes (P4 and P8) bind to A-type histo-blood group antigens (5, 6). Integrin 2β1 has also been reported to serve as an attachment receptor for some RV strains (7), although this integrin, as well as integrins vβ3 and xβ2 and the heat-shock protein 70 (HSC70), have been implicated mostly in a postattachment interaction of the virus that might be involved in cell internalization (7). Nevertheless, RV strains whose infectivity does not depend on integrins have also been reported. RRV enters cells by an endocytic pathway that is independent of clathrin and caveolin, whereas other RV strains have been shown to enter cells through a clathrin-dependent endocytosis (8). It has also been reported that RV cell entry depends on dynamin and cholesterol (8), although contradicting results were recently reported in Madin Darby canine kidney cells (9). RRV infection of monkey kidney MA104 cells has been shown to depend on the small GTPase RAB5, but not on RAB4 or RAB7 (10). The interaction of the RV spike protein VP4 with surface receptors determines the endocytic pathway used by RVs to enter cells (11). This protein has also been proposed to undergo structural changes during the entry process (9, 12); nonetheless, a functional correlation of the proposed structural changes with cellular factors that trigger these changes is not known.

Recently, several studies have reported the use of genome-wide RNAi screens to unravel virus–host cell interactions (13). We recently developed a robust high-throughput screening assay to assess RV replication in cell culture (14). In this study we report a genome-wide siRNA screen that allowed us to identify more than 500 proteins and several biological processes potentially involved in various steps of the RV life cycle. Of particular interest, the endosomal sorting complex required for transport (ESCRT) complex, a major pathway for the lysosomal degradation of monoubiquitinated membrane proteins (15) and also involved in the abscission step of cytokinesis and in the budding process of several enveloped viruses, was found to be involved in RV cell entry. This report describes the use of the ESCRT complex by a nonenveloped virus and highlights the complex mechanism that RVs use to enter cells.

Results

Identification of Cellular Proteins Required for RV Infection Using a Genome-Wide siRNA Screen. The assay involved RRV infection of MA104 (*Cercopithecus aethiops*) cells previously transfected with an siRNA library that targets 21,181 individual human genes, followed by evaluation of virus replication by an optimized immunofluorescent detection of viral protein synthesis (14). The

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screening was performed in two steps (Fig. S1*A* and *Materials and Methods*). Using this experimental design, 522 genes whose expression is required for RV infection were identified (Table S1). Analysis of the data by functional clustering showed that the genes that scored as positives are implicated in a broad array of cellular functions and also suggested several statistically enriched biological pathways that could be involved in the virus life cycle (Table S2). Among the pathways identified are several that have been reported to be relevant for, and in some cases highly regulated during, RV infection, including tight junction (TJ) function (16), endocytosis (8, 10), and calcium signaling (17), as well as the protein-ubiquitination pathway (18, 19).

The functional protein clusters initially identified with the Ingenuity Systems software were enriched with several protein-interaction databases. We initially characterized three cellular processes: the ubiquitin-proteasome pathway, the TJ network, and the endocytosis-related protein system. Regarding the proteasome-ubiquitin components, in silico proteomics showed a strong cluster in our data set: E3 ligase hits, deubiquitinase PAN2, and heat-shock proteins. Components of the 26S proteasome were also present in these hits (Fig. S1*B* and Table S2). These findings are supported by the recent demonstration of the involvement of the proteasome-ubiquitin pathway in the life cycle of RVs (18, 19).

Fifteen proteins belonging to the TJ network were recognized by protein databases among the positive hits, including JAM-A (junctional adhesion molecule) and claudins [human F11 receptor (F11R), claudin 6 (CLDN6), CLDN14, and CLDN9) (Fig. S1*C* and Table S2). Several cytoplasmic proteins involved in the assembly and maintenance of TJs were also present in our data set (PARD6A, CNKSR3, MYL9, MYH1, and MYH8), suggesting that TJs are important for RV infection. Protein interaction analysis predicted several proteins that might also participate in RV replication, such as occludin and ZO-1. Preliminary results substantiate the relevance of the TJ proteins for RV infection (Fig. S1*E*). TJ proteins have also been reported as receptors or coreceptors for reovirus and hepatitis C virus (20).

In agreement with the endocytic pathway reported for RRV cell entry (8, 10), 13 endocytosis-related proteins were identified in the screening (Table S2), including RAB GTPases such as RAB30, a Golgi traffic regulator; RABL2A, whose function is not well characterized; and CDGAP, the activator of GTPase CDC42. Two subunits of the endosomal vacuolar H^+-ATPase (v-ATPase; V0 subunit C and V1 subunit B1) were also detected in agreement with previous results that showed that bafilomycin, an inhibitor of the v-ATPase, affects RV infection (8, 10, 21). Coatomer complex elements (COPB1, COPA) and the GTPase ARF1—whose depletion has been associated, among other phenotypes, with defects in the function of early endosomes (EE) (22)—were tightly clustered in our data set. Of particular interest, two components of the ESCRT complex (VPS25 and VPS37D) (15) were present in the output data, and in silico proteomics predicted these and other ESCRT components to be associated with other cellular proteins identified in the screening (Fig. S1*D*).

Characterization of Endocytosis-Related Proteins in RV Cell Entry by RNAi. To determine if the selected cellular proteins were required for virus entry or during a postentry step, RRV DLPs were introduced into siRNA-transfected cells by lipofection. DLPs are noninfectious because they lack the outer protein layer; however, if introduced into the cells by lipofection, bypassing the virus entry step, they are able to start a replication cycle.

Silencing the expression of two subunits of the coatomer complex I (COPB1, COPA), and ARF1 required for its function, inhibited virus infectivity by more than 70%. These proteins, however, seem to be required at a stage downstream from the virus entry point because the number of infectious foci produced by transfecting DLPs was also reduced by the siRNA treatment (Fig. 1*A*). In contrast, the v-ATPase was shown to be involved exclusively at the virus entry step because siRNAs against the two proton pump subunits notoriously reduced virus infectivity

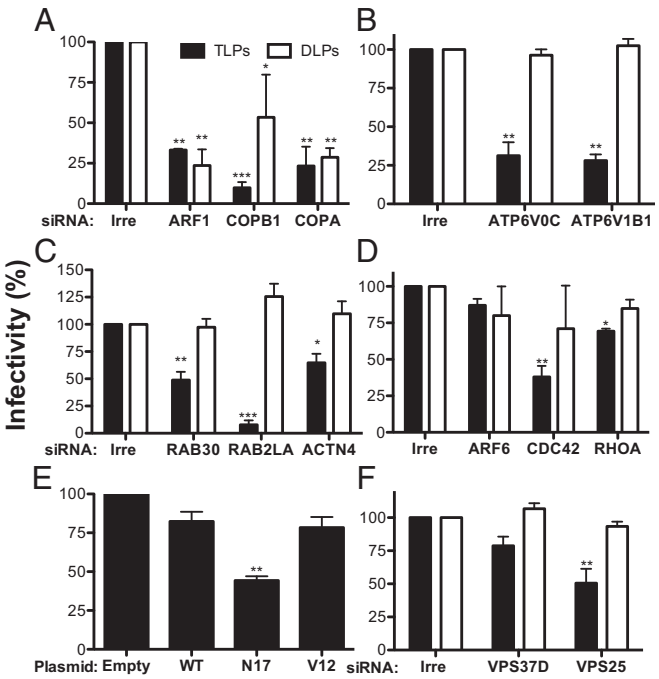

Fig. 1. Characterization of proteins involved in endocytosis and intracellular traffic pathway. (*A*, *B*, *C*, *D*, and *F*) MA104 cells were transfected with the indicated siRNAs and 72 hpt were either infected with RRV [multiplicity of infection (MOI) 3] or transfected with RRV DLPs. (*E*) MA104 cells were transfected with plasmids encoding CDC42 (WT), CDC42N17 (N17), or CDC42V12 (V12) and at 24 hpt were infected with RRV (MOI 5). At 6 hpi, cells were fixed and processed for IF using anti-NSP5 antibodies to detect infected cells, as described in *Materials and Methods*. The infectivity is expressed as a percentage of that observed in cells transfected with an irrelevant siRNA (Irre), which was taken as 100%. The relative infectivity of cells expressing CDC42 DN was obtained by normalizing against its wild-type counterpart. All experiments describing RNAi or DN results (Figs. 1, 2, 5, and 6) represent the arithmetic means and SEM of three independent experiments performed in duplicate. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

whereas they did not affect the replication of transfected DLPs (Fig. 1*B*). RAB30 and RABL2A were also required for virus entry, as well as α–actinin-4 (ACTN4), relevant for endosome maturation and vesicular trafficking (Fig. 1*C*).

We also knocked down the synthesis of three GTPases with a well-characterized regulatory function on endocytosis. Although the siRNA against RHOA (siRHOA) decreased the infectivity of RRV about 25%, the siARF6 had no effect on viral replication. Silencing the expression of CDC42 decreased RRV infectivity by about 50% (Fig. 1*D*), consistent with the findings of the screening that showed that CDGAP, the activator of CDC42, was required for RRV entry. Additionally, the dominant-negative (DN) mutant CDC42N17 caused a 50% decrease in virus infectivity, whereas the constitutively active variant CDC42V12 had no affect (Fig. 1*E*), confirming the participation of CDC42 in RV entry.

Of particular interest, the VPS25 component of the ESCRT machinery was also found to be involved in virus entry because the siVPS25 reduced the number of infected cells by about 40%. In addition, the siVPS37D consistently decreased RRV infectivity but without reaching statistical significance (Fig. 1*F*). Because the existence of VPS37 isoforms could mask the effect of silencing the expression of this protein, the role of other components of the ESCRT complex in RRV cell entry was further explored. The efficiency of RNAi on gene expression was verified by real-time quantitative RT-PCR of the target mRNA and/or by Western blot analysis of the silenced protein (Fig. S2).

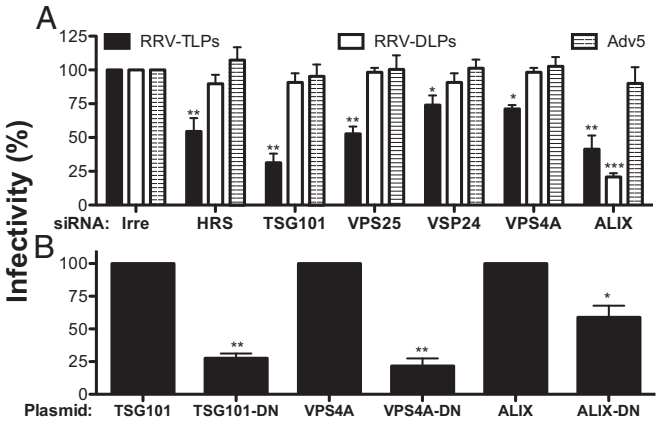

Fig. 2. ESCRT machinery is required for RV infectivity. (*A*) RNAi was carried out as described in Fig. 1, using the indicated siRNAs. Cells were infected with RRV (MOI 3), transfected with RRV DLPs, or infected with human adenovirus 5 (Adv5). (*B*) MA104 cells were transfected with the indicated plasmids and infected with RRV (MOI 5). Cells were processed for IF and analyzed as described in Fig. 1 at 6 or 20 hpi, respectively. Adv5-infected cells were detected using a mAb to the adenoviral protein DBP as described in *SI Material and Methods*. The arithmetic means and SEM of three independent experiments performed in duplicate are shown.

ESCRT Machinery Is Involved in RRV Cell Entry. The ESCRT system consists of four protein complexes (0, I, II, and III) and accessory elements that function in coordination and sequentially to form multivesicular bodies (MVB) (15). Transfection of cells with siRNAs specific for components of all four ESCRT complexes [HRS (ESCRT-0), TSG101 (ESCRT-I), VPS25 (ESCRT-II), VPS24 (ESCRT-III)], for VPS4A (the ATPase associated to ESCRT-III), and for the accessory protein ALIX impaired the infectivity of RRV in MA104 cells (Fig. 2*A*). All siRNAs inhibited virus cell entry, except siALIX, because it also decreased the infectivity of transfected DLPs. To confirm the role of TSG101, VPS4AA, and ALIX in RRV infection, DN mutants of these proteins fused to fluorescent proteins were overexpressed in MA104 cells. Expression of DN mutants TSG101 1–157, VPS4AA E228Q, and ALIX 176–869 decreased RV infectivity between 40% and 75%, compared with their wild-type counterparts (Fig. 2*B*). These results suggest that the function of the ESCRT machinery, including the ESCRT-associated ATPase VPS4A involved in fission of intralumenal vesicles (ILVs), is required for RRV cell entry. On the other hand, even though a role for ALIX in RV entry cannot be discarded, this protein seems to be rather required at a postentry step.

The possibility that interfering with the function of the ESCRT complex could prevent the transport or recycling of a RV receptor to the plasma membrane was ruled out by showing that RRV cell binding and internalization was not reduced under these conditions (Fig. S3). RRV binding and internalization was also not affected by transfection of cells with siRAB5, siRAB7, siEEA1, or si-v-ATPase (Fig. S3*A*). In addition, inhibition of the ESCRT subunits did not seem to alter the general endosomal vesicular traffic because neither the infectivity of human adenovirus 5 (Fig. 2*A* and Fig. S4*A*)—which, like certain RV strains, enters cells via a clathrin-dependent pathway (8, 11)—nor the cellular uptake of transferrin (Fig. S4*B*)—known to occur through an ESCRT-independent endocytic process—were affected by the siESCRTs. Furthermore, neither siESCRTs nor DN mutants modified the intracellular distribution of EEA1, a generic marker for EE, suggesting that the inhibition of ESCRT components (Fig. S4 *C* and *D*) does not have a general negative effect on endocytic processes or on the stability of EE compartments in MA104 cells.

Rotavirus Uncoating and Colocalization with EE and ESCRT Components. To visualize the early steps of RV cell entry, purified RRV TLPs were detected by immunofluorescence (IF) using antibodies to TLPs or mAb 159 directed to VP7 that recognizes the protein only when it is assembled into viral particles (Fig. 3*A* and Fig. S5). RRV TLPs were adsorbed for 1 h at 4 °C, and the cells were then shifted to 37 °C for the indicated periods of time. When the virus was detected with anti-TLP antibodies it showed a progressive aggregation pattern that started to be evident at 20 min postinfection (pi). The signal was maintained up to 40 min pi, and then it started to decline (Fig. S5*C*). The signal of mAb 159 showed a statistically significant decrease at 60 min compared with the 40 min time point (Fig. 3*C*), suggesting that RRV uncoats between 40 and 60 min pi.

We found that EEA1, involved in EE formation, is required for RRV cell entry and confirmed that RRV infectivity is dependent on RAB5 and independent of RAB7, as recently described (10) (Fig. S6). Using the same IF assay described above, RRV particles were found to colocalize with EEA1 starting at 15 min pi, increasing thereafter to reach a maximum at 40 min pi (Fig. 3 *B* and *D*); this colocalization was no longer detectable after 100 min of incubation, suggesting that virus particles reach the EE compartment at about 15 min pi and then become enriched at later times in the EE. These data were confirmed by

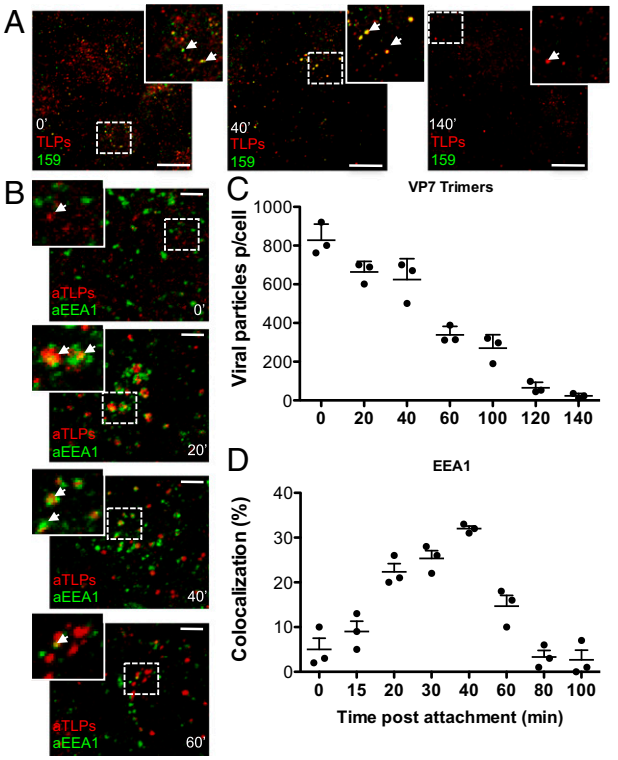

Fig. 3. Kinetics of rotavirus cell entry. (*A* and *B*) MA104 cells were incubated for 1 h at 4 °C with purified RRV TLPs (MOI of 50) and quickly shifted to 37 °C for the indicated periods of time; cells were fixed and processed for IF using anti-TLP antibodies and either (*A*) mAb 159, for detection of VP7 trimers or (*B*) a mAb to EEA1. [Scale bars: (*A*) 5 μm and (*B*) 2 μm.] (*C*) Viral particles were quantified in three different experiments by confocal microscopy. In each experiment the viruses present in five randomly selected cells per time point were scored and averaged. Viral particle counts were determined as described in *Materials and Methods*. (*D*) The level of colocalization of EEA1 and TLPs was evaluated by confocal microscopy and reported as the percentage of the total number of RRV particles per cell at the indicated time points. The colocalization events were scored and reported as described in *SI Material and Methods*.

colocalization of virus particles with RAB5, which was found at essentially the same times pi observed for EEA1 (Fig. S7).

Colocalization of RRV particles with components of the ESCRT machinery was investigated by overexpression of fluorescent fusion proteins (FP) (Fig. 4 *A* and *B*). Infected MA104 cells expressing FP-HRS showed a clear colocalization already at 15 min pi that reached a maximum at 30 min; FP-VSP4A had a maximum colocalization with the virus at 40 min pi. The FP-TSG101 and RRV particles showed a colocalization kinetics similar to those of EEA1 and RAB5. The colocalization of FP-TSG101 with RRV was not as abundant as that observed with FP-HRS and FP-VPS4A, most likely due to its low level of expression in MA104 cells.

ESCRT-Dependent Pathway Is also Required by Human RV Strains. To determine if the ESCRT complex used by RRV is also used by other RV strains, we evaluated the effect of siRNAs and DN mutants on the infectivity of human RV Wa and DS-1 in MA104 cells. Both strains enter cells by clathrin-dependent endocytosis (11). We observed a significant decrease of Wa and DS-1 infectivity in cells transfected with siHRS, siTSG101, siVPS25, and siVPS4A, as well as a clear decrease in the infectivity of Wa with DN mutants of TSG101, VPS4AA, and RAB5 (RAB5S32N) (Fig. 5 *A* and *B*). The effect of DN mutants on DS-1 infectivity could not be evaluated, given the low infectious titer of this strain combined with the low plasmid transfection efficiency (25% compared with an siRNA transfection efficiency of 90%) achieved in MA104 cells.

A more specific cellular component involved in protein and lipid sorting through the MVB is the phospholipid lysobisphosphatidic acid (LBPA) that is found in the membrane of ILVs and is crucial for ILV formation. We addressed the role of LBPA in the cell entry of RRV and Wa using a function-blocking mAb. Pretreatment of cells with the anti-LPBA antibody allows its uptake by fluid-phase endocytosis with accumulation in endosomes (23). Cells were treated with anti-LBPA antibody or an isotype (IgG1) control and subsequently infected with RRV or Wa. This treatment specifically reduced the infectivity of both RV strains by 50% and 80%, respectively (Fig. 5*C*), providing further evidence for the requirement

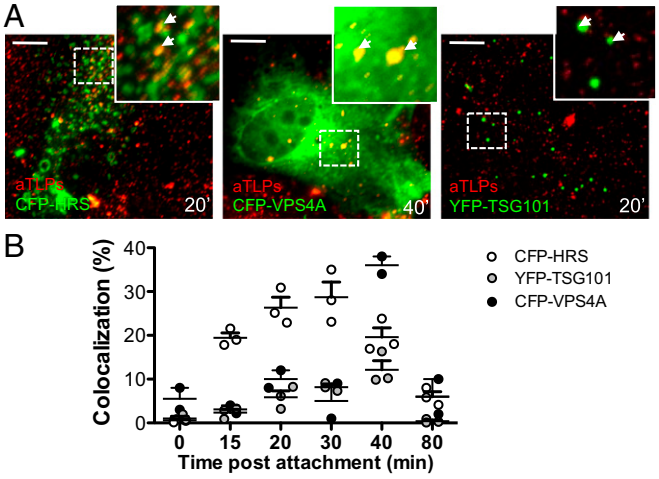

Fig. 4. RRV particles colocalize with ESCRT components. (*A*) MA104 cells transfected with plasmids encoding cyan fluorescent protein (CFP)-HRS, CFP-VPS4A, or yellow fluorescent protein (YFP)-TSG101 were incubated with RRV for 1 h at 4 °C and then at 37 °C for the indicated periods of time, fixed; processed for IF, and analyzed by confocal microscopy. RRV particles were detected with anti-TLP antibodies. (Scale bars, 5 μm.) (*B*) The percentage of colocalization of RRV particles with the ESCRT proteins is shown relative to total RRV particles detected in each cell. Quantification of viral particles and determination of the colocalization events were done as described in Fig. 3.

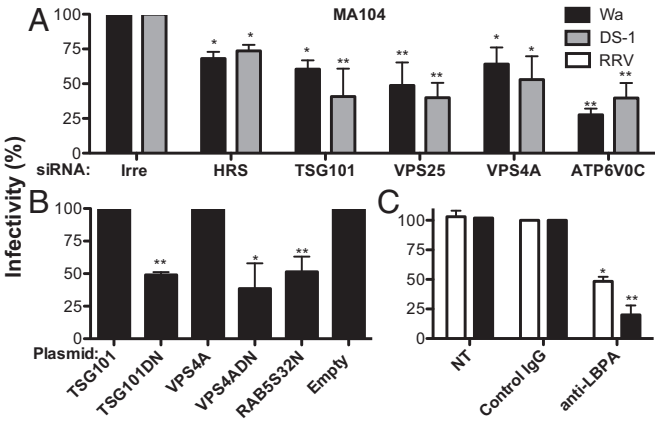

Fig. 5. ESCRT machinery and ILV formation is required for cell entry of human RV strains. (*A*) RNAi in MA104 cells was carried out as described in Fig. 1. (*B*) MA104 cells were transfected with the indicated plasmids, and at 24 hpt were infected with RRV (MOI 5). At 6 hpi, the cells were fixed and processed for IF. The relative infectivity of cells expressing ESCRT DN mutants was calculated by normalizing against their wild-type counterpart. (*C*) MA104 cells were mock-incubated (NT) or incubated with either 50 mg/mL of a mAb to LBPA or a control isotype antibody for 16 h. Cells were then incubated with RRV or Wa at 500 pfu per well for 1 h at 4 °C in the presence of mAb, the unbound virus was washed out, and the cells were transferred to 37 °C; at 6 hpi cells were fixed and processed for IF as described in Fig. 1.

of formation of ILVs for RV infection and highlighting the relevance of the ESCRT machinery in the entry process RVs.

ESCRT Machinery Is a Conserved Requirement for RV Infection of Intestinal Cells. The relevance of the ESCRT complex for RV infection of the human intestinal epithelial cell line Caco-2 was evaluated by RNAi and the use of DN mutants. As shown in Fig. 6*A*, siTSG101, siVPS25, siVPS4A, and siVPS32 inhibited the infectivity of RRV, Wa, and DS-1 to different levels, ranging from 20 to 60%. The least inhibitory effect was that found for siVPS4A and siVPS32; siVPS4A inhibited the infectivity of Wa by only about 20%, whereas siVPS32 blocked the infectivity of RRV and Wa but not that of DS-1 (Fig. 6*A*). On the other hand, the infectivity of RRV was decreased by about 40% with the DN mutants of TSG101 and VPS4A (Fig. 6*B*), indicating that the lack of or low inhibition by siRNAs in Caco-2 cells could be due to a lower transfection efficiency in these cells compared with MA104 cells.

Discussion

In this work we report an RNAi genome-wide screen carried out to recognize cellular proteins involved in the replication cycle of RVs. Several cellular proteins related to endocytic processes were identified in the screen that were characterized in more detail, including actin-related proteins, the v-ATPase, and various components of the ESCRT machinery. ACTN4, a calcium-dependent actin-interacting protein, was found among the positive hits; RHOA and CDC42, two small GTPases that have been reported to regulate clathrin-independent endocytosis of different cargoes by its actin modeling function (24), were also shown to be required for RRV infection, as well as the CDC42 activator CDGAP. Furthermore, it has been shown that RV infection leads to a structural rearrangement of actin filaments, and it was recently described that RHOA is phosphorylated at 0.5 h post-infection (hpi), suggesting its participation in the observed early rearrangements of the actin cytoskeleton (25). Additionally, direct colocalization between incoming RRV particles and the actin network was recently described (9). Altogether these results suggest that RRV might use actin network-related proteins for cell entry.

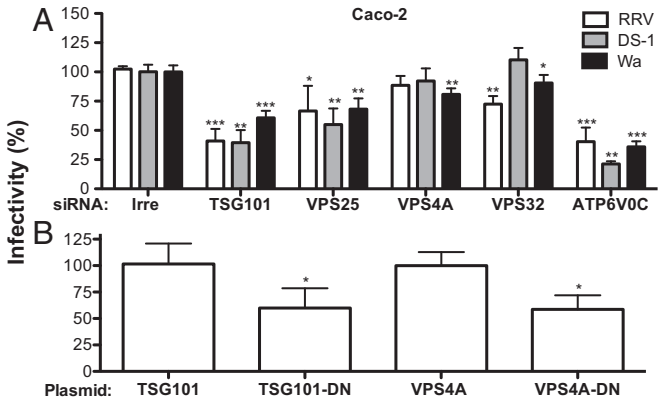

Fig. 6. Rotavirus enters human intestinal cells in an ESCRT-dependent manner. (*A*) RNAi in Caco-2 cells was carried out as described in Fig. 1 and then infected with RV strains RRV, DS-1, or Wa (MOI 1). (*B*) Caco-2 cells were transfected with the indicated plasmids and infected with RRV (MOI 5). At 6 hpi, cells were fixed and processed for IF as described in Fig. 1. The relative infectivity of cells expressing ESCRT DN mutants was calculated by normalizing against their wild-type counterpart.

A highlight of the RNAi screen was the identification of components of the ESCRT complex as important for RV infection. Here we provide evidence for a role of this cellular machinery in RV cell entry, in particular the ESCRT components HRS, TSG101, VPS25, VPS24, and VPS32. The observation that the siRNA against VPS4A and the LBPA-blocking mAb reduced RV infectivity suggests an important role for ILVs. Interestingly, in addition to RAB5, EEA1, and v-ATPase, the cell entry of the human RV strains Wa and DS-1, as well as that of RRV, appears to require the function of the ESCRT machinery and, most importantly, the formation of ILVs both in monkey kidney epithelial cells and in human intestinal cells (Fig. 7). These data strongly suggest that regardless of the cell molecules that different RVs use to bind to the cell surface, and the different endocytic pathway that they use, the vesicular traffic of the endocytosed RV particles converges in EEs. These findings also suggest that the ESCRT-dependent virus entry might be a general mechanism for RV to infect different types of cells.

Moreover, the colocalization data between RRV and ESCRT components suggest that the infecting virus transiently passes through HRS- and TSG101-positive compartments (TSG101 is known for its participation in cargo sorting through its interaction with HRS) and subsequently colocalizes strongly with VPS4A. The formation of ILVs during RV infection is most likely required in EEs or in a maturing endosomal compartment, at least for RRV, because this RV strain does not seem to reach late endosomes (LEs), as judged by its independence from RAB7 and RAB9. The infecting virus presumably enters the cytosol from ILV-containing maturing endosomes (MEs) to start transcribing the viral genome (Fig. 7). In this regard it is important to point out that, although mature MVBs are generally considered as synonymous with LEs, the formation of ILVs begins in EEs (22); the ESCRT machinery is already in place in the cytosolic surface of the EE membrane, the lumen of the vacuolar EE domains often contains several ILVs (26) and, although in minor amounts, LBPA is also present in EE (27). Few other viruses have been reported to use the ESCRT complex during cell entry. Old World arenaviruses (23), echovirus 1 (28), and vesicular stomatitis virus (23) have been suggested as undergoing sorting into ILVs of MVB mediated by the ESCRT complex during cell infection. It will be interesting to determine if this entry mode is shared with other members of the Reoviridae family.

On the other hand, ALIX, an accessory protein of the ESCRT machinery, seems to be required during cell infection at a post-entry step. In this regard, ALIX has been shown to help in the cell exit of hepatitis B virus through an ESCRT-independent mechanism and has also been suggested as a participant in autophagy events (29). This observation raises a potential role for ALIX in the autophagy hijacking mechanism recently described for RV replication (3).

One important question that remains to be answered is how the ESCRT machinery is involved in RV cell entry and why the formation of ILVs is required. We suggest two possible scenarios. In the first scenario, ILVs could be the sites where a RV receptor becomes enriched and clustered, and this clustering could be required to prime a conformational change of VP4 to induce disruption of the EE membrane. Several integrins have been proposed as RV-binding and postbinding receptors (7). These cell-surface

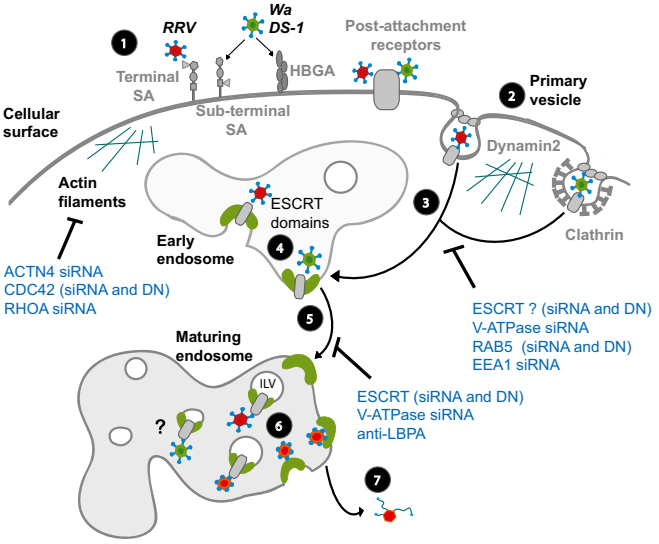

Fig. 7. Working model for rotavirus cell entry. (1) RVs attach to the cell surface through the VP8 domain of VP4. After this initial attachment, the VP5 domain of VP4, as well as VP7 of some RV strains, has been proposed to interact with integrins, and all strains seem to interact with HSC70 at lipid rafts. All known postattachment receptors, and potentially other so-far-unidentified cell molecules, are represented as a single large rectangle. (2) RRV is internalized into the cell by a clathrin- and caveolin-independent endocytic process. All other RV strains so far characterized enter cells by clathrin-dependent endocytosis. The traffic of the RRV endocytic vesicle is probably driven by the actin cytoskeleton and regulated by proteins such as ACTN4, CDC42, and RHOA. The role of these proteins has not been tested for RV strains other than RRV. (3) Regardless the endocytic pathway used, the delivery of coated and uncoated primary vesicles to EEs depends on RAB5, EEA1, and probably on HRS and the v-ATPase (34). (4) At the EE, the endocytic vesicle carrying RV as cargo interacts with components of the ESCRT machinery (specifically HRS, TSG101, VPS25, VPS24, and VPS32) and fuses with the membrane of the EE. At this stage, the virus probably begins to be internalized into the endosomal lumen through the action of VPS4A. (5) EEs progress to MEs by acquiring a lower pH and a lower intraendosomal calcium concentration through the function of the v-ATPase; during this process the formation of ILVs increases. (6) The virus-receptor complex continues to be internalized in domains rich in the phospholipid LBPA with the participation of the VPS4A ATPase; this internalization might cause a clustering of virus receptors or the inhibition/induction of signaling pathways (see text) that causes a conformational change in the spike protein that promotes its interaction with the endosomal membrane, causing a permeabilization of the membrane that leads to the exit of intraendosomal calcium (35). In nanomolar calcium concentrations, the RV particle uncoats and a further and more drastic conformational change of VP4 occurs—referred to as a fold-back structure (35)—that promotes the exit of DLPs from the MEs. (7) The cytosolic DLPs start transcribing the RV genome to continue the replication cycle of the virus. It is important to note that RV strains other than RRV might require to reach LEs, where a lower pH and lower calcium concentration exist, to uncoat and for a successful translocation of the viral DLPs into the cytosol.

proteins are known to be constitutively endocytosed, transported to endosomes, and then recycled back to the plasma membrane by different mechanisms. For integrin β1, a covalent ubiquitin modification is required for its endocytosis and lysosomal degradation orchestrated by the ESCRT complex (30). In addition, several integrins, such as β1, appear to be enriched in MBV after their internalization (28). Thus, it is possible that infectious RV particles are internalized together with integrins or with another so-far-unidentified cell molecule in a virus-receptor complex that uses the ESCRT machinery. Once this complex is enriched in the MVB compartment, the virus may interact with other nearby known or unknown molecules, integrins included, triggering RV structural changes that promote the disruption of the endosomal membrane. Interestingly, JAM-A was also identified in this work as a factor required for RV entry, suggesting that TJ proteins could also be associated with virus internalization.

In the second scenario, the requirement for the ESCRT machinery and the involvement of ILVs in RV entry could result from the need to either attenuate or activate a signaling cascade. It is known that RV activates several signal transduction pathways during cell infection. Some of these signaling events seem to be triggered during cell entry because noninfectious particles and virus-like particles promote NF-κB and STAT activation, IL8 secretion, and JNK phosphorylation (31, 32). Many cellular signaling events are governed by internalization of ligand-activated receptors by endocytosis. This internalization has classically been thought to attenuate signaling by targeting receptors for degradation in lysosomes. However, it can also retain the signals in early signaling endosomes and may even participate in the generation of signals. In these cases, signal transduction ends when the signaling complex is sequestered by ILVs into endosomes. In contrast, in the case of the canonical WNT signal transduction, it has been reported that sequestration of an enzyme from the cytosol inside ILVs of MVBs activates the signaling pathway (33). Thus, an exciting possibility is that ILV formation could be needed for RV entry to regulate a signaling event required for efficient virus replication.

In summary, this study presents a comprehensive genome-scale analysis of cellular proteins and pathways relevant for RV infection. It represents a useful resource for further studies and has the potential to provide insights into possible therapeutic targets to control rotavirus infection. These results also revealed the outstanding requirements and the complex mechanisms that RV employs to enter the cell and supports the use of an endocytic process for cell entry of nonenveloped viruses.

Materials and Methods

An RNAi library composed of 21,121 pools of four siRNAs, each targeting the whole human genome (Dharmacon siARRAY siRNA Library, Human Genome, G-005000–05, Thermo Fisher Scientific), was used. For the secondary screen, individual siRNAs used as the pool in the first screen were assayed. Reverse transfection with oligofectamine (Invitrogen) was used for delivering siRNAs into MA104 cells in triplicate in a 384-well plate format as described (14). Additional details can be found in *SI Materials and Methods*.

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