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# The actin cytoskeleton is important for rotavirus internalization and RNA genome replication

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## ABSTRACT

Numerous host factors are required for the efficient replication of rotavirus, including the activation and inactivation of several cell signaling pathways. One of the cellular structures that are reorganized during rotavirus infection is the actin cytoskeleton. In this work, we report that the dynamics of the actin microfilaments are important at different stages of the virus life cycle, specifically, during virus internalization and viral RNA synthesis at 6 h post-infection. Our results show that the actin-binding proteins alpha-actinin 4 and Diaph, as well as the Rho-family small GTPase Cdc42 are necessary for an efficient virus entry, while GTPase Rac1 is required for maximal viral RNA synthesis.

## 1. Introduction

The actin cytoskeleton is a highly dynamic structure that allows important cellular processes, such as cell division, movement, distribution of organelles, endo- and exocytosis and intercellular communication (Pollard and Cooper, 2009). Its organization mainly relies on assembly and disassembly of actin, which is driven by an extensive catalog of regulatory proteins. Rho-family GTPases are small monomeric G proteins that fluctuate between inactive and active states, depending on their binding to either GDP or GTP (Bustelo et al., 2007). Members of this family are responsible for regulating several processes of the cytoskeleton dynamics, including actin polymerization/depolymerization and formation of high-order actin structures (Blanchoin et al., 2014; Bustelo et al., 2007). Among the small GTPases, RhoA, Cdc42, and Rac1 represent the most widely distributed and best-characterized members. Rac1 and Cdc42 proteins control actin organization during the formation of cell membrane protrusions; Rac1 improves actin polymerization on the periphery of the cell surface producing lamellipodia or membrane ruffles, while Cdc42 regulates the formation of filopodia (Etienne-Manneville and Hall, 2002). RhoA promotes the formation of stress fibers and of complexes associated to focal adhesions (Hall, 1998). Furthermore, the actin filaments are elongated by other cellular proteins such as members of the formins family, as Diaph, the Wiskott-Aldrich syndrome protein (WASP) and cortactin, while proteins such as gelsolin and cofilin improve actin depolymerization

(Carlier et al., 1997; DesMarais et al., 2004; Goode and Eck, 2007; Weisswange et al., 2009). Profilin, another member of the formins family, promotes both processes, contributing to the assembly and disassembly of the actin filament, enhancing its turnover (Didry et al., 1998). Besides, the actin filaments can form networks through proteins such as Actn4 and fimbrin, which allow the formation of contractile fibers and structures such as cortical actin (Bretscher, 1981; Djinovic-Carugo et al., 1999). Not surprisingly, viruses have developed strategies to rearrange the actin cytoskeleton, especially by manipulating the Rho-protein signaling system to successfully infect cells (Taylor et al., 2011).

Rotaviruses, major etiologic agents of acute gastroenteritis in children under three years of age, are non-enveloped virus particles whose capsid is composed of three concentric layers of protein that surround the viral genome, the viral RNA-dependent RNA polymerase VP1 and the capping enzyme VP3 (Estes and Greenberg, 2013). The innermost layer is constituted by the viral protein VP2, while the intermediate layer is formed by VP6. VP7 forms the surface protein layer from which trimers of VP4 are projected forming the viral spikes. VP4 can be cleaved by trypsin into two subunits, VP5\* and VP8\*, and this cleavage is required for the virus to enter cells (Arias et al., 1996; Estes et al., 1981). Although considerable information about different stages of the rotavirus life cycle, like entry, transcription and translation, viroplasm formation, morphogenesis, genome replication, maturation and release has been obtained, the role of the actin cytoskeleton in rotavirus infection has been poorly characterized. A recent study described the

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actin-binding protein Drebrin1 as a restriction factor for rotavirus infection (Li et al., 2017), and it has been reported that changes in microfilaments occur at early (Zambrano et al., 2012) and late (Gardet et al., 2006) times of rotavirus infection; it has also been shown that rotavirus release from polarized and non-polarized cells is sensitive to the action of drugs that disrupt actin filaments (Gardet et al., 2007; Trejo-Cerro et al., 2018). However, the potential role of microfilaments in virus entry and genome replication has not been explored. Of interest, in this work, we show that the small GTPase Cdc42, as well as alpha-actinin 4 (Actn4) and Diaph, are required for rotavirus internalization in MA104 cells, and that Rac1 facilitates viral genome replication.

## 2. Material and methods

### 2.1. Cells and viruses

The rhesus monkey epithelial cell line MA104 (ATCC) was grown in Dulbecco's modified Eagle's medium (DMEM)-reduced serum (Thermo Scientific HyClone, Logan, UT) supplemented with 5% fetal bovine serum (FBS) at 37 °C in a 5% CO<sub>2</sub> atmosphere. The rhesus rotavirus strain RRV (G3[P3]) was kindly provided by H. B. Greenberg (Stanford University, USA), and was grown as described previously (Altenburg et al., 1980). Before infection, rotavirus lysates were activated with trypsin (10 µg/mL) (Gibco Life Technologies, Carlsbad, CA) for 30 min at 37 °C. RRV triple-layered (TLPs) and double-layered (DLPs) particles were purified by CsCl density centrifugation as reported previously (Pando et al., 2002).

### 2.2. Antibodies and reagents

Monoclonal antibodies to Actn4 and Rac1 were purchased from Abnova (Taipei, TW) and Cytoskeleton, Inc. (Denver, CO), respectively. Polyclonal antibodies to Diaph and Cdc42 were from Abcam (Cambridge, UK) and Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies to purified rotavirus particles (anti-TLPs) and to vimentin were produced in our laboratory. Horseradish peroxidase-conjugated goat anti-rabbit polyclonal was purchased from PerkinElmer Life Sciences (Boston, MA). Goat anti-mouse IgG coupled to Alexa 448 and goat anti-rabbit antibody coupled to Alexa 568 were from Invitrogen (Eugene, OR). The siRNAs were obtained from Dharmacon-Thermo Scientific (Pittsburg, PA).

### 2.3. Reverse lipofection of siRNAs

The indicated siRNAs were transfected into MA104 cells by a reverse lipofection method (Lopez et al., 2012). Briefly, each siRNA (1 µM) was diluted in MEM in a final volume of 15 µl and was mixed with 90 µl of MEM containing 1.35 µl of oligofectamine (Invitrogen, Carlsbad, CA). As control, an irrelevant siRNA (#Cat D-001206-13-05) was used. After 20 min of incubation at room temperature, 200 µl of a cellular suspension of 100,000 cells/ml was added to each well and incubated at 37 °C. At 24 h post transfection (hpt) the transfection mixture was removed and fresh DMEM-5% FBS was added; at 72 hpt the transfected cells were processed for analysis.

### 2.4. Cell viability

To determine the cell viability, an LDH release assay was performed using a commercial kit (Sigma, St. Louis, MO). The release of LDH was determined following the manufacturer instructions.

### 2.5. Virus infection

MA104 cells, transfected with the respective siRNA, were washed with MEM and infected with RRV (MOI = 0.025). The virus was

adsorbed for 1 h at 37 °C and subsequently the inoculum was removed, MEM was added, and the infection was left to proceed at 37 °C for 14 h. An immunoperoxidase focus-forming units (FFU) assay (Arias et al., 1987) was performed to quantify the infected cells. Briefly, infected cells were fixed with 80% acetone in PBS and viral antigen was detected with anti-TLPs polyclonal antibodies followed by a peroxidase-conjugated goat anti-rabbit polyclonal antibody, and the FFU were counted. In the case of quantification of viral RNA by RT-qPCR, siRNA-transfected cells were infected with RRV (MOI = 3) as described above. Infected cells were lysed after 6 h post-infection (hpi) and were processed as described below.

### 2.6. Lipofection of DLPs

MA104 siRNA-transfected cells were transfected with RRV DLPs (Bass et al., 1992) using Lipofectamine (Invitrogen, Carlsbad, CA). Briefly, a MEM-Lipofectamine mix was incubated with CsCl-purified DLPs (500 ng) for 20 min at room temperature; afterward, the mix was added to MA104 cells for 90 min at 37 °C, and then the cells were washed with MEM. At 14 h post-lipofection, the cells were fixed, and the infected cells were detected by an immunoperoxidase assay as described above.

### 2.7. Immunoblot analysis

Cells transfected with the indicated siRNA were lysed in Laemmli sample buffer. Proteins were denatured by boiling for 5 min, and were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Bedford, MA). The membranes were blocked with 5% nonfat dry milk in PBS and incubated with primary antibodies diluted in PBS-containing 5% nonfat dry milk, followed by incubation with secondary, species-specific, horseradish peroxidase-conjugated antibodies. The peroxidase activity was detected using the Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences, Boston, MA) according to the manufacturer's instructions. The level of those proteins whose expression was knocked down by RNA interference was analyzed by Western blot, and was normalized with respect for the levels of vimentin using the Image J software.

### 2.8. Radiolabeling of proteins

MA104 cells transfected with the siRNA to Rac1 or with an irrelevant siRNA were infected with RRV strain (MOI = 3) for 1 h at 37 °C. After this time the inoculum was removed and the monolayers were washed and incubated for 5 h. At this time cells were starved in methionine and cysteine-free medium for 30 min and were labeled for 30 min with 25 µCi/ml of a mixture of amino acids <sup>35</sup>S (PerkinElmer Life Sciences, Boston, MA). Cells were washed with MEM and lysed in Laemmli sample buffer. The samples were analyzed by SDS-PAGE as described above and treated for fluorography.

### 2.9. Binding assay

MA104 cells transfected with the respective siRNA were grown to confluence on 96-well plates, washed twice with MEM and then incubated with PBS-BSA (2%) for 1 h at room temperature. After this, the cells were incubated with CsCl-purified RRV particles (MOI = 50) for 1 h at 4 °C. Unbound virus was removed by washing the cells with cold PBS-BSA (0.5%) and then were lysed with lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 0.1% Triton X-100). The bound virus was quantitated by an ELISA as described previously (Zarate et al., 2000).

### 2.10. Virus internalization assay

MA104 cells grown in 24-well plates were transfected with the indicated siRNA. To quantify the internalized virus, cells were incubated

with purified TLPs (MOI = 50) for 1 h at 4 °C. The cells were washed to remove unbound virus and then were shifted to 37 °C for 1 h. After this period, the cell bound-virus that had not been internalized was detached from the cell surface by three washes with EGTA (3 mM). To detect the internalized fraction of the virus, the cells were lysed with TRIzol and the total RNA was extracted according to the manufacturer's instructions. cDNA was generated by reverse transcription using M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA) and qPCR was performed with Real Q Plus Master Mix Green (Ampliqon, Denmark) using an ABI Prism 7500 detection system (Applied Biosystems). The cDNA for the NSP4 gene positive strand, corresponding to the internalized virus particles, was synthesized using the reverse primer as described previously (Ayala-Breton et al., 2009). The primers used in this study were the following: 5'-3', RV-NSP4 (Fw), TCCTGGAATGGC GTATTTTC (nt 122–141; GenBank accession number L41247); RV-NSP4 (Rv), GAGCAATCTTCATGGTTGGAA (nt 191–204; GenBank accession number L41247); 18S rRNA (Fw), CGAAAGCATTGCCAAG AAT; 18S rRNA (Rv), GCATCGTTTATGGTCGGAAC. The level of NSP4 gene was normalized to that of 18S rRNA, as indicator of the input total RNA.

### 2.11. Immunofluorescence assay

RRV-infected cells grown on coverslips were infected or mock-infected with RRV (MOI = 0.5) for 6 h at 37 °C. The cells were fixed with 2% paraformaldehyde in PBS for 20 min and permeabilized by incubation with 0.5% Triton X-100 in blocking buffer (1% bovine serum albumin in PBS with 50 mM NH<sub>4</sub>Cl) for 15 min. The samples were incubated with primary and secondary antibodies for 1 h each at room temperature in blocking buffer. Nuclei were stained with 30 nM DAPI (4',6-diamidino-2-phenylindole) for 15 min. Coverslips were mounted on glass slides by use of Citifluor AF100 antifade solution (Citifluor Ltd., London, United Kingdom), and the images were acquired in a 3I Marianas spinning disk confocal microscope.

### 2.12. Statistical analysis

The statistical significance was determined using a one-way analysis of variance (ANOVA) test and a Tukey's multiple comparison post-test using GraphPad Prism 5.0 (GraphPad Software Inc.)

## 3. Results

### 3.1. Proteins involved in actin dynamics are required for rotavirus replication

In a previous study, through a genome-wide RNAi screening, our lab identified cellular proteins relevant for the rotavirus life cycle, including actin-associated proteins (Silva-Ayala et al., 2013). To characterize the relevance of Actn4, Diaph, Rac1 and Cdc42 in the lifecycle of rotavirus, the expression of these proteins in MA104 cell was knocked down by RNA interference and the replication of the simian rotavirus strain RRV in these cells was assayed. Virus infectivity was reduced by about 40–50% in cells transfected with siRNAs to Diaph, Actn4 and Cdc42 as compared to cells transfected with an irrelevant siRNA, while knocking down the expression of Rac1 reduced RRV infectivity by about 60–70% (Fig. 1A). The expression of the siRNA-responsive cellular proteins was decreased by at least 80%, as evaluated by Western blot (Fig. 1B), and the cell viability of the siRNA-transfected cells was not compromised as determined by an LDH assay (Fig. 1C). These results suggest and confirm that Actn4, Diaph, Rac1 and Cdc42 are required for maximal rotavirus infectivity.

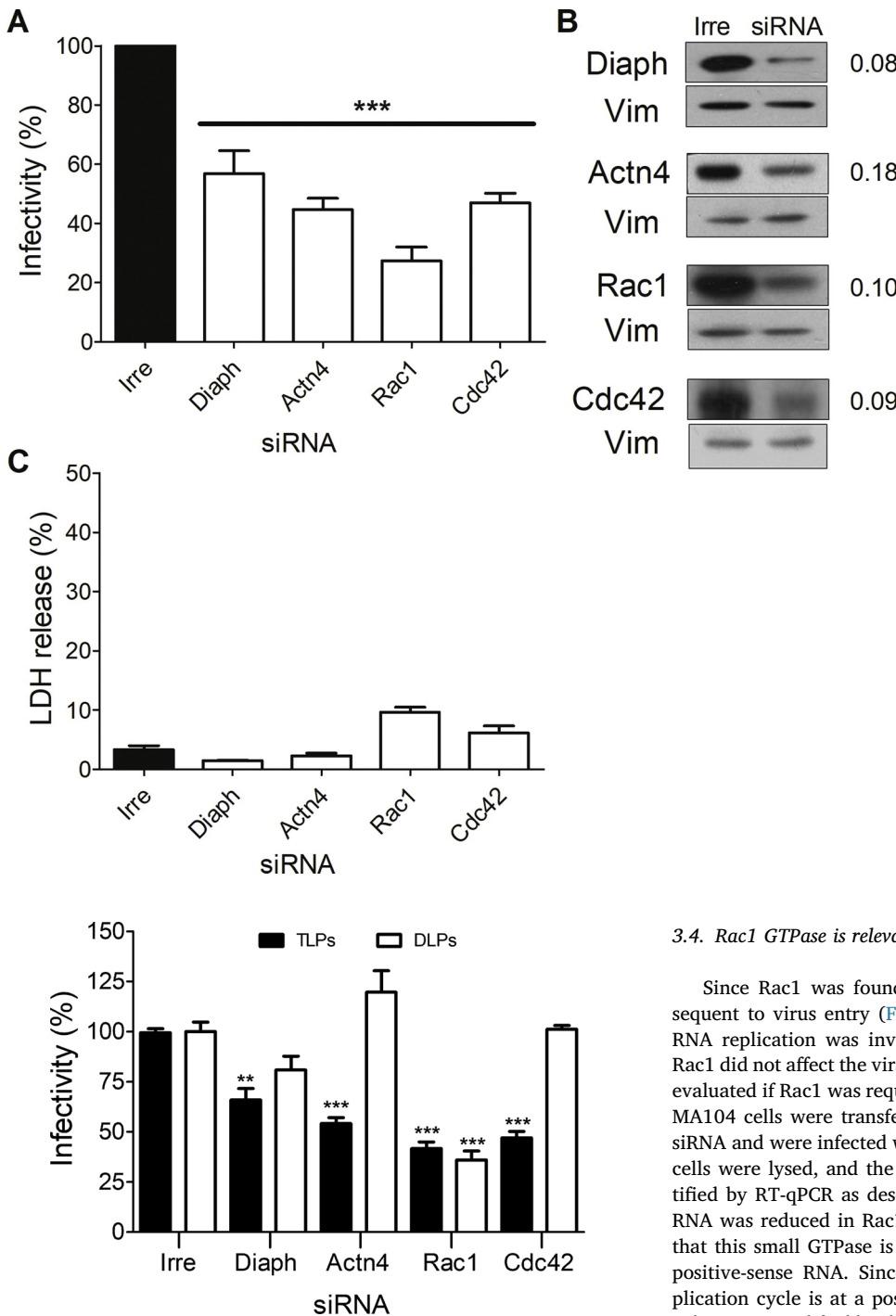
### 3.2. Characterization of the role of actin cytoskeleton regulation and binding proteins during rotavirus entry

Infectious rotavirus particles have a capsid formed by three concentric layers of proteins. However, a productive virus replication cycle can be initiated from transcriptionally active, double-layered particles (DLPs) transfected by lipofection into the cells, thus bypassing the virus entry process. To elucidate if the characterized proteins are required for virus entry, DLPs were transfected into cells where the expression of the various actin related proteins had been knocked down, and the replication of the virus was evaluated. As shown in Fig. 2, Rac1 seems to be required in a step subsequent to virus entry, since the decrease in virus infectivity was maintained when DLPs were transfected. Of interest, the infectivity of RRV was not affected when DLPs were transfected into cells treated with siRNAs to Actn4, Cdc42 or Diaph, indicating that rotavirus requires these cell proteins to efficiently enter MA104 cells, although the recovery of infectivity was less pronounced in cells where the expression of Diaph was silenced as compared to cells transfected with siRNAs to either Actn4 or Cdc42.

### 3.3. Actn4, Cdc42, and Diaph are required for internalization of the virus into cells

Rotavirus has been described to enter cells through a complex process (Lopez and Arias, 2004) that involves its attachment to a receptor on the cell surface, followed by interactions with co-receptors, cell internalization and genome uncoating. The first step involves the interaction of rotavirus with cell binding receptors (López and Arias, 2006). Actn4, Cdc42 and Diaph have mainly a cytoplasmic distribution, although Actn4 can be found at focal adhesion contacts, and Cdc42 can be localized at the plasma membrane (Aksenova et al., 2013; Ocegüera-Yanez et al., 2005). Since these three cellular proteins appear to be important for rotavirus cell entry, we determined the entry step during which they are required. For this, the ability of the virus to bind to MA104 cells transfected with an irrelevant siRNA or with siRNAs directed to either Actn4, Cdc42 or Diaph was initially tested. The ability of the virus to bind to Rac1-silenced cells was also explored in this assay. The siRNA-transfected cells were incubated with purified RRV TLPs (MOI = 50) for 1 h at 4 °C, and then the cells were washed twice to remove the free, unbound virus, and the cell-bound virus was detected by an ELISA as previously reported (Zarate et al., 2000). As shown in Fig. 3A, RRV cell surface binding was not affected by knocking down the expression of Actn4, Cdc42 or Diaph, indicating that these proteins might be required during virus entry, but at a post-binding step. Furthermore, as expected, knocking down the expression of Rac1 did not affect virus binding (Fig. 3A).

The possible role of Actn4, Cdc42 and Diaph during virus internalization (penetration of the plasma membrane by endocytosis) or uncoating (exit from the endosome) was then explored. The internalization of the viral particles was evaluated by RT-qPCR. For this, MA104 cells were transfected with the corresponding siRNA and 72 h post-transfection were incubated with purified TLPs (MOI = 50) for 1 h at 4 °C. The cells were then washed twice to remove the unbound virus, and they were then shifted to 37 °C for 1 h to allow the virus to enter cells. The virus that did not enter the cells, and was thus still bound to the cell surface, was removed by exhaustive EGTA washes and afterwards the cells were lysed and the RNA was extracted, and viral RNA was quantified by RT-qPCR. Interestingly, as shown Fig. 3B, the internalization of the virus was restricted to about 50% in cells where the expression of both Actn4 and Cdc42 was knocked down. This reduction in the amount of internalized viral RNA is similar to the reduction of infectivity observed under the same conditions (Fig. 1A), strongly suggesting that in MA104 cells Actn4 and Cdc42 are important for the virus to be internalized. The internalization data for virus in Diaph-silenced cells was not as clear-cut, since although a tendency for a reduced virus internalization was observed in these cells, the data shown



**Fig. 1.** Actin related proteins are important for rotavirus infection. A) MA104 cells were transfected with the indicated siRNA or an irrelevant (Irre) and 72 hpt were infected with RRV (MOI = 0.025). At 14 hpi cells were harvested and virus infectivity was determined as described under Material and Methods. Virus infectivity is expressed as the percentage of infected cells observed in cells transfected with an irrelevant (Irre) siRNA, which was taken as 100%. B) Western blot showing the knocked down expression of Diaph, Actn4, Rac1 and Cdc42 in MA104 cells transfected with the indicated siRNA, using vimentin (Vim) as loading control. The reported value indicates the mean fold-expression protein relative to the loading control, which was taken as 1. C) MA104 cells were transfected with the indicated siRNA and LDH release was determined as described under Material and Methods. The arithmetic means  $\pm$  standard deviations from three independent experiments performed in triplicate is shown. \*\*\*,  $P < 0.001$ .

**Fig. 2.** Identification of the cellular proteins involved in rotavirus cell entry. The expression of the actin-related proteins was silenced by siRNA transfection as described in the legend for Fig. 1, and the cells were infected with RRV TLPs (MOI = 0.025) or transfected with RRV DLPs (500 ng) as described under Material and Methods. Virus infectivity is expressed as the percentage of infected cells observed in cells transfected with an irrelevant (Irre) siRNA, which was taken as 100%. The arithmetic means  $\pm$  standard deviations from four independent experiments performed in triplicate is shown. \*\*,  $p < 0.01$ ; \*\*\*,  $p < .001$ .

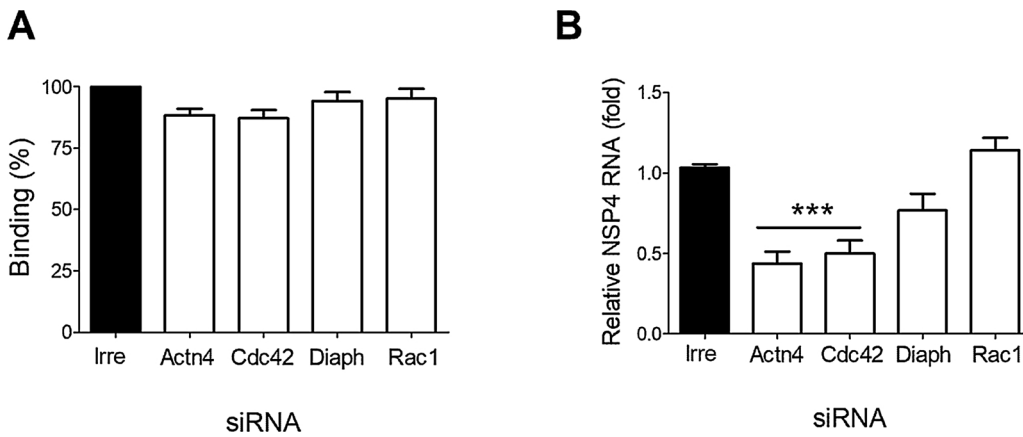
in Fig. 3B did not reach a statistically significant value, suggesting that in these cells the virus might be only partially internalized. As expected, in cells transfected with the siRNA to Rac1 the internalization of the virus was not affected, serving also as an excellent negative control for the assay.

### 3.4. Rac1 GTPase is relevant for viral RNA replication

Since Rac1 was found to be involved in a replication stage subsequent to virus entry (Fig. 2), its role in viral protein synthesis and RNA replication was investigated. Knocking down the expression of Rac1 did not affect the viral *de novo* protein synthesis (Fig. 4A); thus, we evaluated if Rac1 was required for viral RNA synthesis. To this purpose, MA104 cells were transfected with Rac1 siRNA or with an irrelevant siRNA and were infected with RRV (MOI = 3). At 6 h post-infection the cells were lysed, and the viral gene 10 positive-sense RNA was quantified by RT-qPCR as described above. As shown in Fig. 4B, the viral RNA was reduced in Rac1 silenced cells by about 70–80%, indicating that this small GTPase is necessary for maximal synthesis of rotaviral positive-sense RNA. Since the involvement of Rac1 in the virus replication cycle is at a post-entry step, we evaluated if its cellular distribution is modified by the virus infection. As can be seen in Fig. 4C, its cellular localization does not seem to be affected in virus-infected cells, what it is not surprising considering its role as a regulatory protein that probably benefits virus replication through an indirect, regulatory mechanism.

## 4. Discussion

Viruses are obligate intracellular pathogens that exploit host cellular proteins and pathways to optimize their replication and progeny production; one cellular structure that is commonly subverted by viruses is the actin cytoskeleton (Taylor et al., 2011). Since GTPases of the Rho-protein family are key during cytoskeleton organization and dynamics, it is not surprising that viruses have developed strategies to manipulate this cellular signaling system (Taylor et al., 2011).



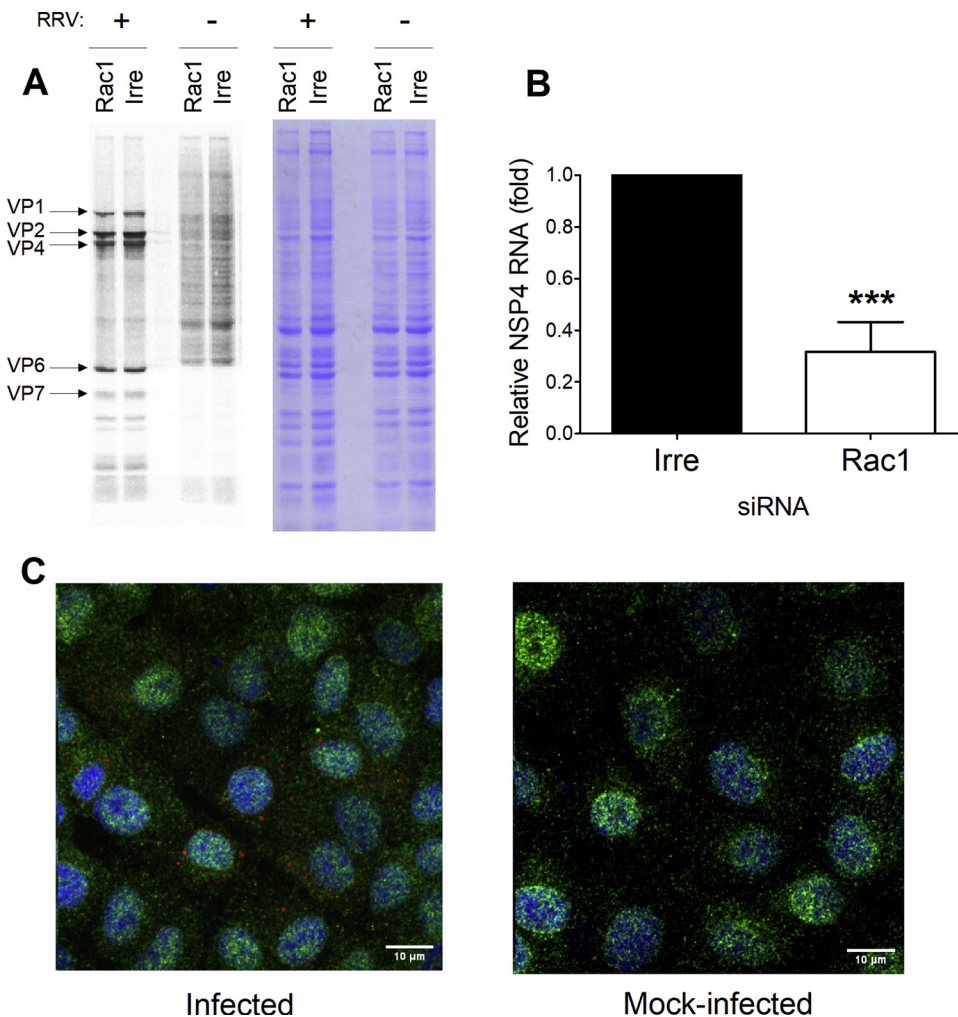
**Fig. 3.** An efficient rotavirus cell internalization is dependent on the expression of Actn4 and Cdc42. MA104 cells were transfected with the indicated siRNA as described above. A) siRNA-transfected cells were incubated with purified RRV TLPs (MOI = 50) for 1 h at 4 °C and the unbound virus was removed by several washes with PBS-BSA (0.5%). The cell-bound virus was detected by an ELISA as previously reported (Zarate et al., 2000). B) siRNA-transfected cells were incubated with purified RRV TLPs (MOI = 50) for 1 h at 4 °C. After exhaustive washes to remove the free virus, the cells were shifted to 37 °C for 1 h. Subsequently, the non-internalized cell bound-virus

was removed by EGTA washes and RNA viral was extracted and quantified by RT-qPCR as described under Material and Methods. Data are expressed as the percentage of the cell bound-virus present in cells transfected with an irrelevant (Irre) siRNA, which was taken as 100%; NSP4 level was normalized to the ribosomal RNA level. The arithmetic means ± standard deviations from four independent experiments performed in triplicate is shown. In the case of Diaph and Rac1 internalization assay (panel B) the results of two experiments performed in triplicate is shown. \*\*\*, p < .001.

In this work, we found that Cdc42 and Rac1, two important members of the Rho-family, are required for efficient rotavirus infection. Previously, we had also shown that RhoA, an additional member of the Rho-family, was important for rotavirus infectivity (Silva-Ayala et al., 2013), indicating that these three members of this GTPase family are necessary for rotavirus to efficiently infect cells. It has also been previously shown that RhoA is activated at early times of rotavirus

infection through the interaction of rotavirus VP5\* with α2β1 integrin, what induces stress fiber formation (Zambrano et al., 2012); however, whether the formation of stress fibers is relevant for rotavirus infection was not explored.

In this work, we also found that Diaph, a RhoA downstream effector belonging to the formin family, is important for rotavirus infection. The activities of formins include the formation of actin networks, cell



**Fig. 4.** Rac1 is required for maximal viral RNA synthesis. A) MA104 cells were transfected with Rac1 or with an irrelevant (Irre) siRNA and infected or not with RRV (MOI = 3). At 5 hpi the cells were incubated with methionine- cysteine-free medium and labeled for 30 min with <sup>35</sup>S. A representative fluorography is shown (left panel) and as loading control the Coomassie blue stained gel is shown (right panel) B) siRNA-transfected cells were infected as described above. After 6 hpi, infected cells were lysed and the viral RNA was extracted and quantified by RT-qPCR as described under Material and Methods. The level of NSP4 RNA was normalized to the ribosomal RNA level. The arithmetic means ± standard deviations from three independent experiments performed in triplicate is shown. \*\*\*, p < .001. C) Confluent MA104 cells grown in coverslips were infected or mock-infected with RRV at an MOI of 0.5, and at 6 hpi the cells were fixed. Rac1 was stained with a monoclonal antibody and goat anti-mouse IgG coupled to Alexa 448 (green). NSP2 was stained with a rabbit polyclonal antibody and a goat anti-rabbit antibody coupled to Alexa 568 (red). Nuclei, shown in blue, were stained with DAPI.

migration, adhesion and intracellular trafficking (Goode and Eck, 2007). Our results showed that knocking down the expression of this protein reduces rotavirus infectivity; however, this block in infectivity was overcome by transfecting DLPs, suggesting that Diaph might have a role during rotavirus entry. In this regard, Diaph has been reported to be present in endosomes where it coordinates with the Arp2/3 complex to promote the formation of the endosome actin coat (Fernandez-Borja et al., 2005). Altogether, these observations suggest the possibility that RhoA might act in concert to Diaph to help rotavirus entry during the endocytic transport.

In the case of Cdc42, we showed that this GTPase is required for rotavirus to be efficiently internalized into cells. Interestingly, it has been described that Cdc42 is important for the cell entry of other RNA viruses, such as respiratory syncytial virus (Krzyzaniak et al., 2013), coronaviruses (Swaine and Dittmar, 2015), and Ebola virus (Quinn et al., 2009). It has also been reported that Cdc42 regulates the endocytic process (Symons and Rusk, 2003), and studies with hepatitis C virus and dengue virus serotype 2 have shown that this cellular protein could facilitate the movement of the virus to interact with the viral receptor and the formation of filopodia, leading to virus entry (Brazzoli et al., 2008; Zamudio-Meza et al., 2009). Considering that rotavirus entry into cells does not seem to involve the formation of protrusions such as macropinocytosis (Gutierrez et al., 2010), a different signaling pathway, such as Cdc42-WASP-Arp2/3, could be involved in this process, especially since the WASP protein was found to also facilitate rotavirus infection (Silva-Ayala et al., unpublished results). It is known that WASP, a downstream protein effector of Cdc42, is involved in the formation of the membrane curvature that occurs during the progression of endocytosis (Tsujita et al., 2006), thus, we hypothesize that the activated Cdc42 interacts with WASP and the Arp2/3 complex, to help promoting rotavirus entry. In this regard, we have previously found that the dominant-negative mutant Cdc42N17 reduced rotavirus infectivity, while the constitutively active variant Cdc42V12 had no effect (Silva-Ayala et al., 2013).

Since RhoA and Cdc42 are important for rotavirus entry, it is possible that a common signaling pathway involving these two proteins may be at work. Of interest, among the diverse functions of the members of the Rho family, is their role in regulation and assembly of cell junctions (Citi et al., 2014). RhoA, mainly through Diaph activation and Cdc42, are essential during initial formation, maintenance and remodeling of adherens and tight junctions (Fukuhara et al., 2003; Sahai and Marshall, 2002). Since it has been reported that rotavirus requires JAM-A, occludin and ZO-1 for entry into MA104 cells (Torres-Flores et al., 2015), it is possible that Cdc42 and RhoA could improve rotavirus internalization through their influence on the tight junction establishment. A recent study by Soliman et al. supports this last hypothesis; they demonstrated that RhoA/Rock signaling is modulated by rotavirus, disrupting tight junctions integrity to enter cells (Soliman et al., 2018). Further studies are necessary, however, to determine the mechanisms involved, and how these GTPases regulate these cellular pathways.

Actn4 is a member of the spectrin superfamily that cross-links actin filaments, and connects the actin cytoskeleton to membrane and focal adhesions (Sjoblom et al., 2008). Although this protein mainly has structural and scaffold functions, it has been described as an important factor in replication and transcription of influenza A and hepatitis C viruses (Lan et al., 2003; Sharma et al., 2014). Here, we confirm the role of Actn4 in rotavirus infection and report its function during virus internalization. Interestingly, Actn4 is up-regulated by Rac1 and Cdc42 (Teramoto et al., 2003) and is associated with the GTPase Rab5 (Lanzetti et al., 2004) and dynamin (Hara et al., 2007), two cellular proteins that have been shown to be involved during rotavirus entry (Silva-Ayala et al., 2013). The localization of Actn4 in tight junctions and stress fibers has also been reported (Honda et al., 1998). Since Actn4 seems to be involved during endocytic processes, it remains to be determined if its function as host cytoskeleton regulator or its interaction with endocytosis-related molecules are necessary for rotavirus

internalization.

Rac1 is a multifunctional protein involved in several cellular processes, as cell motility, polarity, adherens junction formation and membrane ruffles induction (Etienne-Manneville and Hall, 2002). In this work we found that Rac1 is required for maximal viral RNA synthesis (Fig. 4). The cellular distribution of Rac1 was not modified in rotavirus-infected cells as compared to uninfected cells, and neither colocalization with viroplasm (Fig. 4C) nor a decrease of viroplasm number in Rac1 knocked-down cells were observed (data not shown). These data suggest that Rac1 might act through the regulation of other cellular proteins, which in turn might promote viral RNA production. Interestingly, it has been described that inhibition of JNK and p38 protein kinases decreases rotavirus replication in Caco-2 and MA104 cells (Holloway and Coulson, 2006) and, importantly, Rac1 regulates this pathway acting upstream of JNK/p38 activation (Coso et al., 1995; Turkson et al., 1999). Since our data suggest that Rac1 is required for rotavirus genome replication, it can be hypothesized that this process is facilitated through activation of this signaling pathway. On the other hand, a role for the PI3K-Akt-mTOR signaling pathway may also be envisaged, since a recent report showed that blocking this pathway inhibits rotavirus replication (Yin et al., 2018), and Rac1 acts as downstream effector of PI3K (Liu et al., 2004). In addition, since the important role of Rac1 in cytoskeleton regulation, it cannot be discarded that cellular proteins required for viral RNA production are transported to viral replication centers through a Rac1-regulated actin cytoskeleton dynamics. Since Rac1 is involved in many cellular pathways, further studies are required to elucidate which specific signaling pathway(s) are implicated in rotavirus RNA synthesis. Of interest, and other viruses, such as oncolytic Newcastle disease virus, hepatitis B virus and human immunodeficiency virus 1 have been also shown to require Rac1 to promote their replication (Lu et al., 1996; Puhlmann et al., 2010; Tan et al., 2008).

The data presented in this work demonstrate that the actin network structure and its dynamics are important for different stages of the virus life cycle. In this regard, actin has also been shown to be important for rotavirus cell exit (Trejo-Cerro et al., 2018). Understanding the detailed mechanisms through which these proteins facilitate rotavirus replication may reveal cross pathway connections and possible cellular proteins that could be targeted for antiviral developments.

#### Declarations of interest

None.

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