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Virus Research 245 (2018) 44–51

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Most rotavirus strains require the cation-independent mannose-6-phosphate receptor, sortilin-1, and cathepsins to enter cells

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ARTICLE INFO

Keywords:
Rotavirus
Virus entry
Mannose-6-phosphate receptors
Sortilin
Cathepsin

ABSTRACT

Cathepsins, endosomal acid proteases, are transported from the trans-Golgi network to late endosomes by the mannose-6-phosphate receptor (M6PR). We have previously demonstrated that some rotavirus strains, like UK, Wa, WI61, DS-1, and YM, require the cation-dependent (CD-) M6PR and cathepsins to enter from late endosomes to the cytoplasm in MA104 cells, while other strains, like the simian strain RRV, which enter cells from maturing endosomes, do not. However, the role of other trans-Golgi network-late endosome transporters, such as the cation-independent (CI-) M6PR and sortillin-1, has not been evaluated. In this work, we found that several rotavirus strains that require the CD-M6PR for cell entry are also dependent on CI-M6PR and sortilin-1. Furthermore, we showed that the infectivity of all these rotavirus strains also requires cathepsins to enter not only MA104 cells, but also human intestinal Caco-2 cells. This study identifies sortilin-1 as a novel cell factor necessary for the infectivity of a virus; in addition, our results strongly suggest that cathepsins could be common cell factors needed for the infectivity of most rotavirus strains.

1. Introduction

Rotaviruses, members of the family *Reoviridae*, are non-enveloped viruses and major etiologic agents of viral gastroenteritis in infants and young children worldwide (Parashar et al., 2003). These viruses are composed of a triple-layered protein capsid that surrounds the viral genome, the viral RNA-dependent RNA polymerase (RdRp) and the viral capping enzyme. The outermost layer is composed by the glycoprotein VP7 that forms the smooth surface of the virus, and the spike protein VP4 that functions as the virus attachment protein (López and Arias, 2006). Trypsin treatment of rotavirus is essential for virus cell entry, resulting in the specific cleavage of VP4 to yield the products VP8 and VP5 (Arias et al., 1996; Clark et al., 1981; Espejo et al., 1981; Estes et al., 1981; López et al., 1985). It has been proposed that VP4 undergoes conformational changes during the entry process leading to conversion of the mature infectious rotavirus triple-layer particles (TLPs) into transcriptionally active double-layered particles (DLPs) that start transcribing the viral genome in the cytosolic compartment (Dormitzer et al., 2004; Trask et al., 2010; Yoder et al., 2009). However, a direct functional correlation of the proposed structural changes in VP4 with cellular factors that might trigger these changes has not been directly shown.

Several molecules have been involved as cellular receptors and/or co-receptors for rotaviruses, including, sialic acid, gangliosides, integrins, and the heat shock protein hsc70 (Coulson et al., 1997; Guerrero et al., 2002; Guerrero et al., 2000; Keljo and Smith, 1988; López and Arias, 2004; Martinez et al., 2013), and more recently the VP4 of some virus strains was found to attach to human histo-blood group antigens (Hu et al., 2012; Huang et al., 2012; Liu et al., 2012). In addition, it has recently been found that the tight-junction proteins JAM-A, occludin, and ZO-1 are important for cell entry of some rotavirus strains (Torres-Flores et al., 2015). After the initial interactions of the virus with cell surface molecules, different rotavirus strains enter into MA104 cells through distinct endocytic pathways that are determined by the spike protein VP4 (Diaz-Salinas et al., 2013; Gutiérrez et al., 2010; Sánchez-San Martín et al., 2004; Wolf et al., 2012; Wolf

Abbreviations: DLPs, double layered particles; CD, cation-dependent; CI, cation-independent; CTSB, cathepsin B; CTSL1, Cathepsin L1; CTSS, Cathepsin S; EEs, early endosomes; EP, early-penetrating; GM2AP, GM2 activator protein; LDH, lactate dehydrogenase; LEs, late endosomes; LP, late-penetrating; LIMP2, lysosomal integral membrane protein 2; M6PR, mannose-6-phosphate receptor(s); MEs, maturing endosomes; ReoT1L, mammalian reovirus type 1 Lang; SORT1, Sortilin-1; TGN, trans-golgi-network; TLPs, triple-layered particles; Vim, Vimentin

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https://doi.org/10.1016/j.virusres.2017.12.002
Received 10 September 2017; Received in revised form 4 December 2017; Accepted 6 December 2017
Available online 21 December 2017
0168-1702/

Table 1
Characteristics of the rotavirus strains used in this study.

Rotavirus strain	Origin	Genotype	Cytosol entry-level organelle[a]	CD-M6PR requirement[b]	Ref.
RRV	Simian	G3P[3]	ME	No	Diaz-Salinas et al. (2014), Silva-Ayala et al. (2013), Wolf et al. (2012), Wolf et al. (2011)
UK	Bovine	G6P[5]	LE	Yes	Diaz-Salinas et al. (2014), Wolf et al. (2012)
SA11-4S	Simian	G3P[2]	EE	No	Diaz-Salinas et al. (2014)
Wa	Human	G1P[8]	LE	Yes	Diaz-Salinas et al. (2014), Silva-Ayala et al. (2013)
WI61	Human	G9P[8]	LE	Yes	Diaz-Salinas et al. (2014)
DS-1	Human	G2P[4]	LE	Yes	Diaz-Salinas et al. (2014), Silva-Ayala et al. (2013)
YM	Porcine	G11P[7]	LE	Yes	Diaz-Salinas et al. (2014)

[a] Endocytic organelle from which rotavirus enters to the cytoplasm. EE, Early Endosomes; ME, Maturing Endosomes; LE, Late Endosomes.
[b] Require the cation-dependent mannose-6-phosphate receptor (CD-M6PR) for cell entry.

et al., 2011).

Independently of the nature of the cell surface receptor and the endocytic pathway used for cell internalization, all rotavirus strains tested follow a vesicular traffic which converge in early endosomes (EEs) and reach maturing endosomes (MEs) (reviewed in reference (Arias et al., 2015)). Rotavirus strains RRV and SA11-4S presumably reach the cytoplasm from these organelles to continue their replication cycle and they are called early-penetrating (EP) viruses (Diaz-Salinas et al., 2014). However, most other rotavirus strains need to reach late endosomes (LEs) to infect MA104 cells (Table 1), and thus they have been termed late-penetrating (LP) rotaviruses (Diaz-Salinas et al., 2014).

The delivery of newly synthesized lysosomal cell factors, such as cathepsins, from the trans-Golgi-network to LEs (TGN-LEs), is a dynamic process required for obtaining mature LEs (Chia et al., 2013). This action is carried out by specialized transporters, such as the mannose-6-phosphate receptors (M6PRs). Two M6PRs have been described as part of the M6PR-dependent sorting pathways, the 46-kDa cation-dependent CD-M6PR and the 300-kDa cation-independent M6PR (CI-M6PR) (Braulke and Bonifacino, 2009). Both types of M6PRs bind lysosomal enzymes but they can also selectively transport other cargoes, such as the insulin-like growth factor II and the transforming growth factor-β1, among others (Ghosh et al., 2003). Moreover, M6PR-independent TGN-LE transporters have been reported, the most studied being the sorting pathway mediated by the receptor sortilin-1, which transports several non-enzymatic proteins such as prosaposin and the GM2 activator protein (GM2AP), as well as hydrolases like acid sphingomyelinase, and cathepsins D and H (Coutinho et al., 2012).

We have previously demonstrated that the presence of CD-M6PR is important for the cell entry of the LP rotaviruses UK, Wa, WI61, DS-1, and YM, while it is not relevant for EP rotaviruses RRV and SA11-4S (Diaz-Salinas et al., 2014). Furthermore, we also showed that the endo-lysosomal proteases cathepsin B, L1, and S, are important cell factors for the infectivity of LP but not for EP rotaviruses (Diaz-Salinas et al., 2014).

Because TGN-LE transporters have in common the trafficking of cell factors such as cathepsins (Braulke and Bonifacino, 2009; Coutinho et al., 2012), we investigated whether CI-M6PR and sortilin-1 could also have a role in rotavirus infection. Through an RNAi approach we found that the cell entry of LP rotaviruses was significantly decreased in MA104 cells transfected with siRNAs against these two transporters. We also show that the requirement of rotavirus infectivity for the activity of cathepsins is essentially a general trait for LP strains. Furthermore, we showed that acidic proteases are also important for the infection of LP rotaviruses in intestinal Caco-2 cells, suggesting that cathepsins are factors required for the infectivity of these viruses in different cell lines.

2. Material and methods

2.1. Cells and viruses

The rhesus monkey epithelial cell line MA104 and the murine fibroblasts L929 cell line (ATCC) were grown in advanced Eagle's minimal essential medium (MEM) (Invitrogen) supplemented with 5% and 10% fetal bovine serum (FBS), respectively, at 37 °C in a 5% CO2 atmosphere. The human intestinal epithelial cells (Caco-2, clone C2Bb1) were grown in high glucose DMEM supplemented with 10% FBS and non-essential amino acids. The rhesus rotavirus RRV (G3P[3]) and bovine rotavirus UK (G6P[5]) were kindly donated by Y. Hoshino (NIAID-NIH, Bethesda, MD), and have been previously described (Diaz-Salinas et al., 2013; Feng et al., 2009). Human rotavirus strains Wa (G1P[8]) and DS-1 (G2P[4]) were obtained from H. B. Greenberg (Stanford University, Stanford CA), and rotavirus WI-61 (G9P[8]) was obtained from F. H. Clark (Wistar Institute, Philadelphia PA; through M. K. Estes). Simian rotavirus SA11-4S (clone 3; G3P[2]) was obtained from M. K. Estes (Baylor College of Medicine, Houston Texas), and porcine strain YM (G11P[7]) was isolated in our laboratory (Ruíz et al., 1988). All rotavirus strains were propagated in MA104 cells as previously described (Pando et al., 2002). Rotavirus cell lysates were activated with porcine trypsin (10 μg/ml) for 30 min at 37 °C. The mammalian reovirus type 1 Lang (ReoT1L) was a kind donation of T. S. Dermody (Vanderbilt University, Nashville TN). ReoT1L was propagated in L929 cells and the viral title was determined in MA104 cells as previously described (Cuadras et al., 1997; Gutiérrez et al., 2010).

2.2. Reagents and antibodies

Porcine trypsin powder 1:250 was purchased from Gibco™ (Invitrogen Corporation) and it was dissolved in PBS to obtain a stock solution of 1 mg/ml. The small interfering RNAs (siRNA) to the luciferase gene, and the smart pool siRNAs against the CI-M6PR, sortilin-1, cathepsin B, L1, and S, were purchased from Dharmacon Research (Lafayette, CO). Rabbit polyclonal sera against cathepsin B, L1, and S were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX). Horseradish peroxidase-conjugated goat anti-rabbit polyclonal antibodies were from Perkin Elmer Life Sciences (Waltham, MA). The rabbit polyclonal sera, raised against purified rotavirus TLPs, purified reovirus particles (α-Reo), and the rabbit anti-vimentin serum against recombinant vimentin (α-Vim) were produced in our laboratory and their use has been reported (Diaz-Salinas et al., 2013; Gutiérrez et al., 2010). Cathepsin B inhibitor (CA-074) was obtained from Sigma-Aldrich Co. (St. Louis, MO), and cathepsin L inhibitor (Z-FF-FMK) and dimethyl sulfoxide (DMSO) were purchased from Calbiochem, Merck KGaA (Darmstadt, Germany). Leupeptin was obtained from Roche Diagnostics Corporation (Indianapolis, IN).

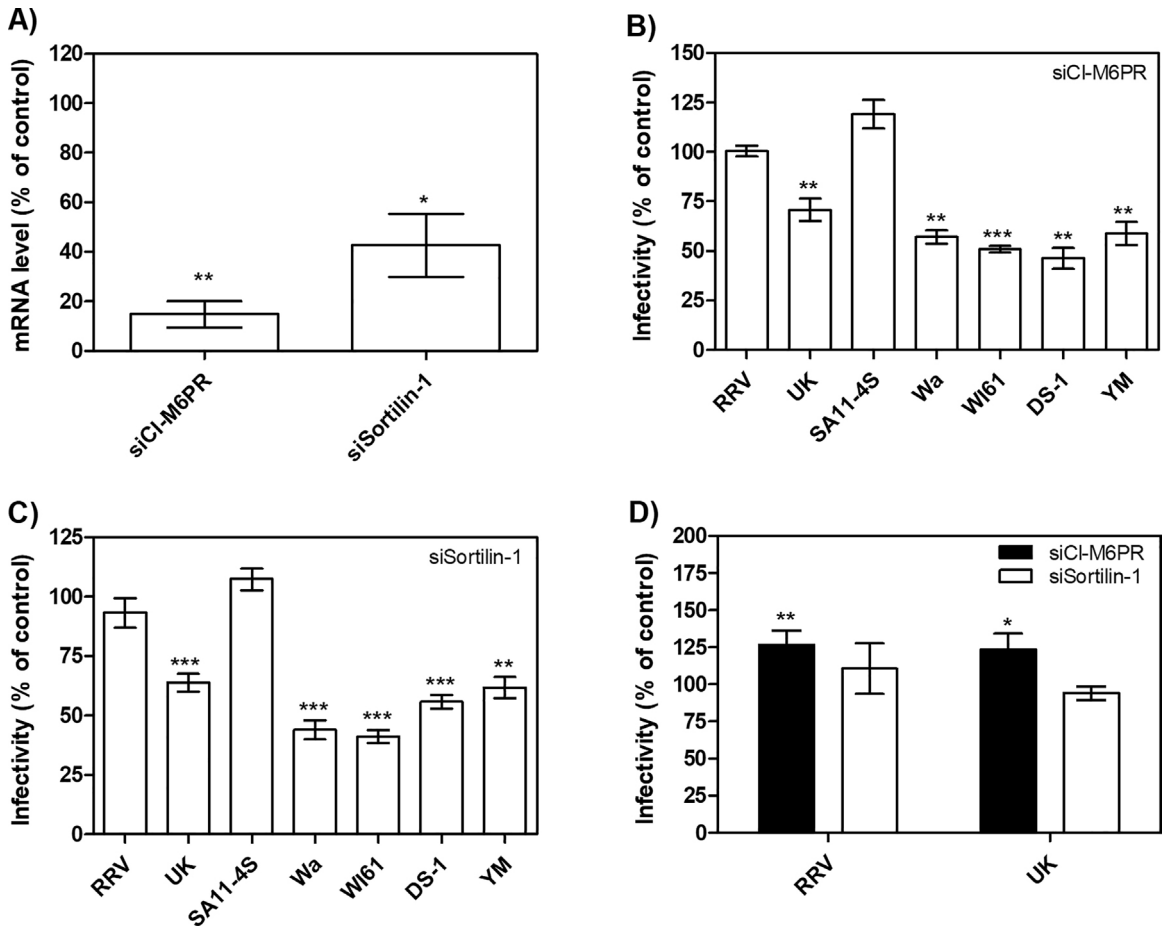

Fig. 1. Effect of silencing the expression of TGN-LE transporters on rotavirus infectivity. **(A)** MA104 cells transfected with siRNAs against CI-M6PR (siCI-M6PR), sortilin-1 (siSortilin-1), or with an irrelevant siRNA were lysed 86 h post-transfection and total RNA was purified for qRT-PCR assay as described in Material and Methods, Section 2.7. The CI-M6PR or sortilin-1 mRNA values were normalized to those of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA in cells transfected with the respective siRNA. Data are expressed as the percentage of the CI-M6PR or sortilin-1 observed in cells transfected with an irrelevant siRNA, which represents 100% of the mRNA expression. The asterisks indicate significant differences between the mRNA expressions detected in the experimental conditions (siCI-M6PR or siSortilin-1 siRNAs) compared to that observed in cells transfected with an irrelevant siRNA. *, $P < .05$; ** $P < .01$. **(B)** MA104 cells transfected with siRNAs against CI-M6PR (siCI-M6PR) or **(C)** sortilin-1 (siSortilin-1), were infected with the indicated viruses at an MOI of 0.02. At 14 hpi the cells were fixed and immunostained as described in Section 2.5. **(D)** DLPs from the indicated rotavirus strains were lipofected into MA104 cells previously transfected with siRNAs against the indicated TGN-LE transporters. At 14 hpi the cells were fixed and immunostained as described in Section 2.5. Data are expressed as the percent infectivity of each virus compared to the infection of cells transfected with an irrelevant siRNA, which represents 100% infectivity. The arithmetic means ± SEM of at least two independent experiments performed in duplicate are shown. The asterisks indicate significant differences between the infectivity of each virus in siRNA-transfected cells, compared to that observed in cells transfected with an irrelevant siRNA. *, $P < .05$; ** $P < .01$; ***, $P < .001$.

2.3. Transfection of siRNAs

siRNAs were transfected into MA104 cell by a reverse method, as described (Gutiérrez et al., 2010). 72 h post-transfection (hpt) the cells were washed twice with MEM and subsequently infected with the indicated rotavirus strain. None of these transfections was toxic for the cells, as determined by a lactate dehydrogenase (LDH) release assay, using a commercial kit (Sigma-Aldrich Co.; St. Louis, MO).

2.4. Treatment of cells with endo-lysosomal protease inhibitors

MA104 or Caco-2 cells were pre-treated with 25 μM leupeptin (in water) or with 5 μM cathepsin B or L inhibitors (in DMSO) for 1 h at 37 °C as previously described (Diaz-Salinas et al., 2014). As controls, the cells were mock-treated for leupeptin and cathepsin inhibitors. Virus inoculum at an MOI of 0.02 was adsorbed for 1 h at 37 °C and the infection was left to proceed for 14 or 16 h, as described below. The indicated protease inhibitor was maintained the entire time of infection. None of the cell treatments with protease inhibitors was toxic for the cells, as determined by the LDH release assay.

2.5. Infectivity assays

MA104 cells transfected with the indicated siRNA, or both MA104 and Caco-2 cell lines treated with the indicated endo-lysosomal proteases, were washed twice with MEM and infected with an MOI of 0.02 using cell lysates of the indicated rotavirus strain. After 1 h of adsorption, the virus was removed, and the cells were washed twice with MEM. At 14 or 16 h post-infection (hpi) for rotavirus or reovirus infection, respectively, the cell monolayers were fixed and the infected cells were stained by an immunoperoxidase focus detection assay as previously described (Pando et al., 2002).

2.6. DLPs Lipofection

MA104 cells transfected with the indicated siRNAs were lipofected with rotavirus DLPs as previously described (Gutiérrez et al., 2010). At 14 hpi the cells were fixed and the infected cells were detected by an immunoperoxidase focus detection assay as described above.

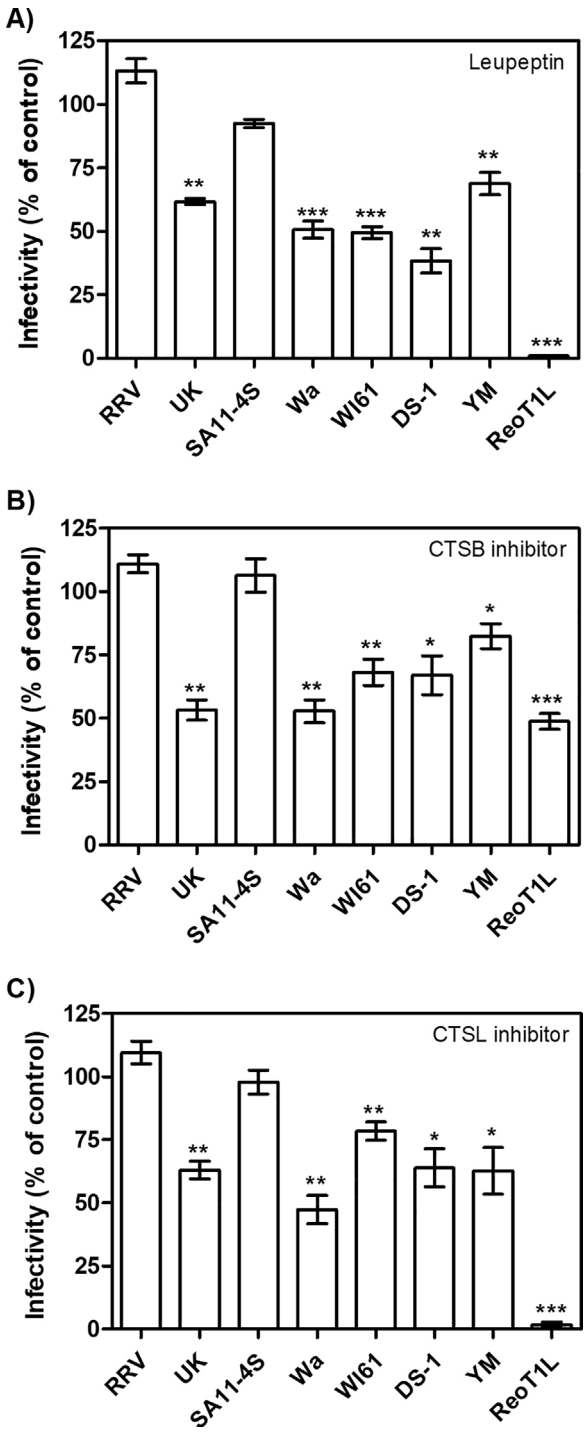

Fig. 2. Effect of cathepsin inhibitors on the infectivity of rotavirus in MA104 cells. MA104 cells were treated or mock treated with **(A)** leupeptin (25 μM), **(B)** the cathepsin B inhibitor CA-074 (5 μM) or, **(C)** the cathepsin L inhibitor Z-FF-FMK (5 μM) for 1 h at 37 °C, and then infected with the indicated viruses at an MOI of 0.02. Cells infected with the indicated rotavirus strain or reovirus type 1 (ReoT1L) were fixed and immunostained at 14 or 16 hpi, respectively, as described in Section 2.5. Data are expressed as the percent infectivity of each virus compared to the infectivity obtained in mock-treated cells. The arithmetic means ± SEM of at least two independent experiments performed in duplicate are shown. The asterisks indicate significant differences between the infectivity of each virus in inhibitor-treated cells with respect to mock-treated cells. *, $P < .05$; **, $P < .01$; ***, $P < .001$.

2.7. Immunoblots

The cells were lysed in Laemmli sample buffer and denatured by boiling for 5 min. The cell lysates were then subjected to SDS-10% PAGE and transferred to Immobilon NC (Millipore) membranes. The membranes were processed for immunoblotting as previously described (Gutiérrez et al., 2010).

2.8. qRT-PCR

The efficiency for silencing the expression of CI-M6PR and sortilin-1 by RNAi was determined by quantitative reverse transcription-PCR (qRT-PCR) as previously described (Ayala-Breton et al., 2009; Silva-Ayala et al., 2013). The forward primer 6333-F (5′-GCAGAAGCTGGG TGTCATAGG-3′) used to evaluate the CI-M6PR mRNA expression was previously described (Rose et al., 2007), while the sequence of the reverse primer was 5′-GTGCAGCTGTCGATATCAAACCTC-3′. The sequences of the forward and reverse primers used to evaluate the sortilin-1 mRNA expression levels were 5′-CTGGTCACAGTCGTAGCAGG-3′ and 5′-CAAGAGGTCCTCATCTGAGTCATC-3′, respectively. The primers to detect GAPDH mRNA have been previously described (Ayala-Breton et al., 2009).

2.9. Statistical analysis

Statistical significance was evaluated by using a two-tailed paired t test with GraphPad Prism (version 5.0) software (GraphPad Software, Inc.). P values of less than 0.05 were considered significant.

3. Results

3.1. CI-M6PR and sortilin-1 are important for the cell entry of LP rotaviruses in MA104 cells

With the aim of investigating whether the CI-M6PR and sortilin-1 are involved in the cell entry of rotaviruses, we evaluated the infectivity of EP (strains RRV and SA11-4S) and LP (strains UK, Wa, WI61, DS-1, and YM) rotaviruses (Table 1) in MA104 cells transfected with siRNAs directed to either CI-M6PR or sortilin-1 (Fig. 1). The level of sortilin-1 and CI-M6PR mRNAs was decreased by 60% and 85%, respectively, when cells were transfected with the corresponding siRNA (Fig. 1A). The cell viability, as determined by an LDH release assay, was not affected by the siRNAs treatment (data not shown).

The infectivity of RRV and SA11-4S was not affected when cells were transfected either with CI-M6PR (Fig. 1B) or sortilin-1 (Fig. 1C) siRNAs, while the infectivity of all LP rotaviruses assayed was significantly decreased, by 30–60%, as compared to cells transfected with an irrelevant siRNA used as control. To investigate if the decreased infectivity of LP rotaviruses was at the entry level, transcriptionally active double-layered particles (DLPs) of RRV or UK rotavirus strains were lipofected into MA104 cells previously transfected with siRNAs against either CI-M6PR or sortilin-1. As we and others authors have previously shown, lipofection of DLPs bypasses the virus cell entry process, since the actively transcribing particles are directly delivered in the cytoplasm (Bass et al., 1992; Diaz-Salinas et al., 2014; Gutiérrez et al., 2010; Silva-Ayala et al., 2013). As can be seen it in Fig. 1D, the infectivity of RRV and UK was not decreased by the siRNA treatment when DLPs were transfected, suggesting that CI-M6PR and sortilin-1 are cell factors needed during the cell entry of LP rotaviruses.

3.2. LP- but not EP-rotaviruses require cathepsins to enter MA104 cells

We have previously shown that the LP-rotavirus UK requires cathepsins to infect MA104 cells, while the EP-rotavirus RRV does not (Diaz-Salinas et al., 2014). This observation led us to investigate whether other LP-rotavirus strains (Wa, WI61, DS-1, and YM) need

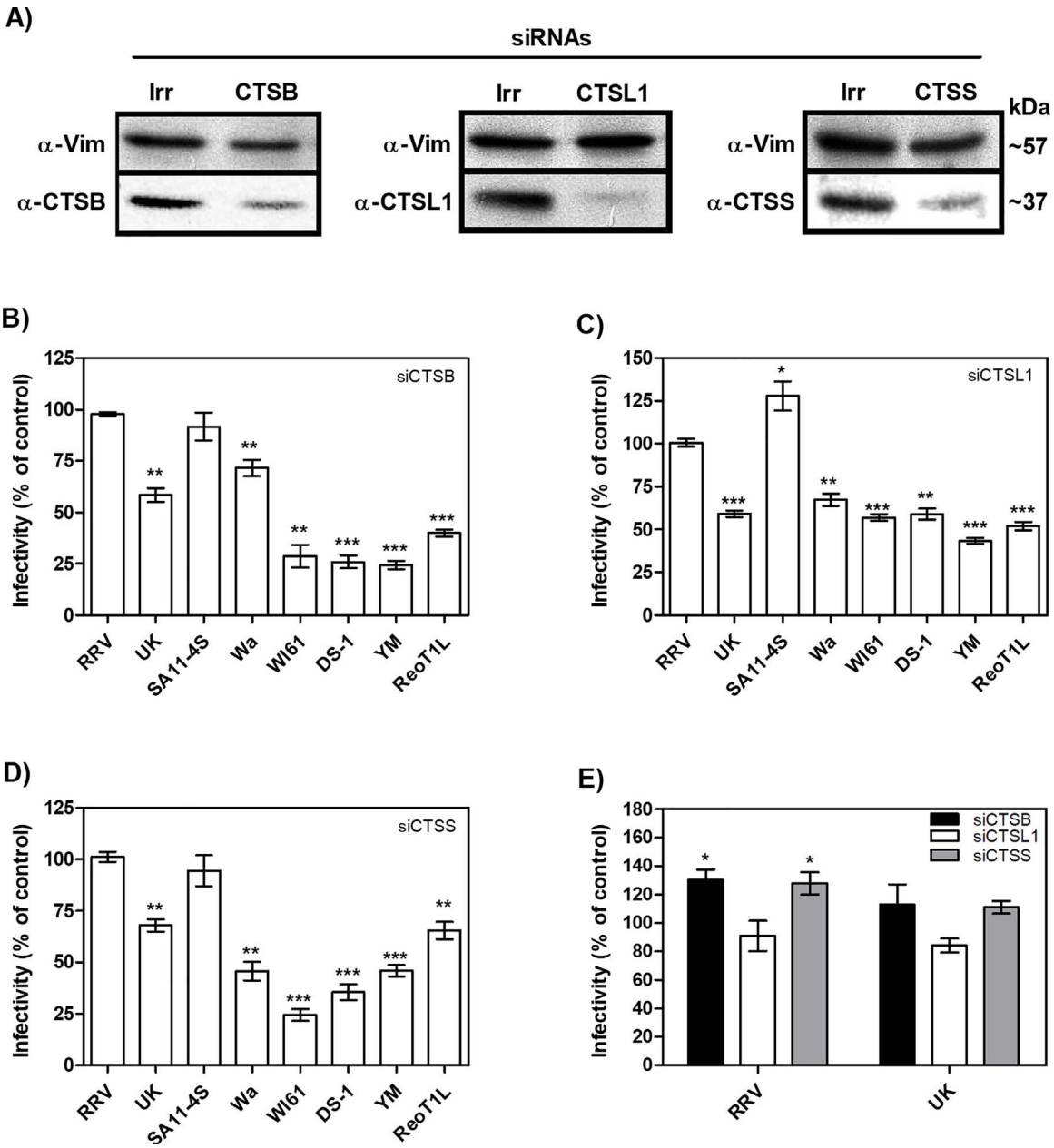

Fig. 3. Effect of silencing the expression of endosomal cathepsins on the infectivity of different rotavirus strains. **(A)** Representative immunoblots to detect the abundance of cathepsins B, L, and S in MA104 cells transfected with either irrelevant, cathepsin B, L, or S siRNAs (Irr, CTSB, CTSL1 and CTSS, respectively). Vimentin (Vim) was used as loading control. The antibodies used are indicated. MA104 cells transfected with siRNAs against cathepsin B **(B)**, cathepsin L **(C)**, or cathepsin S **(D)** were infected with the indicated viruses at an MOI of 0.02. Cells infected with the indicated rotavirus strain or reovirus type 1 (ReoT1L) were fixed and immunostained at 14 or 16 hpi, respectively, as described in Section 2.5. **(E)** DLPs from the indicated rotavirus strains were lipofected into MA104 cells that were previously transfected with siRNAs against the indicated cathepsin proteases. At 14 hpi the cells were fixed and immunostained as described in Section 2.5. Data are expressed as the percent infectivity of each virus as compared to their infectivity in cells transfected with an irrelevant siRNA. The arithmetic means ± SEM of at least two independent experiments performed in duplicate are shown. The asterisks indicate significant differences between the infectivity of each virus in control and experimental conditions. ** $P < .01$; ***, $P < .001$.

cathepsins for infection. For this, the infectivity of the indicated rotavirus strains was evaluated in cells treated with leupeptin, a broad-spectrum inhibitor of endo-lysosomal proteases, or with a specific inhibitor of cathepsin B (CA-074), or with Z-FF-FMK an inhibitor of cathepsin L (Fig. 2). Reovirus type 1 strain Lang (ReoT1L) was used as positive control, since it is well known it requires cathepsins during cell-entry (Ebert et al., 2002; Golden et al., 2004). The infectivity of this control virus, as well as that corresponding to the LP-rotavirus strains were significantly decreased in cells treated with all three inhibitors as compared to control, untreated cells (Fig. 2). Of interest, similarly to RRV, the infectivity of the EP-rotavirus SA11-4S was not affected. To confirm these observations, we silenced the expression of cathepsins B, L1, or S by RNAi (Fig. 3A) and the infectivity of the indicated viruses was evaluated (Fig. 3B–D). Although the siRNAs employed only partially silenced the expression of cathepsins, the infectivity of the control virus ReoT1 and rotavirus UK, but not that of RRV, was significantly decreased under these conditions, as compared to cells transfected with an irrelevant siRNA, as previously reported (Diaz-Salinas et al., 2014). In contrast, the infectivity of all four LP-rotavirus strains tested (Wa, WI61, DS-1, and YM) was decreased by 30–75% under these conditions (Fig. 3B–D). The infectivity of EP-rotavirus SA11-4S was not affected by the silencing of neither cathepsin B nor S (Fig. 3B and D, respectively). Interestingly, the infectivity of this rotavirus strain was increased by almost 30% when the expression of cathepsin L1 was silenced (Fig. 3C).

Next, we evaluated if transfection of DLPs will surmount the inhibition of infectivity caused by knocking down the expression of the various cathepsins. For this, we evaluated rotavirus strains RRV and UK, as representatives of EP- and LP-rotaviruses. The infectivity of UK was not significantly affected when DLPs were used (Fig. 3E), suggesting that the decreased infectivity of mature virions observed in cells treated with these siRNAs (Fig. 3B–D) was due to a blockage in the cell entry of this rotavirus strain. As expected, the infectivity of transfected RRV DLPs was not decreased; on the contrary, RRV DLP transfection in cells where the expression of either cathepsin B (Fig. 3E, RRV, black bar) or cathepsin S (Fig. 3E, RRV, gray bar) had been silenced, increased by about 30% as compared to control cells treated with an irrelevant siRNA. These results suggest that LP-rotaviruses require cathepsins during cell-entry into MA104 cells, and raise the question whether endo-lysosomal proteases could act as anti-viral factors for EP-rotaviruses, such as RRV.

3.3. LP-rotaviruses require cathepsins to infect human intestinal cells

Most research on rotavirus cell entry has been carried out in MA104 cells, which are derived from monkey kidney epithelium. Human intestinal Caco-2 cells have also been used to study some rotavirus-cell interactions (López and Arias, 2006), but the vesicular traffic during rotavirus cell entry has not been characterized in this model cell line. It is known that the infectivity of the EP-rotavirus RRV in Caco-2 cells, as well as the LP-rotaviruses Wa and DS-1, depend on several ESCRT components (Silva-Ayala et al., 2013), suggesting that these rotavirus strains follow a vesicular traffic after their internalization by endocytosis. To investigate if cathepsins are necessary for rotavirus infection of Caco-2 cells, we evaluated the role of these cysteine-proteases on the infectivity of EP-rotaviruses (RRV and SA11-4S) and LP-rotaviruses (UK, Wa and YM) using a pharmacological approach (Fig. 4). ReoT1L was also used as positive control for these assays. As expected, the infectivity of ReoT1L in cells treated with leupeptin (Fig. 4A), as well as with cathepsin B and (Fig. 4B) cathepsin L (Fig. 4C) inhibitors was decreased by about 80%, 40%, and 90% respectively. The infectivity of the EP-rotaviruses RRV and SA11-4S was not affected by treatment with either of the protease inhibitors employed (Fig. 4A–C), in contrast with LP-rotaviruses UK, Wa, and YM, the infectivity of which was decreased by 45%–65% in cells treated with the different endo-lysosomal protease inhibitors (Fig. 4). Interestingly, the infectivity of SA11-4S showed a significant increment of about 30% in cells treated with cathepsin B inhibitor (Fig. 4B). These data suggest that cathepsins are important cell factors for the infectivity of LP-rotaviruses in both MA104 and Caco-2 cells. Altogether, the data presented in this work strongly suggest that LP-rotaviruses require TGN-LE transporters and their cargoes, the cysteine-proteases cathepsins, to infect different cell lines.

4. Discussion

Independently of the nature of the cell surface receptor and the endocytic pathway used for cell internalization, all rotavirus strains evaluated so far seem to converge in EEs during cell entry (Table 1) (Arias et al., 2015). On the other hand, some rotavirus strains enter the cytoplasm from EEs, while most of them traffic to LEs before reaching the cytosol (Diaz-Salinas et al., 2014). The discovery of the need of CD-M6PR and cathepsins as important cell factors in the cell entry of the LP-rotavirus strain UK, and the close relationship between the endo-lysosomal acidic proteases and the CD-M6PR in the maintenance of a correct LE environment (Braulke and Bonifacino, 2009; Coutinho et al., 2012; Chia et al., 2013), encouraged us to investigate whether other TGN-LE transporters, such as CI-M6PR and sortilin-1 could be involved in the infectivity and cell entry of LP-rotaviruses. In this study we have demonstrated that these two transporters are indeed relevant cell factors for the entry of these rotavirus strains, in agreement with the previous observation that CD-M6PR and cathepsins are necessary for UK rotavirus infection in MA104 cells (Diaz-Salinas et al., 2014).

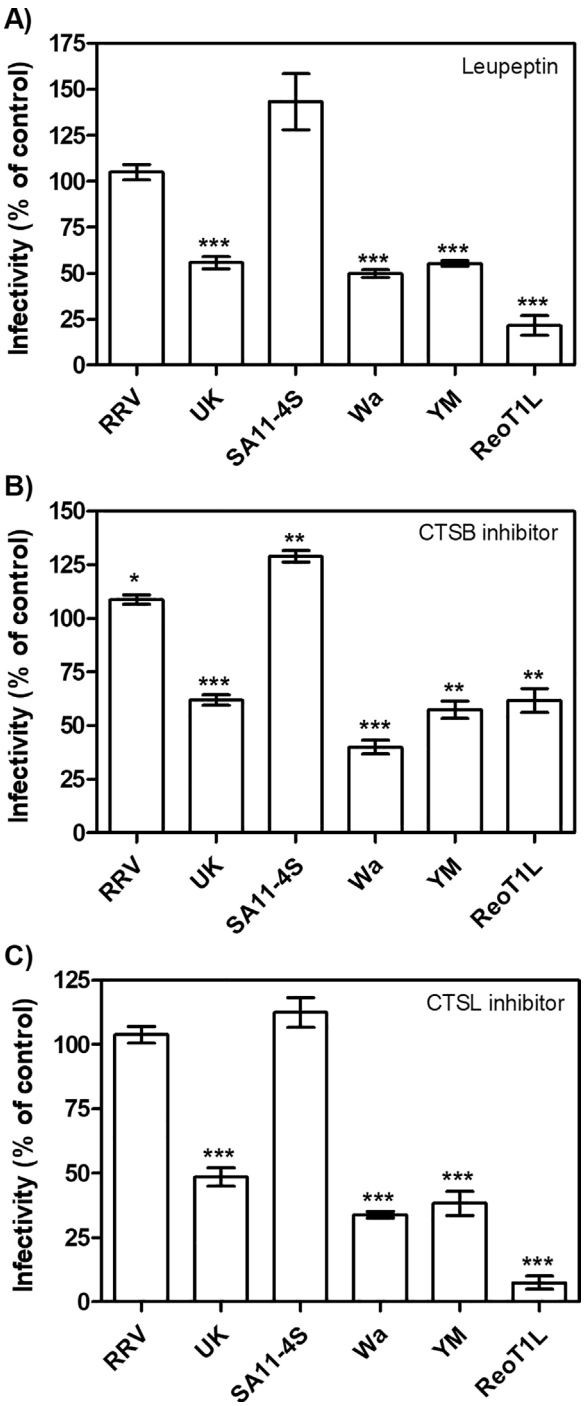

Fig. 4. Effect of protease inhibitors on the infectivity of different rotavirus strains in Caco-2 cells. Caco-2 cells were treated or mock-treated with **(A)** leupeptin, **(B)** the cathepsin B inhibitor CA-074 or, **(C)** the cathepsin L inhibitor Z-FF-FMK, and infected with the indicated viruses at an MOI of 0.02. At 14 or 16 hpi (for assays with rotaviruses or reovirus, respectively) cells were fixed and immunostained as described in Section 2.5. Data are expressed as the percent infectivity of each virus compared to the infectivity obtained in mock-treated cells. The arithmetic means ± SEM of at least two independent experiments performed in duplicate are shown. The asterisks indicate significant differences between the infectivity of each virus in inhibitor-treated cells with respect to mock-treated cells. **, $P < .01$; ***, $P < .001$.

The M6PRs have been shown to be involved in the life-cycle of some non-enveloped viruses. Both CD-M6PR and CI-M6PR have been reported to function as cell receptors during the cell-entry and cell-to-cell

spread of the alpha herpesviruses HSV-1 and HSV-2 in Vero cells (Brunetti et al., 1995), as well as in the case of Varicella-Zoster virus (Chen et al., 2004; Finnen et al., 2006; Gabel et al., 1989; Zhu et al., 1995). It has also been documented that CI-M6PR has a role in HIV-1 infection of human and murine microglia, macrophages, and non-macrophage cells (Suh et al., 2010), and that this transporter is used as cell receptor for HIV-1 to cross the cell blood-brain barrier (Dohgu et al., 2012), favoring virus spread in the central nervous system.

In the case of non-enveloped viruses, we have previously shown that all LP-rotaviruses require CD-M6PR to infect MA104 cells. In the present study, we identify CI-M6PR and sortilin-1 as novel cell factors necessary for the infectivity of most rotavirus strains. It would be interesting to investigate if, besides sortilin-1, the M6PR-independent sorting pathway conformed by the less characterized lysosomal integral membrane protein 2 (LIMP2) (Coutinho et al., 2012), could also be involved in rotavirus infection and/or in the replication of other viruses. We also report that cathepsins, the cargoes that are probably the cause for the requirement of the TGN-LE transporters, are not only needed for the cell-entry of the LP-rotavirus UK, as previously reported (Diaz-Salinas et al., 2014), but they are also required for the cell entry of LP-rotaviruses Wa, WI61, DS-1, and YM, suggesting that these proteases are a general requirement for the infectivity of these rotavirus strains. However, it remains to be determined if these acidic proteases act indirectly, or directly on the virus particle as shown for reovirus (Ebert et al., 2002; Golden et al., 2004). Considering that leupeptin treatment of MA104 and Caco-2 cells significantly decreased the infectivity of LP-rotaviruses (Figs. Figure 2A and Figure 4A), and that sortilin-1 also participates in targeting cathepsins D and H to endo-lysosomes (Canuel et al., 2008; Coutinho et al., 2012), it is possible than other proteases could be involved in the infectivity and cell entry of rotaviruses.

Interestingly, an increase in the infection of the EP-rotavirus strain SA11-4S was observed in cells transfected with siRNAs against cathepsin L1 (Fig. 3C), as well as in cells treated with the cathepsin B inhibitor (Fig. 4B), suggesting a potential role of these cellular molecules as anti-viral factors for this virus strain, and probably for other EP-rotavirus strains, at a non-identified post-entry step. This observation is supported by the increase in the infectivity of RRV DLPs lipofected in cells where the expression of either CI-M6PR (Fig. 1D, black bars), cathepsin B (Fig. 3E, black bar) or S (Fig. 3E, grey bar) was silenced. Furthermore, we also demonstrated through a pharmacological approach that, unlike EP-rotaviruses, cathepsins have a relevant role in LP-rotaviruses infection in Caco-2 cells, suggesting that these viruses could require them to get access into cells using a conserved mechanism between different cell lines. In this regard, it would be interesting to determine whether these cysteine-proteases could be required for the *in vivo* infection of LP-rotaviruses.

5. Conclusions

Altogether, the data presented in this work strongly suggest that LP-rotaviruses require TGN-LE transporters and their cargos cathepsins, to efficiently infect MA104 and Caco-2 cells. These observations open up the possibility for the evaluation of low-toxicity novel inhibitors of cathepsins to block LP-rotaviruses entry and viral infection, as it has been suggested for other cathepsin-dependent viruses like Ebola virus, and the coronaviruses agents of SARS and MERS (Plebanek et al., 2016; van der Linden et al., 2016; Zhou et al., 2016).

Acknowledgements

We thank Rafaela Espinosa for excellent technical assistance with cell culture and virus growth, and Marco Antonio Espinoza for technical assistance with the qRT-PCR assays. This work was partially supported by DGAPA/UNAM [grant IG200317] and the National Council for Science and Technology-Mexico (CONACYT) [grant 221019]. M.A.D-S. and L.A.C. were recipients of a scholarship from CONACYT.

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