

# Protein disulfide isomerase and heat shock cognate protein 70 interactions with rotavirus structural proteins using their purified recombinant versions

## Interacciones de las proteínas disulfuro isomerasa y de choque térmico Hsc70 con proteínas estructurales recombinantes purificadas de rotavirus

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DOI: 10.15446/rev.colomb.biote.v18n1.57714

### Abstract

**Introduction.** Rotavirus entry into cells seems to be mediated by sequential interactions between viral structural proteins and some cell surface molecules. However, the mechanisms by which rotavirus infects target cell are still not well understood. There is some evidence showing that rotavirus structural proteins VP5\* and VP8\* interact with some cell surface molecules. The availability of recombinant rotavirus structural proteins in sufficient quantity has become very important for the identification of the specific virus-cell receptor interactions during the early events of the infectious process.

**Objective.** The aim of the present work is to perform an analysis of the interactions between recombinant rotavirus structural proteins VP5\*, VP8\* and VP6, and cellular proteins Hsc70 and PDI using their purified recombinant versions.

**Materials and methods.** Rotavirus recombinant VP5\* and VP8\*, and cellular recombinant proteins Hsc70 and PDI were expressed in *E. coli* BL21(DE3) while VP6 was expressed in recombinant vaccinia virus-transfected MA104 cells. The interaction between rotavirus and cellular proteins was studied using ELISA, co-immunoprecipitation and SDS-PAGE/Western blotting analysis.

**Results.** The optimal conditions for expression of recombinant proteins were determined and antibodies were raised against them. The findings suggested that viral proteins rVP5\* and rVP6 interact with Hsc70 and PDI *in vitro*. These viral recombinant proteins were also found to interact with raft-associated Hsc70 in a cell culture system. The treatment of cells with either rVP6 or DLPs produced significantly inhibition of rotavirus infection.

**Conclusion.** The results allow us to conclude that rVP5\* and rVP6 interact with Hsc70 and PDI during the rotavirus infection process.

**Key words:** rotavirus; recombinant proteins; protein disulfide isomerase; heat shock cognate protein 70

### Resumen

**Introducción.** La entrada de rotavirus a las células parece estar mediado por interacciones secuenciales entre las proteínas estructurales virales y algunas moléculas de la superficie celular. Sin embargo, los mecanismos por los cuales el rotavirus infecta la célula diana aún no se comprenden bien. Existe alguna evidencia que muestra que las proteínas estructurales de rotavirus VP5\* y VP8\* interactúan con algunas moléculas de la superficie celular. La disponibilidad de las proteínas estructurales de rotavirus recombinantes en cantidad suficiente se ha convertido en un aspecto importante para la identificación de las interacciones específicas de los receptores virus-célula durante los eventos tempranos del proceso infeccioso.

**Objetivo.** El propósito del presente trabajo es realizar un análisis de las interacciones entre las proteínas estructurales de rotavirus recombinante VP5\*, VP8\* y VP6, y las proteínas celulares Hsc70 y PDI utilizando sus versiones recombinantes purificadas.

**Materiales y métodos.** Las proteínas recombinantes de rotavirus VP5\* y VP8\* y las proteínas recombinantes celulares Hsc70 y PDI se expresaron en *E. coli* BL21 (DE3), mientras que VP6 se expresó en células MA104 con virus vaccinia

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recombinante transfectada. La interacción entre el rotavirus y las proteínas celulares se estudió mediante ELISA, co-inmuno-precipitación y SDS-PAGE/ Western.

**Resultados.** Las condiciones óptimas para la expresión de proteínas recombinantes se determinaron y se generaron anticuerpos contra ellas. Los resultados sugirieron que las proteínas virales rVP5\* y rVP6 interactúan con Hsc70 y PDI *in vitro*. También se encontró que éstas proteínas virales recombinantes interactúan con Hsc70 en las balsas lipídicas ("Rafts") en un cultivo celular. El tratamiento de las células, ya sea con DLP o rVP6 produjo significativamente la inhibición de la infección por rotavirus.

**Conclusión.** Los resultados permiten concluir que rVP5\* y rVP6 interactúan con Hsc70 y PDI durante el proceso de la infección por rotavirus.

**Palabras clave:** rotavirus; proteínas recombinantes; proteína disulfuro isomerasa; proteína de choque térmico Hsc70

**Recibido:** marzo 15 de 2015

**Aprobado:** abril 15 de 2016

## Introduction

Rotavirus infection has been recognized as the leading cause of severe dehydrating diarrhea in infants and young children worldwide (Parashar *et al.*, 2006; Esposito *et al.*, 2011), being responsible for more than half a million annual deaths globally (Parashar *et al.*, 2006). Rotaviruses are non-enveloped viruses containing an 11-segmented double-stranded (ds) RNA genome enclosed by a triple-layered protein capsid (Pesavento *et al.*, 2006; Estes *et al.*, 2007). The outermost protein layer is composed by VP4 and VP7 while the intermediate layer consists of VP6. The innermost layer is built from VP2 which encloses the dsRNA segmented genome, and VP1 and VP3. VP4 requires to be cleaved into two trypsin proteolytic products, VP5\* and VP8\*, for the virus becomes infectious (Estes *et al.*, 1981; Benureau *et al.*, 2005).

Rotaviruses exhibit a remarkable tropism for polarized mature enterocytes from the small intestinal villus epithelium (Ramig 2004). However, primary small intestinal epithelial cell (IEC) cultures have been little used for rotavirus infection purposes (Kaushik *et al.*, 2008; Coulson *et al.*, 2002; Macartney *et al.*, 2000); most studies about rotavirus infection mechanism have been conducted in cultured cell systems such as MA104 cells, a non-differentiated kidney epithelial cell line (Guerrero *et al.*, 2000; Londrigan *et al.*, 2000; Halasz *et al.*, 2008). The polarized epithelial cell line Caco-2 derived from a human colon adenocarcinoma (Fogh *et al.*, 1977) has been also used as a model for studying the mechanisms by which rotaviruses affect intestinal functions (Servin *et al.*, 2003; De Marco *et al.*, 2009; Beau *et al.*, 2007). It has been shown that rotavirus can replicate in Caco-2 cells being then released without destroying them (Jourdan *et al.*, 1997).

The mechanisms by which rotavirus infects target cell are still not well understood. Rotavirus entry seems to be mediated by sequential interactions between viral structural proteins and some cell surface molecules (Lopez *et al.*, 2006). There is evidence showing that sialic acid (SA) interacts with VP8\*, the smaller trypsin cleavage product of VP4, during the initial virus binding to cell surface (Isa *et al.*, 2006; Haselhorst *et al.*,

2009). Integrin  $\alpha 2\beta 1$  has been also found to mediate binding to cells for some rotavirus strains through direct interaction with VP5\*, the larger trypsin proteolytic product of VP4 (Méndez *et al.*, 1993; Zárate *et al.*, 2000). Rotavirus interaction with other integrins such as  $\alpha\beta 2$  and  $\alpha\beta 3$  through VP7 has been demonstrated during post-binding events (Graham *et al.*, 2003; Guerrero *et al.*, 2000; Zárate *et al.*, 2004). Cell surface heat shock cognate protein 70 (Hsc70) has been shown to interact with VP5 and VP6 during rotavirus entry (Jolly *et al.*, 2001; Guerrero *et al.*, 2000; Zárate *et al.*, 2004; Gualtero *et al.*, 2007). VP5\* implication in rotavirus entry has been further extended by proposing that this viral protein could assume fusion-inducing conformational changes similar to those characterized in fusion proteins from enveloped viruses, leading to lipid bilayer perforation (Dormitzer *et al.*, 2004; Trask *et al.*, 2010). Rotavirus interaction with cell surface protein disulfide isomerase (PDI) has been suggested based on the sensitivity of rotavirus infection to DTNB [5,5-dithio-bis-(2-nitrobenzoic acid)], bacitracin, and anti-PDI antibody treatment (Wolf *et al.*, 2011). However, the identity of the rotavirus cell surface receptor and entry mechanisms continuous to be a controversial issue (Trask *et al.*, 2010; Wolf *et al.*, 2011; Ruiz, 2009; Arias, 2002).

Although the presence of Hsc70 and PDI has been documented in MA104 cells and their implication in rotavirus infection has been suggested by different strategies including the use of synthetic peptides derived from rotavirus proteins (Jolly *et al.*, 2001; Guerrero *et al.*, 2002; Zárate *et al.*, 2003; Calderon *et al.*, 2012) and antibodies against these cellular proteins (Calderon *et al.*, 2012), a study of rotavirus-cell interaction using the entire rotavirus structural proteins will further provide important insights into how rotavirus enters into the host cell. The aim of the present work is to perform an analysis of the interactions between recombinant rotavirus structural proteins VP5\*, VP8\* and VP6, and cellular proteins Hsc70 and PDI in the context of rotavirus entry. Interactions between viral proteins and cellular proteins were followed by ELISA and SDS-PAGE/ Western blotting analysis of the immunoprecipitated complexes. Our findings confirm the usefulness of re-

combinant proteins in the identification of protein interactions during virus entry into the host cell. We show that addition of recombinant VP6 to MA104 cells led to the inhibition of rotavirus infection, suggesting its participation in the virus entry process. The results also extend the knowledge about rotavirus VP5\* and VP6 interaction with the cellular proteins Hsc70 and PDI which have been found to be implicated in virus entry into the host cell.

## Materials and methods

### **Expression of recombinant VP5\*, VP8\*, Hsc70 and PDI**

Recombinant VP5\* and VP8\* fused with glutathione-transferase (GST) were expressed in *E. coli* BL21(DE3) that had been transfected with pGEX-4T-VP5\* and pGEX-4T-VP8\* (Pharmacia, Stockholm, Sweden), respectively. Plasmids were kindly donated by Dr. C. F. Arias (Instituto de Biotecnología, Universidad Nacional Autónoma de México - UNAM). VP5\* and VP8\* are encoded by RRV (G3 P5B [3]) VP4 sequences spanning nucleotides 1 to 750, and 749 to 2347, respectively (Zárate *et al.*, 2000; Isa *et al.*, 1997). Conditions of transfection, expression and purification were previously described (Zhang *et al.*, 2010). The purified VP5\*-GST and VP8\*-GST in Tris buffer (50 mM Tris-HCl, pH 8.0, 10 mM CaCl<sub>2</sub>) were treated with thrombin (CleanCleave kit, Sigma, St. Louis, MO, USA) to cleave the GST tag from the fusion protein.

Recombinant Hsc70 and PDI were expressed in *E. coli* BL21(DE3) which had been transfected with pET-28a(+)-Hsc70 or pET-28a(+)-PDI expression vector (kindly donated by Dr. C. F. Arias, Instituto de Biotecnología, UNAM, Cuernavaca, Mexico). A cDNA for human Hsc70 in plasmid pETHSC (GenBank accession No. M11717) (kindly provided by R. T. Morimoto, Northwestern University) containing the complete sequence of Hsc70 protein was subcloned into the pET28 expression vector (Novagene) to generate a fusion protein with a six-histidine tail at its COOH-terminus (Vargas *et al.*, 2006). The rat liver PDI cDNA, taken as a *TthIII-SalI* fragment from pMAL5.1, and kindly donated by Dr. R.T. Raines (Department of Chemistry, University of Wisconsin-Madison) was subcloned into the *NdeI-SalI* sites of pET28a (Novagen) (after blunting the *NdeI* site) to produce a fusion protein with a tail of six histidines at its NH<sub>2</sub>-terminus (Edman *et al.*, 1985; Laboissiere *et al.*, 1995).

BL21(DE3) competent *E. coli* cells were prepared as previously described (Chung *et al.*, 1989; Moreno *et al.*, 2013) with some modifications. An *E. coli* colony was resuspended in LB medium and grown overnight at 37 °C with orbital shaking (150 rpm). An aliquot (1 ml) of the culture was inoculated in LB medium (100 ml) to reach an OD<sub>600 nm</sub> of 0.3 - 0.4 and then centri-

fuged (12000 g) for 10 min to collect the cells which were resuspended in 5 ml of freezing medium [10 % polyethylenglicol (PEG) 3350, 5 % dimethyl sulfoxide (DMSO), 100 mM NaCl, 10 mM MgCl<sub>2</sub>, 10 mM MgSO<sub>4</sub>, and 10 % glycerol in 60 % LB medium, pH 6.7 - 7.0] and aliquots were kept on ice for 5 min before being stored in liquid nitrogen. An aliquot was mixed with 10 ng of pET-28a(+)-Hsc70 or pET-28a(+)-PDI expression vector dissolved in 100 ml of a solution containing 100 mM KCl, 30 mM CaCl<sub>2</sub> and 50 mM MgCl<sub>2</sub>. The suspension containing bacteria and plasmid DNA was gently agitated and incubated on ice for 20 min before heat shock treatment at 42 °C for 2 min. The transfected bacteria were seeded in LB-agar plates with 50 µg/ml kanamycin and incubated for 14 h at 37 °C.

A colony from bacteria transfected with pET-28a(+)-Hsc70 was inoculated in 25 ml of LB medium containing 50 µg/ml kanamycin and incubated for 12 h at 37 °C with orbital shaking (150 rpm). An aliquot (5 ml) from this culture was inoculated into 500 ml of LB medium supplement with 50 µg/ml kanamycin and 2 % glucose followed by incubation at 37 °C with orbital shaking (150 rpm) until the OD<sub>600 nm</sub> profile indicated that the exponential growth phase was reached. Isopropyl thiogalactoside (IPTG) (0.5 to 2 mM final concentration) was added to the culture at this growth phase followed by further incubation for 2, 4, 6 or 8 h. In the case of bacteria transfected with pET-28a(+)-PDI, the procedures were as those described for Hsc70 expression vector, except that 2XYT medium was used. Recombinant protein expression was assessed by SDS-PAGE/Western blotting analysis using non-transfected BL21(DE3) cells and transfected BL21(DE3) cells without IPTG treatment as a control. The total protein loaded into each gel well was spectrophotometrically quantified at 280 nm using a BSA standard curve.

### **Solubilization and purification of recombinant Hsc70 and PDI**

Solubilization of rHsc70 and rPDI from BL21(DE3) lysates was performed following the procedure reported (Frangioni *et al.*, 1993) with some modifications. Bacteria were collected by centrifugation at 8000 g for 10 min and washed twice with PBS. Cell pellets were resuspended in 50 ml PBS, treated with 100 µg/ml of lysozyme for 15 min at 4 °C with constant agitation. The suspension was made 5 mM in EDTA, 1% in sodium N-lauroylsarcosine and sonicated for 4 cycles of 15 sec at 20% amplitude before centrifugation at 12000 g for 15 min at 4 °C. Triton X-100 at 1% was added and the presence of rHsc70 and rPDI was analyzed by SDS-PAGE and Western blotting.

Recombinant proteins were purified using a 10 ml His Trap (BioRad) affinity column. The column was washed with 5 volumes of 50 mM phosphate buffer containing 300 mM NaCl and equilibrated with 5 volumes of the same buffer added with 10 mM imidazole. Cell



lysates were also added with 10 mM imidazole before applying on the column. Following washing with phosphate buffer containing 10 mM imidazole until no protein was detected with Bradford's method, recombinant proteins were eluted from the column using increasing imidazole concentrations (0.25 - 1 M) in phosphate buffer. The column was finally washed with 10 volumes of H<sub>2</sub>O and 5 volumes of 30% ethanol.

### Expression and purification of recombinant VP6

Recombinant VP6 was expressed in MA104 cells that had been infected with a recombinant vaccinia virus containing the rotavirus SA11 (G3 P5B (Esposito *et al.*, 2011) gene encoding structural VP6 (kindly donated by Dr. M. Franco, Instituto de Genética, Universidad Javeriana). Recombinant VP6 containing 397 amino acids is encoded by a VP6 sequence spanning nucleotides 1 to 1362 (Tang *et al.*, 1997). Cells were cultured in Eagle Dulbecco's modified medium (DMEM) supplemented with 2% fetal bovine serum (FBS) and 2 mM L-glutamine at 37 °C in a 5% CO<sub>2</sub> incubator. Cells at 80 - 100% confluence were washed twice with PBS and infected with the recombinant vaccinia virus present in a MA104 cell lysate for 1 h at 37 °C. The remaining cell lysate containing the virus was removed and cells were incubated in Eagle minimal essential medium (MEM) for 24 h at 37 °C.

To identify the subcellular fraction in which VP6 is located, the vaccinia virus-infected cells were frozen and thawed 3 times at - 70 °C, sonicated for 2 cycles of 15 sec at 20% amplitude and centrifuged at 15000 g for 15 min at 4 °C. The soluble fraction (SF) was kept and the insoluble fraction (IF) washed twice with Tris buffer (20 mM Tris-HCl, pH 8.0, 150 mM KCl). The IF was treated with 1% Triton X-100 and 8 M urea before SDS-PAGE/Western blotting analysis. Solubilization of VP6 from the previously Tris buffer-washed IF was attempted by resuspension in 1 ml RIPA buffer (10 mM Tris-HCl, pH 7.4, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate and 150 mM NaCl 150) and incubation for 1 h at 4 °C before centrifugation at 12000 g for 10 min at 4 °C. The precipitated fraction was treated with 500 µl of Tris buffer containing 1% N-lauroylsarcosine, CHAPS, β-octylglucoside, Triton X-100, Tween 20, Nonidet P40 or sodium deoxycholate. The solubilized pellets were incubated overnight at 4 °C and then centrifuged at 12000 g for 10 min at 4 °C. The remaining pellet was further treated with 500 µl of 8 M urea dissolved in buffer Tris (20 mM Tris-HCl, pH 8.0, 150 KCl, 1% sodium lauroyl sarcosine). Proteins in each fraction were separated by SDS-PAGE, analyzed by Western blotting and compared with samples from uninfected cells.

Purification of rVP6 was also attempted by a sequential treatment of the IF from recombinant vaccinia virus-infected cell lysates. The IF was sequentially treated with RIPA buffer followed by treatment with 1%

β-octylglucoside, 8 M urea, and 8 M urea plus 1% N-lauroylsarcosine in Tris buffer. The sequential pellets were obtained by centrifugation at 12000 g for 10 min at 4 °C. The different solubilized fractions were analyzed by SDS-PAGE/Western blotting using rabbit polyclonal antibodies against rotavirus particles. The solubilized fractions containing rVP6 were dialyzed against a decreasing gradient of urea in PBS, diluted 10 times in PBS and finally concentrated using Amicon Ultra - 4 (cut-off 10 kD; Millipore, Billerica, MA, USA).

### Production of antibodies against rVP6 and rHsc70

Purified rHsc70 or partially purified rVP6 were separated in preparative 12% SDS-PAGE. Proteins in a gel lane were transferred onto a PVDF membrane and rHsc70 or rVP6 were identified by Western blotting using rabbit polyclonal antibodies against either of these proteins, HRP-conjugated secondary goat anti-rabbit antibodies and 3-amino-9-ethylcarbazole (AEC) substrate. Gels were stained with Coomassie blue and the identified proteins bands were excised from gels and destained in methanol-acetic (50% - 12.5%) solution before being equilibrated in PBS. Gel pieces were crushed in PBS and the slurry passed several times through an 18-, 21- and then 23-gauge needle. Three month-old New Zealand rabbits were subcutaneously inoculated in the back with 750 µg of either recombinant protein emulsified in complete Freund's adjuvant. Each rabbit received 3 injections spaced at 20 days intervals. Rabbits were bled 10 days after the final immunization and sera were obtained and mixed (1:1; v/v) with glycerol before storing at - 20 °C.

The antibodies raised against recombinant proteins were characterized by ELISA, immunocytochemistry and SDS-PAGE/Western blotting. ELISA plates were coated with growing concentrations (2.5 - 20 µg/ml) of rVP6 in PBS, blocked with 1% BSA and reacted with rabbit antibodies against rVP6 diluted (1: 1000) in PBS containing 0.05% Tween 20 and 0.1% BSA. Detection was performed with HRP-conjugated goat anti-rabbit antibodies and AEC in 50 mM Na-acetate buffer, pH 5.0, and 0.04% H<sub>2</sub>O<sub>2</sub>. Immunocytochemistry analysis was carried out by infecting MA104 cell monolayers in 96-well culture plates with purified trypsin activated RRV triple layered particles (TLPs) (MOI of 0.05). Cells were incubated for 1 h at 37 °C, washed twice with MEM and further incubated in MEM for 12 h at 37 °C. Cells were fixed in ice-cold methanol for 40 min and treated with dilutions (1:250 - 1:2000) of rabbit anti-rVP6 antibodies in PBS for 1 h at 37 °C. HRP-conjugated secondary antibody and AEC substrate in 50 mM Na-acetate buffer, pH 5.0, and 0.04% H<sub>2</sub>O<sub>2</sub> were used for developing the reaction. Western blotting analysis of rabbit antibodies to rVP6 and rHsc70 was conducted using as antigen both purified recombinant proteins and cell lysates containing the recombinant proteins. Purified

RRV TLPs (500 ng/well) were also used as antigen to test anti-rVP6 antibodies. Lysates (25 – 100 µg protein/well) from non transfected cell were used as a control. Rabbit antibodies diluted (1:1000) in PBS containing 0.05% Tween 20 and 0.1% skimmed milk were added to membranes. Detection limit for anti-rVP6 serum was determined using dilutions (1:250 – 1:1000) of this serum to detect 250 ng/well of purified rVP6. HRP activity present in the secondary antibody used was visualized as described above for the immunochimistry assay.

### **Interaction between rotavirus structural proteins and cellular Hsc70 and PDI**

Rotavirus structural proteins rVP5\*, rVP8\* and rVP6 were tested for their interaction with cellular rHsc70 and rPDI. ELISA was performed by coating 96-well plates with 40 µg/ml of rHsc70 or rPDI in PBS at 4 °C overnight and blocking with 1% BSA in PBS at 37 °C for 2 h. After washing with PBS, increasing concentrations (1 – 20 µg/ml) of either rotavirus recombinant protein in the same buffer were added and incubated at 37 °C for 2 h. Wells were washed with PBS before addition of rabbit polyclonal antibodies (1:1000) against rotavirus proteins in blocking buffer and incubation at 37 °C for 2 h. Antibodies against rVP5\* y rVP8\* were produced in our animal facilities (Zhang *et al.*, 2010). Reaction was detected using HRP-conjugated goat anti-rabbit antibodies and ortho-phenylenediamine dihydrochloride (OPD) in substrate buffer (25 mM citric acid, 50 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 5.0, containing 0.03% H<sub>2</sub>O<sub>2</sub>). For negative controls, 1% BSA was used instead of antibodies or rotaviral and cellular proteins. Data were expressed as mean of two independent experiments performed with duplicate samples. The plate readings were done at 492 nm and the ΔOD values were calculated by subtracting mean values of control samples containing 1% BSA instead of antibodies or rotaviral and cellular recombinant proteins.

Co-immunoprecipitation analysis for interactions between rotavirus proteins and cellular proteins was conducted by incubating equal volumes (100 µl) of 2.5 µg/ml of rVP5\*, rVP8\* or rVP6 with 10 µg/ml of rHsc70 or rPDI in the presence of 0.1% BSA. Each mix of proteins was maintained at 4 °C overnight prior addition of rabbit polyclonal antibodies (1:200) against rotavirus proteins and incubation for 1 h at 37 °C. BSA (4%)-treated Affi-Gel protein A-agarose (10 µl) (Bio-Rad, Hercules, CA, USA) was added to each mix (200 µl) of antibody-treated proteins followed by incubation for 1 h at room temperature, according to the manufacturer's instructions. After incubation at room temperature for 1 h, the resin was washed 3 times with PBS-Tween 20 (0.05%) and collected by centrifugation at 2000 g for 10 min. Protein in the precipitated complex was analyzed by SDS-PAGE/Western blotting using primary anti-Hsc70 or anti-PDI goat polyclonal antibodies fo-

llowed by addition of HRP-conjugated rabbit anti-goat antibodies and AEC substrate. rHsc70 or rPDI were incubated with antibodies against rVP5\*, rVP8\* or rVP6 and then immunoprecipitated to be used as a control. Conversely, the mix of viral and cellular proteins was immunoprecipitated with goat polyclonal antibodies (1 µg/ml, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) against either Hsc70 or PDI and the Western blotting analysis was performed using rabbit primary antibodies against rVP5\*, rVP8\* or rVP6. These rotavirus proteins were incubated with antibodies against Hsc70 or PDI and then immunoprecipitated to be used as a control.

### **Interaction of VP5\*, VP6 or VP8\* with raft-associated Hsc70**

The interaction between rotavirus proteins and Hsc70 in lipid microdomains (rafts) was determined by mixing a MA104 cell suspension ( $9.2 \times 10^6$ ) (600 µl) with 100 µl of rVP5\* (15 µg/ml), rVP8\* (15 µg/ml) or rVP6 (1.5 µg/ml) followed by incubation for 1 h at room temperature. The cell viability was assessed using the Tripan blue exclusion test after treatment with recombinant proteins. Cells that had not been treated with rotavirus proteins were used as a control. Cells were washed twice with PBS and incubated with 120 µl of lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM CaCl<sub>2</sub>, 1 mM DTT, 1 % Triton X-100, 1 mM PMSF) during 30 min at 4 °C. The lysate was mixed (1:1; v/v) with 80% sucrose in buffer TNC (25 mM Tris-HCl, pH 7.5, 150 mM NaCl; 1 mM CaCl<sub>2</sub>) in a centrifuge tube. A discontinuous sucrose gradient (30 – 5%) in TNC was layered onto the lysate and then centrifuged (Sorvall TST 60.4 at 170000 g for 8 h at 4 °C. The gradient was fractionated and fractions were mixed with β-cyclodextrin (10 mM final concentration) and octylglucoside (0.8% final concentration) and incubated at 37 °C for 1 h. The fractions were incubated with goat polyclonal antibodies against Hsc70 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 37 °C for 1 h before addition of 30 µl Affi-Gel protein A-agarose and incubation for 30 min at room temperature. Proteins in the immunoprecipitated fractions were analyzed using Western blotting and the separated proteins detected with rabbit polyclonal antibodies against rVP5\*, rVP8\* or rVP6.

### **Infectivity inhibition assay**

The effect of rVP5\*, rVP8\* or rVP6 on rotavirus infectivity was assessed by adding either protein (1.2 – 40 µg/ml) to a MA104 cell monolayer in 96-well culture plates and incubating for 1 h at 37 °C. Following the treatment of cells with the recombinant proteins, cell viability was assessed using the Tripan blue exclusion test. RRV TLPs (50 µl) containing  $5 \times 10^4$  FFU/ml were inoculated into cells followed by incubation for 1 h at 37 °C. Cell monolayers were washed with PBS and incu-

bated in MEM for an additional 12 h at 37 °C. Infectivity was determined by fixing cells in ice-cold methanol for 40 min and incubating them with rabbit anti-RRV antibodies (1:1000) in PBS for 1 h at 37 °C. HRP-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was used at 0.133 µg/ml and developed with AEC substrate. Infectivity was expressed as mean percentage of infected cells.

## Results

### *Expression and purification of rHsc70, rPDI and rVP6*

The optimal conditions for expression of pET28a-Hsc70 in *E. coli* BL21(DE3) were determined. These conditions included bacterial concentration (OD at 600 nm) before induction, induction time, and inducer concentration. Induction assays were conducted in bacterial cultures showing OD<sub>600 nm</sub> values that ranged from 0.1 to 0.7 which encompassed the exponential growth phase and the starting of the stationary growth phase. A typical bacterial growth curve is shown in Fig. 1A. Expression of the rHsc70 was followed by SDS-PAGE and Western blotting analysis using rabbit anti-Hsc70 antibodies and taking into account the expected molecular mass (65 kDa) for this protein. In the absence of IPTG, no expression for rHsc70 was found either in the soluble or in insoluble fraction (Fig. 1B, lanes 2, 3). Addition of IPTG (1 mM final concentration) at OD<sub>600 nm</sub> 0.1 – 0.4 for 4 h resulted in induction of basal expression of rHsc70 which was detected as a Western blot band present in either the soluble or insoluble fractions (Fig. 1B, lanes 4 – 11). IPTG induction at OD<sub>600 nm</sub> 0.5 – 0.7 for 4 h led to rHsc70 expression only in the insoluble fraction (Fig. 1B, lanes 13, 15, 17). The highest amount of rHsc70 expression was obtained when the bacterial culture at OD<sub>600 nm</sub> 0.5 was induced with IPTG (1 mM) for 4 h. In this case, rHsc70 represented 1.02% of the total insoluble protein loaded in SDS-PAGE wells (Fig. 1B, lane 13).

Recombinant Hsc70 was found in both the soluble and insoluble fractions when cultures were induced with IPTG at concentrations that ranged from 0.5 to 2 mM (Fig. 1C, lanes 4 – 9) when the culture was induced at OD<sub>600 nm</sub> 0.5. However, a higher proportion of rHsc70 was always observed in the lysate insoluble fraction from cultures irrespective of the IPTG concentration used (Fig. 1C, lanes 5, 7, 9). Cultures induced with 1 - 2 mM IPTG showed the highest total accumulation of rHsc70 (1.15%) relative to the total protein content in both fractions (Fig. 1C, lane 6 and 7). Variations in the IPTG concentration produced slight changes in the rate of rHsc70 accumulation and its location. In order to increase the rHsc70 accumulation and its location in the soluble fraction, the induction time effect was assessed between 2 and 8 h by treating the culture at

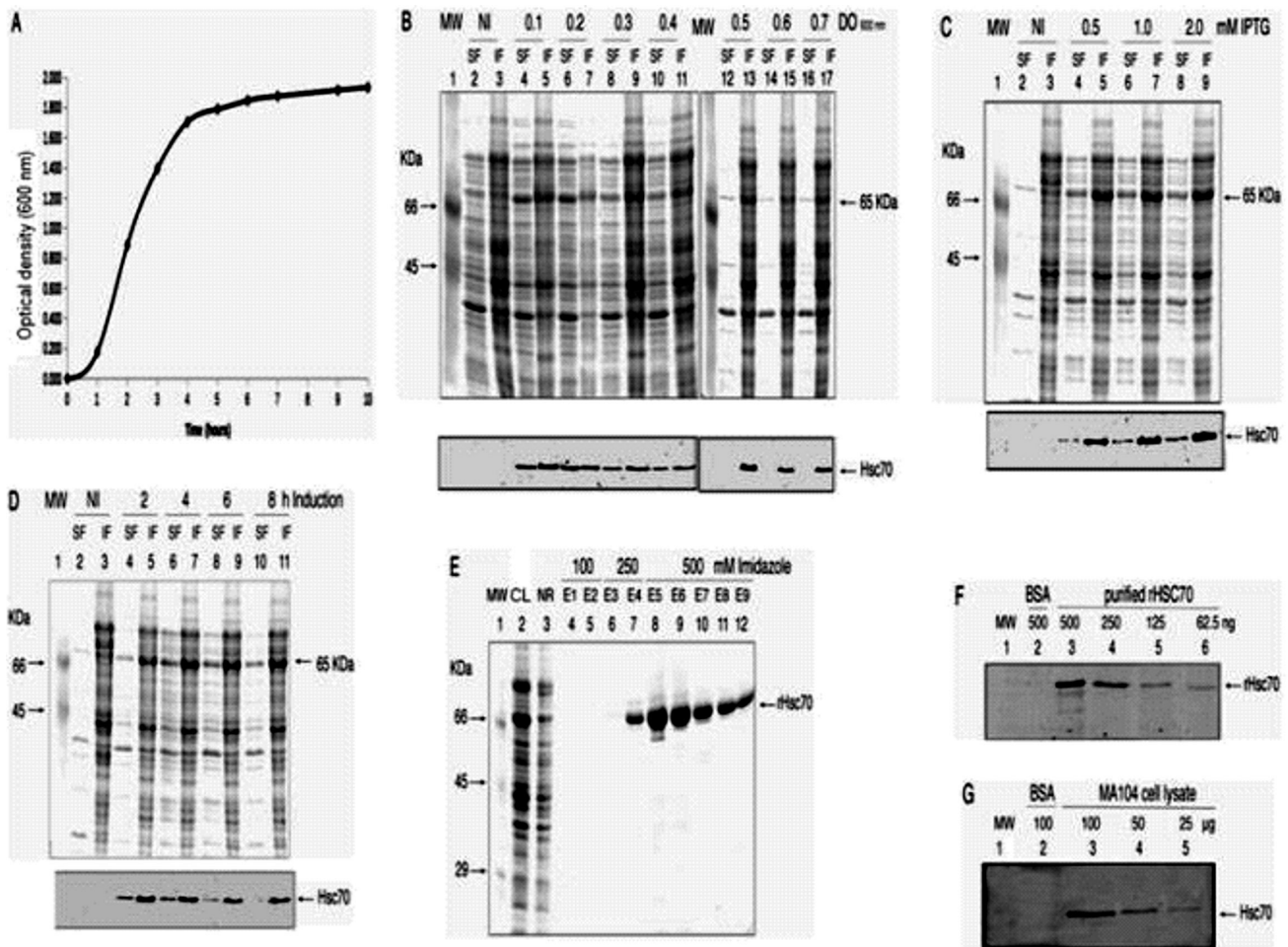
OD<sub>600 nm</sub> 0.5 with 1 mM IPTG. The recombinant protein was identified in all lysate fractions from cells induced during the times indicated (Fig. 1D, lanes 4 -11), but a higher accumulation of rHsc70 continued to be observed in the insoluble fraction. However, the highest rHsc70 accumulation (1.39%) was detected in the insoluble fraction relative to the total protein present in both lysate fractions when induction with 1 mM IPTG was initiated at OD<sub>600 nm</sub> 0.5 and maintained during 4 at 37 °C. The rHsc70 contained in the insoluble fraction was solubilized by treatment with PBS containing N-lauroylsarcosine and DTT but without EDTA to avoid Ni<sup>2+</sup> chelating which interferes with rHsc70 binding to the resin during its purification. Additional treatment with 1% Triton X-100 was performed for refolding the recombinant protein and reducing nonspecific interaction between bacterial proteins and the resin used for rHsc70 purification.

The rHsc70 was efficiently purified as demonstrated by the comparison of the electrophoretic profiles corresponding to the proteins present in the total lysate and the eluted fractions from the resin (Fig. 1E, lanes 2, 3). The analysis of fractions eluted from the resin with an imidazole gradient is shown in figure 1E (lanes 6 – 12). Fractions analyzed in lanes 8, 9 and 10 show the presence of some non-specific protein bands located both in the high and low molecular weight region. Western blotting analysis using rabbit polyclonal antibodies (1:1000) against Hsc70 showed that these antibodies specifically recognized the purified rHsc70 at amounts as low as 62.5 ng/well (Fig. 1F, lane 6), whereas rHsc70 was detected in cell lysates only when samples contained at least 50 µg/well of total protein (Fig. 1G).

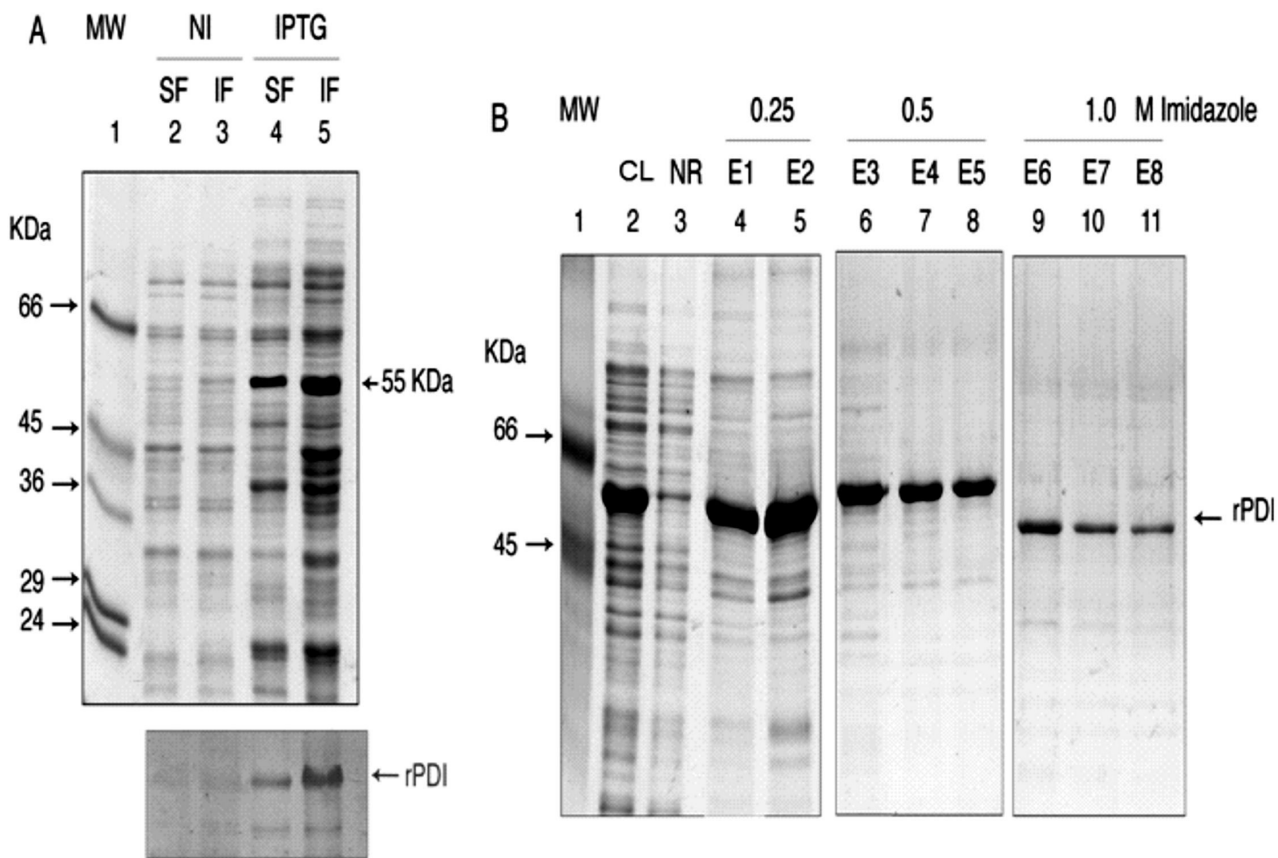
PDI expression was efficiently induced with 0.5 mM IPTG during 4 h at 37 °C. The SDS-PAGE/Western blotting analysis of lysates from the induced cells showed a 55 kDa protein band which was identified as being PDI (Fig. 2A, lanes 4, 5). The PDI purification was carried out by treating cell lysates with PBS containing DTT and sodium N-lauroylsarcosine before passing them through a Ni<sup>2+</sup> column. The profile of the fractions eluted with the imidazole gradient (0.25 – 1 M) is shown in Fig. 2B. The highest amount of PDI eluted with 250 mM imidazole although some non-specific protein bands showing higher and lower molecular weight than PDI were observed (Fig. 2B, lanes 4, 5). Lower amounts of PDI were obtained at higher imidazole concentrations (Fig. 2B, lanes 6 - 11).

Recombinant VP6 was expressed in MA104 cells after the infection with a recombinant vaccinia virus vector. The Western blotting analysis indicated that a protein band of 39 kDa corresponding to rVP6 was identified predominantly in the IF from the vaccinia virus-infected cell lysates (V-IL) (Fig. 3A, lane 5). Less rVP6 accumulation was detected in the soluble fraction (Fig. 3A, lane 4). This protein band was absent from non-infected cell lysates (N-IL) (Fig. 3A, lanes 6 and 7). Solubilization of





**Figure 1. The optimal conditions for expression of recombinant Hsc70 in *E. coli* BL21(DE3).** (A) Growth curve of *E. coli* BL21(DE3) transfected with pET-28a(+)-Hsc70 was determined in 2XYT medium by turbidimetry measurements at 600 nm. (B) Expression of rHsc70 was analyzed in both soluble (SF) and insoluble (IF) fractions by SDS-PAGE/Western blotting using primary rabbit antibodies against Hsc70. Proteins were stained with Coomassie blue. The anti-Hsc70 reaction is shown in the lower panel blot. Protein expression was determined following IPTG (1 mM) induction at the cell density (OD 600 nm) values indicated at the top of the upper panel. Non-induced (NI) cells were used as a control. Molecular weight markers (MW) are indicated. (C) Expression of rHsc70 in SF and IF was analyzed by SDS-PAGE/Western blotting after induction with the different concentrations of IPTG indicated at the top of the upper panel. Detection was performed using a primary rabbit antibody against Hsc70 as shown in the lower panel blot. (D) Expression of rHsc70 was analyzed by SDS-PAGE/Western blotting of SF and IF following the times (h) of IPTG (1 mM) induction indicated at the top of the upper panel. Detection was performed using a primary rabbit antibody against Hsc70. Anti-Hsc70 reaction is shown in the lower panel blot. (E) Purification of rHsc70 was carried out using a HisTrap (BioRad) affinity column followed by elution with an imidazole gradient at the concentrations indicated. Fractions eluted (E) from the column were analyzed by SDS-PAGE. Total lysate (CL) and non-retained (NR) fraction were used as a control. (F) Characterization of rabbit polyclonal antibodies against recombinant Hsc70. Different amounts (ng/well) of purified rHsc70 were analyzed in SDS-PAGE and then transferred to PVDF membranes. Purified rHsc70 was detected with primary rabbit polyclonal antibodies against Hsc70 (1:1000). BSA was used as a control. (G) Different amounts ( $\mu$ g/well) of protein from MA104 cell lysates were separated by SDS-PAGE and then transferred to a PVDF membrane. Hsc70 was detected with primary rabbit polyclonal antibodies (1:1000) against Hsc70. BSA was used as a control.



**Figure 2. Expression of recombinant PDI in *E. coli* BL21(DE3).** (A) Expression of rPDI in *E. coli* cultures induced and non-induced (NI) with IPTG (1 mM) was analyzed in both soluble (SF) and insoluble (IF) fractions by SDS-PAGE/Western blotting. Proteins in the gel were stained with Coomassie blue (upper panel) and PDI was detected using primary rabbit antibodies against PDI (lower panel). (B) Purification of rPDI was carried out using a HisTrap (BioRad) affinity column followed by elution with an imidazole gradient at the concentrations indicated. Eluted fractions (E) were analyzed by SDS-PAGE/Western blotting using primary rabbit anti-PDI antibodies. Total lysate (CL) and non-retained (NR) fraction were used as a control.

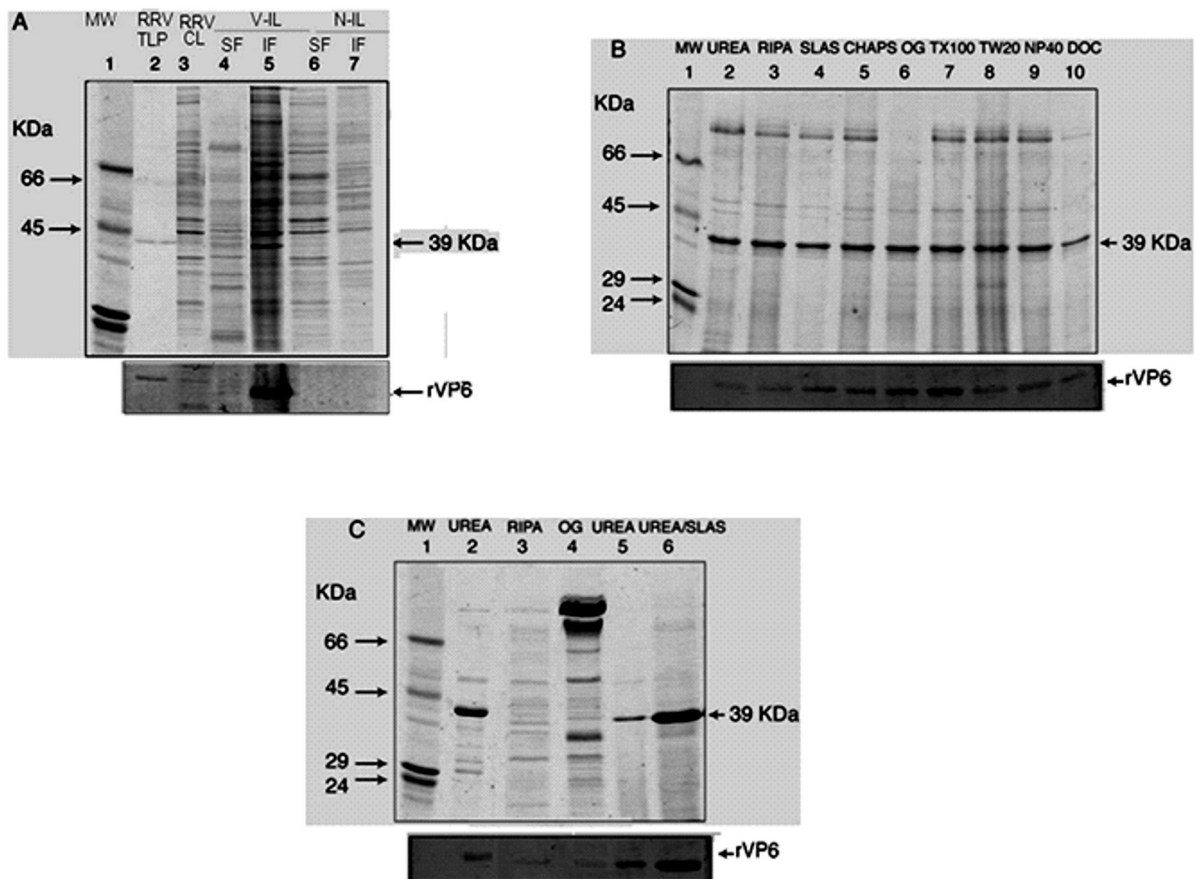
rVP6 from the IF was attempted by treatment with RIPA buffer followed by the recovery of a secondary insoluble fraction which was further treated with 1% sodium N-lauroylsarcosine, CHAPS,  $\beta$ -octylglucoside, Triton X-100, Tween 20, Nonidet P40 or sodium deoxycholate. The treatment of the remaining pellets from these detergent treatments with 8 M urea in Tris buffer showed that, despite some contaminant proteins, all the urea-solubilized fractions were enriched in rVP6 (Fig. 3B). In order to eliminate additional contaminant proteins, the standard procedure for rVP6 purification started with RIPA treatment to solubilize some contaminant proteins from the cell lysate insoluble fraction (IF) (Fig. 3C, lane 3), followed by treatment of the resultant pellet with 1%  $\beta$ -octylglucoside (Fig. 3C, lane 4). Then, the remaining pellet was treated with 8 M urea (Fig. 3C, lane 5) and finally with 8 M urea plus 1% sodium N-lauroylsarcosine (Fig. 3C, lane 6). Urea treatment of the IF in the absence of other treatments

(Fig. 3C, lane 2) showed that the previous detergent treatments of the IF contributed to decrease contaminant proteins. The solubilized VP6 was dialyzed against PBS and concentrated by ultrafiltration (Amicon Ultra 4, Millipore).

#### **Characterización of antibodies against VP6 and Hsc70**

Western blotting analysis of anti-VP6 antibodies showed that these antibodies recognized the corresponding viral antigen in lysates from vaccinia virus-infected cells expressing recombinant VP6 when gel wells were loaded with 25 - 100  $\mu$ g of total protein (Fig. 4A, lanes 3 - 5). Purified rVP6 (500 - 62.5 ng/well) was also recognized in this analysis (Fig. 4B, lanes 3 - 6). However, a positive reaction corresponding to a lower molecular weight band was seen which could be explained in terms of a degradation product from rVP6





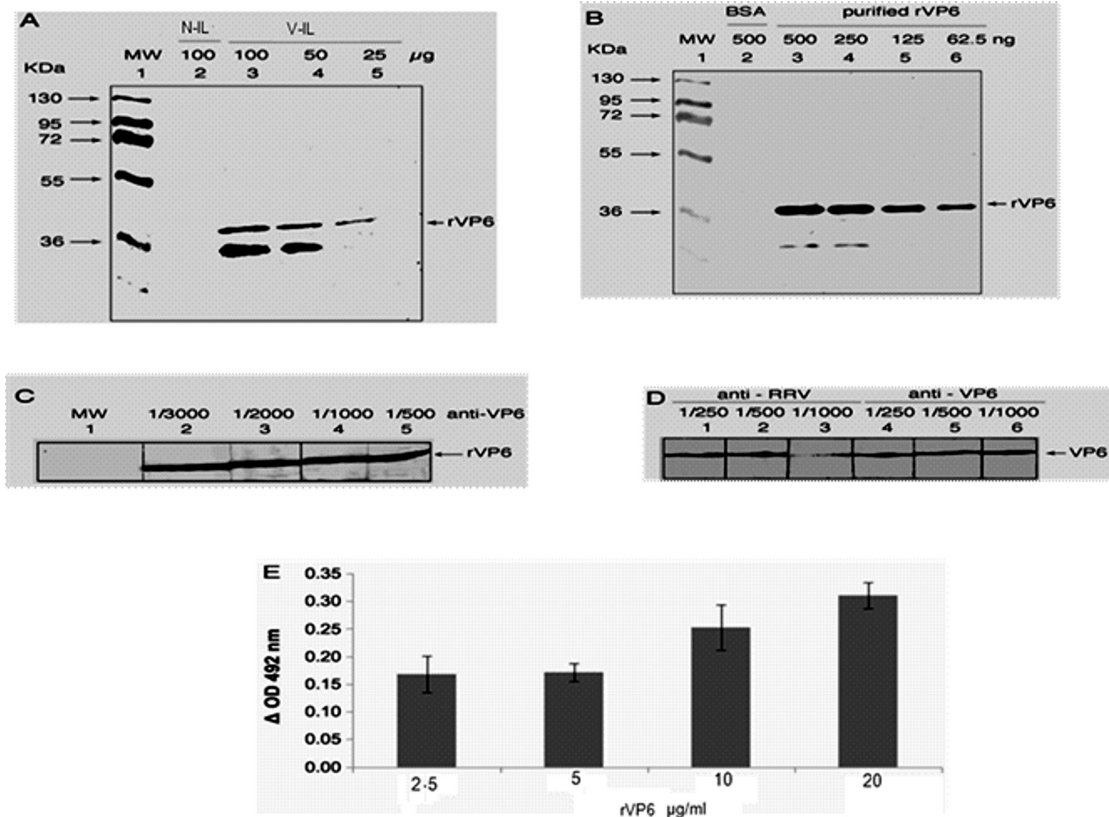
**Figure 3. Expression of recombinant VP6 in MA104 cells.** (A) Cell monolayers were infected with a recombinant vaccinia virus containing a SA11 nucleotide sequence encoding VP6. Soluble (SF) or insoluble (IF) fractions of lysates from both vaccinia-infected (V-IL) and RRV infected (RRV-IL) MA104 cells were analyzed by SDS-PAGE/Western blotting. The membrane shown in the lower panel was reacted with rabbit polyclonal antibodies against RRV particles. Purified RRV TLPs, total RRV-infected cell lysate (RRV CL) and total non-infected cell lysates (N-IL) were used as a control. Molecular weight markers (MW) are indicated. Insoluble fractions (IF) (lanes 5 and 7) were solubilized by treatment with 8 M urea before analysis. (B) Insoluble fractions (IF) from recombinant vaccinia virus-infected cell lysates were solubilized in RIPA buffer or in Tris buffer containing the indicated detergents at 1%. SLAS: Sodium N-Lauroylsarcosine; OG: Octyl  $\beta$ -D-glucopyranoside; TX100: Triton X-100; TW20: Tween 20; NP40: Nonidet P-40; DOC: Sodium deoxycholate. Molecular weight markers (MW) are indicated. The solubilized proteins were submitted to SDS-PAGE/Western blotting analysis. Proteins in the gel were stained with Coomassie blue (upper panel) and rotavirus proteins detected using rabbit polyclonal antibodies against RRV particles (lower panel). (C) Insoluble fractions (IF) from recombinant vaccinia virus-infected cell lysates were sequentially treated with RIPA buffer,  $\beta$ -octylglucoside, and 8 M urea plus N-lauroylsarcosine. The different solubilized fractions were analyzed by SDS-PAGE and stained with Coomassie blue. The arrow indicates the purified VP6. Total SF and IF from cell lysates (CL) were used as a control. Molecular weight markers (MW). IF solubilized with 8 M urea (lane 2); IF solubilized with RIPA at 4 °C for 4 h (lane 3); Remaining pellet from the previous treatment that was solubilized with OG at 1% (lane 4); Remaining pellet from the previous treatment that was solubilized with 8 M urea (lane 5); Remaining pellet from the previous treatment that was solubilized with urea plus SLAS at 1% (lane 6).

(Fig.4A and B, lanes 3 and 4). Partially purified rVP6 (250 ng) was detected by the highest dilution (1:3000) of the antiserum against rVP6 (Fig. 4C, lanes 2 – 5). VP6 from purified TLPs (500 ng/well) was also detected by Western blotting using anti-VP6 antibodies at dilutions ranging from 1:250 to 1:1000 (Fig. 4D, lanes 4 – 6). ELISA showed increasing absorbance values for samples containing rVP6 (2.5 – 20  $\mu$ g/ml) (Fig. 4E). Anti-VP6 antibodies at a dilution of 1:2000 showed to be useful for detecting RRV TLP-infected MA104 cells

that had been inoculated with a MOI of 0.8 since more than 50% of cells were found to be positive to the viral antigen as detected by the immunocytochemistry assay (Data not shown).

#### ***In vitro* interaction of VP5\*, VP8\* and VP6 with Hsc70 and PDI**

To assess the interaction between rotaviral proteins (VP5\*, VP8\* or VP6) and cellular proteins (Hsc70 or



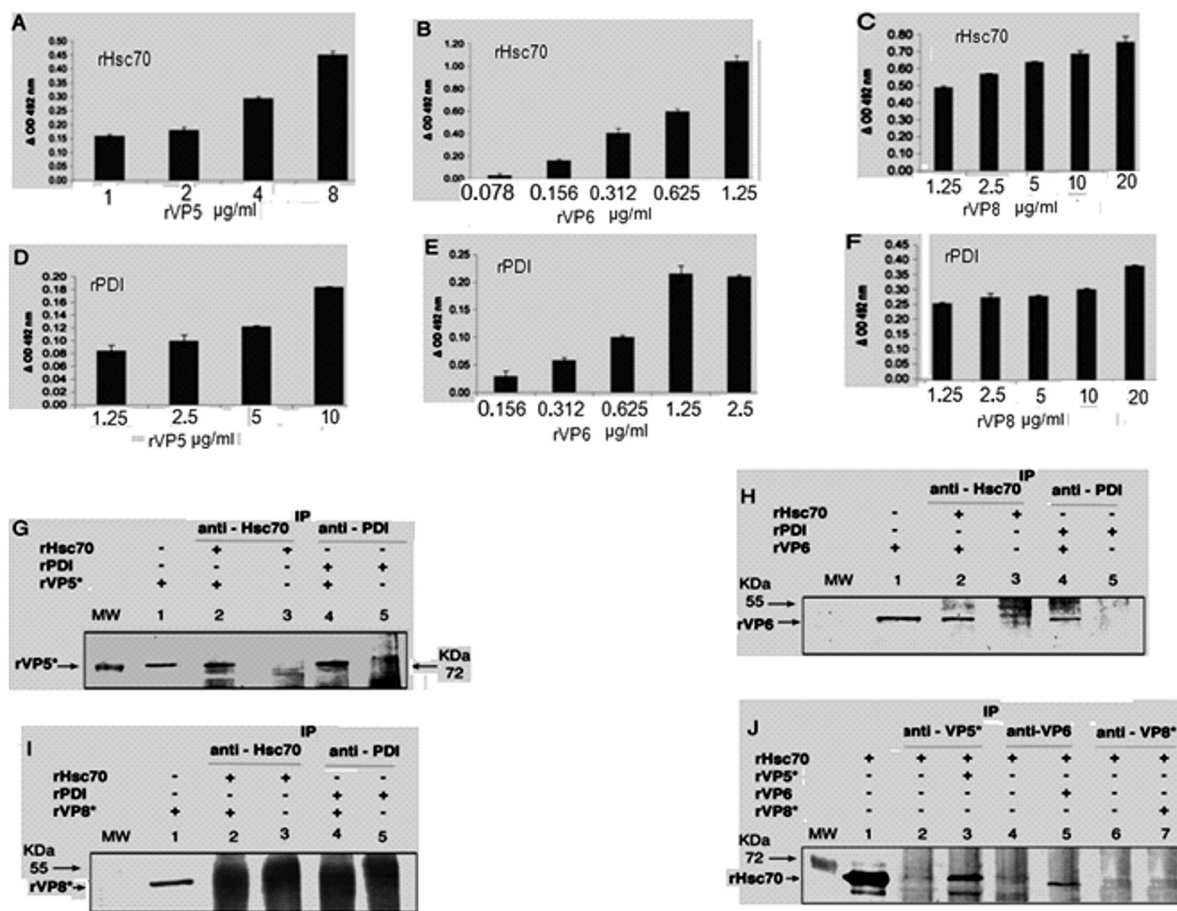
**Figure 4. Characterization of rabbit polyclonal antibodies against recombinant VP6.** (A) Different amounts ( $\mu\text{g}/\text{well}$ ) of lysate protein from vaccinia virus infected-cells expressing recombinant VP6 were separated by SDS-PAGE and then transferred to a PVDF membrane. Non-infected cell lysates were used as a control (N-IL). rVP6 was detected with primary rabbit polyclonal antibodies against VP6 (1:1000). (B) Different amounts ( $\text{ng}/\text{well}$ ) of purified rVP6 were analyzed in SDS-PAGE and then transferred to PVDF membranes. Purified rVP6 was detected with primary rabbit polyclonal antibodies against VP6 (1:1000). (C) rVP6 (250  $\text{ng}/\text{well}$ ) were analyzed in Western blotting. Membranes were probed with decreasing dilutions of rabbit polyclonal antibodies against VP6. (D) TLP proteins (500  $\text{ng}$ ) were separated in SDS-PAGE and then transferred to PVDF membranes. Proteins were detected with increasing dilutions of rabbit polyclonal antibodies against VP6 or rabbit anti-RRV TLP antibodies used as a control. (E) ELISA plates were coated with increasing amounts ( $\mu\text{g}/\text{ml}$ ) of rVP6 and were detected with primary rabbit polyclonal antibodies against VP6 (1:1000) and HRP-conjugated goat anti-rabbit antibody.

PDI), ELISA plates were coated with either of the latter proteins (40  $\mu\text{g}/\text{ml}$ ) and reacted with either rotaviral proteins at the concentrations indicated. Absorbance values showed an increasing profile which was directly proportional to the growing rVP5\* concentrations (1 – 8  $\mu\text{g}/\text{ml}$ ) added to rHsc70 coated wells (Fig. 5A). A similar interaction profile was observed when rHsc70 coated wells were reacted with increasing rVP6 concentrations (0.078 – 1.25  $\mu\text{g}/\text{ml}$ ) (Fig. 5B). These findings suggest that viral rVP5\* and rVP6 interact with Hsc70 at least in the *in vitro* conditions used. When rPDI coated ELISA wells were reacted with increasing rVP5\* concentrations (1.25 – 10  $\mu\text{g}/\text{ml}$ ), and also with different rVP6 concentrations (0.156 – 2.5  $\mu\text{g}/\text{ml}$ ), a positive linear correlation with the absorbance values was found (Fig. 5D and 5E). Although absorbance values were relatively low mainly for VP5\* (less than 0.2), these results suggest that rVP5\* and rVP6 are directly interacting with PDI *in vitro*. ELISA results for rVP8\* in-

teractions with Hsc70 or PDI were negative (Fig. 5C and 5F).

An immunoprecipitation assay was conducted to confirm the interactions found by ELISA. rPDI or rHsc70 (10  $\mu\text{g}/\text{ml}$ ) were separately incubated with either rVP6 (2.5  $\mu\text{g}/\text{ml}$ ), rVP5\* or rVP8\* (10  $\mu\text{g}/\text{ml}$ ). Immunoprecipitation was performed using specific antibodies against either cellular protein. Western blotting analysis of the immunoprecipitated proteins showed the presence of recombinant viral proteins rVP5\* and rVP6 which were detected with the respective polyclonal antibodies (Fig. 5G and 5H). The immunoprecipitation results were not conclusive for VP8\* since its molecular weight was shared with that of immunoglobulin heavy chains which were recognized by the secondary antibody used in this assay (Fig. 5I).

Reciprocal immunoprecipitation of the viral and cellular protein mix was also conducted using antibodies



**Figure 5. Interaction between rotavirus structural proteins and Hsc70 and PDI.** ELISA plates were coated with 40 µg/ml Hsc70 (A, B, C) or PDI (D, E, F) and then reacted with rVP5\* (A and D), rVP6 (B and E) or rVP8\* (C and F). Plate wells without cellular proteins, viral proteins or primary antibodies were used as controls. BSA instead of cellular or viral proteins was also used as a control. Values were expressed as ΔOD 492 nm, calculated as the difference between mean OD values from experiments and those from negative controls (A, B, D and E). VP6 (2.5 µg/ml) or VP5\* or VP8\* (10 µg/ml) were separately incubated with Hsc70 or PDI (10 µg/ml) and then co-immunoprecipitated and analyzed by SDS-PAGE/Western blotting. Proteins were co-immunoprecipitated with anti-Hsc70 or anti-PDI antibodies and the co-immunoprecipitated protein (IP) identified with anti-rVP5\* (G), anti-rVP6 (H) or anti-rVP8\* antibodies (I). (J) Co-immunoprecipitation was performed with antibodies against the viral proteins and identification of the co-immunoprecipitated protein was identified with antibodies against Hsc70.

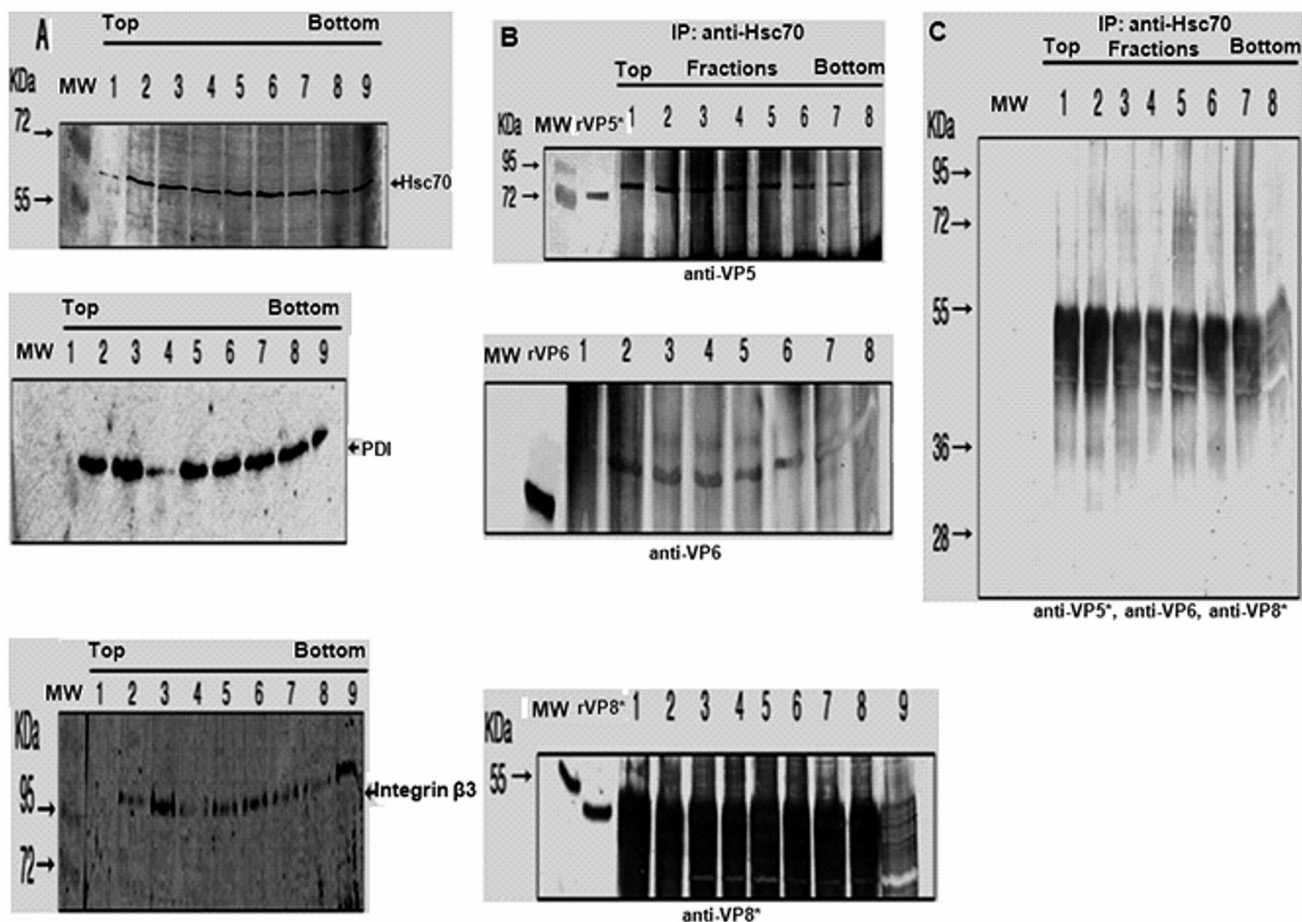
specifically directed against viral proteins. As shown in Fig. 5J (lanes 3 and 5), Hsc70 co-immunoprecipitated with rVP5\* and rVP6 but no with rVP8\* (lanes 7). Hsc70 was not bound to the immunoprecipitation resin in the absence of viral protein addition (Fig. 5J, lanes 2, 4 and 6). These results confirm the interaction between viral proteins (VP5\* and VP6) and cellular proteins Hsc70 and PDI. In the absence of antibody addition, no viral proteins were bound to the resin used in the immunoprecipitation assay (Data not shown).

#### **Interactions between cell surface Hsc70 and viral proteins rVP5\*, rVP8\* and rVP6**

To test interactions between viral proteins and Hsc70, MA104 cell monolayer were separately incubated with

rVP5\*, rVP8\* (15µg/ml) or rVP6 (1.5 µg/ml). Cells were lysed with Triton X-100 at 4 °C and the lipid microdomains (rafts) fractionated by centrifugation through a sucrose gradient. After treatment of gradient fractions with β-cyclodextrin and β-octylglucoside, fractions were immunoprecipitated with antibodies against Hsc70 and analyzed by SDS-PAGE/Western blotting. Viral proteins were detected with specific antibodies against them. Cellular proteins Hsc70 (Fig. 6A, upper panel), PDI (Fig. 6A, middle panel) and integrin β3 (Fig. 6A, lower panel) were detected in lipid raft fractions from control cells that had not been treated with viral proteins. These cellular proteins have previously been found to be associated in rafts from MA104 cells and also involved in rotavirus infection (Calderon *et al.*, 2012). It was also found that viral proteins VP5\* and



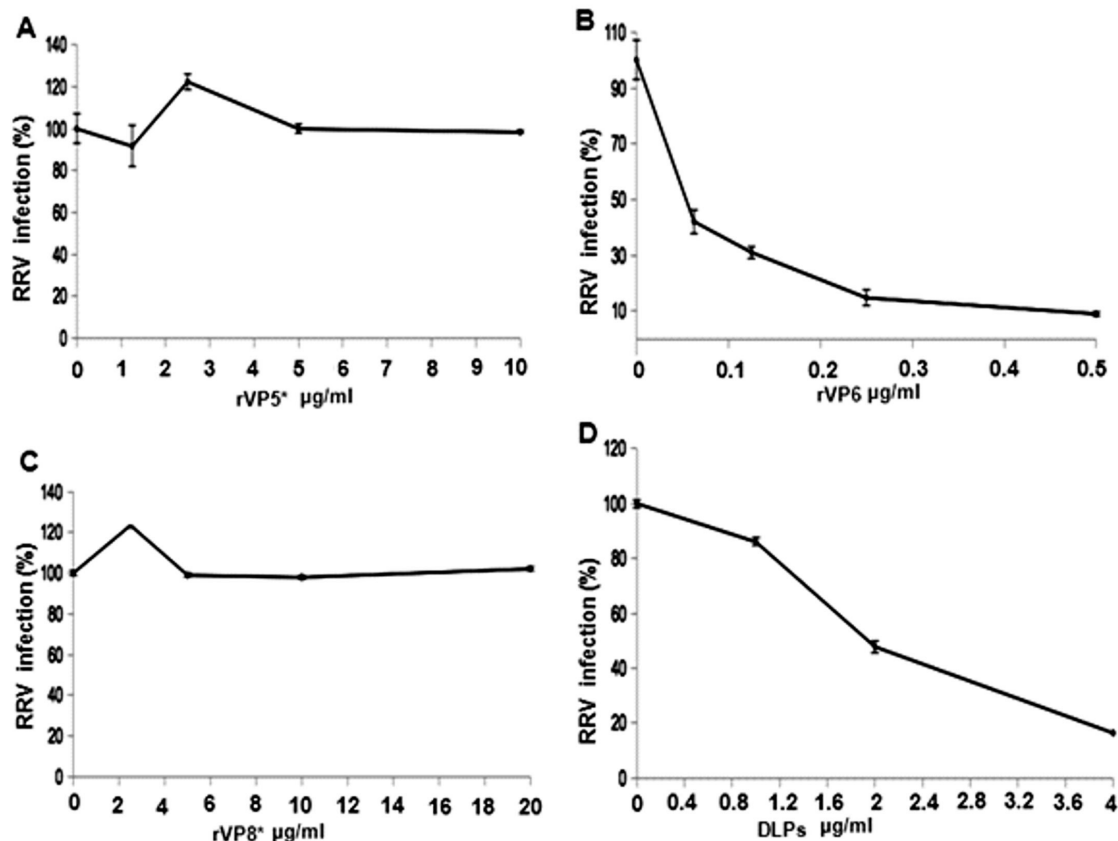


**Figure 6. Identification of Hsc70, integrin  $\beta$ 3 and PDI in lipid microdomains (rafts) and the interaction between raft-associated Hsc70 and recombinant viral proteins rVP5\*, rVP6 and rVP8\*.** MA104 cell monolayers were lysed at 4 °C and centrifuged at 38000  $\gamma$  in a sucrose gradient (30 - 5 %) for 8 h at 4 °C. The sucrose gradient fractions containing rafts were analyzed by SDS-PAGE/Western blotting. (A) Cellular proteins were identified using polyclonal antibodies against Hsc70 (upper panel), PDI (middle panel) or integrin  $\beta$ 3 (lower panel). (B) MA104 cell monolayers were incubated with viral proteins rVP5\* (15  $\mu$ g/ml), rVP6\* (15  $\mu$ g/ml) or rVP6 (1.5  $\mu$ g/ml) and the raft-containing sucrose gradient fractions were treated with 10 mM  $\beta$ -cyclodextrin and 1% octyl-glucoside before being immunoprecipitated with rabbit polyclonal antibodies against Hsc70. The co-immunoprecipitated protein (IP) was analyzed by SDS-PAGE/Western blotting and viral protein identified with HRP-conjugated goat anti-rabbit antibodies (2 ng/ $\mu$ l) against rVP5\*, rVP6 and rVP8\*. (C) Cells without addition of viral proteins were used as a control. Cells were processed as indicated in (B), except that blots were reacted with a mix of antibodies against VP5\*, VP8\* and VP6.

VP6 co-immunoprecipitated with cellular Hsc70. These results show that rVP5\* (Fig. 6B, upper panel) and rVP6 (Fig. 6B, middle panel) were located in lipid rafts interacting directly or indirectly with Hsc70. Again, identification of rVP8\* resulted difficult as its molecular weight was similar to that from antibody heavy chains which reacted with the secondary antibody used (Fig. 6B, lower panel). Gradient fractions from cells that had not been treated with viral proteins, were not reactive with antibodies against VP5\* or VP6 after immunoprecipitation with anti-Hsc70 antibodies (Fig. 6C). This result excludes any non-specific reaction of antibodies against viral proteins.

#### ***Inhibitory effect of VP5\*, VP8\* or VP6 on RRV infection of MA104 cells***

Previous work showed that MA104 cell infection with RRV TLPs was inhibited by treatment of cells with DLPs (double layered particles) (Dormitzer 2004). This suggested that VP6 located on the surface of DLPs is involved in rotavirus penetration to host cell. In order to confirm this hypothesis, MA104 cell were incubated with viral proteins rVP5\*, rVP8\* or rVP6 for 1 h at 37 °C and then infected with RRV TLPs. Immunochimistry detection after 12 h infection showed that infection was decreased by more than 90% when cells were previously incubated with rVP6 (0.5  $\mu$ g/ml) (Fig.



**Figure 7. Effect of rVP5\*, rVP8\*, rVP6 and DLPs on RRV infection.** MA104 cells were incubated with different concentrations of viral proteins rVP5\* (A), rVP6 (B), rVP8\* (C) or DLPs (D) for 1 h at 37 °C. After, the cell monolayers was inoculated with RRV (1 MOI) for 1 h at 37 °C and the incubation was maintained for 12 h at 37 °C. Immunocytochemistry analysis was carried out using polyclonal antibodies against RRV particles. Virus infectivity was determined as mean percentage of infected cells relative to the control untreated cells. Cell viability was determined with the Trypan blue exclusion test and the concentrations of viral proteins used were measured at 280 nm against a BSA calibration curve. The mean percentage of infection for untreated cells was normalized to 100%.

7B). rVP5\* or rVP8\* treatment of cells was unable to interfere with virus infection even at much higher concentrations than those used for rVP6 (Fig. 7A and 7C). Increasing DLP concentrations led to inhibition of virus infection when cells were DLP-treated before TLP infection (Fig. 7D). Trypan blue exclusion assay showed that cell viability was not affected by DLP treatment. These results confirm the implication of VP6 in the rotavirus infection process in MA1|04 cells.

## Discussion

Protein expression in heterologous systems has been a widely used strategy for investigating the molecular mechanisms underlying protein interactions. Expression vectors have been very useful in allowing uptake of genetic foreign material followed by its transcription and translation in heterologous systems (Sorensen *et al.*, 2005; Sahdev *et al.*, 2008). However, proteins

expressed in heterologous systems usually are accumulated forming insoluble inclusion bodies and their solubilization requires detergent treatments and procedures aimed at their *in vitro* refolding (Sahdev *et al.*, 2008). Insolubilization of VP5\* and VP8\* in heterologous environments has been shown to depend on the expression systems used (Frangioni *et al.*, 1993; Tang *et al.*, 1997). In the present study, the conditions for expression of recombinant Hsc70 and PDI were compared in terms of bacterial concentration at the time of induction, time of induction and inductor concentration. Therefore, the optimum conditions for expression of the cellular proteins Hsc70 and PDI were established in an *E. coli* system. Optimum conditions for vaccinia virus expressing recombinant VP6 in the MA104 cell system were also defined. The urea and detergents assayed for solubilization of recombinant proteins in combination with the 6-his-tagged expression of Hsc70 and PDI allowed their efficient purifica-

tion after using His Trap affinity columns. On the other hand, the specificity of the rabbit antibodies raised against recombinant Hsc70 and VP6 was demonstrated by Western blotting analysis.

The mechanisms associated with the rotavirus entry into the host cell continue to be a very controversial subject. The high level of purity of the recombinant proteins obtained from the expression and purification procedures described in the present work allowed their use to gain further insight into rotavirus-cell interaction mechanisms. It had been previously shown that RRV DLPs were able to interfere with rotavirus infection in MA104 cells (Gualtero *et al.*, 2007). This finding suggested that VP6 might be involved in rotavirus entry. The results shown here indicated that addition of rVP6 to MA104 cells resulted in more than 90% inhibition of virus infection. This new result gives further support to the hypothesis that this most abundant rotavirus structural protein could be implicated in rotavirus entry. Llama-derived single-chain antibody fragments directed to rotavirus VP6 have been found to possess broad neutralizing activity both *in vitro* and *in vivo* against rotavirus infection (Garaicoechea *et al.*, 2008). The inhibition of rotavirus infection by either DLPs or VP6 could be explained by their binding to cell surface Hsc70 since this protein was found to interact with free or DLP-associated VP6 in ELISA (Gualtero *et al.*, 2007) or co-immunoprecipitation assay. Differential binding strength of Hsc70 to TLPs and DLPs has been previously reported (Guerrero *et al.*, 2002). This finding could be explained by the apparent existence of two exposed Hsc70 binding sites in TLPs which are represented by the VP5\* domains 642-658 (Zárate *et al.*, 2003) and 531-554 (Gualtero *et al.*, 2007). In contrast, the DLP-associated VP6 seems to have less Hsc70 binding sites (Gualtero *et al.*, 2007).

Hsc70 has been shown to interact with VP5\* and DLPs in previous inhibition and competition studies (Guerrero *et al.*, 2000; Zárate *et al.*, 2004; Gualtero *et al.*, 2007), and cell surface PDI has been found to be needed for rotavirus infection as thiol/disulfide inhibitors and antibodies to PDI interfere with virus infection (Calderon *et al.*, 2012). In the present work, ELISA and co-immunoprecipitation analysis led to conclude that Hsc70 or PDI certainly interact physically with VP5\* and VP6. The inhibitory binding of VP6 to Hsc70 following incubation of VP6 with MA104 cells, as evidenced in the co-immunoprecipitation assay, suggests that VP6 binds to Hsc70 during the infectious process which results in a competition for rotavirus binding to cell surface Hsc70. Similarly, these interactions were also found in raft-associated Hsc70 or PDI, although whether these interactions are direct or are mediated by an intermediary protein remains to be established. We propose that rotavirus entry involves the cell surface PDI reducing activity which probably generates thiol groups in VP5\* and/or VP6 that contribute either to virion disassembly or priming of viral

proteins to interact with other cell surface receptors. The endoplasmic reticulum (ER)-associated oxidant PDI has been reported to interact with VP7 to generate the disulfide bridges which are needed for the proper VP7 folding during its assembly in TLPs (Svensson *et al.*, 1994; Maruri-Avidal *et al.*, 2008). Despite VP8\* and immunoglobulin heavy chains could not be resolved by Western blotting analysis, and interaction between VP8\* and Hsc70 or PDI remains to be elucidated. The treatment of MA104 cells with VP5\* or VP8\* did not affect rotavirus infection. This finding would suggest that the binding of these proteins to the cell surface is dependent of conformational changes that are only produced when these proteins are associated to viral particles and interact with cell surface molecules during the infectious process. VP5\* and VP8\* failed to compete with viral particles probably because they lacked the conformational changes required to interact with cellular receptors. Our results give support to the hypothesis that conformational rearrangements in rotavirus particle proteins occur during the entry process which are similar to those generated in the HIV gp120 envelope glycoprotein conformation (Markovic *et al.*, 2004) or in the non-enveloped reovirus (Madren *et al.*, 2012) or poliovirus (Brandenburg *et al.*, 2007) structural proteins in order to expose critical proteins to cell receptors.

Taken together, the results presented here confirm the usefulness of recombinant proteins in the identification of protein interactions during virus entry into the host cell. However the sequence in which rotavirus VP5\* and VP6 interact with Hsc70, PDI or other cellular proteins during entry remains to be determined in more detail.

## Acknowledgements

This study was supported by a grant (No. 201010018595) awarded by Universidad Nacional de Colombia to support the strengthening of research and artistic creation groups.

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