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Amino acid domains 280–297 of VP6 and 531–554 of VP4 are implicated in heat shock cognate protein hsc70-mediated rotavirus infection

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Summary

The rotavirus infection mechanism seems to be a multi-step process which is still not fully understood. The heat shock cognate protein hsc70 has been proposed as being a co-receptor molecule for rotavirus entry into susceptible cells. In this work, an attempt was made to determine the existence of possible domains for VP4 and VP6 binding to hsc70. We selected amino acid sequences 531–554 from VP4 and 280–297 from VP6 on the basis of already recognized sequences for binding to hsc70. This study determined that DLPs and synthetic peptides from VP6 (aa 280–297) and VP4 (aa 531–554), individually or in combination, inhibited rotavirus RRV, YM and WA entry into MA104 and Caco-2 cells in an additive and dose-dependent manner. Hyperimmune sera against these synthetic peptides blocked infection by infectious TLPs. Capture ELISA results showed that DLPs interact with hsc70, probably through VP6 as the specific interaction between hcs70 and DLPs was disrupted by a VP6 peptide. These results suggest that VP6 takes part during rotavirus cell entry by binding to hsc70. This, as well as previous work, provides insight concerning the function of hsc70 within a multi-step model of rotavirus entry.

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Introduction

Rotaviruses are doubled-stranded RNA viruses belonging to the family *Reoviridae* and are the leading ethological agents of acute dehydrating gastroenteritis in human infants and young animals [10]. These viruses are responsible for 352,000–592,000 human deaths each year [29]. Rotaviruses have very restricted *in vivo* cell tropism as they primarily infect mature enterocytes in the intestine; however, a range of human and monkey cell lines have been shown to support rotavirus replication [3, 21]. Monkey kidney epithelial (MA104) and human colonic adenocarcinoma (Caco2) cell lines are fully permissive to rotavirus infection [21]. Their icosahedral and non-enveloped virions are characterized by having three concentric protein layers surrounding a genome composed of 11 segments of double-stranded RNA. The outermost layer is composed of VP7 glycoprotein and VP4 spike-

forming haemagglutinin. The intermediate layer consists of VP6, comprising 51% of viral mass. The nucleocapsid core consists of VP2, containing the viral genome and the minor viral proteins VP1 and VP3 [10]. VP4 cleavage with trypsin to render VP5* and VP8* subunits is required to make the virus infective.

The hsc70 heat shock cognate protein has been recently proposed as being a co-receptor molecule in rotavirus entry based on experiments in which rotavirus entry was blocked by a VP5* synthetic peptide and by antibodies targeted against human recombinant hsc70 [30, 41]. A VP5* synthetic peptide, comprising amino acids 642–658, competed with the binding of the rotavirus RRV and its neuraminidase-resistant variant nar3 to hsc70 [41]. Similar synthetic peptide strategies have been used to locate recognition sites for hsc70 and integrins in specific rotavirus structural protein domains [9, 14, 39, 42]. Research conducted on integrins has suggested that the presence of tripeptide sequences in extracellular matrix proteins is essential for adhesion to integrins [9]. The DGE amino acid sequence present in subunit VP5* has been characterized as being a recognition site for the $\alpha 2\beta 1$ integrin, while GPR and LDV sequences in VP7 have been reported as binding sites for $\alpha \times \beta 2$ and $\alpha 4\beta 1$ integrins, respectively. Based on these findings, the mechanism for rotavirus entry into the cell seems to be conducted mainly by VP4 and VP7 proteins from the outermost layer [10, 23].

Establishing multiple interactions between cell surface molecules and the viral particle seems to be a crucial event during infection of animal cells by viruses, with or without a lipidic envelope [4, 18]. The rotavirus entry mechanism seems to be a complex multistep process in which at least four different cell surface molecules present in lipidic microdomains or ''rafts'' sequentially interact with VP4 and VP7 viral structural protein domains [1, 15, 20, 26, 42]. It has been proposed that neuraminidase-sensitive rotavirus strains bind to a sialic acid-containing receptor during a first step [25]. After this initial VP8*-mediated interaction, both neuraminidase-sensitive and -resistant strains apparently share a second VP5*-mediated step involving interaction with $\alpha 2\beta 1$ integrin [39, 40]. Additional interactions have been reported, suggesting that $\alpha v\beta 3$ and $\alpha \times \beta 2$ integrins and hsc70 are involved in a later stage of rotavirus entry into the host cell [16, 17, 41, 42].

VP6, the major rotavirus capsid protein, is highly antigenic and immunogenic but does not induce antibodies that are neutralizing *in vitro*, even though some VP6-specific IgA immunoglobulins are protection-inducing *in vivo*, probably via transcytosis through epithelial cells [2, 13, 32]. Vaccine development studies have shown a high level of protection when VP6 has been used as immunogen in different strategies [5–7]. Synthetic peptides comprising the VP6 283–307 region induced full protection against viral challenge when rats were immunized with these peptides [7, 8]. However, when VP6-based vaccines have been used in pigs, conflicting results have been presented depending on both inoculation strategy and inoculum composition [8, 37, 38]. The mechanism by which VP6 protein protects against rotavirus challenge has not been well established; several studies have suggested that this protection is due to intracellular neutralization of DLP particles by IgA immunoglobulins, preventing viral transcript release and translation in the cytoplasm [13, 32].

The specific rotavirus-host cell interaction is not well understood as yet and no convincing entry pathway has been established [22]. Our purpose was therefore to establish whether VP6 is involved in this process by studying its association with hsc70, a protein able to recognize hydrophobic regions [34], such as those characterizing VP6 [10]. The present work shows that hsc70 is probably associated with viral proteins VP6 and VP4 during rotavirus infection. It demonstrates that synthetic peptides spanning VP4 and VP6 amino acid sequences (individually, in combination, or in variable molar ratios with DLP particles) were able to block rotavirus Wa, RRV and YM infection in an additive and dose-dependent manner in inhibition assays carried out with MA104 and Caco-2 cells. The probable implication of these VP6 and VP4 amino acid sequences in rotavirus entry is stressed as antibodies raised against the synthetic versions of these sequences inhibited rotavirus infection.

Materials and methods

Cells and viruses

MA104 cells were grown at 37 °C in Eagle's MEM supplemented with 10% fetal bovine serum (FBS) (Gibco). Caco-2 cells were cultured in Dulbecco's MEM supplemented with 20% FBS. Rotavirus strains Wa (G1 P1A [8]) (human), RRV (G3 P5B [3]) (simian), YM (G11 P9 [7]) (porcine) were propagated in confluent MA104 cells in serum-free medium. Rotaviruses were activated with 10 μg/ml bovine type I trypsin (Sigma) prior to inoculation in cultured cells. Virus strains were obtained from Dr. C. F. Arias (Instituto de Biotecnología, UNAM, Cuernavaca, Mexico).

Virus purification

RRV rotavirus strain double- and triple-layered viral particles were purified from confluent infected MA104 cells showing a complete cytopathic effect. Purification was essentially carried out as described previously [12]. Briefly, infected cells were washed with Tris-buffered saline (TBS) (10 mM Tris–HCl pH 7.4, 15 mM NaCl, 1 mM $MgCl_2$ and 5 mM $CaCl_2$), lysed by freezing and thawing and then briefly sonicated. Lysates were emulsified by vortexing with one-third volume of trichlorothrifluoroethane (Sigma); the phases were separated by low-speed centrifugation. The organic phase was extracted three times with an equal volume of TBS; the aqueous phase was centrifuged through 1 ml 30% (w/v) sucrose in TBS at 150,000 × *g* (Sorvall TST 60.4 rotor) for 1.5 h at 4 °C. Viral particles were suspended in TBS and then layered onto a discontinuous gradient consisting of 1.4157 g/cm^3 (0.5 ml), 1.3039 g/cm^3 (1 ml) and 1.2070 g/cm^3 (0.5 ml) CsCl and an upper layer of 30% (w/v) sucrose. Isopycnic centrifugation was carried out in a Sorvall TST 60.4 rotor at 280,000 × *g* for 1.5 h at 4 °C. Visible DLP and TLP bands were collected and then diluted with TBS before pelleting at 100,000 × *g* for 1.5 h at 4 °C. TLPs were suspended in TBS, and when increased DLP yield was needed, both TLPs and DLPs were suspended in TBS without $MgCl_2$ and $CaCl_2$, but containing 50 mM EDTA (TBSE) [11]. The particles were left for 1 h at 37 °C. The DLP preparation was subjected to a second cycle of sedimentation at 100,000 × *g* followed by viral particles being suspended in TBSE. DLPs were quantified spectrophotometrically at 260 nm, and TLPs were determined densitometrically using purified DLP bands as standards in SDS-PAGE [38].

Peptides

Synthetic peptides spanning regions of VP4 (aa 531–554) and VP6 (aa 280–297) of RRV (G3 P5B [3]) (simian) strain were prepared at Fundacion Instituto de Inmunologia de Colombia (FIDIC) using standard solid-phase procedures [9, 27] and purified by high-performance liquid chromatography (HPLC) and kept lyophilised. Unrelated peptides (VII, VI) of the same size and random sequence peptides having the same amino acid composition and size were synthesized to be used as control. Peptides were suspended in MEM before use in infectivity assays. The peptide effect on cell viability was assessed on MA104 cells using Trypan blue and MTT as described previously [28].

Antisera preparation

Peptides specific for VP4 and VP6 were used as antigen (0.5 mg) in combination with the FIS (FISEAAIIHVLHSR) peptide (0.5 mg) used as adjuvant [31]. Rabbits were injected subcutaneously with 1 ml of an emulsion of the respective peptide combination and Freund's complete adjuvant. Rabbits were injected with the same amount of each antigen emulsified in Freund's incomplete adjuvant twenty and forty days later. Rabbits were bled on day 60 after the first injection, and sera containing sodium azide (0.04%) were diluted two-fold with glycerol and kept at −20 °C until use. Preimmune sera collected on day 0 were used as control.

Capture ELISA

Microtitre 96-well plates (Nunc) were coated with 200 μl polyclonal rabbit antiserum against rotavirus RRV (G3 P5B[3]) (simian) diluted 1:1500 in PBS and incubated overnight at 4 °C. Different concentrations of recombinant hsc70 (0.074–1.4 mg/ml) were mixed with DLPs (1 μg/ml) and incubated overnight at 4 °C. This mixture was added to wells that were previously blocked with 3% skimmed milk and 2% ovalbumin in PBS containing 0.05% Tween 20 (v/v) (PBST), followed by overnight incubation at 4 °C. Plate wells were washed three times with PBST prior to adding polyclonal goat serum against hsc70 (0.4 μg/ml) (Santa Cruz Biotechnology) prepared in blocking-PBST. The reaction was allowed to proceed for 1 h at 37 °C before extensive washing with PBST and adding rabbit anti-goat-HRP conjugate (Santa Cruz Biotechnology) (1:5,000) in PBST. Plates were washed five times with PBST following incubation for 1 h at 37 °C, prior to adding colorimetric substrate (phenylendiamine dihydrochloride, Pierce) diluted in peroxidase stable buffer. The reaction was quantified spectrophotometrically at 493 nm using an ELISA reader. Different VP6 (IV) synthetic peptide concentrations (1–200 μg/ml) were incubated with a mixture containing hsc70 (30 μg/ml) and DLPs (2 μg/ml). The mixture was incubated overnight at 4 °C before adding it to capture ELISA plates as described above.

Infectivity assay

Optimal peptide and DLP concentration ranges were determined as described previously [9, 41]; all inhibition assays were performed in duplicate. Confluent MA104 and Caco2

cell monolayers in 96-well polystyrene plates (Nunc) were treated with different VP4 (0.125–4 mg/ml), VP6 (0.015–2 mg/ml) peptide or DLP (0.01–4 mg/ml) concentrations for 1 h at 37 °C in a 5% CO_2 atmosphere. Cells (5.0×10^4/well) were incubated with trypsin-activated virus-cell lysates from each rotavirus strain (Wa, RRV, YM) at 1000 FFU (ca. 0.02 multiplicity of infection, MOI) for 1 h at 37 °C. MA104 cells were incubated with reovirus (strain 1) cell lysates at 1000 FFU as control. Cells which had been washed twice with MEM were incubated at 37 °C for 12 h in the same medium before fixing with cold methanol for 45 min. Fixed cells were washed three times with PBS and incubated with polyclonal rabbit antiserum against rotavirus for 1 h at 37 °C. The cells were rinsed with PBS, and a 1:3000 dilution of goat anti-rabbit immunoglobulin HRP-conjugate (Santa Cruz Biotechnology) was layered over the cells and incubated for 1 h at 37 °C. Peroxidase activity was detected by using 3-amino-9-ethyl carbazole (Sigma), and the degree of inhibition was expressed in FFU observed and counted using a Euromex inverted microscope. Random VP4 or VP6 peptide sequence and unrelated peptides were used as control.

Inhibition assay

Confluent MA104 cells in 96-well plates were washed twice with MEM prior to adding 1000 FFU of CsCl-gradient-purified TLPs previously activated with trypsin (10 μg/ml). The cultures were then incubated for 45 min at 4 °C. After adsorption, the viral inoculum was removed, the cells were washed twice with MEM, and different hyper-immune antiserum dilutions against VP4 or VP6 peptide were added to the cultured cells. Reovirus strain 1 at 1000 FFU was used as control under the same conditions. Cultures were kept for 30 min at 4 °C, followed by a further 45-min incubation at 37 °C. Cells which had been washed twice with MEM were incubated for 12 h at 37 °C in a 5% CO_2 atmosphere. The FFU were counted after infected cells were fixed and immuno-stained as described above. The degree of inhibition was expressed as mean percentage ± standard deviation.

Results

VP4 and VP6 contain amino acid sequences potentially involved in hsc70 recognition

Previous work has shown that *in vitro* triple-layered rotavirus particles can bind to purified hsc70 protein immobilized on ELISA plates, but double-layered ones cannot do so [17]. A particular domain located between VP5* amino acids 642 and 658 is involved in this interaction [41]. To explore other possible hsc70-rotavirus interactions, cell lysates infected with rotavirus RRV were immunoprecipitated using an anti-hsc70 rabbit polyclonal serum. This allowed the detection of VP4 and VP6 among other immunoprecipitated rotavirus structural proteins (data not shown). Since these results do not necessarily indicate that a direct interaction took place between hsc70 and the immunoprecipitated structural proteins, an attempt was made to determine the existence of possible domains for VP4 and VP6 that bind to hsc70.

The amino acid sequences of 40 peptides already reported in the literature [34] as being able to bind to hsc70 were aligned with sequences present at VP6 and VP4 to determine this. Different human and animal rotavirus structural proteins were aligned using Clustal X software [35]. Once aligned, sequences were selected bearing four characteristics: having high homology with viral proteins, overlapping with more than one of the 40 peptides in the same viral sequence, having selected region conservation in the different types of rotavirus, and the sequence in VP4 and VP6 structural

Table 1. Amino acid sequence of the synthetic peptides

Protein	Peptide	Amino acid sequence
VP4	I	^{531}FSGIKSTIDAAKSMATNVMKKFKK^{554}
M-VP4[a]	II	FSGIKSTLEGAKSMATNVMKKFKK
S-VP4[b]	III	KGKMMIVFFNAIKSTKSTAKASKD
VP6	IV	^{280}IVARNFDTIRLSFQLRPP^{297}
S-VP6[b]	V	RIPQIRLVFLARTSDPNF
FIS[c]	VI	FISEAIIHVLHSR
Non related	VII	KMNMHLENVPWIMNKQGLFK

[a] Modified version of VP4.
[b] Scrambled version of VP4 or VP6.
[c] T-helper epitope [31].

proteins being exposed on the TLP and DLP, respectively. The VP4 (I) FSGIKSTIDAAKSMATN VMKKFKK sequence (aa 531–554) and VP6 (IV) IVARNFDTIRLSFQLRPP sequence (aa 280–297) were thus selected on such criteria (Table 1). The VP4 peptide II (531–554) was synthesized since the VP4 (I) peptide sequence contains the IDA (Ile-Asp-Ala) tripeptide reported as being able to

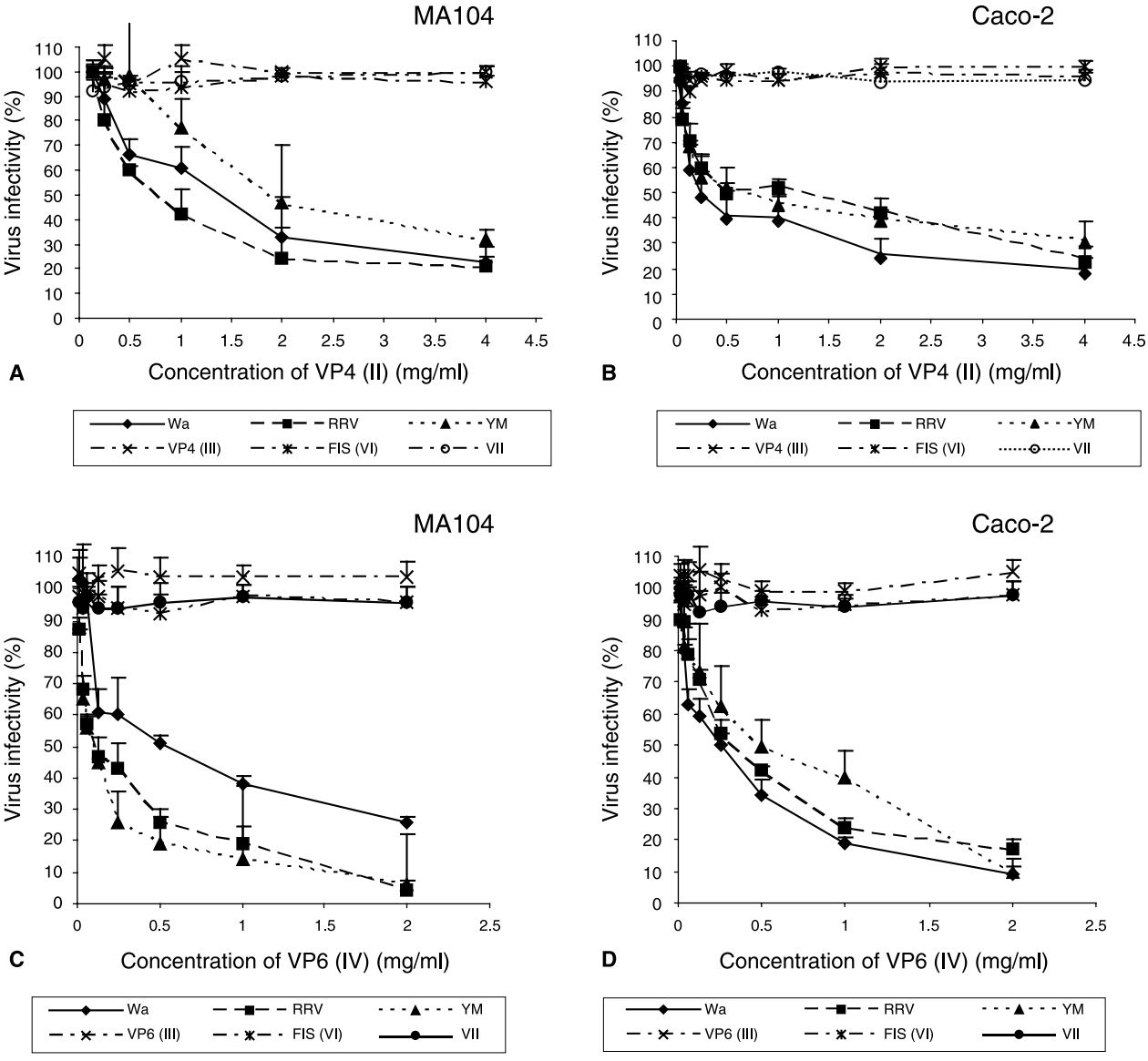

Fig. 1. Effect of VP4 andVP6 synthetic peptides on Wa, RRV and YM rotavirus infectivity. Confluent MA104 (**A** and **C**) and Caco-2 cells (**B** and **D**) in 96-well plates were incubated with different peptide concentrations for 1 h at 37 °C. (**A** and **B**) VP4 (II) and VP4 (III), (VI) and (VII) control peptides were added at 0.125–4 mg/ml concentrations. (**C** and **D**) VP6 (IV) peptide and VP6 (V), (VI) and (VII) control peptides were added at 0.015–2 mg/ml concentrations. Afterwards, cells were infected with 1000 FFU of the corresponding virus and incubated for 1 h at 37 °C. The excess unbound virus was removed and infection was allowed to continue for an additional 14 h at 37 °C. Cells were fixed and immunostained as described in Materials and methods. Results are expressed as percentage of virus infectivity relative to control cells infected with the corresponding rotavirus but without peptide treatment. Curves represent the results of at least three independent experiments, each done in duplicate

bind to integrin α4β1, a receptor for the SA11 rotavirus strain [9]. This tripeptide was replaced by LEG (Leu-Glu-Gly), these being amino acids maintaining the same functional groups in their side chains. Peptides having the same type and number of amino acids, but with a random sequence, were used as control peptides. Control peptides having unrelated sequences were also synthesized. To test the effect of peptides on cell viability, 1 and 2 mg/ml peptide were added to cell cultures for 1 h at 37 °C. The viability test using Trypan blue and MTT showed that the VP4 (I) peptide had a cytotoxic effect at the two concentrations tested. VP4 (II), VP6 (IV) and control peptides (III, V, VI and VII) had no effect on cell viability as compared with cells maintained only in MEN (data not shown).

VP4 and VP6 peptides inhibit rotavirus infectivity

Confluent cells were incubated at increasing VP4 (II), VP6 (IV) or control peptide concentrations and then infected with rotaviruses (Fig. 1) to evaluate whether VP4 (aa 531–554) and VP6 (aa 280–297) synthetic peptides were able to interfere with Wa, RRV and YM rotavirus infectivity in MA104 and Caco-2 cells. The VP4 (II) peptide inhibited MA104 and Caco-2 cell infection by WA, RRV and YM rotaviruses in a dose-dependent and saturable manner. Inhibition was around 79% for Wa and RRV and 68% for YM in MA104 cells (Fig. 1A). Inhibition was 80% for Wa, 66% for RRV and 68% for YM in Caco-2 cells (Fig. 1B). The VP6 (IV) peptide inhibited MA104 cell infection by the three rotaviral strains in a dose-dependent manner. Inhibition was around 94% for RRV and YM and 74% for Wa in MA104 cells (Fig. 1C). Inhibition was 91% for Wa, 83% for RRV and 90% for YM in Caco-2 cells (Fig. 1D). Control peptides had no effect on infection with the three rotavirus strains in either cell line. MA104 cells pre-treated with these peptides were infected with reovirus strain 1 (which enters cells by clathrin-dependent

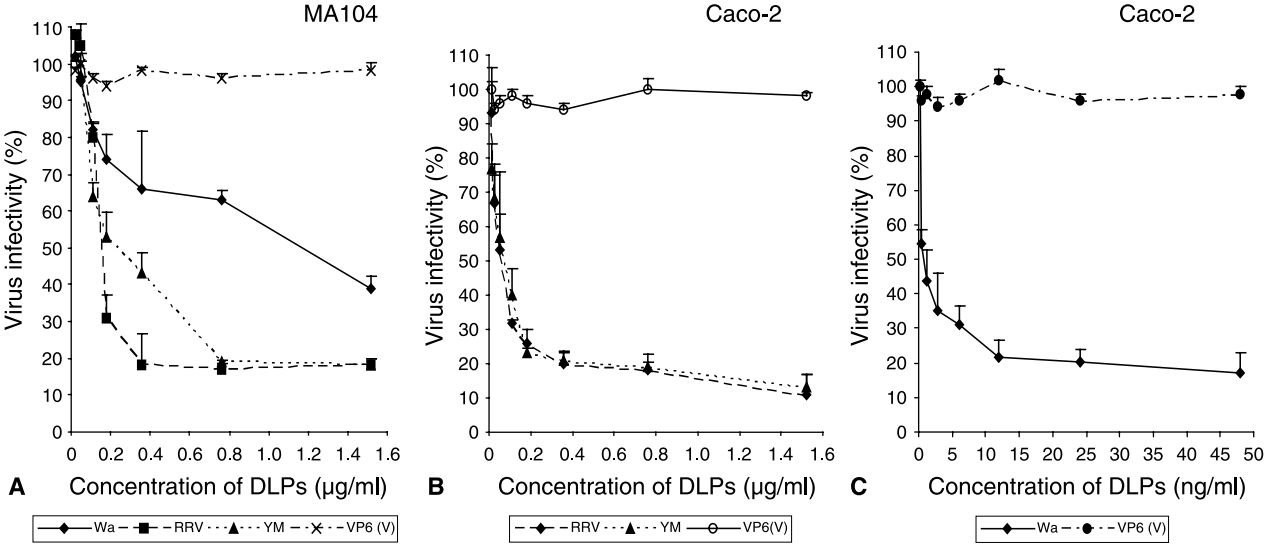

Fig. 2. Effect of RRV DLPs on Wa, RRV and YM rotavirus infectivity. (**A**) Confluent MA104 cells in 96-well plates were incubated for 45 min at 37 °C with 0.02 to 1.52 µg/ml DLP concentrations, or with 0.5 mg/ml VP6 (V) control peptide. Afterwards, cells were infected with 2000 FFU of the corresponding virus and incubated for 1 h at 37 °C. (**B** and **C**) Caco-2 cells as in **A**, except that 1.0–50 ng/ml DLP concentration was used in **C**. Excess virus was removed and the infection was allowed to continue for 14 h at 37 °C. Cells were then fixed and immunostained as described in Materials and methods. The control curve in **A** is expressed as an average percentage of the inhibition produced by VP6 (V) control peptide in the assays in which cells were independently infected by the rotavirus strains Wa, RRV and YM (**A**) or RRV and YM (**B**). Results are expressed as percentage of virus infectivity relative to cells infected with the corresponding virus strain but without VP6 (V) and DLP treatment. Curves are the mean of at least three independent experiments, and the standard deviations are indicated by bars

endocytosis) at 1000 FFU to test the synthetic peptides' inhibition specificity. No effect on reovirus infection was observed when compared to mock-infected cells (data not shown). Synthetic peptides were added after 1 h pi at 37 °C to examine the possible effect of VP4 (II) and VP6 (IV) synthetic peptides on rotavirus infection after entry. No effect on infection with the same three rotavirus strains was observed when compared to mock-infected cells (data not shown). The fact that VP4 (II) and VP6 (IV) synthetic peptides inhibited rotavirus infection but not reoviral infection could suggest that these peptides were specifically interfering with rotavirus infection, probably by competing for cell-surface receptor molecules and thereby inhibiting rotavirus entry into the cell, rather than post-entry events.

Non-infective rotavirus DLPs block infection by TLPs

Since the VP6 (IV) synthetic peptide (aa 280–297) significantly inhibited rotavirus infection, it was decided to determine whether VP6 forming non-infective double-layer particles (DLPs) was able to inhibit infection during rotavirus entry into the cell. Confluent MA104 and Caco-2 cells were thus incubated with different concentrations of DLP (obtained from CsCl-purified RRV rotavirus particles) and then challenged by each rotavirus (Fig. 2). It was found that DLPs inhibited MA104 cell infection by YM and RRV in a dose-dependent manner, reaching about 81% inhibition for these rotavirus strains when the assays were conducted between 0.02 and 1.52 μg/ml DLP. Inhibition for RRV reflected a saturable reaction after 0.36 μg/ml DLP, whereas inhibition for YM showed a similar behavior after 0.76 μg/ml) DLP. On the other hand, inhibition for Wa reached 61% at the highest DLP concentration tested (1.52 μg/ml DLP), although a more linear pattern was exhibited, suggesting a higher probability of a less specific reaction (Fig. 2A). DLPs inhibited Caco-2 cell infection by Wa, RRV and YM in a dose-dependent manner, reaching 87% inhibition for RRV and YM strains at 0.36 μg/ml when assays were conducted between 0.02 and 1.52 μg/ml DLP concentration (Fig. 2B). Interestingly, 78% inhibition for the Wa rotavirus strain was reached at a much lower concentration, 12 ng/ml DLP (Fig. 2C), indicating that the Wa strain required about 30 times less DLP in Caco-2 cells than the RRV and YM rotavirus strains to show a comparable degree of inhibition. The results were similar irrespective of whether or not the cells were washed following incubation with DLPs and challenge with rotaviruses. At the highest DLP concentrations used for each rotavirus strain, no effect was observed on virus infectivity when DLPs were added after 1 h pi (data not shown). In order to further test the DLP activity on virus infectivity, we treated MA104 cells with DLPs that had been pre-incubated with rabbit polyclonal anti-VP6 antibodies. In this way, DLPs inhibited virus infectivity about 10% for rotavirus WA and RRV, and about 18% for YM (data not shown). These results strengthen the hypothesis that VP6 present in DLPs is involved in the virus entry stage.

The infection inhibition results produced by prior incubation of cells with DLPs did not exclude the possibility that inhibition could have been a consequence of steric hindrance associated with non-specific and massive DLP binding to the cell surface. To test this, an infection assay was conducted in MA104 cells (5.0×10^4) in which cells were first incubated with decreasing quantities of RRV DLPs (0.256–0.004 μg/ml) and then challenged with a constant amount (50 μl/well) of RRV TLPs (32 ng/ml; 0.04 MOI) corresponding to the dose required to obtain an infection reference level to which 0% inhibition was assigned when no DLPs were added. Molar ratios between TLPs and DLPs were thus obtained according to the quantifications referred to above [36]. Calculations led to determining that 32 ng/ml TLP corresponded to 3.3×10^8 particles/ml and to 330 particles/cell for the preparation used. Then, an infection assay was done taking the TLP/DLP molar ratio into consideration. The results showed that DLPs blocked TLP infection by 98, 83, 62 and 13% for TLP/DLP molar ratios of 0.125, 1, 4 and 8, respectively (Fig. 3). A monoclonal neutralizing antibody (MAb VP7) raised against a VP7 epitope was used as positive rotavirus infection inhibition control [33]. These results supported the hypothesis that inhibi-

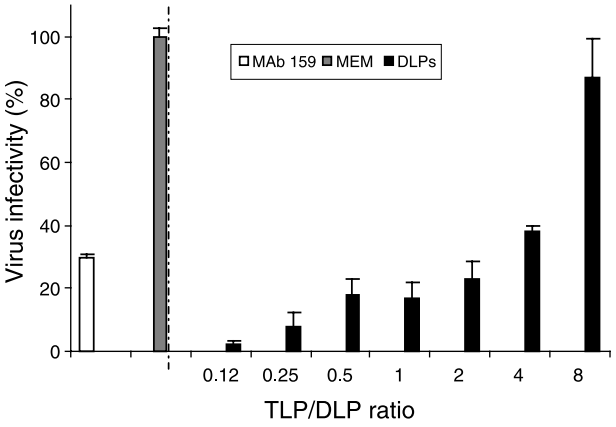

Fig. 3. Inhibition of RRV TLP infectivity at different RRV TLP/DLP molar ratios. MA104 cells in 96-well plates were incubated with DLPs (0.256–0.004 μg/ml; $26–0.41 \times 10^8$ particles/ml). Cells were then infected with trypsin-activated TLPs (32 ng/ml; 3.3×10^8 particles/ml; 0.04 MOI). Data are presented as percentage of TLP infectivity compared to TLP-infected cells in MEM without DLPs (*grey bar* on the left). TLPs treated with neutralizing monoclonal antibody (MAb VP7) were used as control (*white bar* on the left). Each bar represents the mean of experiments performed in duplicate. The standard deviations are indicated at the top of the bars

tion of Wa, RRV and YM rotavirus infection caused by DLPs was not due to steric hindrance but to specific binding to cell-surface molecules which are probably used by TLPs during their entry to the cell.

Synthetic VP4 and VP6 peptide and DLP combinations inhibit rotavirus infection

Experiments were carried out to determine whether or not the inhibitory activity exhibited by synthetic peptides was additive as this could (in principle) give some insight regarding synthetic peptide and DLP binding in terms of using the same or a different receptor molecule. Alternatively, if it were assumed that rotavirus entry into a cell is a sequential multi-step process (analyzing the inhibitory effect produced by peptides and DLPs), then this might lead to suggesting the presence of more than one rotavirus entry pathway. Synthetic peptides or DLPs at half-maximum inhibition concentration were thus used when testing this to try to restore the maximum inhibition value. Confluent MA104 cells were incubated with 1 mg/ml VP4 (II) peptide plus 0.25 mg/ml VP6 (IV) peptide and then infected with rotavirus. The sum of peptides VP4 (II) and VP6 (IV) at half-maximum inhibition concentration produced a cumulative 75% inhibition effect for the Wa strain and 83% for the RRV strain. No cumulative inhibition effect was observed for

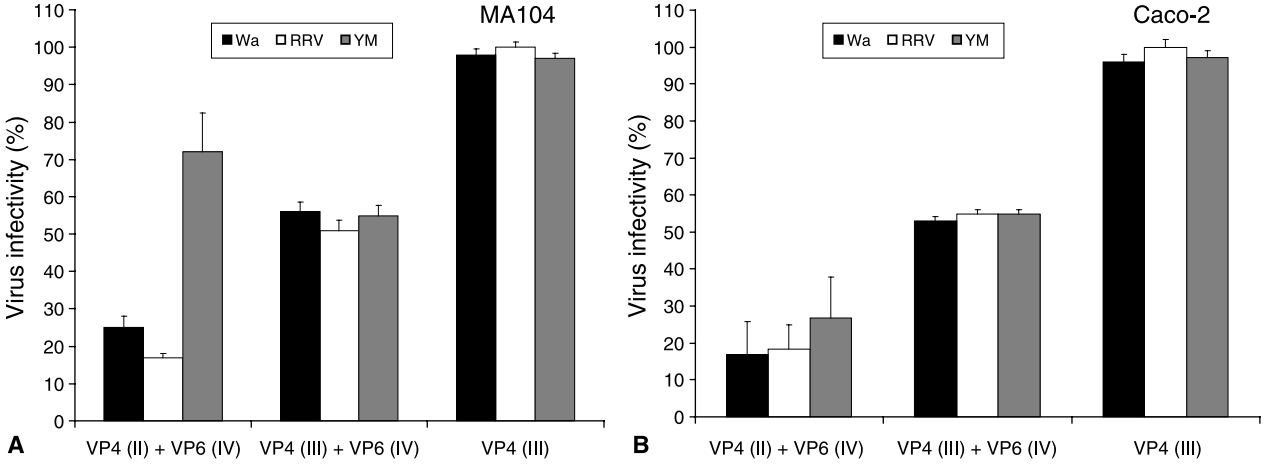

Fig. 4. Effect of synthetic peptide combination on Wa, RRV and YM rotavirus infectivity. MA104 cells (**A**) in 96-well plates were incubated with 1 mg/ml of VP4 (II) plus 0.25 mg/ml of VP6 (IV) and then infected with the corresponding rotavirus. The same procedure was followed with Caco-2 cells (**B**), except that 0.5 mg/ml of VP4 peptide (II) was used instead of 1 mg/ml. Peptide VP4 (III) at 1 mg/ml (**A**) and 0.5 mg/ml (**B**) was used as a control. Plates were developed as described in Fig. 3

the YM strain when the sum of peptides was used, obtaining only 28% inhibition (Fig. 4A). The sum of peptides had a cumulative effect in Caco-2 cells which amounted to 83% for the Wa strain, 82% for RRV and 73% for YM (Fig. 4B). These results showed that the sum of peptides at half-maximum inhibition concentration had a cumulative effect for the three rotavirus strains in Caco-2 and MA104 cells, with inhibition percentages being reached that were similar to those obtained at the maximum concentration for each individually considered peptide, except for the sum of VP6 (IV) and VP4 (II) peptides in MA104 cells when infected with YM rotavirus. Results obtained with cells treated with control III plus VP6 (IV) peptide confirmed that such peptides' half-maximum inhibition concentration produced infection with values close to 50% inhibition with the three viral strains being studied.

MA104 and Caco-2 cells were incubated with the VP4 (II) peptide plus DLPs or VP6 peptide (IV) plus DLPs at half-maximum inhibition concentration and then infected with Wa, RRV or YM rotaviruses (Fig. 5). DLPs plus VP4 (II) peptide blocked infection by Wa, RRV and YM strains; infection inhibition was 81%, 82% and 72%, respectively, for MA104 cells (Fig. 5A) and 82%, 78% and 76%, respectively, for Caco-2 cells (Fig. 5B). The Wa viral strain was slightly more sensitive to infection inhibition in Caco-2 cells than RRV and YM, similar to the results obtained in the experiment taking the sum of peptides.

DLP plus VP6 (IV) peptide inhibited Wa rotavirus infection by 79% and around 84% by RRV and YM in MA104 cells (Fig. 5C). Maximum inhibition of infection in Caco-2 cells was 91% in the case of Wa, 81% with RRV and 79% with YM

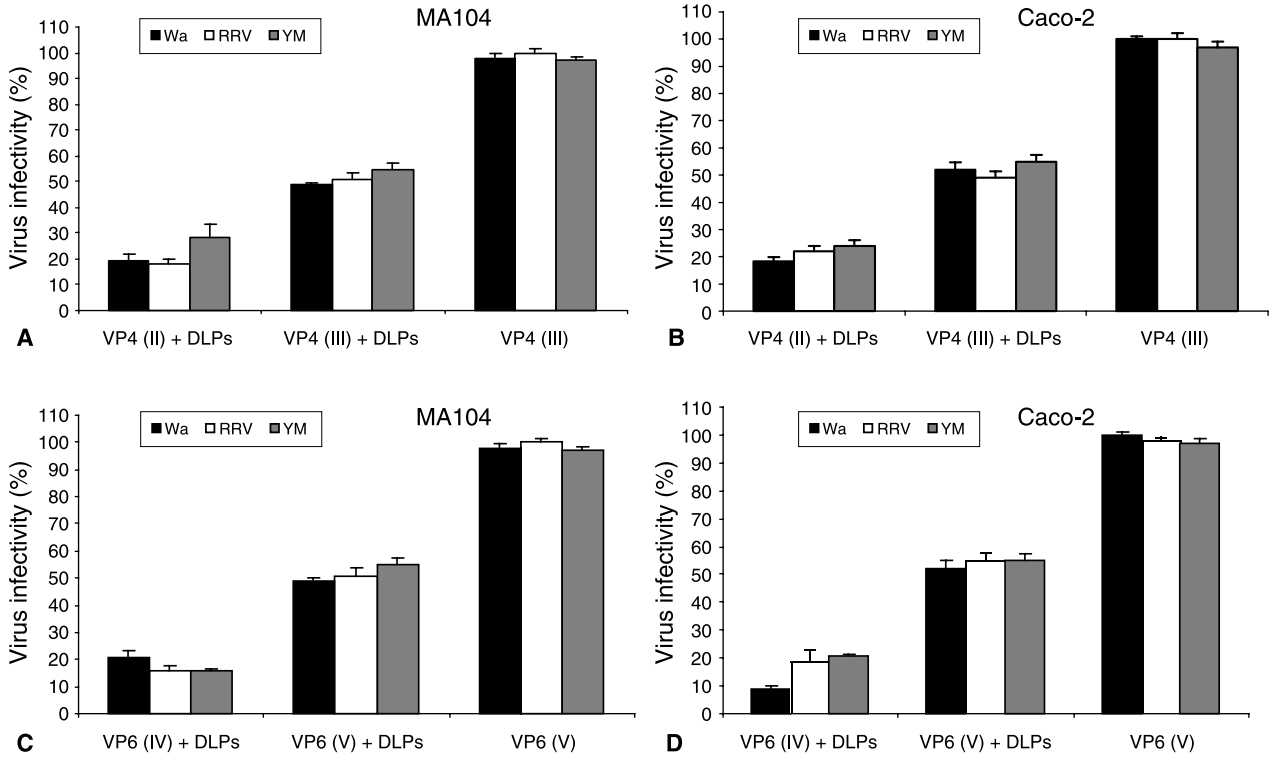

Fig. 5. Effect of synthetic peptide and DLP combination on Wa, RRV and YM rotavirus infectivity. (**A** and **C**) MA104 cells in 96-well plates were incubated with peptide VP4 (II) (0.5 mg/ml) plus DLPs (120 ng/ml) (**A**) and with peptide VP6 (IV) (0.25 mg/ml) plus DLPs (120 ng/ml) (**C**). (**B** and **D**) Confluent Caco-2 cells were incubated with peptides VP4 (II), VP6 (IV) and DLPs as in (**A** and **C**). Peptides VP4 (III) and VP6 (V) at 0.25 mg/ml were used as a control. Cells were then infected with the corresponding rotavirus. Results are expressed as percentage of rotavirus infectivity when MEM without peptides and DLPs was added. Lines over bars represent the results of at least three independent experiments performed in duplicate

(Fig. 5D). The percentage infection inhibition in cell cultures treated with DLPs plus control peptide (III) was approximately one half of that exhibited by all rotavirus strains in the two cell lines tested. No inhibition was observed when only the control peptide was added. The results obtained with the two cell lines showed that the combination of DLPs and VP4 and VP6 peptides at half-maximum inhibition concentration had a additive effect, reaching inhibition percentages similar to those obtained in individual application of such inhibitors at maximum concentration. Taken together, these results suggest that synthetic peptides and DLPs could bind to the same cell surface molecule without it being ruled out that an additive inhibitory effect could also imply the presence of more than one pathway, even though these results do not provide direct evidence to support such a hypothesis.

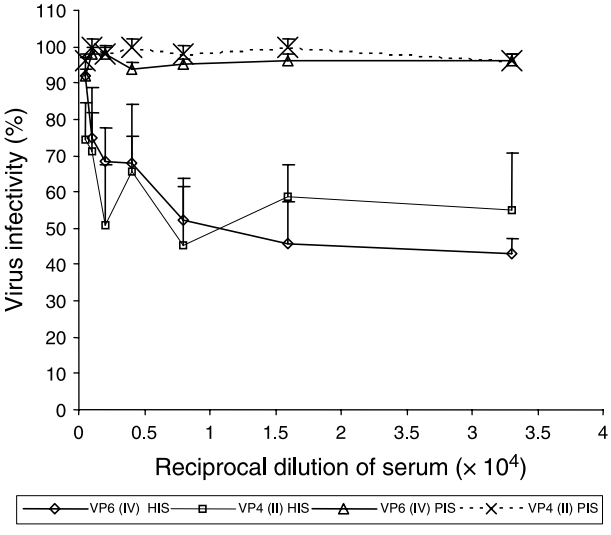

Fig. 6. Effect of antibodies to VP4 (II) and VP6 (IV) synthetic peptides on Wa rotavirus infectivity. MA104 cells in 96-well plates were incubated at 4 °C for 30 min with trypsin-activated Wa TLPs (0.32 μg/ml). Then, hyperimmune serum (*HIS*) against peptides VP4 (II) or VP6 (IV) or the corresponding pre-immune serum (PIS) was added at 4 °C for 30 min. The cells were then incubated for 1 h at 37 °C, washed with MEM and incubated for 12 h at 37 °C. Cells were fixed and immunostained as described in Materials and methods. Results are expressed as percentage infectivity relative to mock-treated cells. Curves represent the results of at least three independent experiments performed in duplicate

VP4 (aa 531–554) and VP6 (aa 280–297) domains are used during rotavirus entry

VP4 (II) and VP6 (IV) synthetic peptides inhibited infection by rotaviruses, suggesting that rotaviruses use these domains during cell entry. To explore this further, hyperimmune sera produced against peptides spanning the amino acid domains 531–554 and 280–297 present in VP4 and VP6, respectively, were used to determine whether serum antibodies were able to block infection. Confluent MA104 cells were incubated at 4 °C for 45 min with RRV rotavirus TLPs and then incubated with different pre-immune or hyperimmune serum dilutions recognizing VP4 (II) and VP6 (IV) peptides (Fig. 6). Hyperimmune sera against VP4 (II) and VP6 (IV) peptides inhibited TLP infection by 45% and 57%, respectively.

Reovirus was used as a control to test the specificity of inhibition by hyper-immune sera directed against synthetic peptides. No effect on reovirus infection was observed when compared to mock-infected cells (data not shown). Pre-immune or hyper-immune serum was added to cell cultures after 1 h pi at 37 °C to evaluate the effect of sera against synthetic peptides on post-entry infection events. Antibodies present in both pre-immune and hyper-immune serum in these assays had no effect on either rotavirus or reovirus infection, since no differences in infection percentages were observed compared to infected cells incubated in just MEM (data not shown). These results, together with those obtained for synthetic peptide inhibition, support the hypothesis that rotaviruses use the 531–554 amino acid domain in VP4 and the 280–297 amino acid domain in VP6 during cell entry.

Indirect ELISA was used to confirm that hyperimmune sera against each of the synthetic peptides recognized their respective antigens. Antibodies produced against the VP4 (II) peptide recognized TLPs, and those produced against VP6 (IV) peptide recognized DLPs (data not shown). Immunocytochemistry and fluorescence assays revealed that hyperimmune sera against synthetic peptides also recognized the viral antigens present in infected cells, although at lower intensity than polyclonal antibodies used against the rotavirus in determining FFU (data not shown). These results indicate that antibodies raised against the synthetic peptides were able to

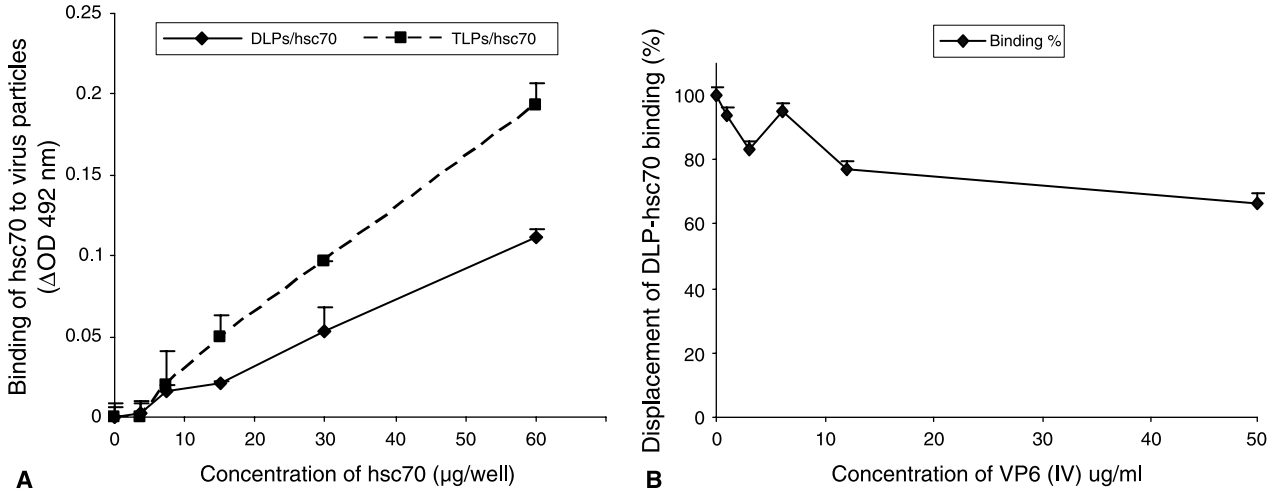

Fig. 7. DLP and TLP binding to hsc70. (**A**) RRV DLPs or TLPs (1 μg/ml) and hsc70 human recombinant protein (3.7–70 μg/50 μl/well) were incubated overnight at 4 °C, and the complex was added to ELISA plates coated with capture polyclonal rabbit serum (1:500) against a mixture of RRV, YM and Wa rotavirus purified particles. Hsc70 was detected by incubating first with a polyclonal goat serum (Santa Cruz SC-1059) and then with an anti-goat-HRP conjugate. Plates were developed with OPD and read at 493 nm. OD values are presented after subtracting control readings. (**B**) RRV DLPs (2 μg/ml) and hsc70 (30 μg/ml) were incubated with peptide VP6 (IV) (0–50 μg/ml) overnight at 4 °C, and the complex was added to ELISA plates as in **A**. Data are presented as percentage displacement of the complex DLP-hsc70 binding. Curves represent the results from at least two independent experiments performed in duplicate; standard deviations are indicated by *bars*

recognize epitopes present in both purified viral particles and infected cell viral antigens. These findings suggest that the 531–554 amino acid domain in TLP VP4 and the 280–287 amino acid domain in DLP VP6 are exposed in the respective viral particle and take some part in infection.

DLPs interact with hsc70

Hsc70 has been detected on the surface of MA104 and Caco-2 cells, and its association with TLPs has been determined by ELISA [17]. As shown above, inhibition of rotavirus infection produced by DLPs suggested that these viral particles bind to cell-surface molecules, thereby interfering with virus entry into the cell. In this context, DLP-conforming VP6 binding to recombinant hsc70 was assessed by using a sandwich-type ELISA in which DLPs were added to wells previously coated with polyclonal serum against rotavirus particles. The results showed hsc70-concentration-dependent interaction with DLPs and TLPs, this being stronger with the latter particles as indicated by reaction readings at 493 nm (Fig. 7A). The specific interaction between hcs70 and DLPs was further confirmed by a displacement test in which peptide VP6 (IV) (50 μg/ml) was able to reduce hsc70-DLP interaction by about 30%. Higher VP6 (IV) concentrations did not show any additional effect on the interaction (Fig. 7B). These *in vitro* results suggest that VP6 (IV) binds specifically to hsc70.

Discussion

The mechanism of rotavirus entry into a cell seems to be a multi-step process in which sequential interactions between proteins on the outermost virus capsid and several cell surface molecules take place during adherence and cell penetration [1, 22]. Hsc70, a protein belonging to the hsp70 family, has been proposed as being a post-adherence receptor for both neuramidinase-sensitive and neuraminidase-resistant rotavirus strains [17], whereas VP7 mediates the binding of rotaviruses to integrin αvβ3 [42]. The activity of a VP5* peptide (aa 642–658), previously identified as being a hsc70 binding site [41], was not compared to the activity of the peptide identified in the present work.

The hypothesis was advanced that the synthetic peptides VP4 (II) (aa 531–554) and VP6 (IV) (aa 280–297) compete for cell-surface molecules, which are used by the rotavirus during entry into cells, to explain the observed inhibitory effect on infection produced by these peptides. It is still not known which molecules on the cell surface are involved in virus binding; however, since these synthetic amino acid sequences were selected for their potential binding to hsc70 [34], they probably bind to the cell through this protein. The methods used in this work did not lead to directly determining whether these amino acid regions were used by the rotavirus to bind to cell receptors during infection. However, when antibodies produced against such synthetic peptides were incubated with cultures where the virus had previously adhered to cells at 4 °C [15–17], they were able to block infection. This suggested that rotaviruses could be using the VP4 531–554 and the VP6 280–297 amino acid sequences during their entry into target cells.

On the other hand, the lack of effect on reovirus infection of both the synthetic peptides and the antibodies directed against them also suggests that VP4 531–554 and VP6 280–297 amino acid sequences are specifically involved in rotavirus infection. The fact that neither synthetic peptides nor antibodies raised against them affected reovirus infection (requiring a clathrin-mediated endocytosis pathway for entry) supports the hypothesis that synthetic peptides probably hamper rotavirus entry into cells and that these synthetic peptides do not inhibit rotavirus infection through a toxic effect on host cells. Synthetic peptides more effectively inhibited RRV and YM than Wa infection in MA104 cells; however, the Wa strain was the most susceptible to inhibition in Caco-2 cells. This could suggest that there are different numbers and arrays of receptor molecules forming an entry pathway for the viral strains as used in the different cell lines. This does not exclude the fact that viral structural proteins involved in recognition and binding can also present differences amongst strains. The possible significance of a difference of human and animal cell hsc70 as rotavirus receptor is not clear at present.

Several studies have suggested that protection against rotaviral infection in animals achieved by VP6-based vaccines has been due to the secretion of IgA antibodies (in intestinal lumen), thereby preventing the liberation of messenger RNA contained in DLPs [2, 13]. A protection-inducing effect has been observed in other studies in which rats have been inoculated with synthetic peptides comprising VP6 amino acids 289–302, 266–295 and 283–307 (including the sequence used in our study) when challenged both orally and intra-nasally with EDIM rotavirus [6–8]. The 280–297 amino acid region present in VP6 (IV) synthetic peptide forms the G-H loop of the H domain in VP6. The R residue, located at position 296 within this region, makes contact with trimers in VP7, while the N residue, located at position 286 in the external layer of the β-pleated sheets denoted as CHEF in VP6, makes contact with VP4 [24]. The location of the 280–297 amino acid sequence in the virion is probably crucial for facilitating contact with hsc70 during the conformational changes the viral particle appears to undergo when binding to the host cell.

The inhibition of infection produced by DLPs could be explained by their binding to hsc70, and this is supported by the fact that these particles specifically bind to the hsc70 recombinant protein *in vitro* and that the VP6 (IV) synthetic peptide reduces hsc70-DLP interaction, as determined by ELISA. These two facts prove the interaction between these two proteins. The DLP/TLP molar ratio assays ruled out a steric effect as being a possible explanation for inhibition of infection by DLPs and suggested that DLP binding to receptor molecules on the cell surface interfered with specific TLP binding, most probably during a second step during rotavirus binding to cells. Moreover, the nonlinear inhibition profile obtained with the DLP/TLP molar ratio assays suggested that a specific and complex interaction could be taking place between cell-surface molecules and virions.

Assays aimed at blocking infection by the combination of VP4 (II) and VP6 (IV) synthetic peptides or the combination of either VP4 or VP6 synthetic peptide and DLPs, showed an additive effect on rotavirus infection in MA104 and Caco-2 cells when RRV and Wa rotavirus strains were used. Although no titration experiments were con-

ducted, this result could suggest that synthetic peptides bound to the same receptor molecule, but it is also possible that the additive inhibitory effect observed involved more than one pathway. On the other hand, a non-additive inhibitory effect was observed with synthetic peptides when MA104 cells were challenged with the YM rotavirus strain. This could suggest that the YM strain in MA104 cells was using different pathways that were susceptible to being differentially hampered by the synthetic peptide used.

Previous work has shown that rotaviruses interact with hsc70 through a domain located in the VP5* subunit of VP4 (aa 642–658) [43]. An additional VP4 domain (aa 531–554) was found in the present work to interact with hsc70, adding new insight regarding the key role that hsc70 seems to play in rotavirus infection. It is tempting to speculate that VP4-hsc70 interaction would favor conformational changes in viral proteins, which could enable the exposure of VP6 domains (such as amino acids 290–297), permitting binding to hsc70. This work has also reinforced the hypothesis that hsc70 functions as one of the receptor molecules during rotavirus entry into cells, suggesting that hsc70 makes contact with VP4 from the outermost capsid and subsequently with VP6 in the virus particle's intermediate capsid during the entry process. Rotaviruses thus seem to make multiple use of hsc70, involving VP4 and VP6. This, in addition to multiple integrin use [1, 9], could extend the range of possible alternative cell-binding and entry mechanisms, enabling rotaviruses to efficiently infect different hosts and cell types.

Acknowledgements

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