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Rotavirus receptor proteins Hsc70 and integrin αvβ3 are located in the lipid microdomains of animal intestinal cells

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Summary. – Several cell surface molecules such as integrins, sialic acid and Hsc70 have been reported to participate in the process of adsorption and penetration of rotaviruses into cells. Some of them have been found in susceptible cell lines but not in epithelial cells of natural hosts so that their real role in the infection process is unclear. In this study, we attempted to confirm the presence of Hsc70 and integrin αvβ3 in the cytoplasmic membrane of isolated intestinal epithelial cells of pig, mouse and cow. Using immunocytochemistry, immunofluorescence, co-immunoprecipitation, ELISA, Western blot analysis and flow cytometry we established that in these cells, (i) Hsc70 and integrin αvβ3 formed a complex in lipid raft microdomains of the cytoplasmatic membrane and (ii) Hsc70 levels increased after rotavirus infection. These results indicate that these molecules act as receptors of rotaviruses in susceptible cells.

Keywords: rotavirus; Hsc70; integrin αvβ3; receptor; animal cell

Introduction

The Hsc70 protein has been proposed as a co-receptor in rotavirus entry (Gualtero *et al*., 2002, 2007; Zárate *et al*., 2003; López *et al*., 2006). Integrin αvβ3 has been found in MA104 and Caco-2 cells (Guerrero *et al*., 2000a; Isa *et al*., 2008) and it has also been proposed as a rotavirus co-receptor, together with integrins α4β1, and αxβ2 (Coulson *et al*., 1997; Ciarlet *et al*., 2001; Graham *et al*., 2006). The Hsc70 and integrin αvβ3 are known to be located in the lipid raft microdomains of the cytoplasmic membrane (Isa *et al*., 2004). In this study, we attempted (i) to confirm the presence of Hsc70 and integrin αvβ3 in the cytoplasmic membrane of isolated intestinal epithelial cells of pig, mouse and cow and (ii) establish the association of these molecules with the process of rotavirus adsorption and penetration into host cells *in vivo*.

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Abbreviations: βCD = β-cyclodextrine; MOI = multiplicity of infection; OG = β-octyl glucoside; p.i. = post infection

Materials and Methods

Animals and cell lines. Suckling mice (10–12 days old) were obtained from the Instituto Nacional de Salud (Bogotá, Colombia) and sacrificed by cervical dislocation. Suckling pigs (15 days old) and suckling calves (about 2 years old) were kindly donated by the Instituto de Ciencia y Tecnología de Alimentos (ICTA) of Universidad Nacional de Colombia. The MA104 cells were grown at 37°C in Eagle's minimal essential medium (MEM) supplemented with 10% FBS (Gibco), and Caco-2 cells were cultured in Dulbecco's MEM supplemented with 20% FBS.

Intestinal epithelial cells. The tissue suspensions were processed as described previously (Guerrero *et al*., 2010). Suckling pig or suckling calf intestines were placed in iced MEM culture media (Sigma, St. Louis MO) and transported to the laboratory. The organs were washed first with RPMI-1640 (Sigma) without phenol red, and subsequently with the same solution plus 1 mmol/l DTT, penicillin (100 U/ml), streptomycin (0.1 mg/ml) and amphotericin B (Sigma; 0.25 mg/ml).

Lipid raft isolation. This procedure was adapted based on reported techniques (Naoki *et al*., 2008). First, 2×10^7 of MA104, Caco-2 or isolated intestinal villi cells were lysed in 200 µl of lysis buffer. Control cells were pre-treated with 10 mmol/l β-cyclodextrine

(βCD) (Sigma) in PBS (1 hr, 37°C). Sucrose density centrifugation of intestinal villi cell lysates was performed using cell lysates layered on 4-ml continuous 5–30% (w/v) sucrose gradient and centrifuged in a SW60 Sorvall rotor for 16 hrs at 200,000 × g, 4°C. Fractions were collected from the top of the gradient and each fraction was divided into eight aliquots (440 µl each). β-octyl glucoside (OG), Triton X100 and βCD were added to a final concentration of 0.2%, 1% and 10 mmol/l, respectively, into each fraction and fractions were incubated at 37°C for 1 hr.

Immunocytochemical staining. In order to detect integrin αvβ3 and Hsc70 in the cytoplasmic membrane of isolated intestinal epithelial cells, this procedure was modified based on reported techniques (Guerrero *et al*., 2010). Following blocking, polyclonal goat anti-Hsc70 (Santa Cruz, SC, 1059), goat anti-integrin β3 (SC6627) (both 2 µg/ml) or mouse monoclonal antibody (SC7312; 10 µg/ml) was added to isolated intestinal epithelial cells. As a secondary antibody, a donkey anti-goat HRP conjugate (SC2020; 0.13 µg/ml) or a goat anti-mouse antibody was used. The peroxidase activity was detected using 3-amino-9-ethyl carbazole (Sigma), and samples were analyzed under a light microscope (VanGuard).

Immunofluorescence assay and flow cytometry. The procedure was modified from reported techniques (Guerrero *et al*., 2000b). Primary antibodies goat anti-Hsc70 (SC1059) or anti-integrin β3 (SC6627) were added to isolated intestinal epithelial cells (both 2 µg/ml) following blocking. To visualize the signal, donkey anti-goat FITC conjugate (SC2024; 1:350) was added and samples were analyzed under a fluorescence microscope (VanGuard) or in a Cyan Dako flow cytometer.

ELISA. The procedure was performed according reported techniques (Zarate *et al*., 2003). The capture antibody was polyclonal rabbit hyperimmune antiserum anti-Hsc70 or polyclonal guinea pig antibody against rotavirus structural particles (both produced in our laboratory, 1:500). Next, the previously obtained sucrose gradient fractions or lysates of infected cells were added. As a detection antibody, goat anti-integrin β3 (SC6627 0.2 µg/ml) or rabbit anti-rotavirus (1:3,000) antibody was added. Finally, the secondary antibody – donkey anti-goat (SC2020) or anti-rabbit HRP conjugate (SC2317) – was added (both 0.133 µg/ml). The ELISA was developed using an OPD system and examined at 492 nm in a Stat Fax 303/Plus ELISA reader. Non-infected cell lysate (for rotavirus detection) or no lysate (for Hsc70 detection) was used as a negative control. The same procedure was then repeated with capture and detection antibodies reversed.

Co-immunoprecipitation. Two×10^7 MA104 cells were lysed in 500 µl of the lysis buffer used in the lipid raft isolation and divided into five aliquots. Laemmli buffer ×2 was added to the first aliquot; the second contained only lysis buffer; 10 mmol/l βCD was added to the third aliquot and 0.2% OG was added to the fourth and fifth aliquots. Next, the second, third and fourth aliquots were incubated with anti-Hsc70 hyperimmune serum (5 µl/100 µl of lysate), and the fifth aliquot was incubated with an irrelevant hyperimmune serum. Except for the first aliquot, all aliquots were immunoprecipitated. The lipid raft microdomains were isolated as described above. The fractions were incubated with hyperimmune anti-Hsc70 serum (5 µl/100 µl of lysate, 37°C, 1 hr) and co-immunoprecipitated. Samples from both procedures were analyzed by SDS-PAGE electrophoresis and Western blot.

Western blot. Proteins separated by SDS-PAGE were transferred to a polyvinylidine fluoride (PVDF) membrane. Membranes were blocked, incubated with goat anti-Hsc70 antibody (SC1059, 0.2 µl/ml) and afterwards with a donkey anti-goat HRP conjugate (SC2020 0.13 µl/ml). The peroxidase activity was developed using luminol (Santa Cruz). Subsequently, the membranes were incubated with goat anti-integrin β3 antibody (SC6627; 0.2 µg/ml) and an anti-goat HRP conjugate (SC2020). Again, the peroxidase activity was developed using luminol.

Rotavirus infection of MA104 cells and isolated intestinal epithelial cells. The cells were infected according to a previously described technique (Guerrero *et al*., 2000b, 2010). The cells were incubated with trypsin-activated virus-cell lysates of rotavirus strain Wa (G1, P1A) at MOI of 1 for MA104 cells and MOI of 5 for the isolated intestinal epithelial cells. The cells were harvested 12 hrs post infection (p.i.), incubated with rabbit anti-rotavirus antiserum (1:3,000) and goat anti-rabbit HRP conjugate (SC2317) was added. The peroxidase activity was detected using 3-amino-9-ethyl carbazole. Subsequently, the cells were incubated with goat anti-Hsc70 (SC1059 2 µg/ml) and anti-rabbit FITC conjugate (SC2024, 1:350). In order to correlate Hsc70 expression with the rotavirus infection in MA104, the cells were cultured and harvested at 0, 2, 4, 6, 8, 10, 12, 14, and 16 hrs p.i., lysed with RIPA buffer and analyzed using capture ELISA.

Results

Heat shock protein Hsc70 and integrin αvβ3 are present in the lipid raft microdomains of isolated intestinal epithelial cells, forming a protein complex

The immunofluorescence (Fig. 1a), immunocytochemical (data not shown) and flow cytometry tests indicated that the isolated suckling mouse intestinal epithelial cells expressed both Hsc70 and integrin αvβ3 in the cytoplasmic membrane. Integrin was present in a greater percentage of isolated non-permeabilized intestinal epithelial cells (30%) than Hsc70 (about 13%). The Hsc70 protein was only present in 40% of the isolated intestinal epithelial cells that were made permeable (Fig. 1b), which suggests that not all cells express quantities of protein detectable with this technique. It was not possible to detect this protein in cow or pig isolated intestinal epithelial cells, because they formed clusters that blocked the flow system of the cytometer. The difficulty encountered in obtaining the biological material made it impossible to conduct ad-

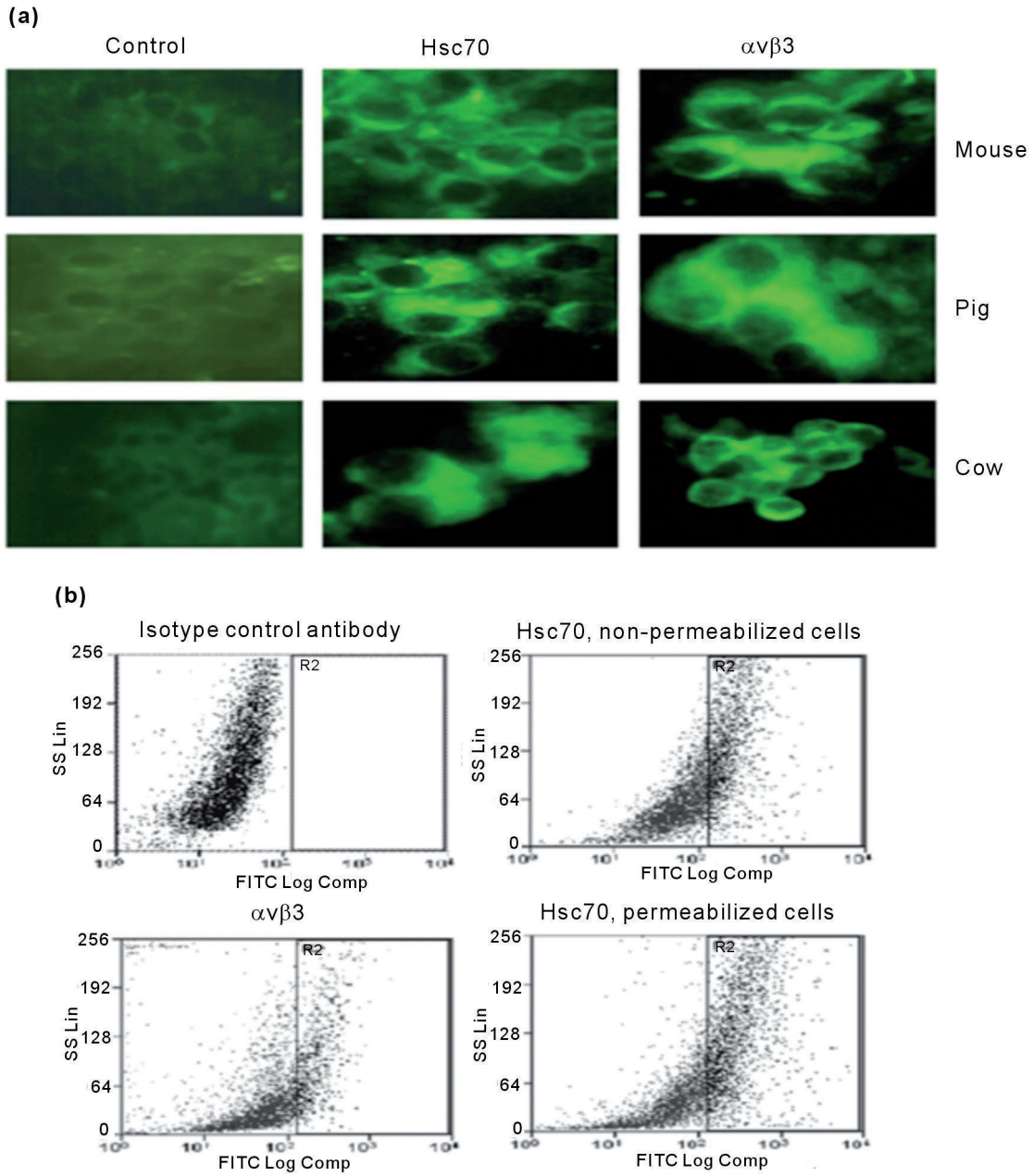

Fig. 1

Hsc70 and integrin αvβ3 were detected in the cytoplasmic membrane of animal intestinal epithelial cells

(a) Immunofluorescence; (b) Flow cytometry.

ditional tests in order to standardize the technique with these cells.

After determining that Hsc70 and integrin β3 are present in the cytoplasmic membrane of isolated intestinal epithelial cells, we wanted to establish whether they are located in the rafts. In sucrose density centrifugation of cell lysates, integrin αvβ3 was distributed along the density gradient, and its presence in the low-density fractions (6, 7, and 8) indicated that it was in the rafts (Fig. 2) from isolated intestinal epithelial cells of suckling and adult mice (data not shown), suckling calf and suckling pig. The MA104 and Caco-2 cell lines were used as controls; the results obtained with these cell lines agree with previous report (Isa *et al.*, 2004).

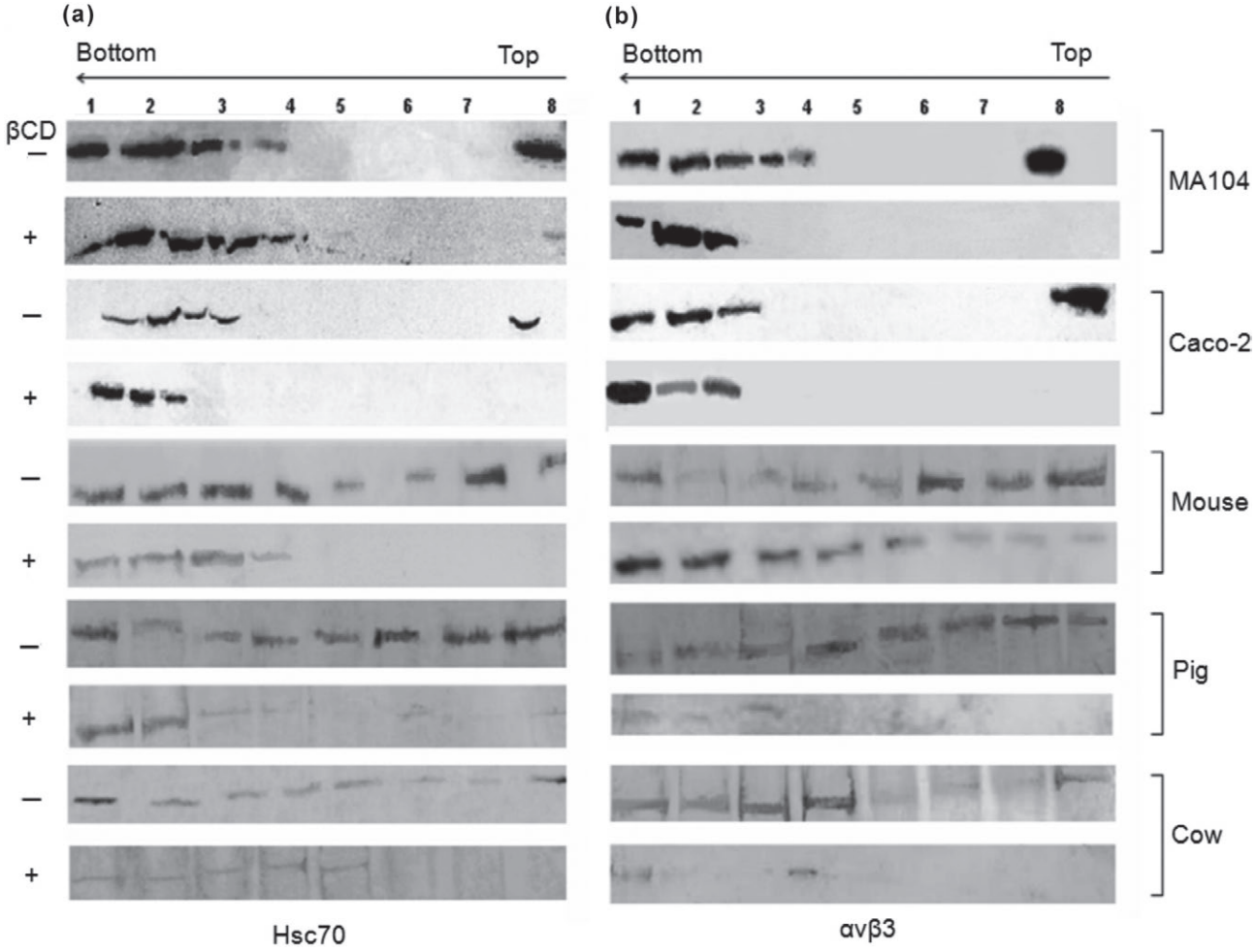

Fig. 2

Hsc70 and αvβ3 were present in the lipid raft microdomains of the cytoplasmic membrane

Western blot analysis of Hsc70 (a) and αvβ3 (b) of lipid raft microdomains from cell lysates treated or not treated (+/-) with βCD. 1–8 = individual fractions from density gradient centrifugation.

In order to determine whether or not Hsc70 and integrin αvβ3 were associated in a complex within the microdomains, we lysed the cells in 1% Triton X100 at 4°C, immediately added anti-Hsc70 antibody and checked for integrin β3 by co-immunoprecipitation. The protein bands observed in the lysate were very dim (Fig. 3a, line 2) or simply absent (results not shown). When OG, a lipid-dissolving detergent, was added to the lysate, the protein bands were clearly observed (Fig. 3a, line 4). These results indicate that the antibodies could not reach the proteins when they were part of the rafts during immunoprecipitation. Therefore, in order to analyze the sucrose gradient fractions by ELISA and Western blot, OG, Triton X100 and βCD were added into each recovered fraction, and fractions were incubated at 37°C for 1 hr with the aim of disaggregating the lipids and disassembling the rafts. The Western blot results showed that both proteins co-immunoprecipitated, suggesting that they were associated in a complex in the microdomains. The ELISA results supported this notion (Fig. 4). Similar results were obtained when either anti-Hsc70 (Fig. 3b) or anti-integrin β3 antibody (data not shown) was used for the co-immunoprecipitation tests. In addition, similar results were also obtained when the sandwich-ELISA plate was coated with hyperimmune anti-Hsc70 capture antibody, or with goat anti-integrin β3 antibody. These results were reproduced in isolated intestinal epithelial cells of young cow, pig and suckling and adult mice (data not shown), and in MA104 and Caco-2 cell lines (Fig. 4).

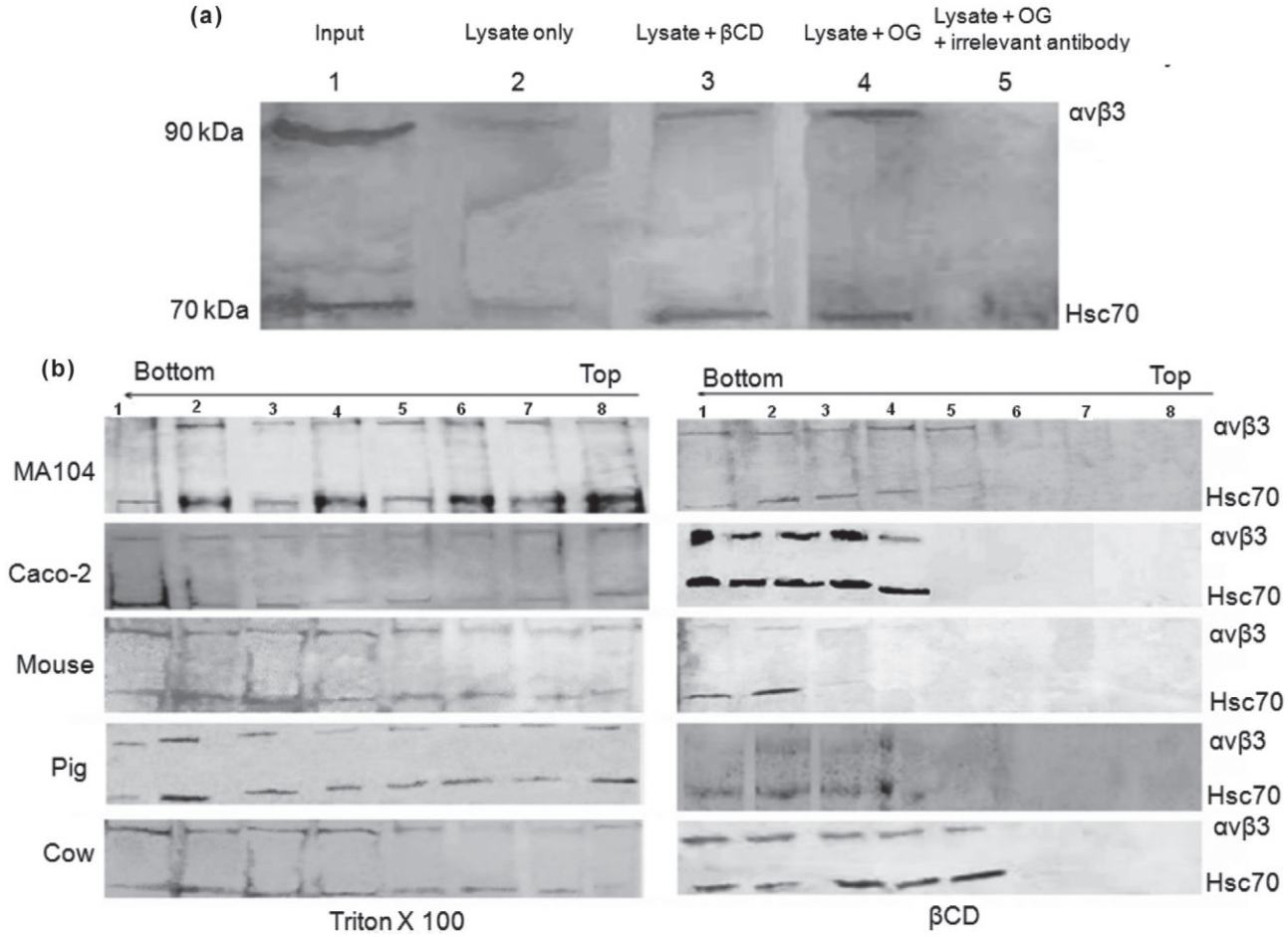

Fig. 3

αvβ3 and Hsc70 formed a complex in the lipid raft microdomains. Western blot analysis of Hsc70 and αvβ3

(a) Co-immunoprecipitation of the MA104 cell lysates treated with lysis buffer only, without density gradient fractions; (b) Co-immunoprecipitation of the density gradient fractions (1–8) of lysates of various cells.

Protein Hsc70 expression increases in MA104 cells and isolated suckling mouse intestinal epithelial cells after rotavirus infection

In order to detect the expression of Hsc70 in cells infected with rotavirus, we used the immunofluorescent staining. Cells infected by rotavirus (stained) as well as uninfected cells (unstained) were observed in both isolated intestinal epithelial cells and the cell lines (Fig. 5a). Infected MA104 cells showed a greater level of fluorescence than the uninfected cells on the same slide and the control (uninfected) slide. A positive correlation was observed in these cells between the intensity of rotavirus infection and Hsc70 expression (Fig. 5a). In the ELISA, Hsc70 was expressed in the infected cell lysate at a three times higher level at 16 hrs p.i. compared to the uninfected cell lysate at 0 hr (Fig. 5b). The increase in the amount of the virus as the infection time progressed correlated with the increase of Hsc70 level (Fig. 5b). This suggested that the rotavirus required this chaperone during its infection process.

Discussion

Several proteins participate in the process that enables rotaviruses to enter cells (Isa *et al.*, 2008). However, until now, research on rotavirus receptor proteins has only been carried out on MA104 and Caco-2 cell lines using rotaviruses adapted to them (Isa *et al.*, 2004). The fact that the fluorescence detection of the membrane protein was only possible with high antibody concentrations (2 μg/ml for anti-Hsc70 and 10 μg/ml for anti-β3) suggests low protein levels, as was reported for integrin αvβ3 (Conrad *et al.*, 1993). The

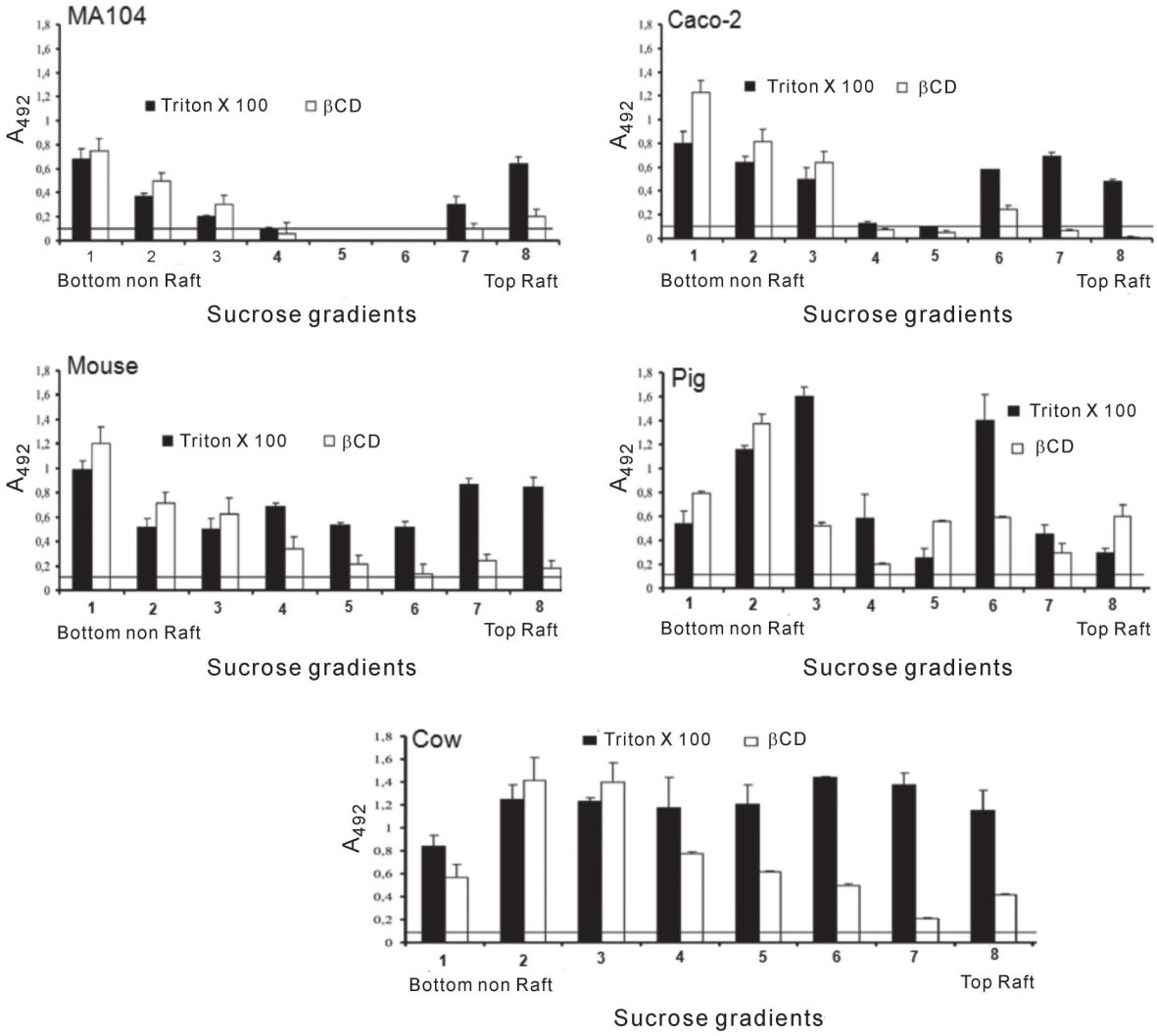

Fig. 4

αvβ3 and Hsc70 formed a complex in the lipid raft microdomains

ELISA of sucrose density gradient fractions (1–8) of lysates of various cells treated with Triton X100 or βCD.

technique used in this work did not allow to determine the real concentration of the proteins in the cytoplasmic membrane of isolated intestinal epithelial cells, MA104 and Caco-2 cells. Flow cytometry of isolated suckling mouse intestinal epithelial cells showed that the expression of the integrin αvβ3 in the cytoplasmic membrane was higher than expression of Hsc70. Nevertheless, the percentage of isolated intestinal epithelial cells that expressed these proteins was relatively low, probably as a result of differential expression in the cells of intestinal villi or of maturation processes.

The distribution of proteins in the sucrose gradient indicates that Hsc70 and αvβ3 were located in the lipid raft microdomains of suckling mouse, cow and pig isolated intestinal epithelial cells, as was found in MA104 cells (Isa *et al.*, 2004). The integrin αvβ3 co-precipitated with Hsc70 when immunoprecipitated by the specific Hsc70 antibody, and they remained together in the capture ELISA after cholesterol was removed with βCD and the lipids were disaggregated with OG and Triton X100. This indicates that they were associated in a complex within the microdomains. Based on the correlation between viral antigen detection by the immunocytochemical analysis and Hsc70 fluorescence detection, our results suggest a connection between the increase in Hsc70 and rotavirus infection in the MA104 cells.

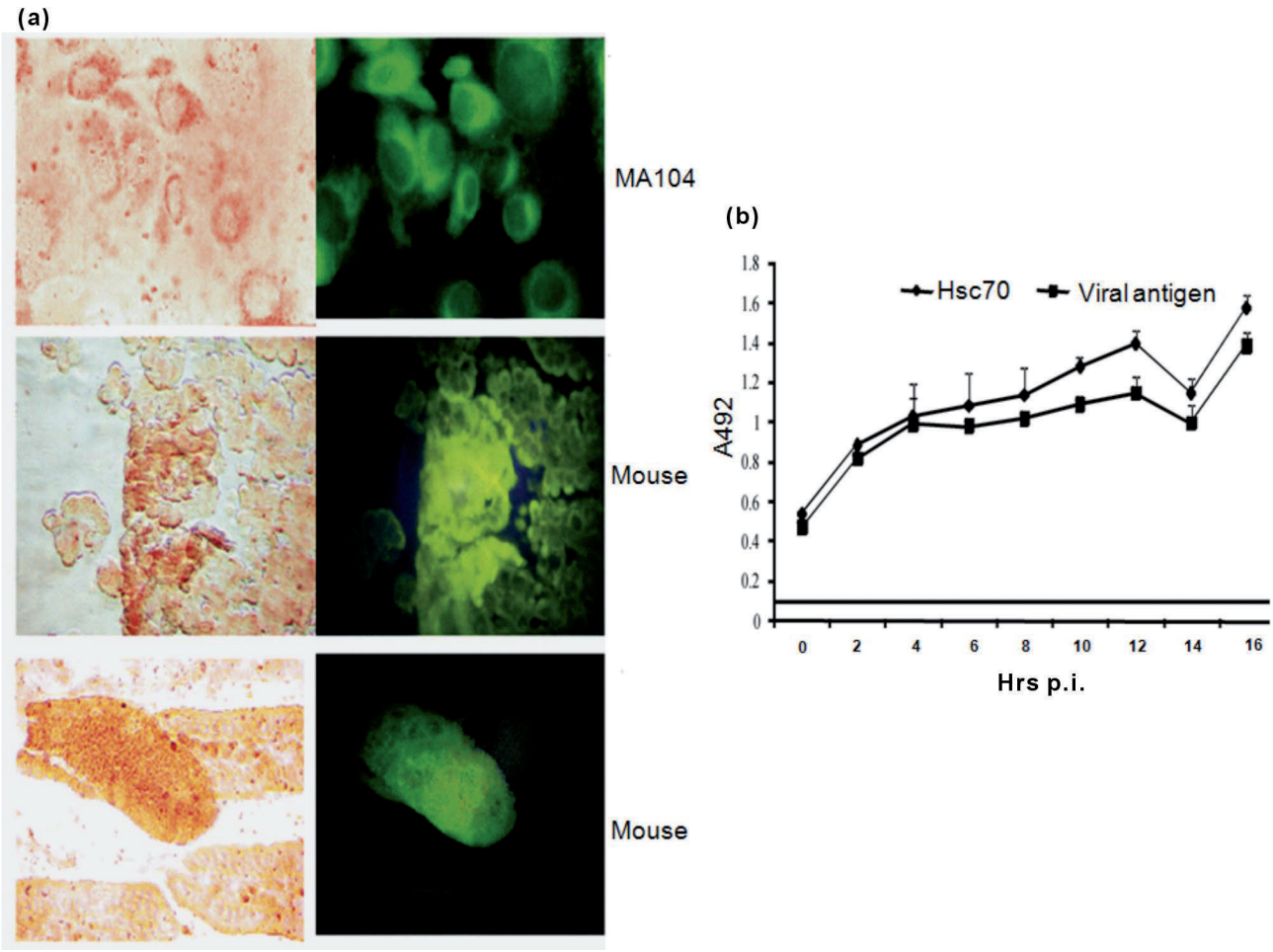

Fig. 5

MA104 and murine intestinal epithelial cells overexpressed Hsc70 after rotavirus infection

(a) Immunofluorescence of Hsc70 and rotavirus antigen in MA104 and murine cells; (b) ELISA of Hsc70 and rotavirus antigen in MA104 cells.

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