HSP70 NEGATIVELY CONTROLS ROTAVIRUS PROTEIN BIOAVAILABILITY IN CACO-2 CELLS INFECTED BY THE ROTAVIRUS RF STRAIN

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ABSTRACT

Previous studies demonstrated that the induction of the heat shock protein Hsp70, in response to viral infection is highly specific and differs from one cell to another and for a given virus type. However, no clear consensus exists so far to explain the likely reasons for Hsp70 induction within host cells during viral infection. We show here that upon rotavirus infection of intestinal cells, Hsp70 is indeed rapidly, specifically and, transiently induced. Using siRNA-Hsp70 transfected Caco-2 cells, we observed that Hsp70 silencing was associated with an increased virus protein level and an enhanced progeny virus production. Upon Hsp70 silencing, we observed that the ubiquitination of the main rotavirus structural proteins was strongly reduced. In addition, the use of proteasome inhibitors in infected Caco-2 cells was shown to induce an accumulation of structural viral proteins. Together these results are consistent with a role of Hsp70 in the control of the bioavailability of viral proteins within the cells for virus morphogenesis.

Key words: Hsp70, rotavirus, intestinal epithelial cells, ubiquitination.
INTRODUCTION

Viral infections of mammalian cells often result in alterations of the synthesis of a group of highly conserved proteins known as heat shock/stress proteins (Hsps). Hsps are ubiquitous molecular chaperones which not only facilitate correct protein folding, assembly, and intracellular transport but also appear to function in intracellular protein degradation (reviewed in (4, 17, 37)). Relationships between the major Hsp, the heat-inducible 72kDa Hsp70, and viral infections have been described in a variety of experimental models with different types of virus and host cells. Cumulative findings indicate that the induction of Hsp70 is not a general response to viral infection but it is instead a highly specific response both with regard to the infecting virus, and to the host cell. A clear example of this concept emerges from the comprehensive study of Phillips et al. (31) on the induction of the 70kDa family of heat shock genes in monkey and human cells infected with different DNA viruses such as adenovirus type 5, herpes simplex virus type-1, simian virus 40, and vaccinia virus. It appeared that only adenovirus type 5, and herpes simplex virus type-1 were able to induce Hsp70 level, and that between three examined Hsps only Hsp70 was induced, thus accounting for a highly specific response. A similar conclusion can be drawn from studies on RNA viruses, though most of these studies have shown a preferential induction of the ER resident Hsps such as the glucose regulated proteins (Grp) Grp78, and Grp94 (reviewed in (34)). Both Grps have been shown to be upregulated in response to infection with rotavirus, a double stranded non enveloped RNA virus that leads to age-dependent diarrhoea (39). Recent studies have identified the constitutive form Hsc70 as a target protein of the multistep process required for the entry of rotavirus into epithelial intestinal cells (15, 21).

Our recent study (3) allowed to detect Hsp70 level in lipid rafts obtained from intestinal epithelial Caco-2 cells, whose well polarized and enterocyte-like phenotype closely corresponds to the \textit{in vivo} natural target of rotavirus (7). In these cells, both rotavirus and
Hsp70 are released before any detectable cell lysis, through an atypical pathway involving lipid rafts (3, 21, 35). In the present work we found that Hsp70 level rapidly increases in response to rotavirus infection in Caco-2 cells and that this virus stress induction is specific to the molecular chaperone Hsp70. In order to clarify the contribution of cellular Hsp70 during rotavirus infection in intestinal epithelial Caco-2 cells, we examine here structural rotavirus protein level in conditions where the intracellular Hsp70 was manipulated. SiRNA-Hsp70 transfection of Caco-2 cells resulted as expected, in a strong decrease of the intracellular level of Hsp70. In that molecular context, the level of the structural rotavirus proteins VP2, VP4 and VP6 was significantly increased 6h post infection of Caco-2 cells, with a correlated increase in progeny virus production. Interestingly Hsp70 silencing was also associated with a strong decrease of the ubiquitination of rotavirus structural proteins. Conversely, inhibition of the proteasomal degradation was also shown to increase the level of VP4, the spike rotavirus protein. Our data are consistent with a model in which the increased level of Hsp70 in rotavirus infected cells may represent a first cellular protective response against infection, through its ability to direct virus proteins towards the ubiquitin-dependent degradation pathway.
MATERIALS AND METHODS

Materials

Trypsin (from porcine pancreas, type IX-S: 13 to 20 Baee units/mg) and SIGMA-Fast o-phenylenediamine dihydrochloride (OPD peroxidase substrate), calpain inhibitor (ALLN), Z-Leu-Leu-Leu-al (MG 132) were from Sigma-Aldrich (Saint Louis, MO). The protease inhibitor cocktail (Complete mini tablets) was from Roche Diagnostics (Mannheim, Germany). The bicinechonic acid reagent (BCA), and bovine serum albumin for protein determination were from Pierce (Rockfort, IL). Acrylamide-Bis-Acrylamide (40% w/v) was from Q-BIOgene (Montreal, Canada). Protein G Sepharose, and Enhanced chemiluminescence (ECL) kit was from Amersham Biosciences (Buckinghamshire, UK). Phosphate buffered saline (PBS), Dulbecco’s modified Eagle medium (DMEM), foetal calf serum (FCS), non-essential amino acids (100X), and antibiotics were from Invitrogen (Paisley, U.K). When not mentioned, reagents were obtained from Sigma Aldrich.

Antibodies used included: monoclonal anti-Hsp70 (SPA-810), polyclonal anti-Hsp70 (SPA-812), monoclonal anti-Hsc70 (SPA-815), monoclonal anti-Hsp90 (SPA-835), monoclonal anti-Hsp110 (SPA-1103) (all from StressGen); monoclonal anti-VP4 from mouse ascite fluids (clone7.7) raised against VP8*, the N-terminal cleavage product of VP4 (5, 9, 10, 29, 40), monoclonal anti VP2 (clone E22), monoclonal anti-VP6 (clone RV133), and polyclonal antirotavirus RF strain (3161) that recognizes several structural viral proteins including VP4, VP2, and VP6 (kindly provided by Jean Cohen), and horseradish peroxidase conjugated secondary antibodies (Rockland, Gilbertsville, PA, and Jackson Immunoresearch, West Grove, PA).

The pCMV-Hsp70 plasmid was kindly provided by Pr. R.I. Morimoto (Northwestern University, Evanston, IL). The pCMV-Neo plasmid control was from Stratagene (La Jolla,
CA). SiRNA-Hsp70 duplex was from Eurogentec (Liege, Belgium), and siRNA scrambled was from Dharmacon Research (Lafayette, IL).

Cell culture

Experiments involved the human colon adenocarcinoma cell line Caco-2 obtained from the European Collection of Cell Cultures (ECACC, Wiltshire, UK) and routinely cultured in DMEM supplemented with 20% FCS, 1% penicillin/streptomycin, and 1% non-essential aminoacid solution in an air/10%CO$_2$ incubator at 37°C. Cells were cultured for 21 days, daily changed(42), and used at passages between 40-60.

Infection

Rotavirus strain RF P6[1] and G(6), a French isolate of bovine rotavirus (isolated from calf faeces) (24), was grown in embryonic African green monkey kidney cells (MA-104) obtained from ECACC. Cells were maintained in DMEM medium supplemented with 10% FCS and antibiotics, infected at low multiplicity, incubated for 72 h until cellular lysis (by freezing and thawing 3 times) to achieve complete virus release. Extracted viral preparations were titered by plaque assay as previously described (30) and used to infect Caco-2 cells at a multiplicity of infection of 1 or 10 plaque forming units per cell (pfu/cell). For infection and in absence of transfection, Caco-2 cells cultured for 21 days were incubated in serum-free medium overnight before exposure to 10 pfu/cell of previously trypsin activated RF rotavirus strain (0.44 µg/ml trypsin, 30 min, 37°C). Following 1 h adsorption to the cell surface at 37°C, the virus inoculum was removed, the cells were washed twice and the infection was continued for the indicated times in fresh medium containing trypsin (0.44 µg/ml). The time of virus removal was taken as time zero in all experiments.
Preparation of cell lysates

Adherent cells were washed in PBS and scraped in cold lysis buffer (TNE: 50 mM Tris pH 7.5, 150 mM NaCl, 1mM EDTA) containing the protease inhibitor cocktail (Roche Diagnostic GmbH, Mannheim, Germany).

Enzyme-linked immunosorbent assay for Hsp70, and VP4

Enzyme-linked immunosorbent assays (ELISA) were performed to determine differences in Hsp70 and VP4 levels. For Hsp70, the monoclonal anti-Hsp70 antibody (SPA 810, 5 µg/well, overnight, 4°C) was used as capture antibody to coat MaxiSorp 96 wells plates (Nunc, Roskilde, Denmark). The wells were postcoated with PBS containing 0.1% Tween-20, and washed before addition of cell lysates or hrHSP-70 standard (StressGen, Victoria, Canada) (6.25-400 ng/ml, diluted in PBS containing 0.1% Tween-20), and incubated for 2 h at room temperature. The polyclonal anti-Hsp70 (SPA 812) was used as detection antibody, diluted to 1/400. The reaction was visualized and quantitated by using colour development followed by horseradish peroxidase-conjugated anti-rabbit IgG (diluted to 1/20,000) substrate OPD. The reaction was stopped by adding 50 µl H₂SO₄ 1M. Absorbance was read at 492 nm in an automated ELISA reader (Labsystems Multiskan MS, Vantaa, Finland). For VP4, a similar system was developed using monoclonal anti-VP4 (7.7, diluted to 1/200) as capture antibody and polyclonal anti-RF (3161, diluted to 1/200) as detection antibody.

pCMV and siRNA plasmids and transfection of Caco-2 cells

The sequence of the 21-nt siRNA-Hsp70 was chosen based on the rules proposed by Elbashir et al. (11) and was designed for interfering with the two mRNA coding Hsp70 (GenBank accession number NM_005345 for HSPA1A and BC057397 for HSPA1B, nucleotide positions 65-85 from the ATG) but not with the two Hsc70 mRNA variants (GenBank accession number NM_006597 and NM_153201). SiRNA-Hsp70 sense: 5’-CACCGCAAGGUGGAGAUA-dTdT-3’; siRNA-Hsp70 antisense: 5’-UGAUCUCACCUUGCCGUG-dTdT-3’. Transfections were performed using Amaxa Nucleofector™ apparatus (Amaxa, Cologne, Germany) according to the manufacturer’s instructions. Caco-2 cells were
harvested at 50-70% cell confluence and resuspended in electroporation buffer T from nucleofector Kit (Amaxa GmbH, Koeln, Germany) at a final concentration of 5x10^6 cells/ml (12). 0.1ml of cell suspension was supplied with either 5µg of pCMV-Neo control or 5µg of pCMV-Hsp70 or 100nM of siRNA scrambled or 2µg of siRNA-Hsp70, and was then transferred into 2mm electroporation cuvette, and electroporated. After electroporation, cells were immediately diluted with 1 ml of complete Caco-2 culture medium and distributed into 24 well culture plates at a density of 5x10^5 cells per well. After 24 hours, medium was changed and cells were cultured 96 hours until experiments. Transfected Caco-2 cells were infected with rotavirus RF bovine strain (1 pfu/cell) either 6h or 18h before the end of the 96 h siRNA-Hsp70 or pCMV recovering period.

**Immunoprecipitation of ubiquitinated proteins and viral protein immunoblot analysis.**

Cell homogenates corresponding to 40µg proteins prepared from Caco-2 cells infected for 6h with rotavirus (1 pfu/cell), were pre-cleared with 70 µl of protein-G Sepharose, 4h at room temperature under rotation. Pre-cleared supernatants were subjected to ubiquitinated protein immunoprecipitation with 2 µl of anti-ubiquitin antibody over-night at 4°C under rotation. Immunopurified ubiquitinated proteins were isolated by centrifugation (8000 x g, 5 min), and 3 washes with lysis buffer. Bound proteins were detached from protein-G Sepharose by boiling for 3 min with sample buffer (62.5 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 0.1% bromophenol blue, and 5% 2-β-mercaptoethanol). Detached samples were loaded onto 7.5% SDS-PAGE gels, subjected to electrophoresis, and electrotransferred to nitrocellulose membranes. After blocking for 1 h at room temperature (polyvinylpyrrolidone 1%, PBS Tween® 0.1%) membranes were sequentially incubated with mouse primary antibodies (dilution 1/5,000): anti-VP4 (clone7.7), anti-VP2 (clone E22)(33) or anti-VP6 (clone RV133)(22), and with the convenient secondary peroxydase-conjugated antibody.
**Additional methods**

**Protein assay**

Proteins were determined using the detergent compatible BCA reagent (Pierce, Rockford, IL) and BSA as standard, according to the manufacturer.

**Blot quantification**

Protein spot levels were determined by using scion® quantification software (www.scioncorp.com)

**Statistics**

Data are presented as mean ± SEM values, and comparisons were performed using the Wilcoxon’s signed rank test. A p value ≤ 0.05 was considered significant.
RESULTS

Rotavirus infection induces a specific increased level of Hsp70 in Caco-2 cells

We initially investigated the impact of viral infection on the level of the stress-inducible protein, Hsp70. Caco-2 cells were infected with the RF strain (10 pfu/cell). Cell extracts were prepared at selected times post infection (p.i.) ranging from 1 to 20 h. As shown in figure 1, Hsp70 level measured by ELISA significantly and rapidly increased in Caco-2 cells following infection, suggesting the building of a stress response. Following infection of Caco-2 cells with rotavirus, trace amounts of the spike protein VP4 were previously observed by immunoblot as early as 3h p.i. (35). This was confirmed here by a quantitative ELISA measurement of VP4 showing detectable levels of VP4 as soon as 1h p.i. while levels were substantial at 5h p.i., and continued to increase until 20h p.i.. In Caco-2 cells, thermal stress has been previously shown to induce an increased level of Hsp70 with a similar kinetics as rotavirus infection though maximal level reached values approximately 6-fold higher (data not shown and (3)). These observations were confirmed here by immunoblot at 6 and 20h p.i., and a representative experiment is shown in figure 2. It should be noted that this Hsp response was specific since the level of other cytosolic heat-stress-inducible proteins (Hsp90 and 110) was not increased upon viral infection (figure 2). Our results also indicated that these three Hsps escape the generalized shut off of host transcription observed during rotavirus infection (32).

In order to analyse the putative function of this specific Hsp70 induction during rotavirus infection, transfection experiments were performed to manipulate the intracellular level of Hsp70.

Specific modulation of cellular Hsp70 amounts after Caco-2 cell transfection with specific siRNA
Figure 3a showed Hsp70 level in Caco-2 cells after siRNA electroporation as a function of time post transfection. The effect of siRNA was time dependent and the lowest amount of Hsp70 was observed after 96h. Then Hsp70 cellular level escaped steadily the silencing by siRNA-Hsp70. Immunoblot analysis (figure 3b) and blot quantification (figure 3c) confirmed the low Hsp70 level at 96h as compared to the siRNA control (“scrambled”) and the specificity of Hsp70 silencing since no change were observed in the amount of either Hsc70, the constitutive form of the heat-stress protein, or actin.

Conversely, transient transfection of pCMV-Hsp70 resulted after 96h in a 4-fold increase of Hsp70, without effect on the constitutive Hsc70 (figure 4a and 4b). Kinetics and dose-effect experiments have been carried out with pCMV-Hsp70 and indicate that it was not possible to obtain a higher over-expression of Hsp-70 in Caco-2 cells because of its toxic effects on cells (not shown).

These transfection models were subsequently used to study the link between rotavirus infection and Hsp70 in intestinal epithelial cells. It should be mentioned that Hsc70, implicated during rotavirus entry steps into the host cells (15), remained mostly unchanged upon Hsp70 down modulation.

*Decrease level of Hsp70 results in increased level of structural viral proteins in infected cells.*

The most striking result observed upon Hsp70 silencing in infected Caco-2 cells (1 pfu/cell, 6h) was a significant increased level of rotavirus structural proteins (figures 5a-5b): VP4 level levels increased by 250%, VP2 by 360% and VP6 by 280%.

Accordingly Hsp70 silencing resulted in an increased virus morphogenesis. This was tested by measuring the progeny virus production 18h post infection in Caco-2 cells transfected with Hsp70 siRNA for 96 hours. Virus production measured by plaque assay increased 8 fold as compared with control cells (figure 5c). This significant increase of virus production
correlated with the increased level of the structural virus proteins, which was of same order of magnitude.

A parallel experiment was performed in which Caco-2 cells were transiently transfected with pCMV-Hsp70 for 90h (resulting in increased Hsp70 level, see figure 4) and subsequently infected by rotavirus (1 pfu/cell, 6h). Interestingly no significant changes were observed, in the amount of VP2, VP4 and VP6 as seen in figure 6a and 6b. Over-expression of Hsp70 did not induce significant change in neo-virus production (figure 6c).

*Increase amounts of viral proteins correlate with their decreased ubiquitination*

It has been established that Hsp70, together with Hsp60 and Hsp40 is directly involved in the transfer of misfolded proteins to the proteasome machinery, through an ubiquitination-dependent process (13). Thus, Hsp70 depletion of Caco-2 cells should result in an increase level of viral proteins due to the lack of an efficient system to transport proteins to sites of degradation. To check this hypothesis, experiments were performed in which siRNA-Hsp70 transfected Caco-2 cells were infected (1 pfu/cell, 6h) and the ubiquitination of viral proteins was measured.

Cell homogenates were submitted to immunoprecipitation with an anti-ubiquitin antibody, and viral proteins were separated by electrophoresis and revealed by immunoblot using antibodies against these structural viral proteins. As illustrated in figure 7 (a and b), depletion of cellular Hsp70 was associated with a decrease of ubiquitinated structural viral proteins VP2, VP4 and VP6 in infected Caco-2 cells at 6h post infection. This result is in good agreement with the idea that Hsp70 may direct viral protein to an ubiquitin-dependent degradation process.

*Proteasome inhibitors increase VP4 level in Caco-2 cells*
To support this hypothesis, we tested whether two proteasome inhibitors, namely ALLN and MG132, might interfere with the level of one of the structural viral protein, VP4 in infected Caco-2 cells. These cells were treated with the inhibitors for the last hours of infection at 12h p.i., i.e. when VP4 synthesis was efficient. As shown in the blots (figure 8), the use of these inhibitors induced an increase of the spike protein VP4 (3 folds with ALLN, and 2.3 folds with MG132 as compared to vehicle DMSO), indicating that VP4 was a regular and natural substrate of the proteasome pathway.
DISCUSSION

This work demonstrates that the level of the major inducible heat shock protein, Hsp70, rapidly and transiently increases upon rotavirus infection of intestinal cells. The present results also suggest an active role for this protein in the control of the level of viral structural proteins and rotavirus morphogenesis through an ubiquitin-dependent process. Other heat shock proteins have already been shown to be involved in rotavirus infection. The 70kDa heat shock cognate protein, Hsc70, whose level is not modified upon rotavirus infection, has been shown to play a role in the entry process of the virus (15, 41) thanks to its well known involvement in the clathrin coated vesicle formation process (18). Previous works have shown that the level of other members of the Hsp super-family, namely Grp78 and Grp94, also increased upon rotavirus infection of MA 104 cells (8, 39), but the exact function of this phenomenon has not been further documented.

Interestingly, other groups have also analyzed the level of Hsp70 in rotavirus infected cells using either transcriptomic (8) or biochemical (39) approaches and do not found significant changes in Hsp70 level. However both sets of results may be reconciled by considering two important factors: the kinetic of Hsp70 level after rotavirus infection and the cell lines used to evaluate Hsp level. In our hands, the rapid and transient increase of Hsp70 level in rotavirus infected Caco-2 cells was an early event (detected as soon as 2h p.i.). In a previous study using a microarray approach on rotavirus infected Caco-2 cells, the authors have shown that Hsp70 mRNA levels do not change, either at 1h or 16h p.i. (8). It is interesting to note that our present results show that Hsp70 only transiently increased between 2 and 7h at the protein level, which is compatible with the microarray data. In another work the level of two other Hsps, namely Grp78 and Grp94, were shown to increase 5h p.i. in MA-104 cells (a non-polarized, non-differentiated monkey kidney cell line) using bi-dimensional gel electrophoresis (39). The authors indicate that Hsp70 level remained essentially unchanged.
However, it has to be pointed out that these two cell lines strongly differ, regarding rotavirus cell cycle, since an efficient rotavirus production followed by cell lysis occurs after 6-10 hours in MA104 cells (21), whereas progeny virions are released at the apical membrane of Caco-2 cells through an atypical trafficking process and without cell lysis only 15-18h p.i. (21). Thus we favour the idea that, in Caco-2 cells, the rapid and transient induction of Hsp70 synthesis is the consequence of rotavirus entry into Caco-2 cells and likely reflects the generation of an adaptive response of the host cell to resist the viral attack. This conclusion is strongly supported here by the observation that silencing Hsp70 level resulted in an increased rotavirus morphogenesis.

A surprising observation was that a forced over-expression of Hsp70, following pCMV-Hsp70 transfection, doesn’t result in significant decrease of viral protein synthesis or virus morphogenesis, contrary to what can be expected from the above conclusion. Over-expression of Hsp70 primarily resulted in a deleterious effect on Caco-2 cell survival. This is likely due to the fact that Caco-2 cells already express rather high basal level of Hsp70 (200ng/mg of proteins). This is expected from an intestinal cell line whose function is to serve as a first line of defence against a large panel of "physiological" stresses (23). Over-expression of Hsp70 in this context will increase interactions of this molecular chaperone with a number of signalling molecules including cell cycle regulators (36) and cell death regulators (1) and may explain why this is toxic for these particular cells. We think that Hsp70 over-expressing Caco-2 cells are no more capable to construct an adaptative response to rotavirus infection, thus indicating that there must be a relatively small concentration window in which Hsp70 levels may be confined.

It is well established that Hsp70 exerts a chaperone function that drive newly synthesized proteins either to a stabilized conformation or to the cellular degradation machineries. While the possibility that Hsp70 could be beneficial for the host has been suggested (2, 34) a pro-
viral role for Hsp70 and other related chaperones has been also described in different models. Evidence that Hsp70 may be involved in virus replication and the assembly of progeny virions has been provided in vaccinia virus (20) adenovirus poliovirus and coxsackievirus B (14, 26, 27). Host Hsp70 is also involved in the assembly of pre-initiation complexes to the origin of DNA replication in human papillomavirus (25). The 70kDa Hsp family from both eukaryotes and prokaryotes was suggested to promote viral capsid assembly in polyomavirus (6). Of note, most of these studies reflect a relatively rapid effect of Hsp70 that preferentially binds nascent viral proteins, mainly from the outer layer, namely external capsids or spikes, and is involved in early steps of the viral replication cycle.

Our results favour the idea that Hsp70 may serve as host factor able to control the amount of viral protein available for virus morphogenesis. Indeed, Hsp70 depletion results in increased viral protein amounts and increased virus morphogenesis that correlate with decreased ubiquitination.

It has been previously shown that several molecular chaperones and particularly Hsp70 are directly involved during the protein ubiquitin tagging process leading to proteasome-dependent protein degradation pathway. Indeed, through its C-terminal EEVD motif, Hsp70 can interact with the protein CHIP, a protein known to favour interactions of protein substrates with ubiquitin ligase enzymes (28). This correlates well with the present results that show that, during rotavirus infection, the major viral structural proteins are ubiquitinated and that this is strongly dependent on the level of cellular Hsp70 level. As a consequence of rotavirus protein ubiquitination, a degradation of these proteins is observed. Indeed, poly-ubiquitination of proteins is known to be associated with their degradation through the proteasome (16). However it remains to explore the exact ubiquitination state of viral proteins and their precise destination since ubiquitination is a multifunctional process that may be involved in various protein degradation pathways (38) as well as intracellular
targeting pathways. More recently it has been proposed that the mono-ubiquitination of proteins may result in their targeting to the endosomal/lysosomal compartment where either degradation or further processing may occur (19). We show here that proteasome inhibitors, in condition in which Hsp70 level remains unaffected, induce an increase of VP4 level. This is in good agreement with the idea that Hsp70 may be involved in an ubiquitin dependent degradation pathway. Whether Hsp70-dependent ubiquitination of rotavirus structural proteins is only involved in this degradation pathway or may also play an additional role in targeting or assembly of progeny virions remains to be clarified. Further studies will tried to delineate how Hsp70 may be involved in the effective control of the bioavailability of rotaviral proteins.
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FIGURE LEGENDS

Figure 1: Rotavirus infection increases Hsp70 level in a time-dependent manner

Hsp70 (white and black histograms) and VP4 (grey curve) level was analysed by ELISA as detailed in materials and methods. Caco-2 cell homogenates were harvested at selected times during the course of infection (1h-20h) (10pfu/cell) in infected cells (black histograms) or in non-infected cells (white histograms). Data are expressed as mean ± SEM n=4 (*: p<0.05). Note that the increased level of Hsp70 is only transient and that VP4 starts to be detectable as soon as after 2h p.i.

Figure 2: Rotavirus infection specifically increases Hsp70 level

Caco-2 cells were infected with RF rotavirus stain (10pfu/cell) for 6h or 20h. Cells were harvested and cell lysates were analysed by immunoblot using monoclonal anti-Hsp70 (SPA-810), monoclonal anti-Hsc70 (SPA-815), monoclonal anti-Hsp90 (SPA-835), monoclonal anti-Hsp110 (SPA-1103) as described in materials and methods. A representative experiment out of 4 is shown. Note that only Hsp70 level increases whereas Hsc70, Hsp90 and 110 level remain unaffected.

Figure 3: Transient and specific inhibition of Hsp70 in Caco-2 cells

(a) Kinetic of inhibition of Hsp70 protein level after specific siRNA targeting (0-14 days).

A Caco-2 cell suspension of 5x 10^5 cells was supplied with 2µg of siRNA-Hsp70 and was then electroporated. Cells were plated and cultured at indicated times after electroporation. Cells were harvested and lysates were analyzed by immunoblot as described in materials and methods. Hsp70 level levels were quantified by immunoblot scanning. Results of this quantification are mean ± SEM (n=4). Note that the maximal inhibition of Hsp70 level was observed four days after si-RNA electroporation.
(b) Specificity of siRNA-Hsp70.

Caco-2 cells were plated and cultured for 96h in the presence of 100pmoles siRNA-scrambled for 100ml of T solution from Amaza and 5 $10^5$ cells submitted to electroporation and 2µg of siRNA-Hsp70. Cell lysates were harvested and analysed by immunoblot as described in materials and methods, using monoclonal anti-Hsp70 or anti-Hsc70 antibodies. A representative experiment out of four is shown. Only Hsp70 level level was affected by siRNA treatment, whereas Hsc70 level remains unaffected.

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(c) Quantitative analysis of Hsp70 levels in siRNA-Hsp70 transfected cells.

Caco-2 cells were treated as described in fig3b and the resulting immunoblots (four independent experiments) were quantified using immunoblot scanning. Hsc70(white histograms) and Hsp70 (black histograms). *: p<0.05. Note that at that time (four days after electroporation) only Hsp70 level decreased, whereas Hsc70 level remains unaffected.

**Figure 4: Transient specific over-expression of Hsp70 in Caco-2 cells**

(a) A suspension of 5x $10^5$ Caco-2 cells was supplied with 5µg of pCMV-Neo (control) or 5µg of pCMV-Hsp70 and was then electroporated. Cells were plated and cultured for 96h. Cells were harvested and lysates were submitted to immunoblot as described above. A representative experiment out of four is shown.

(b) Quantitative analysis of Hsp70 level levels in pCMV-Hsp70 transfected cells.

Caco-2 cells were treated as described in fig 4a, the resulting immunoblots (four independent experiments) were quantified using immunoblot scanning. Hsc70 (white histograms) and Hsp70 (black histograms). *: p<0.05.

Note that Hsp70 level increases after 96h after pCMV-Hsp70 electroporation, whereas Hsc70 level is not affected.
Figure 5: Effects of siRNA-Hsp70 transfection on the rotavirus protein level (a) (b) and on progeny virus production (c) in infected Caco-2 cells

(a) A cell suspension of 5x 10^5 Caco-2 cells was supplied with 2µg of siRNA-Hsp70 and was then electroporated. Transfected cells were infected with RF rotavirus strain (1pfu/cell) for 6h as described in materials and methods. Cells were harvested and lysates were submitted to immunoblot using monoclonal anti-VP4 (clone7.7), anti-VP6 (cloneRV133) and anti-VP6 (clone RV133) as described in materials and methods. A representative experiment out of four is shown.

(b) Quantitative analysis of VP2, VP4 and VP6 levels in siRNA transfected cells.
Caco-2 cells were treated as described in fig 5a, and submitted to immunoblot analysis (a representative experiment out of four is displayed). VP2 (grey histograms), VP4 (white histograms), VP6 (black histograms).

(c) Quantification of virus production by siRNA-transfected and infected Caco-2 cells. Caco-2 cells were treated as described in fig 5a (1 pfu/cell, 18h infection). Supernatants were tittered by standard plaque assay in MA104 cells as described in materials and methods (22). Results are expressed as pfu/ml and represent mean ± SEM from 6 experiments.

Note that in siRNA transfected Caco-2 cells the level of virus structural protein (VP2, VP4 and VP6) is significantly increased and associated to an increase of virus production.

Figure 6: Effects of pCMV-Hsp70 transfection on the rotavirus protein level (a) (b) and on progeny virus production (c) in infected Caco-2 cells

(a) A suspension of 5x 10^5 Caco-2 cells was supplied with 5µg of pCMV-Neo (control) or 5µg of pCMV-Hsp70 and was then electroporated. Cells were plated and cultured for 90h and then infected with RF rotavirus strain (1pfu/cell for 6h), as described in materials and methods.
Cells were harvested and lysates were submitted to immunoblot as described in fig5b. A representative experiment out of four is shown.

(b) Quantitative analysis of VP2, VP4 and VP6 levels in pCMV-Hsp70 transfected cells. Caco-2 cells were treated as described in fig 6a, and submitted to immunoblot analysis (a representative experiment out of four is displayed): VP2 (grey histograms), VP4 (white histograms), VP6 (black histograms).

(c) Quantification of virus production by pCMV-Hsp70 transfected and infected Caco-2 cells. Caco-2 cells were treated as described in fig5a (1 pfu/cell, 18h). Supernatants were tittered by standard plaque assay in MA104 cells as described in materials and methods (22). Results are expressed as pfu/ml and represent mean ± SEM from 3 experiments.

Note that the over-expression of Hsp70 in Caco-2 cells is not followed by any change in viral protein level nor virus production.

Figure 7: siRNA-Hsp70 effect on the virus protein ubiquitination in infected Caco-2 cells.

(a) A cell suspension of 5x 10^5 Caco-2 cells was supplied with 2µg of siRNA-Hsp70 and was then electroporated. Transfected cells were infected with RF rotavirus stain (1pfu/cell) for 18h as described in materials and methods. Infected lysates were subjected to immunoprecipitation using anti-ubiquitin antibodies, as described in material and methods. Immunopurified ubiquitinated proteins were submitted to immunoblot analysis using anti-VP2, VP4 and VP6 antibodies as described in materials and methods.

(b) Quantitative analysis of ubiquitin labelled VP2, VP4 and VP6 levels in siRNA transfected and rotavirus infected Caco-2 cells. Caco-2 cells were treated as decribed in fig 7a and the amounts of ubiquitinated VP2, VP4 and VP6 was quantified using immunoblot scanning. Control (grey histograms), siRNA scrambled (white histograms), siRNA-Hsp70 (black
histograms). Note that the down expression level of Hsp70 in Caco-2 cells results in significant decrease of the ubiquitination of virus structural proteins.

**Figure 8: Effect of proteasome inhibitors on viral structural protein levels.**

Caco-2 cells infected with RF rotavirus stain (10pfu/cell) for 12h, were subjected to proteasome inhibitor treatment, either ALLN (10µM) or MG132 (10µM), or vehicle (lanes C), the last 2 hours of infection. Cells were harvested and cell lysates were analysed by immunoblot using monoclonal anti-VP2, VP6 and VP4 as described in materials and methods. Note that the treatment with proteasome inhibitors results in a significant increase of viral structural protein levels: VP2 (x2,1 for ALLN and 2,8 for MG132), VP6 (x2,2 for ALLN and 2,4 for MG132) and VP4 (x2,3 for ALLN and x3 for MG132).
Figure 1

Figure 2
Figure 7

(a) Western blot analysis of viral proteins VP2, VP4, and VP6 under control, scrambled, and siRNA-Hsp70 conditions.

(b) Quantification of ubiquitinated viral proteins VP2, VP4, and VP6. The y-axis represents the ubiquitinated viral protein level in arbitrary units.

Figure 8

Protein expression analysis of VP2, VP4, and VP6 under control (C), ALLN-treated, control (C), and MG132-treated conditions.
REFERENCES


