HEAT SHOCK INDUCES PREFERENTIAL TRANSLATION OF ERGIC-53 AND AFFECTS ITS RECYCLING PATHWAY.

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**SUMMARY**

ERGIC-53 is a lectin-like transport receptor protein, which recirculates between the ER and the Golgi complex and is required for the intracellular transport of a restricted number of glycoproteins. We show in this paper that ERGIC-53 accumulates during the heat shock response. However, at variance with the unfolded protein response, which results in enhanced transcription of ERGIC-53 mRNA, heat shock leads only to enhanced translation of ERGIC-53 mRNA. In addition, the half life of the protein does not change during heat shock. Therefore, distinct signal pathways of the cell stress-response modulate the ERGIC-53 protein level. Heat shock also affects the recycling pathway of ERGIC-53. The protein rapidly redistributes in a more peripheral area of the cell, in a vesicular compartment that has a lighter sedimentation density on sucrose gradient in comparison to the compartment that contains the majority of ERGIC-53 at 37°C. This effect is specific, as no apparent reorganization of the endoplasmic reticulum, intermediate compartment and Golgi complex is morphologically detectable in the cells exposed to heat shock. Moreover, the anterograde transport of two unrelated reporter proteins is not affected. Interestingly, MCFD2, which interacts with...
ERGIC-53 to form a complex required for the ER-to-Golgi transport of specific proteins, is regulated similarly to ERGIC-53 in response to cell stress. These results support the view that ERGIC-53 alone, or in association with MCFD2, plays important functions during cellular response to stress conditions.
INTRODUCTION

Cells and tissues sense and respond to environmental stress by activating the expression of stress genes encoding molecular chaperones and enzymes that repair and remove cell damage (1, 2, 3). The Heat Shock Response is the most conserved and efficient defence from stress described in living organisms (1). In normal conditions, mammalian cells express constitutive Heat Shock (HS) genes encoding molecular chaperones, which control the folding and translocation of proteins across membranes of different cell compartments (4). During hyperthermia, exposure to heavy metals, amino acid analogues and several other agents, mammalian cells synthesise large amounts of inducible HS proteins, which remove or refold stress-denatured proteins (1). Higher levels of stress-denaturated proteins act as regulators of HS gene expression by binding the HSp70s, which interact with the HS transcription factors in the cytoplasm (5, 6). Unbound HS factors are then able to move into the nucleus and activate transcription of inducible HS proteins by binding promoter sequences named heat shock elements, HSEs (5, 6). In addition to transcriptional activation, some HS proteins are also preferentially translated in a cap- and 5’ end independent manner, through internal mRNA elements termed Internal Ribosomal Entry Sites, IRESs (7), or by the mechanism of “Ribosome Shunting” (8).

The Endoplasmic Reticulum (ER) shows an organelle-specific response to the accumulation of proteins mis-folded by stress (9). ER resident proteins are
specialised in the Quality Control (QC) of folding of newly synthesised proteins (10). Genes of the QC machinery are under the transcriptional control of an inter-organelle signal transduction pathway termed the Unfolded Protein Response (UPR) (9). UPR is conserved from yeast to man and is characteristically induced and regulated by the increase of unfolded or misfolded peptides into the ER lumen (9, 11). In yeast, the UPR results in the up-regulation of many genes involved in QC and also of Golgi-located glycosylating enzymes (12). Conversely, in mammalian cells little is known about the stress response of protein located in post ER compartments of the secretory pathway.

Several glycoproteins exit from the ER through specific transport receptors (13). ERGIC-53 is a mammalian calcium-dependent lectin and is the most descriptive markers of the ER-to-Golgi Intermediate Compartment (ERGIC) (14, 15), which is composed by vesicular and tubular structures localized in the pre-Golgi region and involved in bidirectional protein transport at the ER-Golgi boundary. ERGIC-53 cycles between the ER and the Golgi complex and exports from the ER a small number of glycoproteins (16, 17). It interacts with cathepsin-Z related proteins and is required for the intracellular trafficking of the lysosomal enzyme cathepsin C (18, 19) and for the efficient secretion of coagulation factors V and VIII (20). Indeed, mutations in the ERGIC-53 gene (LMAN1) are responsible for the inherited bleeding disorder referred to as Combined Deficiency of FV and FVIII (FV/FVIIIID), in which the secretion of FV and FVIII is impaired (21, 22).
However, in about 30% of the FVFVIIID patients, the disorder is generated by mutations in other gene having functional properties similar to ERGIC-53 (21). It has been recently shown that mutations in the Multiple Coagulation Factor Deficiency 2 gene (MCFD2) cause a FVFVIIID phenotype that is indistinguishable from that generated by mutations in LMAN1 (23). Interestingly, MCFD2 encodes a protein that interacts with ERGIC-53 and, thus, it was suggested that the two proteins form a specific cargo receptor complex that is necessary for the ER-to-Golgi transport of specific proteins (23).

The specific function of ERGIC 53 in the export from the ER of several glycoproteins prompted us to investigate its role during cell stress. The evidence presented in this paper shows that both expression level and recycling pathway of the protein are modulated by heat shock. While this work was in progress, it was shown that ERGIC 53 is transcriptionally activated during UPR (24). Our results confirm this observation and, interestingly, show that the accumulation of ERGIC 53 during heat shock is driven only by enhanced translation of its mRNA. Moreover, we found that MCFD2 is regulated similarly to ERGIC-53 in response to cell stress, thus supporting the importance of the role of these two proteins in stress conditions.
EXPERIMENTAL PROCEDURES

Antibodies - The following antibodies were used: rabbit polyclonal antibody anti-ERGIC-53 (α-CT) raised against a synthetic peptide corresponding to the last 12 amino acids of the C-terminal end of the cytosolic domain of ERGIC-53 (immuno-blots, immuno-precipitation and immunofluorescence assays); mouse monoclonal anti-ERGIC-53 antibody (immuno-fluorescence analysis) (25); rabbit polyclonal anti-GM130 (26); rabbit polyclonal anti-giantin (Covance); goat polyclonal anti-Sec23, donkey anti-goat and mouse monoclonal anti-α-tubulin (Santa Cruz Biotechnology); mouse monoclonal antibody recognising MCFD2 (immunoblot and immunofluorescence) (23); rabbit polyclonal anti-calreticulin and rabbit polyclonal antibody anti-HSp70 (Stressgen); peroxidase conjugated anti-mouse and anti-rabbit IgG were (Sigma-Aldrich).

Cell culture heat shock and drug treatment – All experiments were performed with the human hepatoma Huh7 cell line. Cells were routinely grown at 37°C in DMEM, 10% FCS in a humidified atmosphere with 5% CO2. For heat shock experiments actively growing cells were fed with medium pre-incubated at 42°C and transferred to a 42°C preset incubator, while control cells were maintained at 37°C.

Cells were incubated with genistein and quercetin (Sigma-Aldrich) for the same times as HS treatment and control cells were cultured in the presence of the same amount of DMSO (Sigma-Aldrich). Thapsigargin (TG) (Sigma-Aldrich) was
used at concentrations ranging between 300 and 500 nM.

**Western Blot analysis** - Cells were lysed in B-buffer containing 10 mM Tris-HCl pH 7.4, 150 mM NaCl, EDTA 1 mM pH 8.0 and 1% SDS. Proteins were separated by 10% SDS of proteins were transferred and blocked o/n in PBS containing 10% non-fat dry milk and 0.5% Tween-20. Membranes were incubated with dilutions of rabbit polyclonal anti-ERGIC-53, anti-HSp70, anti-MCFD2 and anti-α-tubulin antibody for 2 h. Anti-rabbit or anti-mouse IgG horseradish peroxidase conjugated was used as a second antibody. To visualise bands membranes were developed employing the ECL reaction (Amersham International), which was performed according to the instructions of the manufacturer. In Western Blot (WB) experiments we used the concentration of 10 µg/lane, which was chosen by titrating the amount of protein extract that gave signals in the linear range by the ECL method. Anti α-tubulin antibody was used to normalise for equal amounts of proteins and calculate the relative induction ratio. Densitometry of autoradiographs was performed by the NIH image program and values obtained were the mean±standard deviation of three independent experiments.

**RNA extraction, Northern blot and Real Time RT-PCR analyses** - Total RNA was extracted by using the Trizol (Invitrogen) according to the instructions of the manufacturer. 20 µg/lane of total RNA was fractionated on a formaldehyde-1% Agarose gel and transferred on Hybond N⁺ nylon strips (Amersham International). Filters were hybridised with a [³²P]-labelled human ERGIC-53 SacII/HindIII
fragment (27). [\textsuperscript{32}P]-labelled human HSp70 cDNA probe (Stressgen) was used as marker of HS response. GAPDH transcripts were detected by [\textsuperscript{32}P]-labelled EcoRI/EcoRI human cDNA fragment that was used as equal loading control probe.

Serial dilutions corresponding to 0.02 to 2 µg of total RNA were reverse transcribed (GIBCO/BRL) and Real Time RT-PCR was performed using iCycler Apparatus (BioRad). Forty PCR amplification cycles of 60 s (15 s, 95°C; 45 s, 60°C) were run and amplification rates were monitored by the Sybr Green method. For PCR amplification the following primers were used: GAPDH-forward: GAA GGT GAA GGT CGG AGT C; GAPDH-reverse: GAA GAT GGT GAT GGG ATT TC; ERGIC-53-forward: GGG CAG CAT GGG CAG ATT AC; ERGIC-53-reverse: CAT AGA CGC CTC CAG CAG AGC; GRP94-forward: TCC GCC TTC CTT GTA GCA GAT A; GRP94-reverse: TGT TTC CTC TTG GGT CAG CAA T; MCFD2-forward: TGC ATG ATT ATG ATG GCA ATA ATT T; MCFD2-reverse: CAT TAG TGG TGC CTG TTC ACT CC.

Transfection, radioactive labelling and immunoprecipitation of Huh7 cell extract - For metabolic labelling Huh7 cells were pre-incubated 1 h in methionin / cystein free DMEM, 0.5% FCS. Thereafter, cells were incubated for 2 h in the same culture medium supplemented with 100 µCi/ml [\textsuperscript{35}S]-methionin/cystein labelling mix (Perkin-Elmer). 4x10\textsuperscript{7} cpm of cell lysates were incubated with undiluted polyclonal rabbit anti-ERGIC-53 antibody in ice cold buffer o/n. Cells were
chased for variable times with labeling medium containing 5 fold excess cold cysteine/methionine. Expression vectors carrying the cDNAs encoding CD8α (28) and AP-CD8 (29) were transfected in Huh7 cells cultured as described above. Immunoprecipitation, SDS-PAGE and fluorography, were performed as detailed previously (28).

\textit{Indirect immunofluorescence} - Huh7 cells were grown on glass cover slips. Following incubation at 42°C, cells were fixed with 4% formaldehyde for 20 min at room temperature and permeabilized with 0.1% Triton X-100 in PBS. Only in the case of the goat polyclonal anti-Sec23 antibody the cells were fixed in methanol at -20°C for 1 min, followed by 1 min at -20°C in aceton and 5 min in PBS-glycine 0.1 M at room temperature. Cells were labelled with the appropriate antibody and with fluorescein or Texas Red conjugated secondary antibodies. Cover slips were mounted in Moviol and viewed by epifluorescence on a Zeiss axiomatic photomicroscope with a 63x planar objective. Results of quantitative analysis of ERGIC-53 distribution in normal and heat shocked cells were performed by analysing 150 cells/time point and values obtained were the mean±standard deviation of three independent experiments.

\textit{Electron microscopy} - For conventional thin sections electron microscopy, samples were fixed with 2% glutaraldehyde in PBS buffer at room temperature. Samples were post fixed in 1% osmium tetroxide in veronal acetate buffer (pH 7.4) for 1h at 25°C, stained with 0.1% tannic acid in the same buffer for 30 min at 25°C
and with uranyl acetate (5 µg/ml) for 1 h at 25°C, dehydrated in acetone, and embedded in Epon 812. Thin sections were examined unstained or post-stained with uranyl acetate and lead hydroxide.

Discontinuous sucrose gradients - Cells (7-10 x 10⁷) were homogenised by 10 strokes in a Wheaton glass homogeniser in a buffer containing HEPES/KOH pH 7.3 20 mM, sucrose 120 mM. Post Nuclear Supernatant (PNS) was obtained by centrifugation at 500 x g for 5 min in a micro centrifuge and loaded on the top of a discontinuous sucrose gradient (15, 20, 25, 30, 35, 40, 45, % w/v) made up in the same buffer. The gradient was spun in a SW 50.1 rotor for 1 h at 43,000 rpm in a Beckman ultracentrifuge and 13 fractions were collected from the bottom of the tube with a peristaltic pump. Fractions were TCA precipitated and proteins were separated by SDS-PAGE, transferred to ECL membranes and subjected to Western blot analyses (30).
RESULTS

*Cells accumulate ERGIC-53 in response to HS*- To evaluate the expression level of ERGIC-53 in response to HS, the amount of ERGIC-53 in Huh7 cells cultured for different times at 42°C was measured by Western Blot analysis and compared to the level of HSp70, marker for HS response, and α-tubulin, marker for constitutively expressed gene (Fig. 1). These experiments showed that the amount of ERGIC-53 increased with exposure time to HS, in a similar fashion to HSp70 (Fig. 1A and C). Densitometry of autoradiographs showed that the increase in ERGIC-53 was detectable at 4 h of HS with a relative fold of induction of 2.68±0.25 and that such values remained higher (2.86±0.18) after 8 h of continuous HS. Interestingly, HSp70 presented similar rates of induction (2.6±0.45 and 2.94±0.40) at the same time points. Conversely, between 8 and 16 h at 42°C the level of HSp70 stayed high, while the level of ERGIC-53 returned to steadiness (data not shown).

Accumulation of ERGIC-53 was also measured during the transient HS (Fig. 1, B and D). In these experiments, the cells were first incubated for 30 (data not shown) or 60 min at 42°C, then moved to 37°C and analyzed at different time points. Interestingly, increased amounts of either ERGIC-53 (2.06±0.51) or HSp70 (2.6±0.56) appeared 4 h after exposure to transient HS. The ERGIC-53 level returned normal after 8 h, while HSp70 remained higher at 8 h (1.8±0.2) and returned normal after 12 h exposure to transient HS (not shown). Finally, the cells were fully viable after HS treatment and could be normally cultured and
passaged, and similar accumulation of ERGIC-53 in response to HS was detected in a monkey (CV1) cell line (data not shown).

[Fig. 1]

**Differential regulation of ERGIC-53 mRNA expression in response to HS or UPR** – To test whether the increased amount of ERGIC-53 in response to HS was due to increased levels of ERGIC-53 mRNA, we performed Northern Blot analysis. The results showed that the level of ERGIC-53 mRNA remained unchanged both during prolonged and short exposure of cells to HS (Fig. 2, A and B). As expected, the same RNA sample revealed a higher level of HSp70 mRNA and an unaltered level of GAPDH mRNA, a marker of housekeeping genes (Fig. 2: A and B). Conversely, cells expressed a higher level of ERGIC-53 mRNA in response to the ER calcium ATPase inhibitor thapsigargin (TG), a well-known inducer of UPR that was recently shown to enhance the transcription of ERGIC-53 (24). As shown in Fig. 2C, higher levels of ERGIC-53 mRNA were detected, with maximal intensity between 4 and 8 h of TG treatment. To conclusively prove that ERGIC 53 gene transcription is differentially regulated in response to HS or UPR, we switched to quantitative RT-PCR assays, a more accurate method than Northern Blot. These assays confirmed that ERGIC-53 mRNA was stably expressed under both transient and prolonged HS (Fig. 2D). Conversely, higher levels of ERGIC-53 mRNA, and of the UPR marker GRP94, were detectable after 4 and 8 h of TG
treatment (Fig. 2D).

These results strongly suggested that UPR, but not HS, transcriptionally activates the expression of ERGIC 53. This conclusion was further confirmed by the finding that the accumulation of ERGIC-53 protein at 42°C was not inhibited by quercetin, a drug which blocks the activation of HS transcription factors and therefore the heat induced synthesis of heat shock proteins, or by genistein, which inhibits the up regulation of glucose regulated proteins (GRPs) and heat shock proteins (HSPs) (Fig. 3A and B) (31, 32). Conversely, both drugs were able to inhibit the accumulation of Hsp70 protein in response to HS, and had no effect on the level of expression of α-tubulin (Fig. 3A and B).

Heat shock enhances translation of ERGIC-53 – The results reported above suggest that the accumulation of ERGIC-53 protein during HS could depend on an increased synthesis of ERGIC-53 protein, or a decreased degradation, or both. To address these questions, cells were pulse labelled with [35S]-methionine and cysteine in the presence or absence of HS and the amount of newly synthesized ERGIC-53 measured on SDS-PAGE after immunoprecipitation. As shown in Fig. 4B, increased ERGIC-53 synthesis was observed between the second and the fourth h of continuous HS treatment, as well as between the second and fourth h at 37°C after transient HS (60 min at 42°C). Conversely, no effect was revealed in the
synthesis of tubulin that was used as internal control protein (data not shown). In addition, very similar amounts of ERGIC-53 protein were recovered at the end of 8 h chase performed either at 37°C or at 42°C after a 2 h pulse at 37°C (Fig. 4C). Therefore, the increased level of ERGIC-53 protein in response to HS was the result of enhanced translation of its mRNA.

**[Fig. 4]**

*Analysis of the putative promoter and 5′ untranslated region of ERGIC-53 –*

Our results suggested that the 5′ of the ERGIC-53 gene contained cis-acting elements regulating either transcription by UPR or enhanced translation during hyperthermia. Therefore, we analysed the putative promoter and the 5′ untranslated region of ERGIC-53 found in the human genome database (NCBI ref/NT_033908.1/Hs18_34063). Computer analysis to identify transcription factors binding sites (MatInspector, Genomatix Software Gmbh) revealed that the ERGIC-53 putative promoter (500 nts) contained two CCAAT motifs and five GC boxes (Fig. 5A). The main but not the only CCAAT-binding protein is CBF/NFY, while GC boxes are consensus binding sites for factors of the SP1 family. These sequences are encountered at very high frequency in many eukaryotic promoters, in which they mediate constitutive expression of genes (33, 34). Instead, the transcriptional control of UPR responsive genes requires "ER stress" response elements (ERSEs) (34). ERSEs are structured in a tripartite configuration composed by the CCAAT box followed by a 9 bps spacer sequence containing a GGC triplet.
and a CCACG motif at the 3’ end (35, 36). Interestingly, we found that none of the “CCAAT sequences” found in the putative promoter region of ERGIC-53 gene fulfilled all the criteria expected for a functional ERSE sequence (36). Moreover, as we expected, we found no binding sites for eukaryotic Heat Shock Factors (i.e. Heat Shock Elements, HSE) and, interestingly, we did not find either sequences corresponding to known binding sites for the eukaryotic initiation factor TFIID (TATA box) or classic eukaryotic signals for transcription initiation (37).

We also analysed the ERGIC-53 5’untranslated region (21 nts) that was previously identified (38). Interestingly, sequence analysis revealed a striking complementarity to 18S rRNA (Fig. 5B). In addition, RNA structure prediction analysis (39) showed that the 5’untranslated of ERGIC-53 might be able to form a stable RNA/RNA hybrid with 18s rRNA (Fig. 5B).

Since complementarity with the 18s rRNA is an essential requirement for the preferential translation of viral and cellular genes during down regulation of general translation, we suggest that ERGIC-53 could be preferentially translated by a ribosomal shunting mechanism in response to HS.

_Intracellular redistribution of ERGIC-53 in response to HS_ - To test whether HS could affect the intracellular trafficking of ERGIC-53 we assayed the intracellular localisation of the lectin after different times of incubation at 42°C by indirect immunofluorescence. At 37°C, ERGIC-53 is localised in punctuate structures of variable size, dispersed throughout the cytosol, as well as in the central
Golgi region which is concentrated perinuclearly (Fig. 6A, panel a). Conversely, incubation at 42°C clearly generated a redistribution of ERGIC-53 in the cell periphery and a loss of accumulation in the Golgi region (Fig. 6A, panel b). This redistribution required two hours to be completed, as shown by the time course of its appearance (see Fig. 6B), and occurred in the great majority of the cells exposed to HS. Similar redistribution was observed in another mammalian cell line, CV1 (data not shown). The distribution of ERGIC-53 returned progressively to normal within 12 h incubation of the cells at 37 °C after HS (Fig. 6C). In addition, both the redistribution at 42 °C and the return to normal localization at 37 °C occurred in the presence of the protein synthesis inhibitor cycloheximide (data not shown).

Conversely, we found that the Golgi marker giantin and the ER marker calreticulin, did not change their intracellular localization after 2 h of HS (Fig. 6A, panels c-h), thus suggesting that higher temperature does not affect significantly the structure of the Golgi and ER. Similarly, HS did not alter the localization of the COPII coatamer member Sec23, thus suggesting that HS did not have an effect on the distribution and/or the number of the ER-exit sites.

This conclusion was also supported by the electron microscopy analysis of cells exposed to HS (Fig. 7). In cells incubated for 2 h at 42 °C, the number and the dimension of ER, ERGIC and the Golgi complex were indistinguishable from those displayed by control cells (Fig. 7 and Table I). Moreover, HS resulted in a minor decrease in the number of membrane protrusions on the ER (Table I). Membrane
protrusions represent the most reliable morphological marker to visualize ER exit sites in tissue cultured cells (40), thus we concluded that HS did not have major effect on the ER exit sites.

[Fig. 6]

[Fig. 7]

[Table I]

ERGIC-53 accumulates in a different vesicular compartment in response to HS. We next analysed the sedimentation on sucrose gradient of the vesicular fraction containing ERGIC-53 protein in cells exposed to HS. Post Nuclear Supernatant fractions were obtained from control and HS-treated cells and loaded on the top of discontinuous sucrose gradients (30). The sedimentation profile of ERGIC-53 containing fractions was compared to the profiles of calreticuline, GM130, and Sec23, markers for ER, Golgi complex and COPII/ER exit sites, respectively. This analysis showed that the ERGIC-53 enriched fractions obtained from cells incubated for 2 h at 42 °C sedimented at a lighter density than those from control cells (Fig. 8A). Time course experiments revealed that ERGIC-53 progressively shifted to the slower sedimenting fractions and that this effect was maximal after 2 h of incubation of the cells at 42°C (data not shown). At variance with ERGIC-53, all other markers showed very similar sedimentation profiles before and after HS treatment (Fig. 8, B-D). In addition, the distribution of ERGIC-
53 and of the intracellular membrane markers was similar to that obtained after 1 or 16 h centrifugation (data not shown). Therefore, these findings indicated that upon HS treatment the intracellular pathway of ERGIC-53 is specifically altered, confirming and extending the previous evidence based on immunofluorescence and electron microscopy.

[Fig. 8]

**MCFD2 is regulated similarly to ERGIC-53 during stress**

Given the suggestion that ERGIC-53 may form with MCFD2 a cargo receptor complex for the ER-to-Golgi transport of specific proteins (23), we asked whether MCFD2 expression was regulated similarly to ERGIC-53 during HS or UPR. To verify that, we first performed real time RT-PCR on RNA samples obtained from cells cultured under normal condition or following exposure to TG for 4 and 8 h or HS for 1 and 4 h (Fig. 10 A). Our results showed that MCFD2 mRNA accumulated only in response to TG induction, with a maximal accumulation between 4 to 8 h, thus indicating that, in a way similar to ERGIC-53, UPR, but not HS, was able to activate the transcription of MCFD2. Interestingly, Western Blot analyses revealed that the amount of MCFD2 increased in a manner similar to ERGIC-53 in response to HS. (Fig. 10B). In addition, MCFD2 increased of about two fold at either 8 h continuous HS or after 4 h of recovery from transient HS. Finally, as we might expect, MCFD2 level was increased between 4 and 8 h of TG induction (Fig. 10 B).
Finally, we tested whether HS could have an effect similar to ERGIC-53 on the intracellular distribution of endogenous MCFD2. Thus, we performed indirect immunofluorescence assays (Fig. 9C) At 37°C, endogenous MCFD2 localised at 37°C in punctuate structures of variable size, spotted throughout the cytoplasm and more concentrated in the central Golgi region (Fig. 9C, panel a). This pattern was similar, but not completely coincident with that of ERGIC-53 (Fig. 9C, panel b). Interestingly, the incubation at 42°C clearly generated a broader distribution of MCFD2 throughout the cell, with a loss of accumulation at the Golgi region (Fig. 9C, panel c) very similar to the distribution of ERGIC-53 (Fig. 9C, panel d).

**[Fig. 9]**

**HS does not alter the ER to Golgi anterograde pathway**

To evaluate the effect of heat shock on ER to Golgi transport we analysed the rate of glycosylation of CD8α (41) and the rate of terminal N-glycosylation of AP-CD8 (29). Newly synthesised CD8α is not glycosylated: it acquires the initial O-glycans when it reaches an early Golgi region and completes its glycosylation in the *trans*-Golgi. We previously showed that the per cent of the terminally glycosylated form of CD8α labelled in a 30 min pulse with \[^{35}\text{S} \] methionine/cysteine is a reliable measure of the rate of transport of the protein from the ER to the *trans*-Golgi complex (41). This analysis was repeated on transfected cells incubated at 37°C or for 2 h at 42°C (with labelling performed in the last 30 min of HS). As shown in Fig. 10A and C, HS had a negligible effect on the
maturation of the protein. A similar conclusion was obtained analysing the chimeric protein AP-CD8. This protein has the entire ectodomain of the human alcaline-phosphatase joined to the transmembrane and cytosolic domain of CD8α. Alcaline-phosphatase acquires high-mannose N-glycans in the ER and complextype glycans in the medial-trans Golgi region (42), and the two forms are distinguishable on SDS-PAGE by pulse-chase analysis. We performed similar analysis on transfected cells incubated either at 37°C or at 42°C. As shown in Fig. 10B and D, the timing of conversion of the faster migrating form, containing high-mannose glycans, into the slower migrating, terminally glycosylated form of AP-CD8 was identical if not shorter in the cells exposed to HS.

[Fig. 10]
DISCUSSION

Our investigation of the possible role of ERGIC 53 during HS generated three main findings: i) in response to hyperthermia, cells accumulate ERGIC-53; ii) HS stimulates ERGIC-53 expression by enhancing its translation; iii) HS affects ERGIC-53 intracellular trafficking. Moreover, the main features of ERGIC-53 are also shown by MCFD2, which interacts and shares similar functions with ERGIC-53. Therefore, our findings suggest that ERGIC-53 alone, or in conjunction with MCFD2, plays important function during the cell response to stress conditions. This conclusion is confirmed by the finding that two different signal pathways of cell stress control the expression of ERGIC-53 and MCFD2: the Unfolded Protein Response (UPR) and the Heat Shock Response (HSR). Because our analysis was mostly focused on ERGIC-53, we will for the most part discuss the results obtained with this protein.

In mammalian cells, genes increased by heat have a variety of functions and are mainly localized in the cytosol. Heat induction has been also shown for a very small number of genes localised in the ER: the chaperones of the glucose regulated family (GRP78/Bip and GRP94) and the ER lectin calreticuline (43), which is structurally and functionally similar to ERGIC-53 (44). Conversely, GRP78/BiP, GRP94 and calreticuline are induced also by UPR and participate to the stress response in conjunction with many other inducible ER proteins (34). Therefore, to the best of our knowledge, our data describe for the first time the accumulation in
response to HS of proteins mainly localized downstream the ER in the secretory pathway and with a function different from that of the assisting the folding of newly synthesized proteins.

These results raise the interesting question of how cell stress regulates expression of the ERGIC-53 gene (LMAN1). Recent results showed that the induction of ERGIC-53 during the UPR was dependent on the activation of the ERSE binding factor ATF6α (24) and our results confirm that ERGIC-53 is up-regulated during the UPR. However, we also found that the putative ERSEs present in the 5′ of LMAN1 did not match with any known ATF6α functional binding site. Similarly, only one ERSE-like sequence is present in the promoter region of MCFD2 (data not shown), which could account for the increase of the MCDF2 mRNA in response to TG. Therefore, the mechanisms by which ATF6α regulates ERGIC-53 expression and MCFD2 is induced by during UPR remain to be elucidated. In response to hyperthermia ERGIC-53 and MCFD2 accumulate at a higher rate in both prolonged and transitory HS and in the absence of transcriptional activation. This finding goes well along with the observation that both LMAN1 and MCFD2 do not have HS elements in their putative regulatory region. Moreover, hyperthermia did not affect the half-life of ERGIC-53, thus indicating that during HS, ERGIC-53 maintains its native conformation and therefore its functional properties. In addition, ERGIC-53 showed normal folding at 42°C as revealed by WB analyses of non-denaturing gels, which showed that HS did not affect the
formation of ERGIC-53 dimer or examer (data not shown). Heat shock is a major cell stress, in which a central effect is that the general cap dependent translation is down regulated. This impairment is associated with dephosphorylation of the translation initiation factor eIF4E (45). Conversely, our data clearly show that ERGIC-53 translation is markedly enhanced in response to HS, suggesting that translation of ERGIC-53 mRNAs does not require eIF4F activity. Two different mechanisms have been described to explain the preferential translation of stress genes during HS: translation dependant on Internal Ribosomal Entry Sites (IRES) and Ribosomal Shunting. IRES sequences, identified in many cellular and viral mRNAs, are very diverse and the mechanisms by which they facilitate translation is not fully understood (46). However, it has been clearly shown that IRES-dependent translation is essential during HS to ensure high levels of expression of stress genes like the ER chaperones Bip/GRP78 (7) and the BCL2 associated athanogene BAG1 (47). Interestingly, during the HS response, HSp70s are also preferentially translated, but in a different way, known as Ribosomal Shunting that combines features of both scanning and internal initiation (8). It has been clearly shown that preferential translation of HSp70s mRNA by the Ribosomal Shunting mechanism requires the presence of short sequences, in their 5’ untranslated region, with high complementarity to 18S rRNA (8). Our sequence analysis revealed that the 5’ untranslated of ERGIC-53 shows a significant complementarity to 18S human rRNA, thus suggesting that ERGIC-53 may be translated by Ribosomal Shunting.
Some complementarity is also present in the 5’ untranslated of MCFD2 mRNA (data not shown). Therefore, further work will be undertaken to characterise the molecular mechanism responsible for the transcriptional induction during the UPR and the translational control during HS of ERGIC-53 and MCFD2.

ERGIC-53 is distributed between the central Golgi region and the cell periphery at 37°C in pleomorphic tubulo-vesicular elements, which represent the structural and functional boundary between the ER and Golgi complex called ERGIC. Less informations are available on the distribution of endogenous MCFD2 (23), although our results suggest that intracellular localization of the endogenous MCFD2 is similar to that of ERGIC-53. Current knowledge indicates that ERGIC has a complex architecture. Morphological evidence suggests that it comprises elements close to the ER exit sites, central elements, scattered in the cytosol and not close to the ER or Golgi complex, and late elements, closer to the cis-face of the Golgi complex (26). At steady state, ERGIC-53 protein is enriched in central and late elements, but is also present in the early elements as well as in the ER (15). Moreover, by sedimentation profile analysis, all ERGIC-53 positive elements of the ERGIC appear formed by small tubules and vesicles as well as by large-sized structures (14). ERGIC-53 recycles continuously between ER, ERGIC and the Golgi complex, although its transport pathway has not been elucidated in depth, and its preferential accumulation in distinct elements of the ERGIC is most likely due to a dynamic equilibrium between anterograde and retrograde traffic of the protein.
Our results show that this dynamic equilibrium is specifically altered in response to HS. ERGIC-53 changes its overall distribution, moving to a more peripheral area of the cell, prior to and irrespective of its accumulation in response to HS. In this new location, ERGIC-53 is contained in membrane elements of lighter sedimentation profile on sucrose gradient. Thus the redistribution is not simply due to the spread at the periphery of the cell of the elements that contained the majority of ERGIC-53 at 37°C. Immunofluorescence, EM and sedimentation analysis showing that HS does not alter the ER, the ER exit sites or the Golgi complex support this conclusion. Furthermore, the markers we used to monitor the anterograde pathway from the ER to the Golgi have shown that this transport step occurs at normal rates during HS. Therefore, we postulate that HS interferes with the normal recycling pathway of ERGIC-53, either in the anterograde or in the retrograde direction, or both. HS could induce this redistribution in several ways. It could have a direct effect on the folding of ERGIC-53 itself; alternatively, it may have an indirect effect either on the cellular recycling mechanisms or by modifying the glycoproteins that exit the ER thanks to ERGIC-53. At present, only the first hypothesis seems unlikely because the half-life of the protein does not change during HS, and ERGIC-53 shows normal folding at 42°C.

MCFD2 is regulated similarly to ERGIC-53 and in response to HS redistributed in peripheral structures, which also contain ERGIC-53. It has been already demonstrated that the two proteins, which are both implicated in the
secretion of coagulation factors V and VIII, are also able to interact, in a calcium
dependant manner, in the ERGIC (23). Our results confirm that observation.

Therefore, it would be interesting to study the effect of HS on the secretion rate of
factor V and VIII (or of cathepsin Z), which might be affected by the increased
level and the different distribution of ERGIC-53 and MCFD2.
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**FIGURE LEGENDS**

**Fig. 1**  **Time course of ERGIC-53 accumulation in response to HS.**

**A:** Immunoblot analysis after SDS-PAGE of cell lysates obtained after continuous exposure of the cells to 42°C. Lane 1: control cells; lanes 2, 3, 4: cells incubated 2, 4, 8 h at 42°C respectively. **B:** Immunoblot analysis after SDS-PAGE of cell lysates obtained from transient HS (60 min incubation at 42°C). Lane 1: control cells; lane 2, 60 min HS at 42°C; lanes 3 and 4: cells incubated 4 and 8 h at 37°C after 60 min at 42°C, respectively. **C** and **D:** quantitative analysis of ERGIC-53 and HSp70 accumulation in response to continuous or transient HS, respectively. Values were obtained using the NIH Image program for densitometry analysis of the immunoblots. Relative folds of accumulation were obtained by normalising the signals for α-tubulin (Tub) expression. See experimental procedures for details.

**Fig. 2**  **Effect of HS and UPR induction on ERGIC-53 mRNA accumulation.**

**A, B, C:** NB analysis; **D,** real time PCR analysis. See experimental procedures for details. **A:** Prolonged heat shock exposure. Cells were incubated at 42°C as indicated. **C,** control cells. **B:** Follow up of the response to 60 min HS. Cells were incubated for 60 min at 42°C, then either harvested or incubated at 37°C as indicated. **C,** control cells. **C:** Analysis of ERGIC-53 mRNA accumulation in
the cells exposed to HS or 300 nM TG as indicated. C, control cells. D: The RNA samples analysed in panel C were subjected to quantitative real time RT PCR analysis.

Fig. 3 Effect of quercetin and genistein on the accumulation of ERGIC-53 and HSp70 in response to HS.

Immunoblot analysis after SDS-PAGE of cell lysates obtained before (lanes 1 and 2) and after 4 h exposure to 42°C (lanes 3 and 4) in the absence (-) or in the presence (+) of 150 µM quercetin (panel A), or 200 µM genistein (panel B). Lanes 1: control cells in DMSO; lanes 2, 3, 4: heat-stressed cells for 4 and 8 h respectively at 42°C.

Fig. 4 Increased level of ERGIC-53 synthesis during the HS response.

A: SDS-PAGE analysis of the immunoprecipitated product from parallel cultures of cells pulsed with [35S]-methionine/cysteine mix for 2 hours. PI, pre-immune serum. I, immune serum. B: SDS-PAGE analysis of the immunoprecipitated product from parallel cultures of cells labelled for 2 h 37°C (c); HS, cells labelled between the second and the fourth h of continuous HS treatment; T-HS, cells labelled between the second and the fourth h at 37°C after transient HS (60 min at 42°C). KDa of molecular weight marker are reported to the left.

C: Analysis of ERGIC-53 half-life by pulse/chase assay. Cells were pulsed with
[\textsuperscript{35}S]-methionine/cysteine mix for 2 hours at 37°C, then either harvested and processed for immunoprecipitation (c), or chased for 8 h at 37°C or 42°C as indicated. Arrows indicate the position on the gel of ERGIC-53.

Fig. 5 The ERGIC-53 5’ regulatory region

A: Nucleotide sequence of the putative promoter region of ERGIC-53. CCAAT boxes and GC sequences are represented in bold. Putative transcription factors binding to the regulatory sequences are indicated. B: Predicted structure of the RNA hybrids formed between 18s rRNA and the 5’-UTR of ERGIC-53. Nucleotide complementary are underlined. Numbers represent the position of nucleotides of the 18s human rRNA sequence.

Fig. 6 Intracellular localisation of ERGIC-53 during the HS response.

A: Indirect immunofluorescence analysis of the intracellular localisation of ERGIC-53, giantin, CLR and Sec23 at 37°C or 42°C. Cells were incubated at 37°C (panels a, c, e and g) or for 2 h at 42°C (panels b, d, f and h) and subsequently fixed and permeabilized for immunofluorescence analysis. See experimental procedures for details. B: Time course of the ERGIC-53 redistribution during HS. Histograms represent per cent of cells showing redistribution of ERGIC-53 at different times of HS as indicated. C: Recovery to normal distribution of ERGIC-53 after HS. Histograms show the percent of cells showing normal ERGIC-53 distribution, at
the different times indicated of recovery at 37°C, following 2 h HS at 42°C.

**Fig. 7** Incubation of cells at 42 °C does not alter the morphology of ER, ERGIC and the Golgi complex.

Conventional transmission microscopy of epon-embedded Huh7 cells incubated at 37°C (panels A, C) or 42 °C (panels B, D). The asterisks in A and B indicate ERGIC compartments; G, Golgi complex; NM, nuclear membrane; Nu, nucleus; M, mitochondrion; PM, plasma membrane; Bars, 0.5 µ.

**Fig. 8** Analysis of the vesicular fractions containing ERGIC-53 before and after HS by discontinuous sucrose gradient. Post nuclear supernatant fractions obtained from cells incubated at 37°C or for 2 h at 42°C were analysed by discontinuous sucrose gradient followed by SDS-PAGE and immunoblotting to detect the indicated proteins. The percentage of total protein contained in each fraction is reported on the left scale; on the right scale is the percentage (w/v) of sucrose.

**Fig. 9** Analysis of the MCFD2 expression and intracellular localization in response to HS.

A: Quantitative RT-PCR analysis of the MCFD2 mRNA accumulation in response to HS and UPR. Huh7 cells were exposed to HS at 42°C or 300 nM TG for the times indicated. C, control cells. B: Immunoblot analysis after SDS-PAGE
of cell lysates obtained after continuous exposure of the cells to 42°C for 4 and 8 h or from cells incubated 4 h at 37°C after transient HS (T-HS) for 60 min at 42°C, or from cells exposed to 300 nM TG for the times indicated. C: Indirect immunofluorescence analysis of the intracellular localisation of MCFD2 and ERGIC-53 at 37° or 42°C. Cells were incubated at 37°C (panels a and b, and insets) or for 2 h at 42°C (panels c and d, and insets) and subsequently fixed and permeabilized for immunofluorescence analysis. Double staining was performed by using the monoclonal antibody anti-MCFD2 and polyclonal anti-ERGIC-53 antibody (α-CT) recognizing the ERGIC-53 cytosolic domain.

Fig. 10 Terminal O- and N-glycosylation of reporter proteins are not affected by HS. A: SDS-PAGE analysis of the CD8α forms immunoprecipitated from transfected cells labelled for 30 min with [35S]-methionine and cysteine and incubated at 37°C or 42°C. The letters on the left indicate the migration on the gel of the un-glycosylated (u), initially glycosylated (i) and terminally glycosylated (m) CD8α forms, respectively. B: SDS-PAGE analysis of the AP-CD8 immunoprecipitated from transfected cells labeled for 30 min with [35S]-methionine/cysteine, incubated at 37°C or 42°C, and chased at the same temperatures for the times indicated. m, terminally glycosylated; i, initially
glycosylated AP-CD8 forms, respectively. C and D: densitometry analysis of the results shown in A and B, respectively. In C is reported the percentage of CD8 m form (m/u+i+m); in D is reported the percentage of AP-CD8 terminally glycosylated form (m/i+m).

Table I  HuH7 cells were processed for transmission EM analysis as in Fig. 7. 50 different cell sections from cells incubated at 37 °C or at 42 °C for two hours were analyzed at random to measure: the total length of ER membranes, the total number of ER protrusions, the number and the mean area of ERGIC clusters and Golgi complex stacks.
Figure legend to supplemental files

File n. 1  WB analysis of non-reducing SDS-PAGE of cell lysates obtained from control or HS induced cells for 4 and 8 h as indicated. m: ERGIC-53 monomer; d: ERGIC-53 dimer, e: ERGIC-53 examer. Left numbers indicate the KDa of MW markers.

File n. 2  SDS-PAGE analysis of the immunoprecipitated product from parallel cultures of cells labelled for 2 h 37°C (-); HS, cells labelled between the second and the fourth h of continuous HS treatment. ERGIC-53: lanes loaded with cell lysates immunoprecipitated with the anti-ERGIC-53 antibody α-CT; Tubulin: lanes loaded with cell lysates immunoprecipitated with the antibody recognizing the protein tubulin as internal control.
A

CBF/NFY

gctgggtccagaggtcttagggctagCCAATTcctgtccaccaggtattct
-310

aagaagccggagaagaagctccctgcaggtaaaggaccccacggegcttcccg
-261

cacttgcgcgcgggaagcgaagggtgagttggtagcccctttcccaacccggag
-210

SP1
ggtGGGCGGGGgcggaaggacggccacgcgcccaagaCCCAGCCCc
-168

SP1
cctgcgcgtggcCCCGGCCCacagctgcctgctggccggtgceggecgcgcg
-121

SP1
gaaaggggaggccggGGGCGGGGgctcaccctttgcccttgggggtc
-75

CBF/NFY

cccggggctctgCCAATTcagcgagcgccccttgtggccatcgcgcagca
-27

SP1

tccgcgcccccctctctcctCCCCGCCCTctctggegttccagaatcaagg
+21

B

18s rRNA  3' --- ACAUAUU(168) CGAGAUCCUUAGGUUUC(142) ACCCUCU -5'

ERGIC-53 mRNA  5' - CUCCGCGUUCAGAAUCCAAGAGAUG ------- 3'
Fig. 6A

A

37°C

b

42°C

c

d

ergic-53

Giantin

e

f

CLR

g

h

Sec23
**B** Time course of the ERGIC-53 redistribution during HS

![Graph showing time course of ERGIC-53 redistribution](image)

**C** Recovery to normal ERGIC-53 distribution after HS

![Graph showing recovery to normal ERGIC-53 distribution](image)
Fig. 9 A and B
C

37°C

a

42°C

c

b

d
Table I. Effect of HS on the ER exit sites and ERGIC

| T      | Total length of ER membranes | Number of protrusions on ER membranes | Mean area and number (n) of Golgi complex observed | Mean area and number (n) of ERGIC membranes observed |
|--------|------------------------------|-------------------------------------|---------------------------------------------------|---------------------------------------------------|
| 37°C   | 146 μm                       | 78                                  | 0.6 μm² (50)                                      | 0.3 μm² (12)                                      |
| 42°C   | 156 μm                       | 62                                  | 0.6 μm² (54)                                      | 0.2 μm² (10)                                      |

50 different HeLa cell sections were analyzed at random from cells maintained at 37°C or incubated at 42°C for two hours.
Suppl. File n. 2
Heat shock induces preferential translation of ERGIC-53 and affects its recycling pathway

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