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Proteomic Signatures in Thapsigargin-Treated Hepatoma Cells

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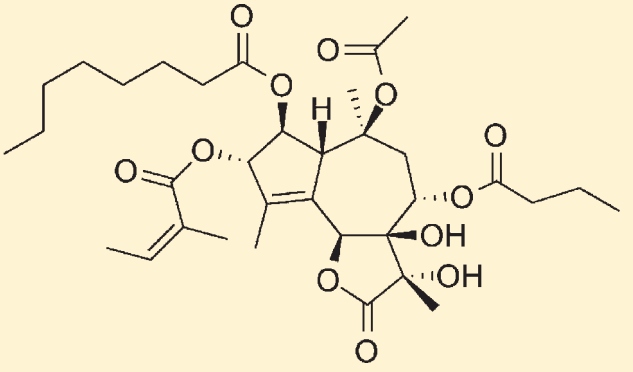
Supporting Information

ABSTRACT: Thapsigargin, an inhibitor of the endoplasmic reticulum (ER) calcium transporters, generates Ca^{2+}-store depletion within the ER and simultaneously increases Ca^{2+} level in the cytosol. Perturbation of Ca^{2+} homeostasis leads cells to cope with stressful conditions, including ER stress, which affect the folding of newly synthesized proteins and induce the accumulation of unfolded polypeptides and eventually apoptosis, via activation of the unfolded protein response pathway. In the present work, we analyzed the proteome changes in human hepatoma cells following acute treatment with thapsigargin. We highlighted a peculiar pattern of protein expression, marked by altered expression of calcium-dependent proteins, and of proteins involved in secretory pathways or in cell survival. For specific deregulated proteins, the thapsigargin-induced proteomic signature was compared by Western blotting to that resulting from the treatment of hepatoma cells with reducing agents or with proteasome inhibitors, to elicit endoplasmic reticulum stress by additional means and to reveal novel, potential targets of the unfolded protein response pathway.



INTRODUCTION

Thapsigargin (TG) is a plant-derived sesquiterpene lactone, which prevents Ca^{2+} transport into the endoplasmic reticulum (ER) by acting as a powerful inhibitor of the ER calcium transporters known as sarcoplasmic- or Ca-ATPases (SERCAs).[1,2] Normally, these enzymes maintain a Ca^{2+} gradient across the ER membrane with concentrations ranging in the cytosol from 10^{-7} to 10^{-6} mol/L and in the ER lumen from 10^{-4} to 10^{-3} mol/L. Thapsigargin modifies this equilibrium by blocking ER Ca^{2+} uptake, which generates the depletion of ER Ca^{2+} stores and simultaneously increases Ca^{2+} level in the cytosol. Variations of Ca^{2+} concentrations within the cytosol directly or indirectly modulate the activity of proteins that control protein folding, chaperone activity, degradation, vesicle trafficking, lipid and physiological processes, such as vision, memory, fertilization, proliferation, transcription, contraction, and immune response.[3] However, ER Ca^{2+} stores are essential for steroid biosynthesis.[4] Thus, decreased calcium level within the ER affects the proper folding of newly synthesized proteins and induces the accumulation of unfolded polypeptides. Cells react to this condition, defined as ER stress, by activating coordinate signal transduction pathways collectively called unfolded protein response (UPR).[5−7] The final purpose of the UPR is to restore protein homeostasis in order to ensure the correct function of the entire secretory pathway.[8]

The UPR is coordinated by three transmembrane proteins, IRE1p, ATF6α, and PERK, which translate, with different mechanisms, stress signals from the ER lumen into the expression of chaperones, enzymes, and ER components required for the processing of newly synthesized proteins.[7−9] In addition, the UPR reduces protein synthesis and enhances the proteasomal-dependent degradation pathway (ERAD).[10] Prolonged ER stress also activates multiple mediators implicated in the execution of cell death, including caspases, Bcl-2 proteins, phosphatases, kinases, and transcription factors.[11,12] The apoptotic events induced by ER stress are under the control of the IRE1p and of the PERK pathways of the UPR.[13−15] However, increased cytosolic Ca^{2+}, as in the case of TG exposure, may directly trigger death effectors and induce mitochondria to apoptotic transitions.[16] Interestingly, ER stress also induces autophagy.[17] Moreover, a number of evidence suggests that ER stress and the UPR are implicated in several common diseases such as diabetes, cardiovascular diseases, viral infection, and tumor growth under hypoxic conditions and in some neurodegenerative diseases.[18]

Thus, in the attempt to obtain a representative picture of the gene expression pattern obtained by the TG induced ER stress, we performed a comparative proteomic analysis by the use of the 2D-DIGE approach combined with mass-spectrometry identification. Comparative analysis of the protein profiles revealed differential expression for a number of proteins, which depend on Ca^{2+} for their function. In the cells exposed to TG, most of the

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proteins showed a reduced expression, while only a few showed increased expression levels. Specific proteins were validated by Western blotting experiments, which were also used to investigate protein expression after treatment with two additional UPR inducers.

MATERIALS AND METHODS

Antibodies. The following antibodies were used in Western blotting experiments: anti-Grp78/BIP antibodies were purchased from Cell Signaling Technology (Danvers, MA); mouse monoclonal anti-ALIX (G-10), goat polyclonal anti-SET (E-15), anticalumenin (H-40), anti-IkB-α (C-15), anti-GAPDH, and anti-α tubulin antibodies were from Santa Cruz Biotechnology (SantaCruz, CA); PLOD3 mouse polyclonal antibody (B01P) was obtained from Abnova (Taipei, Taiwan); and mouse antireticulocalbin 2 (RCN2) antibody was purchased from Abcam (Cambridge, UK).

Cell Cultures and Induction of ER Stress. Human hepatoma Huh7 cells were grown in DMEM containing 4.5 g/L D-glucose, supplemented with 1 mM Na-pyruvate, 2 mM L-glutamine, 10% FCS, at 37 °C, in a humidified atmosphere with 5% CO_2. To induce ER stress, actively growing cells were incubated either with 300 nM thapsigargin (Sigma-Aldrich, St. Louis, MO), 2 mM dithiothreitol (DTT), or the proteasome inhibitor MG132 (10 μg/mL) (Sigma-Aldrich, St. Louis, MO).

Preparation of Protein Extracts and 2D-DIGE Analysis. Huh7 cells were lysed in 10 mM Tris-HCl at pH 7.4, 150 mM NaCl, 1 mM EDTA pH 8.0, containing 1% Triton, on ice, for 20 min. Lisates were scraped off the dishes, and the membrane pellet was removed by centrifugation at 12,000*g* for 15 min. Protein concentration of the supernatant was measured according to the Bradford method (Bio-Rad).

Samples obtained from the lysis of three different preparations of control and Huh7 cells treated with TG for 8 h were precipitated in acetone/methanol (8:1, v:v), for 16 h, at −20 °C, and recovered by centrifugation at 16,000*g* for 30 min, at 4 °C. Proteins were solubilized in 7 M urea, 2 M thiourea, 4% CHAPS, and 30 mM Tris−HCl. Protein concentration was determined using the Bradford method (Bio-Rad Laboratories, Hercules, CA). Before labeling, the pH of the samples was adjusted to a value of 8.5. Each labeling reaction was performed with 50 μg of the protein extracts, in a 10 μL volume, in the presence of 400 pmol of Cy2-, Cy3-, or Cy5-dyes (GE Healthcare, Little Chalfont, Buckinghamshire, UK), for minimal protein labeling at lysine residues. A dye-swapping strategy was used; thus, two Huh7 control lysates were labeled with Cy3, while the third was labeled with Cy5. In a complementary manner, two TG-treated Huh7 lysates were labeled with Cy5, and the third was labeled with Cy3. Three mixtures of the six samples (50 μg each) were labeled with Cy2 dye, as the internal standard required by the 2D-DIGE protocol. The labeling reactions were performed in the dark for 30 min, at 0 °C, and were stopped by the addition of 1 mM lysine. Sample mixtures, including appropriate Cy3- and Cy5-labeled pairs and a Cy2-labeled control, were generated and supplemented with 1% IPG buffer, pH 3−10 NL (GE Healthcare, Little Chalfont, Buckinghamshire, UK), 1.4% DeStreak reagent (GE Healthcare, Little Chalfont, Buckinghamshire, UK), and 0.2% DTT (w/v) to a final volume of 450 μL in 7 M urea, 2 M thiourea, and 4% CHAPS. The mixtures (150 μg of total protein content) were used for passive hydration of immobilized pH gradient IPG gel strips (24 cm, pH 3−10 NL) for 16 h, at 20 °C, in the dark. Isoelectric focusing (IEF) was carried out with an IPGphor II apparatus (GE Healthcare, Little Chalfont, Buckinghamshire, UK) up to 75,000 V/h, at 20 °C (current limit set to 50 μA per strip). The strips were equilibrated in 6 M urea, 2% SDS, 20% glycerol, and 0.375 M Tris−HCl (pH 8.8), for 15 min, in the dark, in the presence of 0.5% (w/v) DTT, and then in the presence of 4.5% (w/v) iodacetamide in the same buffer, for an additional 15 min. Equilibrated IPG strips were transferred onto 12% polyacrilamide gels, within low-fluorescence glass plates (ETTAN-DALT 6 system, GE Healthcare, Little Chalfont, Buckinghamshire, UK). The second-dimension SDS−PAGE was performed using a Peltier-cooled DALT II electrophoresis unit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) at 1.5 W/gel, for 16 h. Gels were scanned with a Typhoon 9400 variable mode imager (GE Healthcare, Little Chalfont, Buckinghamshire, UK) using appropriate excitation/emission wavelengths for Cy2 (488/520 nm), Cy3 (532/580 nm), and Cy5 (633/670 nm). Images were captured in ImageQuant software (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and analyzed using DeCyder 6.0 software (GE Healthcare, Little Chalfont, Buckinghamshire, UK). A DeCyder differential in-gel-analysis (DIA) module was used for spot detection and pairwise comparison of each sample (Cy3 and Cy5) to the Cy2 mixed standard present in each gel. The DeCyder biological variation analysis (BVA) module was then used to simultaneously match all of the protein-spot maps from the gels and to calculate average abundance ratios and statistical parameters across triplicate samples (Student's *t* test).

For preparative protein separations, 600 μg of unlabeled protein samples from control and TG-treated Huh7 cell lysates were used to hydrateT passively two 24 cm strips for first dimension pH 3−10 NL IPG strips (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The first and second dimension runs were carried out as previously described for the analytical separations. After 2-DE, gels were fixed and stained with SyproRuby fluorescent staining (Invitrogen, Carlsbad, CA). After spot matching with the master gel from the analytical step in the BVA module of DeCyder software, a pick list was generated for spot picking by a robotic picker (Ettan spot picker, GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Protein Identification. Picked gel spots were minced and washed with water. Proteins were reduced, S-alkylated, and *in-gel* digested with trypsin, as previously reported.[19] Digest samples were desalted and concentrated on microC18 ZipTips (Millipore Corp., Bedford, MA) using acetonitrile as eluent before MALDI-TOF-MS analysis. Peptide mixtures were loaded on the MALDI target together with α-cyano-4-hydroxycinnamic acid as matrix, by using the dried droplet technique, and analyzed in a Voyager-DE PRO mass spectrometer (Applied Biosystems, Inc., Foster City, CA). Spectra were acquired in reflectron mode; internal mass calibration was performed with peptides derived from trypsin autoproteolysis. The MASCOT software package was used to unambiguously identify spots from the SwissProt human sequence database by peptide mass fingerprint experiments.[20] Candidates with MASCOT scores >62 (corresponding to $p < 0.05$ for a significant identification) were further evaluated by comparison with molecular mass and pI experimental values obtained from 2-DE. The occurrence of protein mixtures was excluded by sequential searches for additional protein components using unmatched peptide masses. Protein identification was confirmed by performing PSD fragment ion spectral analysis of the most abundant mass signal within each MALDI-TOF-MS spectrum.

Western Blot Analysis. Western blot analyses were used to validate the differential expression of mass spectrometry-identified proteins. In particular, Huh7 cells treated for the indicated times with 300 nm TG (Sigma-Aldrich, St. Louis, MO), 2 mM DTT (Sigma-Aldrich, St. Louis, MI), 10 μM MG132 (Sigma-Aldrich, St. Louis, MO), or 10 μg/mL cycloheximide were lysed in RIPA buffer, which gave, for the selected proteins, results similar to those obtained by the 1% Triton lysis buffer used in the 2D-DIGE analysis. Proteins were separated on 10−12% polyacrylamide gels by SDS−PAGE and then blotted on Protran nitro-cellulose membranes (Schleicher&Schuell Bioscience Gmbh, Dassel, Germany). Filters were blocked with PBS containing 10% nonfat dry milk and 0.1% Tween-20, overnight, and then incubated for 2−12 h with the optimal diluition of the primary antibody. Conjugated antirabbit or antimouse IgG horseradish peroxidase was used as

the secondary antibody; bands were visualized by autoradiography of ECL reaction (Amersham International, Amersham, UK). α-Tubulin or GAPDH was used to normalize for equal amounts of proteins loaded on the gels and to calculate the relative induction ratios. Quantitative analyses were performed on protein samples giving signals in the linear range of the ECL assay. Densitometry of autoradiographs was performed by the Image J program; values obtained were the mean ± SD of three independent experiments. Statistical analysis was performed using Student's *t*-test ($n = 6-9$).

Quantitative RT-PCR Analysis. For reverse transcription of DNase-treated total RNA samples (1 μg per sample), the ImProm-II Reverse Transcription System (Promega) was used, with pdN6 primers; reaction was performed in 20 μL. Quantitative real-time PCR was performed on a 7500 Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA) using 12.5 μL of Power SYBR Green PCR Master Mix (Applied Biosystems, Inc., Foster City, CA), 0.2 μM each of forward and reverse primers, and 6 μL of diluted cDNA (1:5). The oligonucleotide primers were designed by the Primer Express software (Applied Biosystems, Inc., Foster City, CA); their sequences were the following: GRP78 forward, 5′-GCCGCACGTGGAATGAC-3′; GRP78 reverse, 5′-AACCACCTTGAACGGCAAGA-3′; RCN2 forward, 5′-TGGGCG-TCCAGGAAGATG-3′; RCN2 reverse, 5′-CTTTATGATCGCCTG-CAGTCTTT-3′; PLOD3 forward, 5′-TGCCCCAGAGGGATGTGTT-3′; PLOD3 reverse, 5′-GATGCCCTTGTCTCGAAAGC-3′; SET forward, 5′-TCTGATGCAGGTGCTGATGAG-3′; SET reverse, 5′-CGGGAAC-CAAGTAGTACTGTAATGG-3′; ALIX forward, 5′-CCTAGTGCTC-CTTCAATTCCTACAC-3′; ALIX reverse, 5′-CTTAGTAGGCGG-CATGGTTCTT-3′; CAL forward, 5′-TCCTTCCCTCAGACTAT-GATCATG-3′; CAL reverse, 5′-GCTTGCCATCCTTGTTTTGG-3′; ACTB forward, 5′-CGTGCTGCTGACCGAGG-3′; and ACTB reverse, 5′-GAAGGTCTCAAACATGATCT-3′. For the calculation of relative mRNA expression, ACTB (β-actin) transcripts were used as the reference mRNA; data analysis was carried out according to the ΔΔCt method. [21,22]

RESULTS

Differentially Expressed Proteins in TG-Treated Human Hepatocytes. In order to investigate the effect of TG treatment on protein expression in Huh7 cells, a differential expression proteomic analysis based on the 2D-DIGE approach was used. Three biological replicates of control and Huh7 cells treated with TG for 8 h were generated for protein extraction and fluorescence labeling with Cy-dyes. Figure 1A shows a merged image of the representative gel (master gel), used to match the 2-D profiles obtained from three sample pairs. Analysis of the images according to the DeCyder bioinformatic software (see Materials and Methods section for details) allowed us to detect about 1,100 matched protein spots within the three gels. Thirty-one spots (circled in Figure 1B) appeared to be deregulated following quantitative and statistical analysis, under parameters defined as relative expression ratio in TG-treated versus control Huh7 cells; +1.25 for spots up-regulated (4 in number) and −1.25 for spots down-regulated (27 in number), with a P value <0.05 (data not shown). These spots were matched with the corresponding ones from the preparative gels stained with fluorescent stain SyproRuby (yellow circles in Figure 1C); the spots were, indeed, individually excised from preparative gels and further subjected to MS analysis for protein identification. Table 1 (see Supporting Information) summarizes their relative expression ratios in TG-treated versus control Huh7 cells as well as the mass spectrometry data for the corresponding 20

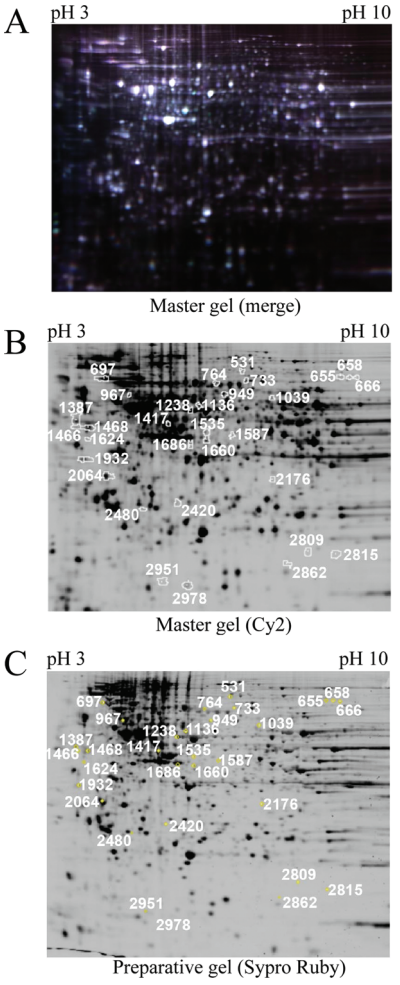

Figure 1. 2-DE map of differentially expressed proteins in TG-treated Huh7 cells. The Figure shows the master gel (panels A and B) and the preparative gel (panel C). In panel A, the master gel is shown as the overlay of the three Cy-dyes used to label protein lysates. In panel B, the relevant deregulated spots (31 in number) are surrounded by a white border and numbered; matched spots within the preparative gel (panel C) are highlighted by a yellow circle, which also indicates the picking surface for the robotic spot picker. The representative image reported in panel B shows the Cy2-labeled proteins on the scanned master gel; protein spots in panel C were stained with Sypro Ruby fluorescent staining.

identified proteins. A multiple identification occurred for spot 531, which showed the concomitant presence of the protein products of *PLOD3* and *PDCD6IP* genes encoding for procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3 (PLOD3), and ALG2-interacting protein X (ALIX).

Validation of 2D-DIGE Results by Western Blot Analysis of Proteins Differentially Expressed in TG-Induced Cells. Biochemical validation of the proteomic results was performed by Western blotting analysis of six out of the 20 identified proteins; such proteins were selected, according to their potential relevance in the cell response to thapisgargin (Figure 2). Thus, we first examined the accumulation of GRP78, the major marker of ER stress, shown as one of the up-regulated proteins in the 2-DE analysis (Table 1, Supporting Information). Grp78, also referred to as BiP, is a major ER chaperone with antiapoptotic properties, which plays a key role in the control of UPR activation through the binding-release of the transmembrane ER stress sensors

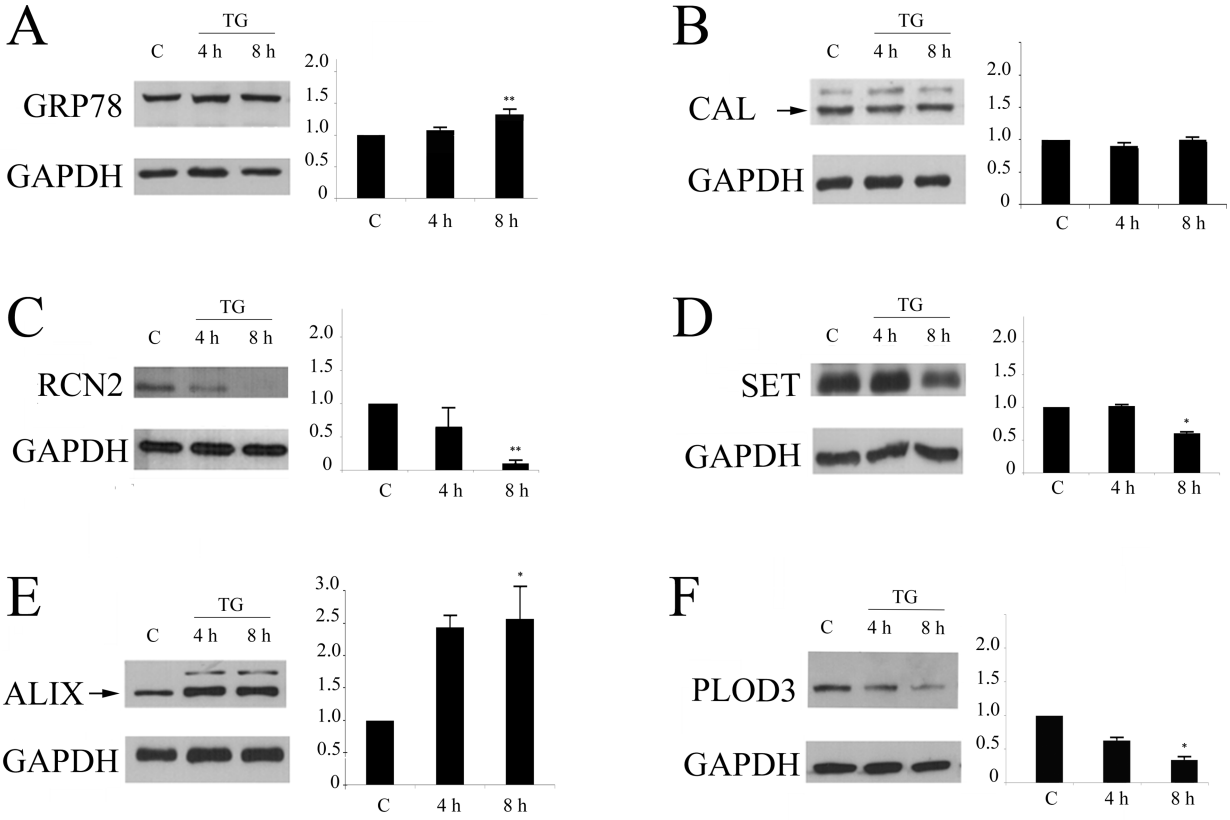

Figure 2. Grp78, CAL, RCN2, SET, ALIX, and PLOD3 expression levels in TG-treated cells. Western blotting analysis of cell extracts obtained from Huh7 cells exposed to 300 nM thapsigargin for the indicated times or the control (C). Protein extracts (10−15 μg) were used to obtain signals in the linear range of the ECL assay. The blots were probed with antibodies against the indicated proteins. One out of three separate experiments is shown. Histograms show the relative normalized abundance of Grp78 (panel A), CAL (panel B), RCN2 (panel C), SET (panel D), ALIX (panel E), and PLOD3 (panel F) in Huh7 cells calculated from the densitometry of immunoblots using GAPDH as the internal standard. Statistical analysis was performed using Student's *t*-test (n = 6−8; * = $P < 0.005$; ** = $P < 0.005$).

(IRE1, PERK, and ATF6). As we would have expected and consistent with our 2-DE data, treatment of Huh7 cells with TG for 4 and 8 h enhanced the Grp78/Bip protein level 1 ± 0.08- and of 1.3 ± 0.1-fold, respectively, compared to those of the control cells (Figure 2, panel A). Next, we examined the quantitative levels of calumenin (CAL) e reticulocalbin 2 (RCN2), two ER resident and functionally related Ca^{2+} binding proteins.[23] Our experiments showed that calumenin was stably expressed either in the 4 h (0.9 ± 0.07) or in the 8 h TG-treated cells (1 ± 0.06) (Figure 2, panel B). Instead, the RCN2 level declined with time in the TG-treated cells, showing a decrease (0.64 ± 0.29 fold) in 4 h and a higher reduction rate in 8 h (0.1 ± 0.07) (Figure 2, panel C). Similarly, we examined the expression level of SET, a multifunctional protein with prosurvival properties.[24] Western blot and densitometry analyses revealed that SET is appreciably down-regulated in the TG-treated cells; such an effect was inconsistent at 4 h (1.02 ± 0.01) but very evident at 8 h of TG-treatment (0.6 ± 0.01) (Figure 2, panel D). Finally, we tested the expression of ALIX and of the PLOD3 protein, which were coidentified in spot 531. Quite intriguingly, Western blotting analysis showed an up-regulation of the ALIX protein in the TG-treated cells giving a fold of induction of 2.33 ± 0.19 and 2.46 ± 0.49 in 4 and 8 h of TG treatment, respectively (Figure 2, panel E) and a down-regulation of PLOD3 (0.63 ± 0.06 and 0.34 ± 0.06) (Figure 2, panel F), which would explain the 2-DE measurement obtained for the 531 spot (−1.42), which includes both proteins.

In addition, since TG induces attenuation of protein synthesis through the PERK pathway of the UPR, we examined whether the inhibition of protein synthesis was sufficient by itself to cause the downregulation observed in the 2D-DIGE analysis. To test that, the expression of the selected proteins was examined by WB analyses performed on the extracts obtained from Huh7 cells exposed for different times to cycloheximide (CHX) (data not shown). The results of the WB experiments (see Supporting Information) show that cell exposure to 10 μg/mL CHX for up to 4 h, prolonged CHX treatments, revealed highly toxic effects (data not shown) and progressively down-regulated the level of the short half-life protein IkBalpha.[25] Interestingly, CHX treatment did not generate significant differences on the level of the selected proteins, suggesting that the down regulation of the calumenin, reticulocalbin 2, SET, and PLOD3 proteins observed in the TG induced cells is not simply the consequence of the protein synthesis inhibition induced by TG. The validations obtained by Western blotting analysis were indeed quite consistent with 2D-DIGE data, with the exception of calumenin, whose levels remained unaltered, following TG treatment, in the immunoblot analysis. Taken together, these experiments highlighted the up-regulation of GRP78 and ALIX, as well as the down-regulation of reticulocalbin 2, SET, and PLOD3 in response to TG.

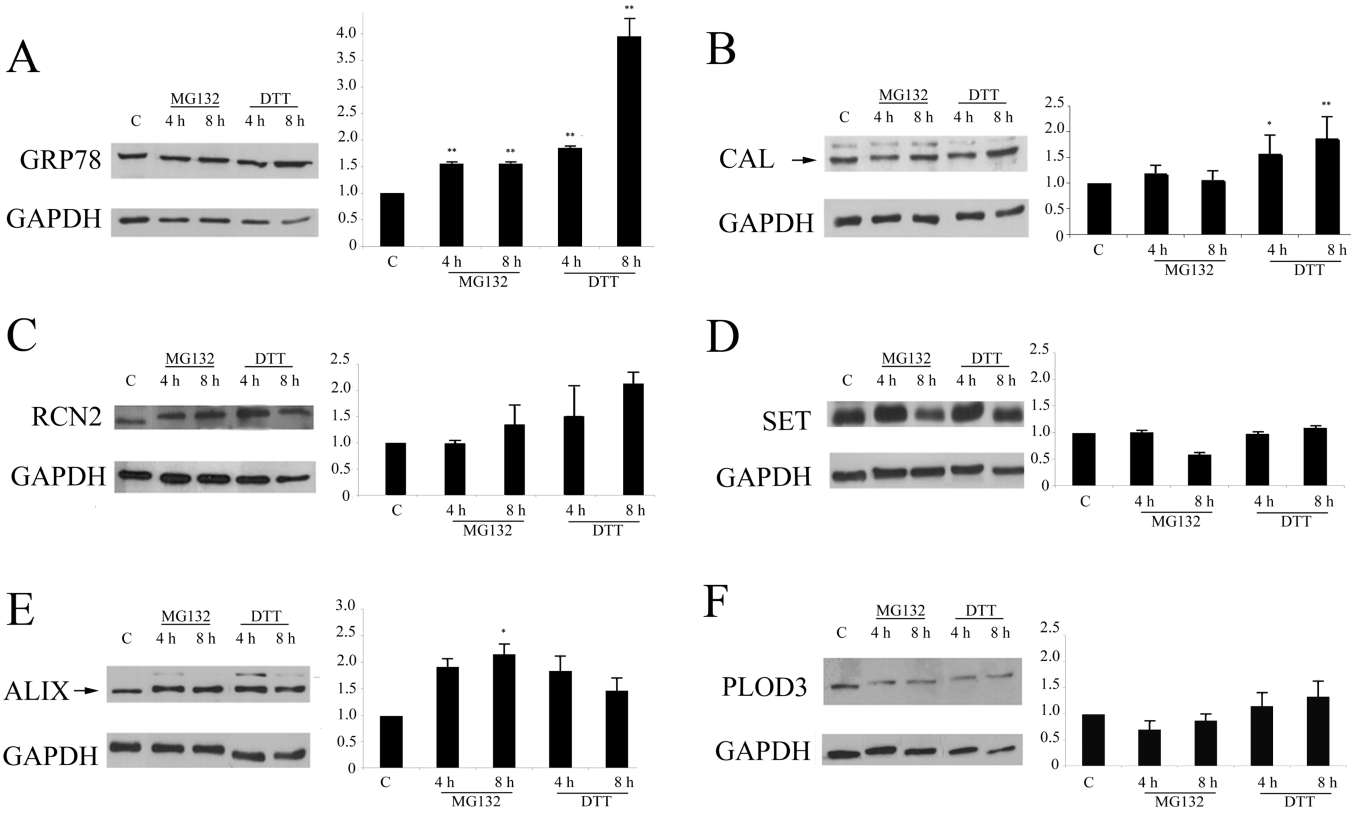

Figure 3. Grp78, CAL, RCN2, SET, ALIX, and PLOD3 expression levels in MG132- and DTT-treated cells. Western blotting analysis of cell extracts obtained from Huh7 cells exposed to 2 mM dithiothreitol (DTT) or MG132 (10 μg/mL) for the indicated times or the control (C). Protein extracts (10−15 μg) were used to obtain signals in the linear range of the ECL assay. The blots were probed with antibodies against the indicated proteins. One out of three separate experiments is shown. Histograms show the relative normalized abundance of Grp78 (panel A), CAL (panel B), RCN2 (panel C), SET (panel D), ALIX (panel E), and PLOD3 (panel F) in Huh7 cells calculated from the densitometry of immunoblots using GAPDH as the internal standard. Statistical analysis was performed using Student's *t*-test (n = 6−8; * = $P < 0.005$; ** = $P < 0.005$).

To test whether the decreased levels displayed by the selected proteins could be explained by transcription suppression or induction of mRNA degradation, we analyzed the mRNA expression of the various selected species by quantitative RT-PCR analysis on total RNA extracted from Huh7 cells treated with TG (see Supporting Information). Interestingly and similarly to the effect shown by the 2D-DIGE and WB analyses, RCN2 and SET mRNAs were reduced in response to TG-induced stress, suggesting a downregulatory effect elicited by the treatment, which involves transcriptional and/or post-transcriptional mechanisms. Furthermore, as expected, TG promptly up-regulated the amount of GRP78 mRNA levels, and this occurrence was also verified for Alix transcripts. In both cases, we observed maximal accumulation of the transcripts 2 h after treatment and a decline at longer times. Intriguingly and consistently with the protein downregulation revealed by the 2D-DIGE, the mRNA levels of calumenin were downregulated in response to TG.

Effect of ER Stress Induced by Dithiotheitol or MG132 on Calumenin, Reticulocalbin 2, SET, ALIX, and PLOD3 Protein Expression Level. In order to evaluate whether the altered expression of the deregulated proteins was actually related to the ER-stress condition, we analyzed their accumulation in Huh7 cells treated with either dithiotheitol (DTT), which induces ER stress by interfering with disulfide bond stability and/or formation, or MG132, which induces ER stress by acting as an inhibitor of the proteasomal degradation pathway. As expected, our results showed that the amount of Grp78 protein increased in MG132-treated cells (4 h TG treatment: 1.55 ± 0.07; 8 h 1.55 ± 0.07) and, particularly, in those treated with DTT for 4 h (1.85 ± 0.07) and 8 h (3.95 ± 0.35) (Figure 3, panel A).

Similarly to what was observed for the TG-induced ER stress condition, in Huh7 cells treated with MG132 (Figure 3, panel B), the immunoblots showed rather constant calumenin levels in the cells treated for 4 h (1.19 ± 0.14) and 8 h (1.06 ± 0.16), while higher levels of this protein were revealed following 4 h (1.57 ± 0.34) or 8 h of DTT treatment (1.87 ± 0.41). Similarly and in clear contrast with the results from 2D-DIGE and Western blotting in TG-treated cells (Figure 2), reticulocalbin 2 was slightly up-regulated in 8 h in MG132 (1.35 ± 0.36)- and, to a higher extent, in DTT-treated cells following 4 h (1.51 ± 0.57) and 8 h exposures (2.13 ± 0.21) (Figure 3, panel C).

As far as SET expression is concerned and similarly to the occurrence of its observed down-regulation in TG-treated cells, its levels were clearly decreased in the MG132-treated cells, after 8 h of stimulation (0.58 ± 0.1). However, SET levels were rather unchanged in DTT-treated cells (0.97 ± 0.1 and 1.1 ± 0.1) (Figure 3, panel D). Finally, as already observed in TG-treated cells, also in the MG132-treated hepatocytes for 4 h (1.91 ± 0.15) and for 8 h (2.15 ± 0.20) as well as in the DTT-treated cells, the ALIX protein accumulated to levels higher than those observed in control cells, both 4 h (1.83 ± 0.29) and 8 h (1.46 ± 0.23) of treatment (Figure 3, panel E); PLOD3

showed decreased levels in the MG132-treated cells (0.70 ± 0.16 m and 0.87 ± 0.12), while in the DTT-treated cells, higher amounts (1.15 ± 0.25 and 1.32 ± 0.31) were detected following DTT exposure (Figure 3, panel F).

DISCUSSION

The proteomic analysis described in this article reports a representative picture of the protein pattern that is generated by acute perturbation of Ca^{2+} homeostasis. TG induces persistent changes of the ratio between cytosolic calcium and ER calcium stores. This imbalance induces profound changes in the gene expression pattern, which are mostly due to the activation of signal transduction pathways involved in the control of cell survival. The findings presented here suggest that perturbation of Ca^{2+} homeostasis, as induced by TG, produces a down-regulatory effect on protein expression and, in particular, on proteins that have a function within the secretory pathways, depend on calcium for their functions or participate in the control of protein synthesis or in the regulation of cell survival.

In agreement with the down-regulatory effect of TG, our results show that important factors involved in regulatory steps of protein synthesis are down-regulated. In particular, we found reduced expression of the translation factor eIF3 (spot 1848), a protein that retains key regulatory functions, including the regulation of translation reinitiation on polycistronic mRNAs and the kinase-dependent control of protein synthesis.[26] Reduced levels were also observed for the nascent polypeptide-associated complex (NAC) (spot 1932), a cytosolic protein that stabilizes the nascent polypeptide emerging from ribosomes and whose expresson is related to ER stress.[27] Interestingly, we also found lower levels of the COP9 signalosome (CSN) (spot 1660), a protein factor that controls the ubiquitin-proteasome-mediated protein degradation in a number of cellular and developmental processes.[28]

Proteomic profiling of TG-induced cells showed decreased levels for a number of ER resident proteins. Calumenin (spot 1468) and reticulocalbin 2 (spot 1387), for example, are two EF-hand proteins belonging to the CREC family of Ca^{2+}-binding proteins.[23] Interestingly, CAL is functionally associated with the release/uptake of Ca^{2+} by interacting either with ryanodine receptors (RyRs), the intracellular calcium channel mediator of calcium-induced calcium release (CICR) in animal cells, or with SERCA2 transporters. Therefore, changes in the expression of calumenin are consistent with the perturbation of Ca^{2+} cycling induced in the TG-treated cells. Instead, the decreased levels observed for RCN2 could be explained by the effect of Ca^{2+} depletion within ER on the stability of the protein, which is a six EF-hand protein and requires Ca^{2+} for its binding to target proteins in the ER. Our results suggest that down-regulation of RCN2 and CAL is most likely calcium dependent. This conclusion is supported by the observation that either MG132 or DTT, which induce ER stress through different mechanisms, is able to effectively up-regulate CAL and RCN2 protein levels.

Among the ER resident proteins, we also found lower amounts of PLOD3 (spot 531), an ER-resident enzyme involved in biosynthesis of the highly glycosylated type IV and VI collagens, whose deficiency is associated with a number of known collagen disorders.[29] More interestingly, down-regulation of PLOD3 seems to be dependent on ER stress at least in the case of MG132, in which PLOD3 was also observed as down-regulated.

Previous observations revealed that TG impairs ER in Golgi transport within the early secretory pathway and alters the morphology of post-ER compartments.[30] In agreement with these findings, 2-DE data reported in this study revealed reduced level of proteins involved in membrane-trafficking events, such as copine 1 (spot 949), a member of the copine family of Ca^{2+}-dependent, phospholipid-binding proteins,[31] and Sec1 family domain-containing protein 1 (spot 733), which is involved in fusion events during vesicular trafficking.[32]

According to the role of calcium in the control of cell survival, we show that TG induces down-regulation of the prosurvival factors mortalin (spot 1848) and SET (spot 1624). Mortalin, also known as hsp70/PBP74/Grp75, is a molecular chaperone that resides in multiple cell compartments, including mitochondria, ER, plasma membrane, transport vesicles, and cytosol, and performs various functions related to cell survival, control of proliferation, and stress response.[33] Interestingly, the SET protein was found down-regulated upon TG treatment and also in the ER stressed cells exposed to MG132. SET retains dual functions, namely, as a histone chaperone and phosphatase (PP2A) inhibitor.[34] Thus, its down-regulation, as observed in ER stress cells, may enhance apoptosis caused by the reactivation of PP2A phosphatase.[35]

Among identified proteins, we observed only three up-regulated proteins: ER chaperone Grp78 (spot 697), serine proteinase inhibitor α-1-antitrypsin (SERPINA1) (spot 1136), and ALIX (spot 531). Quantitative expression of Grp78 is not surprising, given its central role in the ER stress response. Similarly, SERPINA1 accumulates in TG-treated cells in the same way to what is shown in ER-stressed cells after incubation with tunicamicin.[36] Intriguingly, the spot leading to ALIX identification (spot 531) also contained PLOD3, and it appeared to be a down-regulated spot in TG-treated cells. However, Western blotting analysis demonstrated that ALIX is actually up-regulated after TG treatment, also in ER-stressed cells resulting from MG132 or DTT treatment. These results are in perfect agreement with previous reports, which link ALIX expression and its calcium-dependent interaction with the proapoptotic factor ALG2 in TG-induced apoptosis.[37] Besides its proapoptotic properties, ALIX retains important functions in several processes including endocytosis, endosomal sorting, virus budding, cytokinesis, and vesicle budding events at the ER exit sites.[38,39]

In conclusion, this study has provided a representative picture of the changes observed in hepatoma cells' proteomes as a result of the treatment of cells with TG, a powerful inhibitor of Ca^{2+} transporters regulating ion accumulation in the ER. A comparison focused on specific proteins with other inducers of ER stress, either acting on polypeptide disulfide bond stability/formation or inhibiting the proteasomal degradation pathway, highlighted a variable expression response depending on the molecule/mechanism tested. Further comparative proteomic analyses with these and additional UPR modulators may indeed contribute to a detailed characterization of the different stress-responsive pathways and may yield results relevant to human diseases.

ASSOCIATED CONTENT

Supporting Information. Differentially expressed proteins in thapsigargin-treated Huh7 cells as revealed by 2D-DIGE analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

DIGE, differential in-gel electrophoresis; ER, endoplasmic reticulum; TG, thapsigargin; UPR, unfolded protein response.

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