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Changes in the proteome of Huh7 cells induced by transient expression of hepatitis D virus RNA and antigens

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ABSTRACT

Hepatitis delta virus (HDV) infection of human hepatocytes infected with the hepatitis B virus (HBV) is associated with increased liver damage and risk of fulminant disease. Although considerable progress has been made towards the elucidation of the mechanisms of HDV replication and pathogenesis, little is still known about the host factors involved in the different steps of the replication cycle. Here, we made use of a proteomic approach to analyse the global alterations in protein expression that arise in human hepatocytes separately transfected with each of the HDV components. Huh7 cells were transiently transfected with plasmids that code for the small delta antigen (S-HDAg), large delta antigen (L-HDAg), genomic RNA (gRNA), and antigenomic RNA (agRNA), respectively. Total protein extracts were separated by 2-DE and differentially expressed spots were identified by MALDI-TOF followed by database searching. We identified 32 proteins known to be involved in different pathways namely nucleic acid metabolism, protein metabolism, transport, signal transduction, apoptosis, and cell growth. Moreover, the down regulation of hnRNP D, HSP105, and triosephosphate isomerase was further confirmed by Real time PCR.

1. Introduction

Hepatitis delta virus (HDV) infects hepatocytes already infected with the hepatitis B virus (HBV) increasing about ten fold the risk of fulminant hepatitis and cirrhosis [1,2]. It is estimated that about 10% of HBV infected individuals are coinfected with the HDV. Currently, there is no specific treatment for HDV infection. The clinical association between the HDV and the HBV is due to the fact that the HDV outer envelope consists of HBV surface antigens (HBsAgs) which are necessary for virus maturation and propagation of infection [3]. The HDV genome consists of a 1.7 kb negative, circular, ssRNA molecule. Similar to plant viroids, this molecule bears a high degree of internal base pairing, about 70% of the genome. Furthermore, a self cleaving ribozyme activity was found in both genomic RNA (gRNA) and the complementary antigenomic RNA molecule (agRNA). RNA replication is believed to occur via a rolling circle mechanism involving cellular RNA polymerase II [4,5]. As a result, multimeric antigenomes are produced and subsequently self cleaved at precise monomeric intervals. After religation, the antigenomic RNA molecules serve as template for production of monomeric RNA genomes by a similar mechanism.

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A single ORF was identified in the HDV antigenome. Transcription from this ORF results in the synthesis of a 0.8 kb mRNA molecule, and subsequent production of the only virus protein, the delta antigen (p24; S-HDAg) [6,7]. During RNA replication an editing mechanism involving cellular adenosine deaminase 1 (ADAR1) occurs in the antigenome. As a consequence, an amber stop codon (UAA) is converted into a triptophan codon (UAG) extending the ORF by 19 additional aminoacids and resulting in the production of the large form of the delta antigen (p27; L-HDAg) [8]. An RNA binding motif, a dimerization domain, and a nuclear localization signal were identified in both S-HDAg and L-HDAg [4,5]. It has additionally been reported that the L-HDAg is isoprenylated at a cysteine residue in position 210 [9].

Several functions have been assigned to the S-HDAg during virus replication: mediation of nuclear import of HDV RNPs, facilitation of processivity during RNA-directed RNA transcription, and acceleration of HDV ribozyme activities [10–12]. The L-HDAg, in contrast, seems to repress RNA replication, and rather to be necessary for interaction with HBsAgs and virus maturation [9,13].

Despite its simplicity, little is known about the detailed mechanisms of HDV replication and pathogenesis namely the host factors that promote virus RNA-directed RNA synthesis. Until now, only a few proteins were found to bind to either the HDV antigens or RNA during the replication cycle. Among them are the Delta interacting protein (DIPA), the polypyrimidine tract-binding protein-associated splicing factor (PSF), nucleolin, and ADAR 1 [14–17]. However, the mechanisms by which the HDV interacts with the host cell, repressing or stimulating the synthesis of specific proteins is not known.

In this work, we made use of a proteomic approach to identify changes in the expression profiles of host proteins caused by the presence of the different virus components. Huh7 cells were transiently transfected with plasmids coding exclusively for the S-HDAg, L-HDAg, gRNA, and agRNA, respectively. Control mock transfections were performed with plasmids pSVL and PGEM4Z. Total cellular proteins were separated by 2-DE and the differential expressed spots were identified by MALDI-TOF MS and data base searching. Overall, 32 proteins were identified. The down regulation of the heterogeneous nuclear ribonuclearprotein D (hnRNP D), heat shock protein 105 (HSP105), and triosephosphate isomerase (TPI) was further confirmed by Real time PCR.

2. Materials and methods

2.1. Materials

For 2-DE analysis, IPG DryStrips and IPG buffer were purchased from GE Healthcare. 30% Acrilamide/Bis solution and TEMED were purchased from BioRad. APS, Iodoacetamide, Thiourea, Brilliant Blue G, Urea, CHAPS, DTE and SDS were all purchased from Sigma.

Regarding mass spectrometry, DTT was purchased from GE Healthcare. Iodoacetamide, NH_4HCO_3 and 2,5 DHB were obtained from Sigma. ACN LC-MS was purchased from Riedel-de-Häen. CHCA and peptide calibration mix 1 were from Laser Biolabs. Other chemicals were domestic products (analytical grade).

2.2. Cell culture and transfection

The human hepatocellular carcinoma cell line (Huh7) was used in this study. Cells were cultured as monolayers in RPMI 1640 medium (Sigma) supplemented with 10% foetal bovine serum (Invitrogen). Cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2.

For transfection, the Fugene6 transfection reagent (Roche) was used following the manufacture's instructions. Cells were analysed 48 h after transfection.

Four different plasmid constructs were used in this study: pSVLAg-S [13], pSVLAg-L and pDL481 [18], and pDL542 [19]. These plasmids contain the cDNA coding for the S-HDAg, L-HDAg, HDV agRNA, and HDV gRNA, respectively. Transfection efficiency was monitored by indirect immunofluorescence or FISH [20], depending on the plasmid used for transfection. Only samples displaying at least 50% of transfection efficiency were considered for further utilization.

Control mock transfections of Huh7 cells were separately performed as above described, with plasmids pSVL (Pharmacia Biotech) and pGEM4Z (Promega).

2.3. Sample preparation

For preparation of protein samples, cells were first harvested, rinsed with PBS, and centrifuged at 800 g for 10 min, at 4 °C. Protein precipitation was achieved by resuspending the cell pellet in 10% TCA. After precipitation, proteins were pelleted by centrifugation at 12,000 g, for 5 min, and washed with 3 volumes of ice cold acetone, for 2 h at 20 °C. Finally, the precipitated proteins were pelleted again, the acetone was removed, and the samples were stored at 80 °C until use.

For 2-DE, protein extracts were resuspended in IEF buffer (7 M Urea, 2 M Thioureia, 4% CHAPS, 0.5% IPG Buffer, 60 mM DTE and 0.002% Bromophenol Blue).

Protein quantification was performed on cell pellets using a Bradford assay based kit (BioRad).

2.4. 2D electrophoresis

The first dimension, isoelectric focusing (IEF), was performed on precast 18 cm immobilised non-linear pH 3–11 gradient (IPG) strips. Approximately 200 g of protein, resuspended in IEF buffer, were loaded onto each strip. After 12 h rehydratation, IEF was carried out in an Ettan IPGphor Isoelectric Focusing System (GE Healthcare) until a total of 97,000–98,000 Vh was achieved.

Following IEF separation the gel strips were incubated twice with equilibration buffer (50 mM Tris–HCl pH 8.8, 6 M Urea, 30% Glycerol, 2% SDS, 2% DTE), for 15 min at room temperature. The strips were next incubated twice with blocking buffer (50 mM Tris–HCl pH 8.8, 6 M Urea, 30% Glycerol, 2% SDS, 0.002% Bromophenol Blue, 2.5% Iodoacetemide), also for 15 min at room temperature.

The second dimension, SDS PAGE, was performed on 9% polyacrilamide gels in a Hoefer ES 600 Ruby apparatus (GE Healthcare). Proteins were separated overnight, at 50 V, at room temperature, until the bromophenol blue dye front reached the end of the gel.

2.5. Gel staining and image analysis

After electrophoresis, the gels were stained using a Blue Silver staining protocol [21]. Image acquisition was performed using a Sharp JX330 calibrated densitometer, and the gels were analysed using the Phoretix 2D Expression 2005 software (Nonlinear Dynamics). Five gel replicas were analysed for each sample. Only spots present in at least four of the gels were used to analyse expression differences.

Normalized spot volumes were used to compare the different samples. Quantitative analysis was performed using the Student's *t*-test (p 0.05) between protein gels from mock transfected Huh7 cells and each of the Huh7 transfected cells. Spots displaying at least a two fold protein expression increase or decrease were selected for identification by Peptide Mass Fingerprinting (PMF).

2.6. Protein identification

Spots corresponding to differentially expressed proteins were excised, destained with 50% ACN and dried in a Speed Vac centrifuge. The polypeptides were subjected to reduction, alkylation, and digestion with sequencing-grade modified trypsin (Promega) as earlier described [22]. Peptide samples were desalted and concentrated using Gel Loader Tips (Eppendorf) filled with Poros R2 resin (Applied Biosystems). Finally, peptides were eluted directly into the MALDI plate with 10 mg/ml CHCA (Laserbiolabs), and allowed to dry at open air.

For MALDI-TOF mass spectrometry and protein identification, mass spectra were acquired using a Voyager-DE STR MALDI-TOF mass spectrometer (Applied Biosystems) and the Voyager software v5.10. Spectra were analysed using the M/Z program v2001.02.13 (Genomic Solutions, UK) and contaminant peaks were removed using the Peakerasor program (v1.76). The remaining peaks were then matched with the theoretical peptide masses using MASCOT (Matrix Science, UK). The search was performed against a *Homo sapiens* database (MASCOT) with a peptide mass tolerance of 100 ppm, a single missed cleavage allowed, and carbamidomethylation of cysteines and oxidation of methionines as fixed and variable amino acid modifications, respectively. Criteria used to accept the identification were significant homology scores achieved in MASCOT, significant sequence coverage values, and similarity between the protein molecular mass calculated from the gel and for the identified protein [23]. The FindMod software (http://www.expasy.ch/sprot/findmod.html) was used to evaluate the presence of post-translational modifications.

2.7. mRNA extraction and cDNA synthesis

For mRNA extraction the Oligotex Direct mRNA Mini kit (Qiagen) was used, following the manufacturer's instructions. All samples were treated with DNase I using the DNase-free kit (Ambion, UK) according to the manufacturer's instructions.

Synthesis of cDNA from mRNA was accomplished using Revert Aid™ M-MuLV Reverse Transcriptase (Fermentas). Typically, reaction mixtures contained approximately 100 ng of mRNA, 0.5 g of oligo(dT)$_{12–18}$ (Roche), 10 mM dNTP mix (Roche), 20 U RNase inhibitor (Roche), and 40 U M-MuLV Reverse Transcriptase in Reaction buffer (Fermentas). The reactions were allowed to proceed for 1 h, at 42 °C, and stopped by incubating at 70 °C, for 10 min. All cDNA samples were purified using the GFX PCR DNA and Gel Band purification kit (GE Healthcare).

2.8. Real time PCR

Real time PCR was performed using the qPCR Core kit for SYBR® Green I (Eurogentec, Belgium) following the manufacturer's specifications, in 96-well plates with optical caps (Applied Biosystems) on a GeneAmp® 5700 Sequence Detector System (Applied BioSystems). Reaction mixtures, for a final volume of 20 l, contained 1× Reaction buffer, 3.5 mM $MgCl_2$, 200 M dNTPs, 300 nM of each primer, 0.025 U/ l HotGoldStar enzyme, and diluted SYBR® Green. The PCR program used for amplification was: 10 min at 95 °C, 40 cycles with 15 s at 95 °C, and 1 min at 60 °C. Each sample was assayed in triplicate and analysed with the GeneAmp® 5700 SDS (version 1.1.) software and Microsoft Excel.

Relative quantification was performed by the 2 ^{Ct} method earlier described [24]. For the Ct calculation to be valid, the amplification efficiencies of the target and the reference must be approximately equal. A sensitive method for assessing if two amplicons have the same efficiency is to look at how Ct varies with template dilution.

The reference used in this work was the gene -2-Microglobulin (2MG; Genbank accession number P61769) to which all the samples were compared with. Primers for the reference and targets were designed in our laboratory using the Primer Express™ 1.5 software and the bioinformatic tool Oligonucleotide Properties Calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html). GC content, melting temperature, secondary structure and length were taken in consideration for primer design, and cDNA transcript sequences were obtained in Genbank database from NCBI. The primer sequences used in this work to amplify the 2MG, HSP105, hnRNP D, and TPI are displayed in Table 1.

3. Results

3.1. 2-DE characterization of Huh7 cells and Huh7 transfected cells

To perform a comparative analysis of the cellular proteome in cells transiently expressing each of the HDV components separately, we made use of plasmids pSVLAg-S, pSVLAg-L, pDL542, and pDL481, which code for the S-HDAg, L-HDAg, [13,18] gRNA,

Table 1 – Primers used for Real time PCR analysis of mRNA expression levels of hnRNP D, HSP105, TPI, and 2MG

Target gene	Forward primer	Reverse primer
2MG	5'-GGC TAT CCA GCG TAC TCC AA-3'	5'-TCA CAC GGC AGG CAT ACT C-3'
HSP105	5'-ATC GAG ACC ATC GCC AAT G-3'	5'-GAA TGC TCG GCC ATG AAA TC-3'
hnRNP D	5'-AGT TAG ATC CTA TCA CAG GGC GA-3'	5'-CTC TAT GGA TTC CAC CTC ACC A-3'
TPI	5'-GCA CTC AGA GAG AAG GCA TGT-3'	5'-CAA TGC AGG CGA TTA CTC CGA-3'

and agRNA [18,19], respectively to transfect Huh7 cells. Plasmids pDL542 and pDL481 were engineered with 2-nt deletions in order to be unable to support production of HDAg [19].

Since the transfection procedure could affect the protein expression profile of the cells, we performed control mock transfections in Huh7 cells using plasmids pSVL, used to clone the cDNA coding for S-HDAg and L-HDAg, respectively, and pGEM4Z (the backbone of plasmids pDL542 and pDL481).

After 48 h, the efficiency of transfection with plasmids pSVL-AgS, pSVL-AgL, pDL542, and pDL481 was monitored either by indirect immunofluorescence or FISH, using a rabbit polyclonal antibody which recognizes the small and large forms of the delta antigen [25] or digoxigenin labelled plasmid pSVL(D3) which contains a trimer of full length HDV cDNA [20], respectively. Only transfection efficiencies higher than 50% were considered to be adequate for further 2-DE analysis.

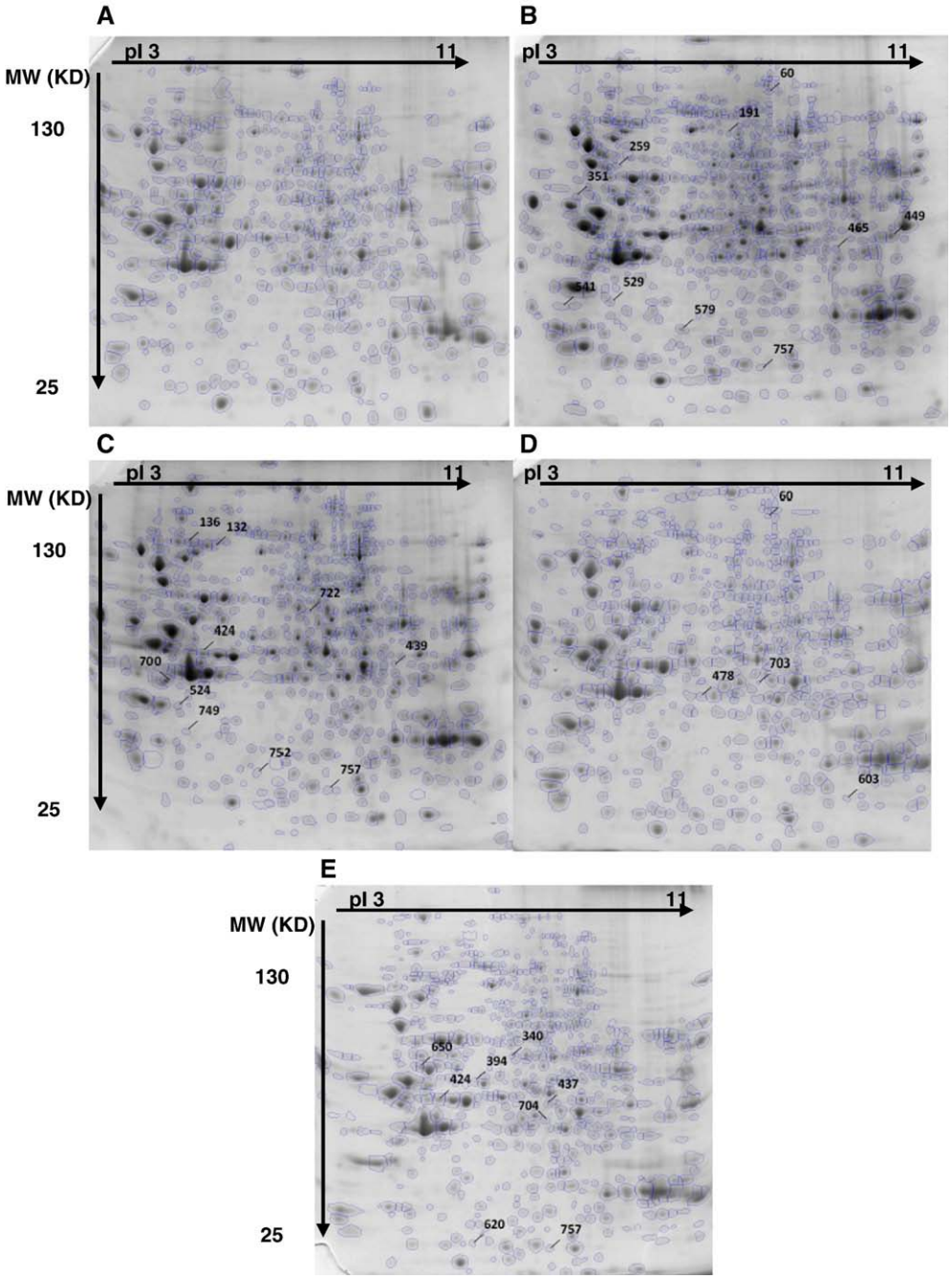

Fig. 1 – 2-DE analysis of proteins extracted from the Huh7 cell line (A) and from Huh7 cells transiently expressing S-HDAg (B), L-HDAg (C), gRNA (D) or agRNA (E). The proteins were separated on pH 3–11 NL IPG strips followed by SDS PAGE on a 9% polyacrilamide gel. The gels were stained using a Blue Silver staining protocol [21]. The detected spots are surrounded by a blue line. The identified proteins corresponding to each numbered spot are shown in the Supplementary Table.

To separate the cellular proteins, 200 g proteins were loaded onto pH 3–11 NL IPG strips. After the first dimension IEF, the second dimension SDS PAGE was carried out on 9% polyacrilamide gels which were subsequently stained using a Blue Silver staining protocol (Fig. 1). Five gel replicas, each corresponding to a different transfection experiment, were obtained for Huh7 and Huh7 transfected samples.

We first investigated if mock transfections with plasmids pSVL and PGEM4Z induced changes in the proteome of Huh7 cells. To do this, the corresponding gel images were uploaded to the Phoretix PG200 software followed by spot detection and matching. No detectable changes were found in the cellular proteome caused by the mock transfection of Huh7 cells in the analysed 2DE gels (data not shown).

One of the mock transfected Huh7 gels was selected as a reference gel to be compared to the other gels corresponding to Huh7 cells expressing S-HDAg, L-HDAg, gRNA, and agRNA, respectively.

The images of the gels were again uploaded to the Phoretix PG200 software followed by spot detection and matching. Compared to Huh7 gels, a total of 32 spots were found to be differentially expressed in Huh7 transfected cells (at least two fold; $p \leq 0.05$). In both S-HDAg and L-HDAg expressing Huh7 cells 10 differentially expressed spots were identified. Concerning gRNA and agRNA expressing Huh7 cells, 4 and 8 spots were found to be differentially expressed, respectively (Table 2).

3.2. *MS analysis and protein identification*

The 32 spots corresponding to proteins found to be differentially expressed in Huh7 transfected cells compared to mock transfected Huh7 cells, were excised, digested with trypsin, and analysed by MALDI-TOF (see Fig. 2). Using PMF we were able to identify all the differentially expressed protein spots (Table 3 and Supplementary table). Protein mixtures were not detected as a direct result from the protein sequence database or after a resubmission of the peptide masses not used for each protein identification. The non-matched subset of ions was re-evaluated for the presence of doubled miss cleavages using MASCOT. As a result, for 13 of the 32 identified proteins one to five doubled miss cleavages were assigned.

In S-HDAg transfected cells, 2 proteins were found to be up regulated and 8 down regulated whereas expression of the L-HDAg induced the up regulation of 4 proteins and down regulation of 6. Concerning Huh7 cells expressing the genomic RNA, 4 spots corresponded to differentially expressed proteins of which 3 were down regulated and 1 up regulated. Finally, expression of the HDV antigenomic RNA altered the expression of 8 proteins, 3 of which displayed lower expression levels and 5 higher expression levels when compared with the mock transfected Huh7 cells.

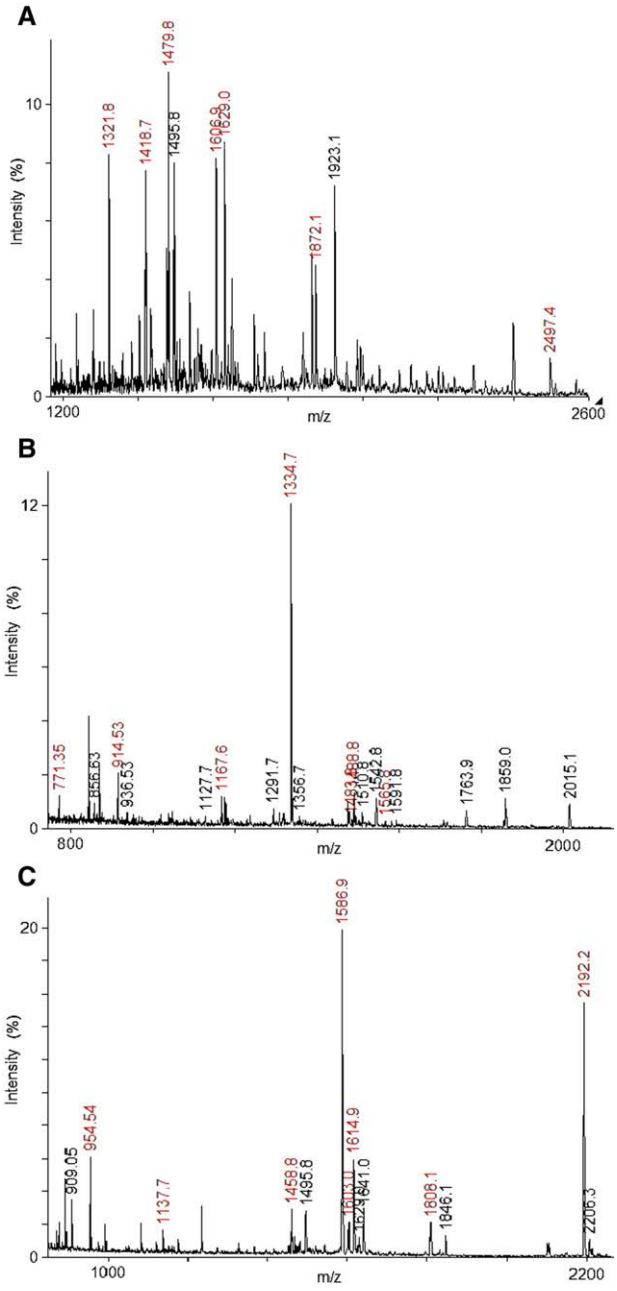

Fig. 2 – Identification of the heat shock 105 kDa (A), heterogenous nuclear ribonucleoprotein D (B), and triosephosphate isomerase proteins (C), in spots 136, 465, and 757, respectively. The ion masses labelled in the spectra were used for search in a protein sequence database. The peptide masses coloured in red match the masses of the peptides used for protein identification.

Table 2 – 2-DE analysis of Huh7 cells and Huh7 cells expressing the HDV antigens and RNA

Spot	Huh7	S-HDAg	L-HDAg	gRNA	agRNA	Sum
Total	641±85	843±80	771±90	704±62	724±138	3683
Differential[a]	–	10	10	4	8	32

[a] Spots differentially expressed in Huh7 transfected cells in comparison with non-transfected Huh7 cells by two fold or more ($p \leq 0.05$).

It was additionally observed that some of the identified proteins, like vigilin, triosephosphate isomerase and dihydropyrimidinase related protein 2, were differentially expressed in more than one group.

Using the FindMod software it was possible to predict the presence of already described post-translational modifications in 4 of the identified proteins: phosphorylation of CKAP4

Table 3 – Distribution of the differentially expressed proteins according to their biological functions

Biological function	L-HDAg	S-HDAg	gRNA	agRNA
Metabolism; energy pathways	Proteasome (prosome, macropain) activator subunit 1; Triosephosphate isomerase; NADH2 dehydrogenase (ubiquinone) flavoprotein 1 precursor	Triosephosphate isomerase	Adenylate kinase 2B	Probable Thiol-specific redutase/Peroxiredoxin 4; Triosephosphate isomerase
Protein metabolism	Eukaryotic translation initiation factor 2 subunit 1; Proteasome (prosome, macropain) activator; Heat shock 105 kDa protein 1	–	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 1	Heat shock 60 kDa protein; Protein Disulfite-isomerase ER60 precursor
Cell growth and/ or maintenance	Keratin 8; Keratin, type I cytoskeletal 19	CKAP4 protein; Tubulin alpha 6	–	Alpha-centractin; Keratin 8
Signal transduction; cell communication	Dihydropyrimidinase-related protein 2	Annexin IV; Proliferating cell nuclear antigen	–	Dihydropyrimidinase-related protein 2
Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	TRIM 28 protein	Heterogeneous nuclear ribonuclearprotein D, isoform C; Zinc finger protein 326; DNA structure specific endonuclease FEN1	–	Ribonuclearprotein La
Transport	N-ethylmaleimide-sensitive factor attachment protein, alpha	High density lipoprotein binding protein (vigilin)	High density lipoprotein binding protein (vigilin); Sorting nexin 5, fragment	–
Apoptosis	–	Dopamine receptor interacting protein 4	–	–

[26] and of the Fen1 endonuclease [27], as well as acetylation of vigilin [28] and of the serine proteinase inhibitor [29].

After identification, the human protein reference database (www.hprd.org) was used to group the proteins according to their biological functions, namely protein metabolism, nucleic acid metabolism, apoptosis, transport, energy pathways, cell growth, and signal transduction (Table 3).

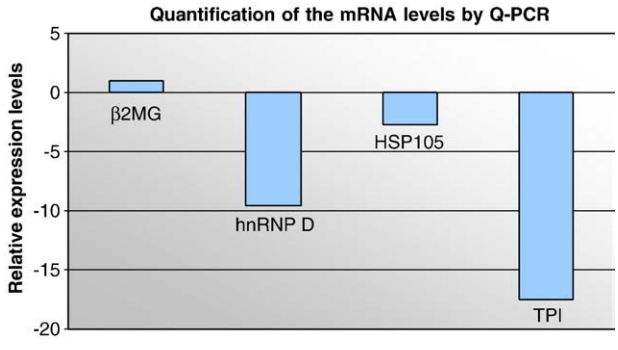

Fig. 3 – Real time PCR analysis for hnRNP D, Hsp105, and TPI in Huh7cells transiently expressing S-HDAg, L-HDAg, and agRNA, respectively. The vertical axis represents the fold change in relative gene expression levels. The 2MG gene was used as a housekeeping internal control. Fold changes were determined as 2 (Ct sample Ct control) (p b 0.05).

3.3. *Validation of proteomic results*

To analyse the reproducibility and validate the proteomic results, we selected the HSP105, hnRNP D, and TPI proteins to be validated by Real time PCR. These proteins, involved in protection from apoptosis, control of RNA stability, and lipid metabolism, respectively, were found to be down regulated in S-HDAg, L-HDAg, and agRNA expressing Huh7 cells, respectively. After transfection of Huh7 cells with the corresponding plasmids, mRNA was extracted and used as template for cDNA synthesis and subsequent Real time PCR experiments. The reference protein was -2-microglobulin, and the obtained results are shown in Fig. 3. Compared to mock transfected Huh7 cells, the HSP105, hnRNP D, and TPI mRNAs display significant lower levels of expression (p b 0.05) in transfected cells (9.6 for the hnRNP D, 2.7 for the HSP105, and 17.4 for TPI). The differences in mRNA expression levels of hnRNP D and TPI, as determined by Real time PCR, are higher then the corresponding protein levels in S-HDAg and agRNA transfected cells (2.4 and 2.6, respectively). In contrast, the difference in the mRNA

expression level of HSP105 was lower then the corresponding difference in protein expression observed in L-HDAg transfected cells (5.6). These results may be indicative of possible alterations in mRNA processing and stability or translation efficiency of the transcripts.

4. Discussion

The identification of host factors involved in HDV replication and pathogenesis has been so far impaired by the lack of an appropriate cell culture system. During HDV replication, four different virus specific molecules are produced: S-HDAg, L-HDAg, gRNA, and agRNA. Both HDAgs and gRNA, together with HBsAgs, are the only components of HDV infectious particles. In an attempt to identify and characterize the changes that arise in host cells as a consequence of HDV RNA replication and protein expression, we used a proteomic approach to compare the global expression profiles between a mock transfected human liver carcinoma cell line Huh7, and Huh7 cells transiently expressing each of the virus specific antigens and RNAs, separately. We were able to identify 32 proteins differentially expressed between Huh7 cells and Huh7 transfected cells. Overall, 12 proteins were found to be over expressed and 20 were down regulated in Huh7 cells as a consequence of synthesis of HDV antigens and RNA. These proteins could all be assigned to functional categories, such as apoptosis, signal transduction/cell communication, nucleic acid metabolism, protein metabolism, transport, energy pathways, and cell growth and/or maintenance proteins.

The HDV circular ssRNA genome shares a number of similarities with plant viroids, namely the high degree of internal base pairing of the rod-shaped RNA molecule. Viroids are known to induce gene silencing in infected plant cells by mechanisms that may include RNA-directed DNA methylation of homologous DNA sequences in the host genome [30]. Our results could indicate that expression of HDV components may also induce gene silencing since over 60% of the identified differentially expressed proteins were found to be down regulated in Huh7 cells. However, most of the down regulated proteins were found in cells expressing one of the virus antigens (14/20). Furthermore, in gRNA and agRNA expressing cells, the overall number of identified down regulated and up regulated proteins was identical (6 down regulated and 6 up regulated in gRNA and agRNA transfected cells). We additionally observed that, globally, host protein synthesis is more affected by the expression of HDAgs rather than virus RNA. In S-HDAg and L-HDAg transfected Huh7 cells, 20 proteins were found to be differentially expressed whether in gRNA and agRNA expressing cells we could only identify 12 differentially expressed proteins.

Despite being a small and simple pathogen, very little is known about the host proteins that are involved in HDV replication and pathogenesis. It has been previously reported that the cellular double-stranded RNA-dependent protein kinase (PKR), nucleolin, the delta-interacting protein (DIPA), a cellular homolog of HDAg, can bind to the HDAg [14,16,31]. Additionally, the small form of adenosine deaminase (ADAR-1), the negative elongation factor (NELF), glyceraldehyde 3-phosphate dehydrogenase (GADPH), PKR, and the polypirimidine tract-binding protein-associated splicing factor (PSF) were also found to bind to the virus RNA [15,17,32–34]. However, the functional significance of most of these interactions is still unclear.

Interestingly, as previously reported for Huh7 cells harboring a hepatitis C virus (HCV) replicon [35], we also found the -soluble N-ethylmaleimide-sensitive factor (NSF), a member of the SNARE superfamily, to be over expressed in the presence of L-HDAg.

Cytokeratin is one of the cellular proteins found to be up regulated in the presence of both L-HDAg and agRNA. In contrast, -tubulin is down regulated in S-HDAg expressing cells. These findings may indicate a possible deregulation of cytoskeleton dynamics during HDV replication as it seems to be the case of other RNA and DNA viruses.

It is generally accepted that viruses not only recruit cellular factors necessary for transcription and replication but also widely subvert the metabolism of the cell influencing the control of the cell cycle and signalling pathways. Our results indicate that a similar picture may arise upon HDV replication. This is supported by two observations. First, the down regulation of proteins involved in DNA replication such as PCNA as well as the Fen1 endonuclease, which interacts with PCNA and is involved in DNA repair and replication [36]. Second, the up regulation of CKAP4, a protein found to bind to the antiproliferative factor (APF) and to be necessary for its activation [37]. Additionally, we observed that annexin IV, a protein that plays a pivotal role in early stages of apoptosis induction, is over expressed in S-HDAg transfected cells. Altogether, these results suggest that HDV infection of liver cells may result in the inhibition of cell proliferation and induction of cell death. This view is also supported by the finding that HSP105 is down regulated in L-HDAg expressing cells, an observation that was further confirmed by Real time PCR. This protein is thought to play different roles in the cell metabolism including the protection from apoptosis and induction of malignant transformation [38]. Furthermore, the inhibition of hnRNP D and of La expression in S-HDAg and agRNA expressing cells, respectively, seems to indicate that host RNA synthesis and processing may also be impaired. This could account for the observed and apparently non-coincident, differences in protein expression, and mRNA levels of HSP105, TPI, and hnRNP D, as detected by Real time PCR. The hnRNP D protein has been reported to be involved in the regulation of several cellular pathways namely by controlling RNA stability. However, it was previously reported that the hnRNP D can function both as an mRNA stabilization factor in NIH 3T3 cells and as an mRNA destabilization factor in human erythroleukemic K562 cells [39,40]. The down regulation of hnRNP D expression in S-HDAg and agRNA expressing Huh7 cells was further confirmed by Real time PCR. Additional experiments are crucial to elucidate the biological significance of this observation.

Finally, the over expression of the HSP60 protein detected in agRNA transfected cells may suggest a possible role of this chaperonin in the correct folding of the HDAgs.

The assessment of specific HDV induced alterations in protein expression profiles in the liver of infected patients is an important task that may provide new insights on the mechanisms of virus pathogenesis, namely those associated with an increased risk of cirrhosis and fulminant disease.

However, accomplishment of this goal is made difficult by the simultaneous presence of HBV and HDV thus not allowing in determining which alterations on host protein expression are due to the specific replication of both viruses. Additionally, HDV and HBV may also compete for some common cellular factors as well as disrupt the same metabolic pathways.

This is the first report on the global alterations caused by the expression of HDV proteins and RNA in human liver cells. We believe it may contribute to a better understanding of the mechanisms of virus replication and pathogenesis. Further studies are mandatory to clarify the precise roles of each of the individually identified proteins in the HDV replication cycle.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jprot.2007.12.002.

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