Identification of p90 Ribosomal S6 Kinase 2 as a Novel Host Protein in HBx Augmenting HBV Replication by iTRAQ-Based Quantitative Comparative Proteomics

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Purpose: The aim of this study was to screen for novel host proteins that play a role in HBx augmenting Hepatitis B virus (HBV) replication.

Experimental design: Three HepG2 cell lines stably harboring different functional domains of HBx (HBx, HBx-Cm6, and HBx-Cm16) were cultured. iTRAQ technology integrated with LC-MS/MS analysis was applied to identify the proteome differences among these three cell lines.

Results: In brief, a total of 70 different proteins were identified among HepG2-HBx, HepG2-HBx-Cm6, and HepG2-HBx-Cm16 by double repetition. Several differentially expressed proteins, including p90 ribosomal S6 kinase 2 (RSK2), were further validated. RSK2 was expressed at higher levels in HepG2-HBx and HepG2-HBx-Cm6 compared with HepG2-HBx-Cm16. Furthermore, levels of HBV replication intermediates were decreased after silencing RSK2 in HepG2.2.15. An HBx-minus HBV mutant genome led to decreased levels of HBV replication intermediates and these decreases were restored to levels similar to wild-type HBV by transient ectopic expression of HBx. After silencing RSK2 expression, the levels of HBV replication intermediates synthesized from the HBx-minus HBV mutant genome were not restored to levels that were observed with wild-type HBV by transient HBx expression.

Conclusion and clinical Relevance: Based on iTRAQ quantitative comparative proteomics, RSK2 was identified as a novel host protein that plays a role in HBx augmenting HBV replication.

1. Introduction

Hepatitis B virus (HBV) is the prototypic member of the Hepadnaviridae family, which contains a group of closely related hepatotropic small DNA viruses with pronounced host specificity.[1] The HBV genome is a 3.2 kb, circular, partially double-stranded DNA with four open reading frames (ORFs) named pre-C/C, pre-S/S, P, and X. The pre-C/C ORF encodes the hepatitis B e antigen (HBeAg) and hepatitis B core antigen (HBcAg); the pre-S/S ORF encodes the hepatitis B surface antigen (HBsAg); the P gene encodes the viral polymerase; the X gene encodes a nonstructural protein known as hepatitis B virus X-protein (HBx).[2] HBx is a 154-amino acid protein with an N-terminal negative regulatory domain and a C-terminal transactivation or coactivation domain. HBx is a multifunctional regulator that modulates gene transcription, signaling pathways, genotoxic stress responses, protein degradation, cell cycle, cell proliferation, and apoptosis and genetic stability by directly or indirectly interacting with host factors.[3]

The role of HBx in the viral life cycle has been investigated by several studies. It has been demonstrated that X-deficient hepadnaviral genomes inoculated into woodchuck livers are unable to initiate productive infections.[4] Moreover, the role of HBx in activating HBV transcription and replication has been recently demonstrated using cellular systems and mouse models. HBx could restore HBV transcription and replication with the X-deficient replicon to wild-type levels in vivo and in vitro.[1,3–7] To further investigate the precise regions of HBx involved in the stimulation of HBV transcription and replication, a study performed by Tang et al. constructed a series of clustered alanine substitution mutants (Cm1–Cm21) of HBx using an alanine scanning mutagenesis strategy. For each mutant, seven consecutive amino acids were replaced by the sequence AAASAAA. The HBx mutants Cm1–Cm7 and Cm10–Cm12 (2–50 aa, 67–87 aa) retained the ability to complement the augmentation effect of HBx on HBV replication. HBx mutants Cm8–Cm9 and Cm13–Cm21 (52–65 aa, 88–102 aa) were unable to do so.
Clinical relevance

HBeX plays an important role in augmenting HBV replication by the transcriptional transactivation function in vivo and in vitro. However, the underlying molecular mechanism by which HBeX enhances HBV replication is not fully understood. The transcriptional transactivation function of HBeX is dependent on pleiotropic protein–protein interactions. The "key" host proteins involved in HBeX enhancement of HBV transcription and replication have not been identified. Clarification of the roles of the host proteins in HBeX argumentation of HBV transcription and replication would help to elucidate the HBV pathogenicity mechanism. In this study, we utilized iTRAQ proteomic methodology coupled with LC-ESI-MS/MS to identify and quantify differentially expressed proteins among HepG2 cell lines stably harboring different functional domains of HBeX. RSK2 was identified as a novel host protein that plays a role in HBeX enhancing HBV replication based on iTRAQ quantitative comparative proteomics. This result may help to better understand the HBV pathogenicity mechanism and may provide new therapy targets for HBV replication.

88–154 aa) were unable to restore the augmentation function of HBeX.\textsuperscript{(7,\textsuperscript{8})} It was also found that the transactivation and coactivation activities of HBeX coincide well with its augmentation function in HBV transcription and replication.\textsuperscript{(7)} Therefore, the regions spanning aa 52–65 and aa 88–154 of HBeX are important for the transactivation or coactivation activities of HBeX and its stimulatory function in HBV transcription and replication. Although the first activity identified for HBeX was the ability to activate transcription of viral and cellular genes, HBeX is unable to directly bind to any HBeX-responsive elements in viral and host genes. The trans-acting transcriptional activity of HBeX in enhancing HBV replication may depend on protein–protein interactions. The regulation of transactivation activity by HBeX relies on the interactions with several components of the basal transcriptional machinery, cellular sequence-specific transcription factors, or activation of signal transduction pathways.\textsuperscript{(7)} However, the underlying molecular mechanism of HBeX activating HBV replication are not fully understood. The "key" host proteins involved in HBeX activating HBV transcription and replication are not fully understood.

Isobaric tags for relative and absolute quantitation (iTRAQ) coupled with liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is a powerful proteomics technique for quantifying protein changes. ITRAQ technology has many advantages compared with 2D gel electrophoresis, including high-throughput capabilities and identification of low-abundance proteins. The advantages of iTRAQ have made it applicable for investigating the underlying molecular mechanisms in many types of scientific research.

To elucidate the molecular mechanisms of HBeX regulation of HBV replication, we utilized iTRAQ proteomic methodology coupled with LC-MS/MS to identify and quantify proteins that are differentially expressed in HepG2 cells lines stably harboring different functional domains of HBeX. In addition, our approach led to the identification of p90 ribosomal S6 kinase 2 (RSK2), which was found to be highly expressed in HepG2 cell lines stably harboring functional domain of HBeX. In addition, the role of RSK2 in HBeX enhancement of HBV transcription and replication was investigated.

2. Experimental Section

2.1. Plasmid Constructions

The plasmid pPAYw1.2 (1.2 wt, subtype ayw) has been described previously,\textsuperscript{(7)} and contains 1.2 copies of the wild-type HBV genome. The HBeX-minus mutant vector pPAYw\textsuperscript{(7)}(1.2x(-)) contains 1.2 copies of HBX-minus HBV genome.\textsuperscript{(7)}

The mammalian expression plasmids pNKF-HBeX expresses full-length HBX (aa 1–154). Alanine scanning mutagenesis was employed to construct a series of clustered alanine substitution mutants (designated Cm) as previously described.\textsuperscript{(7)} The pNKF-Xcm6 plasmid that expresses HBX with a 7-aa residue mutant retained the ability to complement the augmentation effect of HBX (aa 37–43 was changed to AASAAA). The pNKF-Xcm16 plasmid that expresses HBX with a 7-aa residue mutant was unable to complement the augmentation effect of HBX (aa 101–108 changed to AAASAAA).

The mammalian expression plasmids pcDNA3.1-HBeX, pcDNA3.1-Xcm6, and pcDNA 3.1-Xcm16 were derived from pNKF-HBeX, pNKF-Xcm6, and pNKF-Xcm16, respectively. The forward primer contained an EcoRI site (5'-TAGAATTCATGGCTGCTAGGG TGTGC-3'), and the reverse primer contained an XbaI site (5'-GGTTCAGTTAGG-CAGAGGTGAAAAAGTTGC-3').

2.2. Cell Culture

The human hepatocellular carcinoma HepG2 cells were cultured in DMEM with 10% fetal bovine serum, 1 mM glutamate, and 100 units mL\textsuperscript{-1} penicillin and were maintained at 37 °C in a 5% CO\textsubscript{2}–air mixture incubator. The stable HBV replication cell line HepG2.2.15 was cultured in DMEM with 100 μg mL\textsuperscript{-1} G418. HepG2 cells that constitutively express X-wt, X-cm6, and X-cm16 were prepared by transfection with pcDNA3.1-HBeX, pDNA3.1-Xcm6 and pDNA3.1-Xcm16, respectively. Stably transfected cells were selected in the presence of 400 μg mL\textsuperscript{-1} genetin for 2–3 weeks. HepG2-HBeX-Cm6 retained the ability to complement the augmentation effect, whereas HepG2-HBeX-Cm16 did not.

2.3. Protein Sample Preparation

The cells were suspended in the lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM tris-HCl, pH 8.5, 1 mM PMSF, and 2 mM EDTA) and were sonicated on ice. The proteins were reduced with 10 mM DTT (final concentration) at 56 °C for 1 h and then were alkylated with 55 mM IAM (final concentration) in the dark for 1 h. The reduced and alkylated protein mixtures

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were precipitated by adding a 4 x volume of chilled acetone and incubating at −20 °C overnight. After centrifugation at 30,000 g for 4 °C, the pellet was dissolved in 0.5 M TEAB (Applied Biosystems, Milan, Italy) and sonicated on ice. After centrifugation at 30,000 g at 4 °C, an aliquot of the supernatant was taken for determination of protein concentration. The proteins in the supernatant were kept at −80 °C for further analysis.

2.4. iTRAQ Labeling and SCX Fractionation

Total protein (100 μg) each sample was digested with Trypsin Gold (Promega, Madison, WI, USA) at 37 °C for 16 h with the ratio of protein:trypsin = 30:1. After trypsin digestion, peptides were dried by vacuum centrifugation. Peptides were reconstituted in 0.5 M TEAB and processed according to the manufacturer’s protocol with 8-plex iTRAQ reagent (Applied Biosystems). Briefly, one unit of iTRAQ reagent was thawed and reconstituted in 24 μL isopropanol. Samples were labeled with the iTRAQ tags as follows: Sample X-wt (119,121 tag), Sample X-Cm6 (113,115 tag), and X-Cm16 (116,117 tag). Peptides were labeled with the isotopic tags, and were incubated at room temperature for 2 h. The labeled peptide mixtures were then pooled and dried by vacuum centrifugation.

SCX chromatography was performed with an LC-20AB HPLC Pump system (Shimadzu, Kyoto, Japan). The iTRAQ-labeled peptide mixtures were reconstituted with 4 mL buffer A (25 mM NaH2PO4 in 25% ACN, pH 2.7) and loaded onto a 4.6 X 250 mm Ultemex SCX column containing 5 μm particles (Phenomenex). The peptides were eluted at a flow rate of 1 mL min−1 with a gradient of buffer A for 10 min, 5–60% buffer B (25 mM NaH2PO4, 1 M KCl in 25% ACN, pH 2.7) for 27 min, 60–100% buffer B for 1 min. The system was then maintained at 100% buffer B for 1 min before equilibrating with buffer A for 10 min prior to the next injection. Elution was monitored by measuring the absorbance at 214 nm, and fractions were collected every 1 min. The eluted peptides were pooled into 20 fractions, desalted with a Strata X C18 column (Phenomenex) and vacuum dried.

2.5. LC-ESI-MS/MS Analysis Based on Triple TOF 5600

Each fraction was resuspended in buffer A (5% ACN, 0.1%FA) and centrifuged at 20,000 g for 10 min; the final concentration of peptide averaged approximately 0.5 μg μl−1. And 10 μL supernatant was loaded onto an LC-20AD nano HPLC (Shimadzu, Kyoto, Japan) by the autosampler onto a 2 cm C18 trap column. Peptides were eluted onto a 10 cm analytical C18 column (inner diameter 75 μm) packed in-house. The samples were loaded at 8 μL min−1 for 4 min, then the 35 min gradient was run at 300 nL min−1 starting from 2 to 35% B (95%ACN, 0.1%FA), followed by 5 min linear gradient to 60%, then, followed by 2 min linear gradient to 80%, and maintenance at 80% B for 4 min, and finally return to 5% for 1 min.

Data acquisition was performed with a TripleTOF 5600 System (AB SCIEX, Concord, ON) fitted with a Nanospray III source (AB SCIEX, Concord, ON) and a pulled quartz tip as the emitter (New Objectives, Woburn, MA). Data were acquired using an ion spray voltage of 2.5 kV, curtain gas of 30 psi, nebulizer gas of 15 psi, and an interface heater temperature of 150 °C. The MS was operated with an RP greater than or equal to 30,000 FWHM for TOF MS scans. Information-dependent acquisition survey scans were acquired at 250 ms and as many as 30 product ion scans were collected if exceeding a threshold of 120 counts per second with a 2+ to 5+ charge state. Total cycle time was fixed at 3.3 s. The Q2 transmission window was 100 Da for 100%. Four-time bins were summed for each scan at a pulser frequency of 11 kHz by monitoring the 40 GHz multichannel TDC detector with four-anode channel detection. A sweeping collision energy setting of 35 ± 5 eV coupled with iTRAQ adjust rolling collision energy was applied to all precursor ions for collision-induced dissociation. Dynamic exclusion was set for 1/2 of peak width (15 s), and then the precursor was refreshed off the exclusion list.

iTRAQ-based quantitative comparative proteomics were completed by the Beijing Genomics Institute. Protein identification was performed using the Mascot search engine (Matrix Science, London, UK; version 2.3.02). To reduce the probability of false peptide identification, only peptides with significance scores (≥ 20) at the 99% confidence interval by a Mascot probability analysis greater than “identify” were counted as identified. Each confidence protein identification involves at least one unique peptide.

2.6. Bioinformatic Analysis

For protein quantitation, it was required that a protein contains at least two unique peptides. The quantitative protein ratios were weighted and normalized by the median ratio in Mascot. We only used ratios with p-values < 0.05, only fold changes of > 1.5, and double repetition was considered significant.

Functional annotations of proteins were conducted using the Blast2 GO program against the nonredundant protein database (NR; NCBI). The KEGG database (http://www.genome.jp/kegg/) and the COG database (http://www.ncbi.nlm.nih.gov/COG/) were used to classify and group identified proteins.

2.7. Real-Time Quantitative RT-PCR Analysis

Total RNA was extracted using Trizol reagent (Invitrogen, IL, USA). One microgram of RNA was reverse transcribed into cDNA with a reverse transcription kit (Takara, Japan). Quantitative RT-PCR was performed on the LightCycler 96 System (Roche, Germany) and using the FastStart Essential DNA Green Master (Roche, Germany) according to the manufacturer’s instructions. Primers are listed in Table 1.

2.8. Western Blotting

The cells were lysed at 4 °C in lysis buffer (20 mM tris-HCl, pH 7.6, 0.5% NP-40, 250 mM NaCl, 3 mM EGTA, 3 mM EDTA, 1 mM sodium vanadate, 2 mM dithiothreitol, 0.5 mM PMSF, 20 mM β-glycerophosphate, and 1 μg mL−1 leupeptin).
Table 1. Primer sequences used in the quantitative RT-PCR analysis.

| Gene   | Forward primer | Reverse primer |
|--------|----------------|----------------|
| ADH4   | 5'-TCCAGAGGAGCTAATAATCGG-3' | 5'-AAAGGCAGGTATGGGTCA-3' |
| SOD1   | 5'-GGTCTTCTTATTAATCCCTATC-3' | 5'-TTCTTATTTCCACCTTTC-3' |
| CSTB   | 5'-TTACCAGACACGGCAGAG-3' | 5'-AAAGGCAGGTATGGGTCA-3' |
| ACSL4  | 5'-GCGATCTCCCAAGTGGGAC-3' | 5'-TTCTTATTTCCACCTTTC-3' |
| PLIN2  | 5'-GCTGCTTACTAATCCCTAC-3' | 5'-AAAGGCAGGTATGGGTCA-3' |
| APOA1  | 5'-GGTTCTTCTGCGGTACAGGT-3' | 5'-TTCTTATTTCCACCTTTC-3' |
| RSK2   | 5'-GCTGCGACAAGATGGTGTG-3' | 5'-AAAGGCAGGTATGGGTCA-3' |
| PRKAR1A| 5'-TTCTTGAGGAGTCTGGTCTG-3' | 5'-AAAGGCAGGTATGGGTCA-3' |
| RRP1B  | 5'-GCTGCGACAAGATGGTGTG-3' | 5'-AAAGGCAGGTATGGGTCA-3' |
| DPP4   | 5'-GCTGCGACAAGATGGTGTG-3' | 5'-AAAGGCAGGTATGGGTCA-3' |
| FABP1  | 5'-GCTGCGACAAGATGGTGTG-3' | 5'-AAAGGCAGGTATGGGTCA-3' |
| HBsAg  | 5'-GCTGCGACAAGATGGTGTG-3' | 5'-AAAGGCAGGTATGGGTCA-3' |

The protein samples (20 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane, and proteins were detected by Western blot.

2.9. Enzyme-Linked Immunosorbent Assay (ELISA)

HBsAg and HBeAg in the cell culture medium and mouse serum were measured by using ELISA kits (Kehua Bioengineering, Shanghai, China). HepG2.2.15 and HepG2 cells were seeded in 6-well plates at 70–80% confluence. After the cells were cultured for 24 h, they were transfected as indicated in the Figure 4 legend. The culture media was collected, and the levels of HBsAg and HBeAg were measured according to the manufacturer’s instructions.

2.10. Southern Blotting

The isolation and determination of HBV DNA replication intermediates were performed as previously described. These HBV DNA replication intermediates (HBV DNA RI) were resuspended in 30 μL of tris-ethylene diamine tetraacetic acid buffer. The samples were separated by 1% agarose and transferred to Hybond-N+ membrane (Amersham Biosciences, Bucks, UK). The membrane was probed with digoxigenin-labeled full-length HBV DNA sequence and then analyzed using the DIG Luminescent Detection Kit for Nucleic Acids (Roche, Germany).

3. Results

3.1. iTRAQ Analysis of Differentially Expressed Proteins

We utilized iTRAQ proteomic methodology coupled with LC-MS to identify differentially expressed proteins. HepG2 cells lines which harbored different functional domain of HBx were investigated. HepG2-HBx and HepG2-HBx-Cm6 retained the ability to complement the augmentation effect on HBV replication, whereas HepG2-HBx-Cm16 did not. To increase the coverage of protein identification and/or the confidence of the data generated, samples were iTRAQ labeled as follows: HepG2-HBx (119,121 tag), HepG2-HBx-Cm6 (113,115 tag), and HepG2-HBx-Cm16 (116,117 tag). A schematic flowchart of the iTRAQ method is shown in Figure 1.

A total of 548 proteins were identified as being differentially expressed (over 1.5-fold, \( p < 0.05 \)) among three cell lines (Figure 2A). These 548 proteins were classified into 24 functional categories by using cluster of orthologous groups (Figure 2B). To reduce false positives for the selection of differentially expressed proteins, 70 differentially expressed proteins were identified by double repetition among these three groups (119:116,117; 119:113,121:117; Tables 2 and 3). Functional clustering analyses of the identified proteins from HepG2-HBx, HepG2-HBx-Cm6, and HepG2-HBx-Cm16 cells are presented in Figure 2C.

![Figure 1. Schematic summary of experimental and data analysis process of the study.](image-url)
3.2. Validation of Differentially Expressed Proteins

Differential expression levels of the proteins identified by the iTRAQ approach were validated using real-time quantitative RT-PCR analysis. Figure 3A shows the relative mRNA expression levels of ADH4, SOD1, CSTB, ACSL4, CORO1A, PLIN2, APOA1, RSK2, PKR1A, RRPIB, DPP4, and FABP1. The mRNA levels of ADH4, SOD1, CSTB, ACSL4, CORO1A, PLIN2, APOA1, RSK2, PKR1A, and RRPIB were downregulated in HepG2-HBx-Cm16 compared with HepG2-HBx, whereas the mRNA levels of DPP4 and FABP1 were upregulated. These trends were similar with the protein expression levels determined by the iTRAQ approach.
Table 2. iTRAQ analysis of differentially expressed proteins between HepG2-HBx and HepG2-HBx-Cm16.

| No | Protein description | Gene | Function | Acce.no | Score | Mass | Cov | Fold change |
|----|----------------------|------|----------|---------|-------|------|-----|-------------|
| 1  | Glutathione S-transferase A1 | GSTA1 | Transferase | P08263 | 138 | 33277 | 19.8 | 0.131 |
| 2  | Glutathione S-transferase A2 | GSTA2 | Transferase | P09210 | 138 | 32949 | 19.8 | 0.131 |
| 3  | Alcohol dehydrogenase 4 | ADH4 | Oxidoreductase | P08319 | 110 | 53588 | 12 | 0.308 |
| 4  | Hydroxymethylglutaryl-CoA synthase | HMGC51 | Lipid metabolic process | Q01581 | 161 | 66952 | 22.5 | 0.376 |
| 5  | Cathepsin D | CATD | Lysosome | P07339 | 1078 | 52338 | 41.5 | 0.461 |
| 6  | 4-hydroxyphenylpyruvate dioxygenase | HPD | Catabolism | P327542 | 678 | 49712 | 39 | 0.468 |
| 7  | Superoxide dismutase | SOD1 | Oxidoreductase | P00441 | 597 | 19804 | 22.7 | 0.473 |
| 8  | Cystatin-B | CSTB | Thiol protease inhibitor | P04080 | 273 | 14232 | 24.5 | 0.479 |
| 9  | Acyl-CoA synthetase 4 | ACSL4 | Lipid metabolic process | Q8TAF6 | 127 | 61773 | 10.2 | 0.498 |
| 10 | Cathepsin D | CATD | Lysosome | P07339 | 1078 | 52338 | 41.5 | 0.461 |
| 11 | Phenylalanine-4-hydroxylase | PAH | Oxidoreductase | P00441 | 597 | 19804 | 22.7 | 0.473 |
| 12 | Cystatin-C | CST3 | Thiol protease inhibitor | P01034 | 254 | 18451 | 18.5 | 0.498 |
| 13 | Coronin-1A | CORO1A | Structural component | P31146 | 114 | 58675 | 11.3 | 0.517 |
| 14 | Perilipin-2 | PLIN2 | Cellular component | P327542 | 678 | 49712 | 39 | 0.468 |
| 15 | Superoxide dismutase | SOD1 | Oxidoreductase | P00441 | 597 | 19804 | 22.7 | 0.473 |
| 16 | Cystatin-C | CST3 | Thiol protease inhibitor | P01034 | 254 | 18451 | 18.5 | 0.498 |
| 17 | Cystatin-B | CSTB | Thiol protease inhibitor | P04080 | 273 | 14232 | 24.5 | 0.479 |
| 18 | Acyl-CoA synthetase 4 | ACSL4 | Lipid metabolic process | Q8TAF6 | 127 | 61773 | 10.2 | 0.498 |
| 19 | Cystatin-C | CST3 | Thiol protease inhibitor | P01034 | 254 | 18451 | 18.5 | 0.498 |
| 20 | Coronin-1A | CORO1A | Structural component | P31146 | 114 | 58675 | 11.3 | 0.517 |
| 21 | Perilipin-2 | PLIN2 | Cellular component | P327542 | 678 | 49712 | 39 | 0.468 |
| 22 | Superoxide dismutase | SOD1 | Oxidoreductase | P00441 | 597 | 19804 | 22.7 | 0.473 |
| 23 | Cystatin-C | CST3 | Thiol protease inhibitor | P01034 | 254 | 18451 | 18.5 | 0.498 |
| 24 | Cystatin-C | CST3 | Thiol protease inhibitor | P01034 | 254 | 18451 | 18.5 | 0.498 |
| 25 | Coronin-1A | CORO1A | Structural component | P31146 | 114 | 58675 | 11.3 | 0.517 |
| 26 | Perilipin-2 | PLIN2 | Cellular component | P327542 | 678 | 49712 | 39 | 0.468 |
| 27 | Superoxide dismutase | SOD1 | Oxidoreductase | P00441 | 597 | 19804 | 22.7 | 0.473 |
| 28 | Cystatin-C | CST3 | Thiol protease inhibitor | P01034 | 254 | 18451 | 18.5 | 0.498 |
| 29 | Coronin-1A | CORO1A | Structural component | P31146 | 114 | 58675 | 11.3 | 0.517 |
| 30 | Perilipin-2 | PLIN2 | Cellular component | P327542 | 678 | 49712 | 39 | 0.468 |
| 31 | Superoxide dismutase | SOD1 | Oxidoreductase | P00441 | 597 | 19804 | 22.7 | 0.473 |
| 32 | Cystatin-C | CST3 | Thiol protease inhibitor | P01034 | 254 | 18451 | 18.5 | 0.498 |
| 33 | Coronin-1A | CORO1A | Structural component | P31146 | 114 | 58675 | 11.3 | 0.517 |
| 34 | Perilipin-2 | PLIN2 | Cellular component | P327542 | 678 | 49712 | 39 | 0.468 |

The MS/MS spectrum of p90 RSK2 (peptide sequence: LTAALVLR) is presented in Figure 3C. The ratio of 119:116 and 121:117 indicates the relative abundance of RSK2 protein in HepG2-HBx compared to that in HepG2-HBx-Cm16 cells. Similarly, the ratio of 119:113 and 121:115 indicates the relative abundance of RSK2 protein in HepG2-HBx compared to that in HepG2-HBx-Cm6 cells. When the same protein gave two relative quantitative ratios, the quantitation ratio from the experiment with the best P-values was selected.

Figure 3B shows a representative Western blot analysis of RSK2 expression in four cell lines. RSK2 is expressed at higher levels in HepG2-HBx and HepG2-HBx-Cm6, compared with HepG2 and HepG2-HBx-Cm16. HepG2-HBx-Cm6 harboring the HBx retained the ability to complement the augmentation effect, whereas HepG2-HBx-Cm16 did not. This indicates that the host protein RSK2 might play a role in HBx augmenting HBV replication.

### 3.3. Association of RSK2 With HBV Replication

RSK2 expression was silenced by siRNA in HepG2 and HepG2.2.15. (Figure 4A,B). As shown by Western blotting, RSK2 siRNA transfection significantly reduced RSK2 protein...
Table 3. iTRAQ analysis of differentially expressed proteins between HepG2-HBx and HepG2-HBx-Cm6.

| No | Protein description | Gene name | Function | Acce. no | Score | Mass | Cov | Fold change |
|----|----------------------|-----------|----------|----------|-------|------|-----|-------------|
| 1  | Alcohol dehydrogenase 4 | ADH4 | Oxidoreductase | P08319 | 110  | 53588 | 12  | 0.182       |
| 2  | Heat shock protein beta-1 | HSPB1 | Molecular chaperone | P04792 | 438  | 25259 | 39  | 0.274       |
| 3  | Glypican-3            | GPC3     | Protease inhibitor | P51654 | 154  | 77139 | 11.6 | 0.313       |
| 4  | Claudin-6             | CLD6     | Host–virus interaction | P56747 | 129  | 26280 | 5.5  | 0.422       |
| 5  | 3-oxo-5-beta-steroid 4-dehydrogenase | AKR1D1 | Oxidoreductase | P51857 | 160  | 44996 | 8.6  | 0.425       |
| 6  | CTP synthase 2        | CTSP2    | Synthase     | Q9NRF8  | 231  | 77880 | 15   | 0.48        |
| 7  | Liver carboxylesterase 1 | EST1 | Transferase | P23141 | 1014 | 74630 | 32.3 | 0.488       |
| 8  | Dimethylargininedimethylaminohydrolase 2 | DDAH2 | Hydrolase | Q5SSV3  | 221  | 25551 | 19   | 0.527       |
| 9  | Glutathione-S-transferase A1 | GSTA1 | Transferase | P08263 | 138  | 33277 | 19.8 | 0.549       |
| 10 | Glutathione-S-transferase A2 | GSTA2 | Transferase | P09210 | 138  | 32949 | 19.8 | 0.549       |
| 11 | Cystatin-C            | CST3     | Thiol protease inhibitor | P01034 | 254  | 18451 | 18.5 | 0.558       |
| 12 | Aldo-keto reductase family | AKR1B10 | Cellular component | O60218 | 155  | 48851 | 5.1  | 0.558       |
| 13 | Lysosomal-associated membrane protein 1 | LAMP1 | Host–virus interaction | P11279 | 155  | 51147 | 4.8  | 0.558       |
| 14 | Mucosa-associated lymphoid tissue protein 1 | MALT1 | Ubl conjugation | Q9U7Y8  | 168  | 108963 | 10  | 0.575       |
| 15 | cAMP-dependent protein kinase type 1 | PRKAR1A | Celluar component | Q6BQ4  | 523  | 50180 | 29.9 | 0.601       |
| 16 | Putative uncharacterized protein | DKFZp779L0468 | Other | Q6BQ4  | 523  | 50180 | 29.9 | 0.601       |
| 17 | Aldo-keto reductase family 1 member C2 | AKR1C2 | Lipid metabolism | P52895 | 1325 | 45325 | 39.9 | 0.617       |
| 18 | SUB1 homolog     | SUB1     | Transcription | P53999 | 534  | 20471 | 48.8 | 0.642       |
| 19 | Eukaryotic translation initiation factor 4E | EIF4E | Translation | P50730 | 173  | 30481 | 18.4 | 0.586       |
| 20 | Long chain fatty acid CoA | ACSL3 | Fatty acid metabolism | B2R8D | 532  | 99518 | 21.7 | 0.586       |
| 21 | Glucan-branching enzyme | GBE1 | Glycogen biosynthetic process | E9PGM4 | 158  | 87015 | 9.1  | 1.586       |
| 22 | Insulin-like growth factor binding protein 1 | IGFBP1 | Insulin-like growth factor binding | P08333 | 286  | 31954 | 13.1 | 1.642       |
| 23 | Structural maintenance of chromosomes protein 2 | SMC2 | Nucleotide binding | Q97347 | 300  | 18003 | 15.5 | 1.681       |
| 24 | Zinc finger CCHC domain containing 3 | ZCCHC3 | Poly(A) RNA binding | Q9NUSD | 286  | 52299 | 18.6 | 1.705       |
| 25 | Gelsolin           | GSN      | Calcium ion binding | P06396 | 237  | 94869 | 22.2 | 1.798       |
| 26 | Cholecystokinin   | CCK      | Hormone   | P06307 | 104  | 36688 | 7.8  | 1.875       |
| 27 | Histone H1.2     | HIST1H1c | Chromatin DNA binding protein | P16403 | 1508 | 39604 | 30   | 2.042       |
| 28 | Histone H1.4    | HIST1H1E | Chromatin DNA binding protein | P10412 | 1488 | 41017 | 32.9 | 2.042       |
| 29 | RAD23 homolog B | RAD23B | UV excision repair protein | P5472B | 370  | 49590 | 23.2 | 2.193       |
| 30 | Alpha-feto protein | AFP      | Secreted protein | P02771 | 350  | 85062 | 16.2 | 2.383       |
| 31 | Fatty acid binding protein 3 | FABP3 | Transport | P05413 | 138  | 19398 | 34.8 | 3.393       |
| 32 | Metallothionein-1E | MT1E     | Zinc ion binding | P04732 | 71   | 9887  | 32.8 | 4.027       |
| 33 | Fatty acid binding protein 1 | FABP1 | Transport | Q05CP7 | 768  | 22745 | 52.2 | 5.737       |

levels, whereas control siRNA has no effect. The effect of RSK2 siRNA transfection on HBV replication was explored further. The level of HBV replication intermediates in RSK2 siRNA-transfected HepG2.2.15 cells was twofold lower than that in the controls. The levels of HBV DNA, HBcAg, HBsAg, and HBeAg were decreased 37.4, 34.0, 42.3, and 50.2, respectively, after silencing RSK2 in HepG2.2.15 (Figure 4A). This indicates that silence of RSK2 reduced HBV DNA replication and expression.

3.4. Association of RSK2 in HBx Enhancing HBV Replication

As shown by Western blotting in Figure 4A, RSK2 siRNA transfection of HepG2 cells significantly reduced RSK2 protein levels. In HepG2 cells, the levels of HBV DNA replication intermediates synthesized from the HBx-deficient HBV genome were twofold lower than levels synthesized from the wild-type HBV genome. Furthermore, the decreased levels of HBV replication intermediates synthesized from the HBx-minus HBV construct were restored to levels similar to that observed with the wild-type construct by cotransfection of HBx expression plasmid (two- to threefold that of HBx-deficient HBV). These results indicate that HBx has an augmentation role in HBV transcription and replication. In contrast, the levels of HBV replication intermediates synthesized from HBx-minus HBV mutant genome were not restored to levels observed with wild-type HBV by cotransfection of the HBx expression plasmid in RSK2 siRNA-transfected HepG2 cells. The levels of HBV DNA (p < 0.001), HBcAg (p < 0.001), HBsAg (p < 0.01) and HBeAg (p < 0.001) shows the same trend as HBV DNA replication intermediates (Figure 4C).
Figure 3. Evaluation of the differentially expressed proteins. A) Real-time RT-PCR detected the relative mRNA expression levels of ADH4, SOD1, CSTB, ACSL4, CORO1A, PLIN2, APOA1, RSK2, PRKAR1A, RRP1B, DPP4, and FABP1 in HepG2-HBx, HepG2-HBx-Cm6, and HepG2-HBx-Cm16 cells. *p < 0.05 differ from HepG2-HBx and HepG2-HBx-Cm16. B) Western blot for RSK2 in cell line HepG2, HepG2-HBx, HepG2-HBx-Cm6, and HepG2-HBx-Cm16 cell. C) Representative MS/MS spectrum showing the peptides from RSK2 (peptide sequence: LTAALVLR).

4. Discussion

It has been demonstrated that HBx plays an important role in augmenting HBV replication by transcriptional transactivation function in vivo and in vitro. However, the underlying molecular mechanism by which HBx enhances HBV replication are not fully understood. The transcriptional transactivation function of HBx is dependent on pleiotropic protein–protein interactions. The "key" host proteins involved in HBx enhancement of HBV transcription and replication have not been identified. Clarification of the roles of host proteins in HBx augmentation of HBV transcription and replication would help elucidate the HBV pathogenicity mechanism.
Figure 4. Association of RSK2 in HBV replication and HBx augmenting HBV replication. A) Levels of RSK2, HBV DNA replication intermediates, HBcAg, HBsAg, and HBeAg in HepG2.2.15 and RSK2 siRNA-transfected HepG2.2.15. B) RSK2 detected by Western blotting after siRNA transfection of HepG2 cells. C) Levels of HBV DNA RI, HBcAg, HBsAg, and HBeAg in HepG2 which were transfected with wild-type HBV, HBx-minus HBV, and HBx-minus HBV plus ectopic expression of HBx plasmid. Data shown are the means ± SD. Statistical significance was examined by one-way analysis of variance pairwise comparison. *p < 0.05 was considered statistically significant; **p < 0.01; ***p < 0.001. RI: replication intermediates.
In this study, we utilized iTRAQ proteomic methodology coupled with LC-ESI-MS/MS to identify and quantitate differentially expressed proteins among HepG2 cell lines stably harboring different functional domains of HBx. Seventy differentially expressed proteins were identified among HepG2-HBx, HepG2-HBx-Cm6, and HepG2-HBx-Cm16 by two duplicate groups. Many of them, including ADH4, SOD1, CSTB, ACS14, CORO1A, PLIN2, APOA1, RSK2, PRKAR1A, RRP1B, DPP4, and FABP1, were confirmed by qRT-PCR analysis. RSK2 was expressed at higher levels in HepG2-HBx and HepG2-HBx-Cm6, compared with HepG2-HBx-Cm16 and HepG2. These results are consistent with the protein expression level determined by the iTRAQ approach. Tao Zhang et al.\textsuperscript{[10]} performed an integrated proteomics and bioinformatics analysis of HBx interacting proteins. They also identified RSK2 as a novel interactor. Collectively, these data provide evidence that the iTRAQ reagents labeling method for large scale protein quantification is powerful and reliable for HBV-related investigations.\textsuperscript{[13]}

The RSK2 siRNA-transfected HepG2.2.15 cells showed decreased HBV DNA, HBsAg, and HBeAg levels. This indicates that RSK2 is involved in regulating HBV replication. HBx augments HBV replication and expression, because an HBx-minus HBV mutant genome led to decreased levels of HBV replication intermediates, HBsAg, and HBeAg. As a result, HBx decreases the number of proteins synthesized from the HBx-minus HBV mutant genome, were not restored and levels observed in wild-type HBV by transient ectopic expression of HBx. After silencing RSK2 expression, the levels of HBV replication intermediates, HBsAg and HBeAg synthesized from the HBx-minus HBV mutant genome, were not restored to levels observed in wild-type HBV by transient ectopic expression of HBx. This indicates that RSK2 plays an important role in HBx by augmenting HBV replication. RSK2 was identified as a novel host protein in HBx augmenting HBV replication.

The RSK (90 kDa ribosomal S6 kinase) family comprises a group of highly related serine/threonine kinases that regulate diverse cellular processes, including cell growth, proliferation, survival and motility.\textsuperscript{[12]} Members of this family, which are downstream effectors of the Ras/ERK signaling pathway, include four vertebrate isoforms (RSK1, RSK2, RSK3, and RSK4).\textsuperscript{[13]} RSK2 was identified as an important effector of ERK in global transcriptional regulation. Indeed, activated RSK2 was shown to phosphorylate several transcription factors including AP-1, CREB, c-Fos, c-Jun, and others, some of which contribute to the IEG (immediate-early gene) response or are IEG products themselves.\textsuperscript{[12] Some transcription factors play an important role in the transcription of retrovirus. The previous studies demonstrated that HBx transcriptional activity is also linked to its capacity to stimulate MAPKs and JAK/STAT signaling pathways.\textsuperscript{[14]} Despite the fact that RSK2 is a member of MAPKs signaling pathways, no study has investigated the relationship between HBx and RSK2. RSK2 has been implicated in other viral infections, such as HIV, HCV, and influenza.\textsuperscript{[15,16]} Kapoši’s sarcoma-associated herpes virus ORF45 mediates transcriptional activation of the HIV-1 long terminal repeat via RSK2.\textsuperscript{[17–19]} HIV Tat protein interacts with RSK2 and activated RSK2 kinase activity in cells, and might serve to induce early changes in the chromatin organization of the HIV LTR.\textsuperscript{[20]} The regulation and functional role of RSK2 may explain its role in HBx enhancement of HBV replication. In this study, pathway analysis also revealed that HBx-related differentially expressed proteins are associated with lipid metabolism. Previous studies indicated that HBx-induced abnormal lipid metabolism of hepatoma in hepatocarcinogenesis.\textsuperscript{[21]} This indicates that HBx induces abnormal lipid metabolism to meet the bioenergetic demands of extreme cell growth and proliferation. Another study showed that fatty acids increase HBx stabilization and HBx-induced inflammatory gene expression.\textsuperscript{[22]} The differentially expressed protein apolipoprotein A-I (apoA-I), which has a specific role in lipid and cholesterol metabolism, has been proved to be a novel interactor with HBx and may influence HBV secretion.\textsuperscript{[10]}

In conclusion, RSK2 was identified as a novel host protein in HBx augmenting HBV replication by iTRAQ-based quantitative comparative proteomics. These results may help to better understand the HBV pathogenicity mechanism.

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Conflict of interest

The authors have declared no conflict of interest.

Keywords

HBx protein, hepatitis B virus, p90 ribosomal S6 kinase 2, proteomics, replication

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