In Vivo and In Vitro Validation of the Interaction Between Hepatitis B Virus X Protein and TAB1 Protein Postprint

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Abstract

Objective: Leveraging our laboratory' s prior foundation in mass spectrometry analysis techniques and data analysis research, we employed co-immunoprecipitation (Co-IP) and GST pull-down assays to verify the interaction between HBV X protein and Tab1 protein, thereby providing experimental evidence for further investigating the role of HBx in the carcinogenic mechanism of chronic HBV infection. Methods: The pGEX-2TK-GST-HBx plasmid was successfully constructed, the GST-HBx fusion protein was induced and expressed, and incubated with GST-beads; pcDNA3.1/myc-His(-)B-Tab1 was constructed and transfected into 293T cells for expression, followed by GST pull-down in vitro assay to verify their interaction; pcDNA3.1/myc-His(-)B-Tab1 and pcDNA3.1-3×flag-HBx eukaryotic expression plasmids were constructed and co-transfected into 293T and HepG2 cells for expression. Co-IP experiments verified that anti-Myc antibody could precipitate HBx from cell lysates, confirming their interaction in both cell lines. Results: The results demonstrated that HBx and Tab1 can interact under both in vivo and in vitro conditions, laying a foundation for further elucidating the function and mechanism of HBV X protein.

Full Text

In Vivo and In Vitro Validation of the Interaction Between Hepatitis B Virus X Protein and Tab1

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Abstract

Objective: Building upon our laboratory' s previous mass spectrometry analysis and data mining foundation, this study employed co-immunoprecipitation (Co-IP) and GST pull-down assays to validate the interaction between HBV X protein (HBx) and Tab1 protein, providing experimental evidence for further investigation of HBx' s role in HBV chronic infection and carcinogenesis. **Methods:** We constructed the pGEX-2TK-GST-HBx plasmid, induced expression of the GST-HBx fusion protein, and incubated it with GST-beads. The pcDNA3.1/myc-His(-)B-Tab1 plasmid was constructed and transfected into 293T cells for expression, followed by GST pull-down verification of their in vitro interaction. Additionally, eukaryotic expression plasmids pcDNA3.1/myc-His(-)B-Tab1 and pcDNA3.1-3×flag-HBx were co-transfected into 293T and HepG2 cells. Co-IP experiments demonstrated that anti-Myc antibody could precipitate HBx from cell lysates, confirming their interaction in both cell lines. **Results:** Our findings demonstrate that HBx and Tab1 can interact under both in vivo and in vitro conditions, establishing a foundation for further elucidation of HBx protein function and mechanism of action.

Keywords: HBV X Protein; Tab1; Co-IP; GST pull-down; Interaction

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Introduction

Hepatitis B virus (HBV) can establish persistent infection in hepatocytes, posing a serious threat to human health. HBV infection causes acute and chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC). Previous studies have demonstrated that chronic HBV infection is a major risk factor for primary liver cancer, with approximately 80% of HCC cases associated with HBV infection. Following HBV infection, complex mechanisms contribute to the development and progression of HCC. The HBV X protein (HBx), the smallest open reading frame in the HBV genome, consists of 154 amino acids and two functional domains. HBx plays a crucial role in HBV-associated hepatocarcinogenesis, functioning as a transactivator or proto-oncogene to promote tumor development. Studies have shown that HBx interacts with multiple host proteins during HBV infection, cell cycle regulation, apoptosis, and hepatocarcinogenesis, establishing HBx as an important multifunctional effector in HBV pathogenesis.

Tab1 is a critical scaffold protein initially identified through its interaction with TAK1 protein. Human Tab1 comprises 504 amino acids and primarily functions to recruit other proteins into complexes, though it lacks catalytic activity

required for protein activation. Tab1 binds TAK1 to form a complex that induces TAK1 autophosphorylation, and research has shown that TAK1 kinase activity is associated with Tab1 in both yeast and mammals. Activated TAK1 can stimulate multiple signaling pathways, including NF- B and MAPK, which are increasingly recognized for their involvement in human diseases.

As an important transcriptional activator, HBx mediates various signal transduction pathways involved in inflammation, immune response, apoptosis, cell cycle regulation, and differentiation. However, most studies have focused on HBx' s indirect effects on intracellular signaling pathways, with relatively few reports on its direct target proteins. Consequently, research into the molecular mechanisms of HBV infection and pathogenesis remains ongoing. Leveraging our laboratory' s mass spectrometry data, this study aimed to validate the interaction between HBV X protein and Tab1 using Co-IP and GST pull-down assays, laying the groundwork for investigating the molecular mechanisms by which HBx influences cellular signaling pathways following HBV infection.

Materials and Methods

1.1 Materials and Reagents

E. coli DH5 and BL21 strains, 293T cells, HepG2 cells, eukaryotic expression plasmid pcDNA3.1-3×flag-HBx, cellular cDNA template, pcDNA3.1/myc-His(-)B, and pGEX-2TK were maintained in our laboratory. Commercial products included 2×hlingene Pfu PCR masterMix (Shanghai Huiling Biological), EcoR , Sma I, BamH (TaKaRa), T4 DNA ligase (BioLabs, New England), endotoxin-free plasmid extraction and gel recovery kits (Beijing Tiangen), DNA transfection kit (Biotool), ECL detection kit (Suzhou New Cell & Molecular Biotech), Anti-flag Beads (Sigma), Glutathione-Sepharose 4B (GE Healthcare, USA), anti-Myc tag polyclonal antibody (Sigma), HRP-labeled goat anti-rabbit IgG (Cell Signaling), DNA markers (MD2K, MD15K) (Beijing TransGen Biotech), ampicillin, agarose, and other analytical grade chemical reagents. PCR primers were synthesized by Suzhou Genewiz Biotechnology, and sequencing was performed by Shanghai Ruidi Biotechnology.

1.2.1 Cell Culture

293T and HepG2 cells were cultured in DMEM medium supplemented with 10% fetal bovine serum at 37°C in a humidified incubator with 5% CO . Cells were routinely passaged using 0.25% trypsin.

1.2.2 Construction of pcDNA3.1/myc-His(-)B-Tab1 Plasmid

Primers were designed based on the multiple cloning sites of the pcDNA3.1/myc-His(-)B vector and the full-length Tab1 coding sequence. Tab1 forward

primer: 5 -GGAATTCCGCAGGGGTTCCTCCAAG-3 ; reverse primer: 5 -CCCAAGCTTGGCGGTGCTGTCACCACGCTCTG-3 . EcoR I and Hind III restriction sites were introduced at the 5 and 3 ends, respectively. The full-length Tab1 sequence was amplified from 293T cDNA library. PCR products were analyzed by 1% agarose gel electrophoresis, purified, and digested with EcoR I and Hind III. The digested Tab1 fragment was directionally cloned into the similarly digested vector, transformed into *E. coli* DH5 competent cells, and plated. Random colonies were selected for expansion, and plasmids were extracted for double digestion and colony PCR validation. Positive clones were sequenced and compared with GenBank sequences, yielding the recombinant plasmid pcDNA3.1/myc-His(-)B-Tab1.

1.2.3 Construction of pGEX-2TK-GST-HBx Plasmid

Primers were designed based on the pGEX-2TK-GST vector multiple cloning sites and HBx full-length coding sequence. HBx forward primer: 5 -TCCCCCGGGAATGGCTGCTCGGGTGTGCTGC-3 ; reverse primer: 5 -CGAATTCTTAGGCAGAGGTGAAAAAGT-3 . Sma I and EcoR I sites were introduced at the 5 and 3 ends, respectively. The HBx full-length sequence was amplified from laboratory-stored pcDNA3.1-3×flag-HBx template. PCR products were analyzed by 1% agarose gel electrophoresis, purified, and digested with EcoR I and Sma I. The digested HBx fragment was cloned into the vector, transformed into DH5 cells, and validated through double digestion, colony PCR, and sequencing, yielding pGEX-2TK-GST-HBx.

1.2.4 Induction and Purification of GST-HBx Fusion Protein

pGEX-2TK-GST-HBx and empty pGEX-2TK vectors were transformed into *E. coli* BL21 competent cells. Single colonies were cultured overnight in 3 mL LB medium, then inoculated into 200 mL LB at a 1:10 ratio and grown at 37°C, 220 rpm for approximately 5 hours until OD reached 0.6–0.8. Protein expression was induced with 0.5 mmol/L IPTG at 25°C, 160 rpm overnight. Bacteria were harvested by centrifugation at 4°C, 5000g for 10 minutes. Cell pellets were resuspended in lysis buffer, sonicated, and centrifuged at 4°C, 12,000 rpm for 20 minutes. The supernatant was transferred to a new tube and centrifuged again until no pellet remained. Purification was performed using Glutathione-Sepharose 4B according to the manufacturer's instructions, yielding beads bound with GST-HBx fusion protein or GST alone. The beads were stored briefly at 4°C for subsequent incubation with lysates from 293T cells transfected with pcDNA3.1/myc-His(-)B-Tab1.

1.2.5 Co-Immunoprecipitation of Myc-Tab1 and Flag-HBx

pcDNA3.1/myc-His(-)B-Tab1 and pcDNA3.1-3×flag-HBx plasmids were extracted using an endotoxin-free midiprep kit according to the manufacturer' s protocol. For co-transfection experiments, pcDNA3.1-3×flag-HBx (or empty pcDNA3.1 vector) was co-transfected with pcDNA3.1/myc-His(-)B-Tab1 into

293T and HepG2 cells. After 24–36 hours, cells were lysed using Biyuntian Western and IP lysis buffer to harvest total protein. For Western blot input controls, 80 L of lysate was mixed with 20 L SDS-PAGE loading buffer and boiled at 100°C for 10 minutes. The remaining lysate was incubated with pre-washed Anti-flag Beads at 4°C for 4 hours. Beads were collected on a magnetic stand, washed twice with PBS containing protease inhibitors (5 minutes each), then resuspended in 50 L 1×SDS-PAGE loading buffer and boiled at 100°C for 10 minutes. The supernatant (IP sample) was collected for SDS-PAGE electrophoresis alongside input samples. Proteins were transferred to nitrocellulose membranes and analyzed by Western blotting using anti-Myc antibody (rabbit) to detect both input lysates and immunoprecipitated complexes.

1.2.6 GST Pull-Down Assay of Myc-Tab1 and GST-HBx

Glutathione-Sepharose 4B beads (20 L) pre-bound with GST-HBx fusion protein or GST alone were washed three times with PBS, then incubated with lysates from 293T cells expressing Myc-Tab1 at 4°C for 4 hours. After incubation, beads were centrifuged at 3,000 rpm, 4°C for 5 minutes and the supernatant was discarded. Beads were washed four times with lysis buffer (10 minutes each) and the supernatant was completely removed. Washed beads were resuspended in 1×SDS-PAGE loading buffer, boiled for 10 minutes, and analyzed by SDS-PAGE followed by Western blotting with anti-Myc antibody. The GST protein group served as the negative control.

Results and Analysis

2.1 Construction and Expression of pcDNA3.1/myc-His(-)B-Tab1

Using 293T cDNA as template, the full-length Tab1 coding sequence (1,515 nucleotides) was amplified by PCR. Agarose gel electrophoresis confirmed the DNA fragment size matched the expected length [Figure 1: see original paper]. The PCR product was digested and inserted into the vector. Double digestion (EcoR I and Hind III) and colony PCR validation of the recombinant plasmid pcDNA3.1/myc-His(-)B-Tab1 yielded fragments corresponding to the expected size [Figure 2: see original paper]. Sequencing confirmed the insert sequence was correct. To verify expression, the constructed plasmid was transfected into 293T cells and analyzed by Western blotting with anti-Myc antibody [Figure 3: see original paper], demonstrating successful expression of the target protein at the expected molecular weight.

2.2 Construction of pGEX-2TK-GST-HBx Plasmid

Using laboratory-stored pcDNA3.1-3×flag-HBx as template, the full-length HBx coding sequence with restriction sites was amplified by PCR. Agarose gel

electrophoresis confirmed the DNA fragment size matched expectations [Figure 4: see original paper]. The PCR product was digested and cloned into the vector. Double digestion (Sma I and EcoR I) and colony PCR validation of pGEX-2TK-GST-HBx produced fragments of the expected size [Figure 5: see original paper]. Sequencing confirmed the insert sequence was correct.

2.3 Induction and Purification of GST-HBx Fusion Protein

The pGEX-2TK-GST-HBx recombinant plasmid was transformed into BL21 cells. Induction with 0.5 mmol/L IPTG at 25°C overnight yielded a protein band consistent with the expected molecular weight [Figure 6: see original paper]. Following sonication, the supernatant was directly used for bead binding. Analysis of beads after binding showed the GST-HBx fusion protein was expressed at the correct size and could be obtained in sufficient soluble form, though some degradation was observed [Figure 7: see original paper]. The prepared beads were stored briefly at 4°C for subsequent incubation with Myc-Tab1 cell lysates from pcDNA3.1/myc-His(-)B-Tab1 transfected 293T cells.

2.4 Co-Immunoprecipitation of Myc-Tab1 and Flag-HBx

Myc-tagged Tab1 and Flag-tagged HBx plasmids were co-transfected into 293T and HepG2 cells. Experimental groups were co-transfected with pcDNA3.1-3×flag-HBx and pcDNA3.1/myc-His(-)B-Tab1, while control groups received pcDNA3.1-3×flag empty vector with pcDNA3.1-myc-His(-)B-Tab1. Cell lysates were immunoprecipitated with anti-FLAG antibody (mouse) and analyzed by Western blotting with anti-Myc antibody (rabbit) to detect Tab1 in the precipitated complexes. In 293T cells, anti-FLAG antibody successfully precipitated Myc-Tab1 from lysates co-expressing both proteins, with anti-Myc antibody detecting a clear signal. In contrast, control co-transfections with empty vector showed no signal [Figure 8: see original paper]. These results demonstrate specific interaction between Tab1 and HBx, not with the FLAG tag alone. Similar Co-IP experiments in HepG2 hepatocellular carcinoma cells confirmed that Tab1 binds HBx but not the FLAG tag [Figure 9: see original paper], establishing their interaction in this cell line as well.

2.5 GST Pull-Down Assay of Myc-Tab1 and GST-HBx

Glutathione-Sepharose 4B beads pre-bound with GST-HBx fusion protein or GST alone were incubated with lysates from 293T cells expressing Myc-Tab1. GST protein served as the negative control. Results showed that Myc-Tab1 interacted with GST-HBx but not with GST alone [Figure 10: see original paper], demonstrating that Tab1 specifically binds HBx in vitro.

Discussion

HBx is recognized as an important multifunctional factor in HBV pathogenesis. Current in vivo and in vitro studies demonstrate that HBx plays significant roles in controlling cell proliferation and activity, and exerts direct oncogenic effects during HBV-induced hepatocarcinogenesis. For example, HBx transactivates multiple oncogenes including c-myc, ras, and c-fos, and regulates various nuclear transcription factors such as NF- B, AP-1, and c-EBP, thereby stimulating multiple signaling cascades involved in hepatocyte proliferation, transformation, and apoptosis, including PI3K/AKT, JAK-STAT, and MAPK pathways. These interconnected pathways form a complex signaling network that amplifies HBx' s effects on hepatocytes.

Regarding Tab1, it was originally discovered as a TAK1-interacting protein. Tab1 can directly interact with and activate TAK1, leading to IKK activation and NF- B activation. Studies have identified a conserved modified region in Tab1' s carboxyl terminus that enables it to both bind and activate TAK1 (a MAPKKK family member) through the classical three-tiered kinase cascade to regulate p38 activity. This same conserved carboxyl terminus also allows Tab1 to directly bind p38 (a MAPK family member), inducing p38 autophosphorylation and activation. While HBx signaling has been extensively studied, detailed investigations into how HBx mediates signal transduction—particularly regarding its direct interaction partners—remain limited. Based on current understanding of HBx and Tab1 functional regulation, we hypothesize that Tab1 plays an important role in HBx-mediated signaling pathways involved in HCC development, making it significant for further exploration of HBx' s oncogenic mechanisms.

In summary, this study employed Co-IP and GST pull-down assays to validate HBx-Tab1 interactions in vivo and in vitro. We constructed prokaryotic and eukaryotic expression plasmids for HBx and a eukaryotic expression plasmid for Tab1. GST pull-down experiments demonstrated their direct interaction in vitro. Furthermore, co-transfection of HBx and Tab1 into 293T and HepG2 cells followed by Co-IP analysis confirmed their interaction in cellular contexts. These results suggest that HBx may activate relevant signaling pathways through direct binding to Tab1 during HBV infection, providing experimental basis for further investigation of the functional roles of HBx-Tab1 interactions in signaling pathways.

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Note: Figure translations are in progress. See original paper for figures.