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IDENTIFICATION OF GRP75 AS A NOVEL PRES1 BINDING PROTEIN USING A PROTEOMICS STRATEGY

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ABSTRACT

The PreS1 region of the L protein is important in cell attachment and consequently in hepatitis B virus (HBV) infectivity. To identify novel PreS1 interacting protein, we performed Glutathione-S-transferase (GST) pull-down, two-dimensional gel electrophoresis (2-DE) and mass spectrometry assays. Glucose-regulated proteins (GRP) 78 and 75 were found to bind PreS1. The interactions between PreS1 and GRP75 were confirmed by a co-immunoprecipitation experiment. GRP78 and GRP75 may play important roles in mediating HBV virion entering into hepatocyte and regulating proper folding of the L protein due to their critical functions in protein folding and trafficking. The finding of novel PreS1 binding protein enriches our knowledge about molecular mechanism of HBV infection.

Key words: HBV, PreS1, Proteomics, GRP75

INTRODUCTION

The hepatitis B virus (HBV) is a worldwide cause of hepatic disease with variations in prevalence from region to region (13). China is one of the highest HBV prevalence areas. There are approximately 130 million HBV carriers in China. Current treatment for HBV infection has limited efficacy. The attachment to hepatocytes by HBV during infection has long been proposed to be a potential target for antiviral intervention. However, little is known about the molecular events mediating HBV attachment to hepatocytes. HBV PreS1 region, specifically, the aa 21-47 segment, is believed to play an essential role in mediating HBV attachment to the putative receptor on hepatocytes (9,14). In the past two decades, Human immunoglobulin A (IgA) receptor, a 31-kDa protein, interleukin-6, a 44-kDa protein (HBV-BP), Homology to SCCA1, a human squamous cell carcinoma antigen 1 (human serpin), p80 (GRP78), a 35-kDa protein homolous to Glycerinaldehyde-3-phoshate-dehydrgenase (GAPD), a serum glycoprotein of 50-kDa, and an asialoglycoproteinreceptor (ASGPR) have been proposed as PreS1 binding proteins (4), but none of these molecules has been identified as receptor in HBV infection.

With the rapid development in screening technology and bioinformatics, novel receptor or coreceptor candidates have been discovered recently. Deng found that lipoprotein lipase (LPL), a key enzyme in lipoprotein metabolism, might interact with PreS and HBV particles by using phage display library (3). Li *et al*. screened NACA as a novel PreS1 associated protein by using yeast two-hybrid system (10). Recently, proteomic techniques have been used as a new tool for studying protein-protein interaction. In this study, to identify

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novel PreS1 interacting protein, we performed Glutathione-S-transferase (GST) pull-down, high-resolution two-dimensional gel electrophoresis (2-DE) and mass spectrometry assays. GRP75 was identified as a novel PreS1 binding protein and its possible roles in virus infection are discussed. GRP78, a protein previously described to interact with preS1, was also identified in these assays.

MATERIALS AND METHODS

GST fusion construct of PreS1

The PreS1 fragment was amplified by PCR using HBV DNA as template extracted from serum of a chronically infected patient with HBsAg, anti-HBc, and HBeAg positive serology. PCR were performed using the following primers, 5′-AGCGGATCCATGGGAGGTTGGTCTTCCA-3′ (forward) and 5′-ATATCTCGAGTTAGGCCTGAGGATGACTGT-3′ (reverse). The PCR products were subsequently cloned as *BamH* I-*Xho* I fragments into pGEX-4T2 (Pharmacia) and recombinant vectors were introduced into the *E. coli* BL21 (DE3) (Invitrogen). The GST fusion proteins were induced as described previously and were purified from the bacterial cell lysates by affinity binding to glutathione sepharose beads (Pharmacia) as detailed elsewhere (11).

GST pull-down assay

HepG2 cells were lysed in binding buffer (50 mMTris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 1 μg/mL leupeptin, 1 μg/mL aprotinin and 0.1 mM PMSF.), and triplicate independent soluble protein fraction was incubated with GST and GST-PreS1 fusion proteins overnight at 4°C. Then, co-precipitations were performed by addition of 80 μl glutathione–sepharose 4B resin. After washing five times with 600 μl of binding buffer, binding proteins were eluted with 200 μl of 10 mM reduced glutathione in PBS. Eluted samples were precipitated with 4 volumes of cold acetone, centrifuged and the pellets were washed with 200 μl of 20% (v/v) cold methanol. After centrifugation for 30 min at 12,000×g, pellets were air-dried and dissolved in rehydrating solution (8 M urea, 2% CHAPS, 18mM dithiothreitol (DTT), 0.5% IPG buffer pH 3-10) for further 2-DE analysis.

Two-dimensional gel electrophoresis (2-DE)

2-DE was performed as described previously (2). Briefly, samples were subjected to isoelectric focusing (IEF) using IPG DryStrips with immobilized pH gradient, pH range 3-10, 17 cm, linear (Bio-Rad). IEF was performed in an IPGphor (Amersham Biosciences) according to the following protocol: 30 V 6 h, 60 V 6 h, 500 V 1 h, 1000 V 1 h, 8000 V 3-4 h to 30 000 Vh. After IEF, strips were equilibrated in 50 mM Tris-HCl, pH 8.8, 6 M urea, 2.0% SDS, 30% glycerol with 1% DTT for 10 min, and then for 10 min in the same buffer without DTT but with 4% iodoacetamide. Equilibrated strips were placed on top of 10% (w/v) polyacrylamide gel to separate the proteins by molecular mass.

Protein identification

Protein spots were excised from the gels, destained, and subjected to in-gel digestion with trypsin (modified, sequence grade porcine; Promega), as described earlier (2). Tryptic peptides were dissolved in 2 μl of 0.5% TFA containing the matrix (a-cyano-4-hydroxycinnamic acid) and analyzed by matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) (Bruker Daltonics, Germany). Data were screened against the NCBInr databases using the MASCOT search program (www.matrixscience.com).

Immunoprecipitation

PreS1 gene was cloned into the *BamH* I and *Hind* restriction sites of pXJ40 (pXJ40-PreS1). Encoding domain of GRP75 gene was generated by using the forward primer (5'-CTTAAGCTTGCCATGATAAGTGCCAGCCGAGCTG-3') and reverse primer (5'-CGCCTCGAGTATTACTGTTTTTCCTCCTTTTGATC-3') and cloned into *Hind* and *Xho* I restriction sites of pXJ40 (pXJ40-GRP75). pXJ40-PreS1 and pXJ40-GRP75 were co-transfected into COS7 cells using Lipofectamine 2000

(Invitrogen). 48 h after transfection, cells were washed with phosphate-buffered saline and lysed in 0.5 ml lysis buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 % NP-40, 100 µmol/L PMSF, 1 µg/mL leupeptin, 1 µg/mL aprotinin). After brief sonication, the lysate was centrifugated at 15000 rpm for 15 min at 4°C. The supernatant was incubated with mouse monoclonal anti-PreS1 antibody (Feipon Biotech Inc, China) overnight at 4°C, then incubated with a slurry of protein-A-sepharose with rotation for 2 h at 4°C. The beads were pelleted and washed five times with cell lysis buffer. Finally, proteins were solubilized in sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer by boiling.

Western blot

Samples were resolved in 8% or 17.5% polyacrylamide gel, and proteins were transferred to a nictrocellulose membrane. Immunoblotting was carried out using rabbit anti-GRP75 antibody (Santa Cruz) or mouse anti-PreS1 antibody as indicated above and anti-rabbit or anti-mouse IgG-horseradish peroxidase (HRP) conjugates. After rinsing with PBS-T (phosphate-buffered saline [PBS] containing 0.1% Tween 20), the blots were visualized by the enhanced chemiluminescence procedure as recommended by the supplier (Pierce).

RESULTS

Preparation of GST-fusion construct of PreS1

We sought to identify novel PreS1-binding proteins using a GST pull-down assay. To this end, an expression plasmid, pGEX4T2-PreS1 was generated carrying a full-length preS1 sequence. The GST-PreS1 fusion protein was expressed in *E. coli* BL21. SDS-PAGE analysis revealed that GST-PreS1 can be expressed and be co-purified with other proteins (Fig. 1A, lane 3). Similar to the previous study (11), Western blot analysis with anti-preS1 monoclonal antibody indicated a major band of a 39 K protein corresponding to the intact GST-PreS1, several bands of lower molecular weight proteins that most likely represent degradation products of the GST-preS1 fusion protein (Fig. 1B, lane 3).

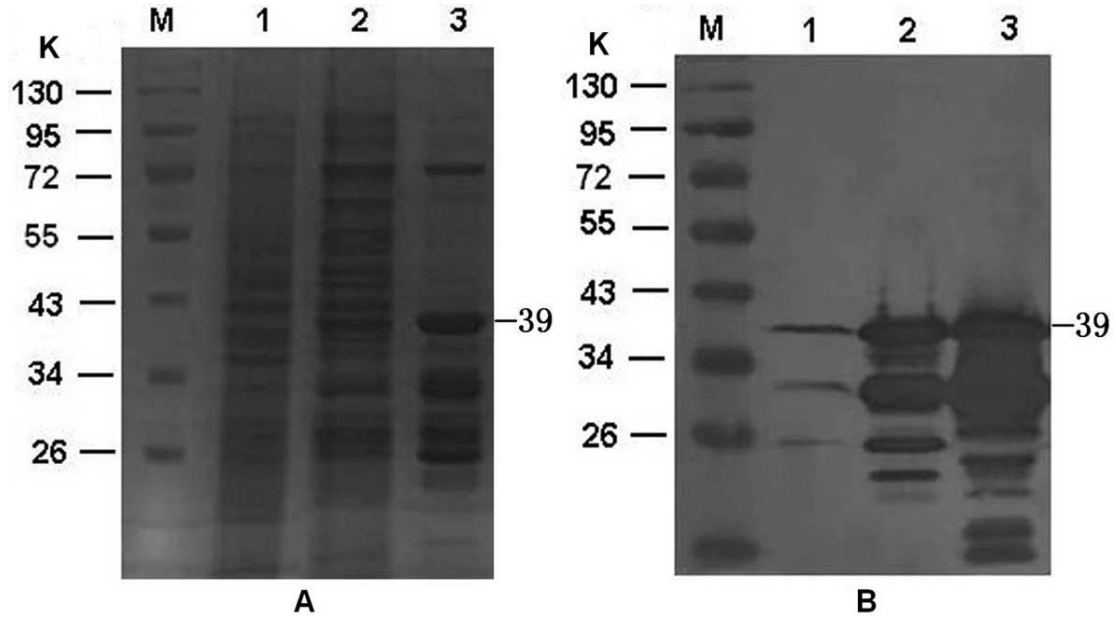

Figure 1. Expression and purification of the preS1. (A) 10% SDS–PAGE. B. Immunoblotting with anti-preS1 monoclonal antibody. Uninduced recombinant cell lysates, induced recombinant cell lysates, and GST-PreS1 protein purified using glutathione-agarose (lane 1, 2, and 3, respectively), molecular size markers (M)

2-DE analysis of proteins binding with PreS1

To identify proteins associated with PreS1, the high resolving power of 2-DE was applied to the analysis. The GST and GST-PreS1 samples incubated with and without HepG2 cell lysate were separated using IPG strip in first dimension and 10% polyacrylamide gels in the second dimension (Fig.2). GST and GST-PreS1 samples incubated with binding buffer (Fig. 2A, B), GST sample incubated with HepG2 cell lysate (Fig. 2C) were set as control. Those protein spots appeared in the pull-down sample gel while not in the control gels were excised and further analyzed by MALDI-TOF-MS. Database searching showed that two proteins of HepG2 could bind with GST-PreS1, including GRP78 and GRP75 (Point 1, 2, Fig. 2D). Peptide masses and further data concerning protein identity are listed in Table 1.

GRP75 associates with PreS1 in vivo

It has been previously demonstrated that GRP78 bound specifically to the PreS1 in vitro and in vivo (1). In this study, we therefore opted to confirm the interaction between GRP75 and PreS1 *in vivo*. The coding sequence of PreS1 and GRP75 was cloned into pXJ40. COS-7 cells were co-transfected pXJ40-GRP75, pXJ40-PreS1, or empty vector. As shown in figure 3, anti-PreS1 antibody coimmunoprecipitated PreS1 and GRP75. It is thus possible that GRP75 is PreS1-associated protein.

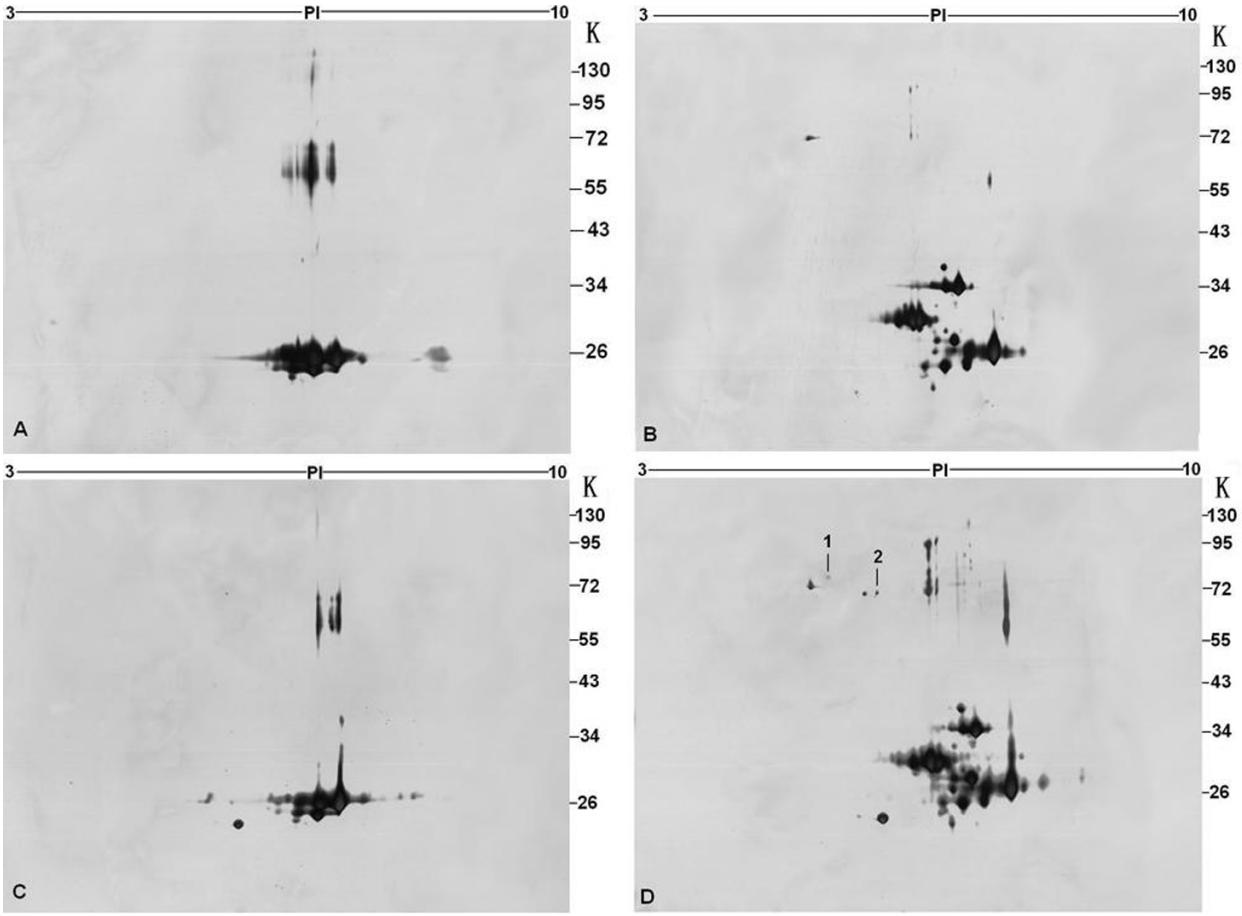

Figure 2. 2-DE analysis of GST-PreS1 binding protein. GST pull down using GST (A), GST-PreS1 (B) with binding buffer, GST (C) and GST-PreS1 (D) with HepG2 cell lysate. IEF on Immobiline DryStrips (pH range 3-10) followed by SDS-PAGE and silver staining. Protein spots 1 and 2 (D) were identified by MALDI-TOF-MS, one of which was GRP78 (point 1), another was GRP75 (point 2)

Table 1. Protein identities and peptide masses

Protein identified	Actual pI/Mw (kDa)	Calculated pI/Mw (kDa)	Sequence coverage (%)	Accession no.	Identified peptide sequences	Experimental masses (Da)	Calculated masses (Da)
GRP78	5.4/76.9	5.07/72.5	38	P11021	NGRVEIIANDQGNR	1554.8192	1554.7862
					ITPSYVAFTPEGERLIGDAAK	2234.1613	2234.1582
					NQLTSNPENTVFDAKR	1832.9256	1832.9016
					TWNDPSVQQDIK	1429.7298	1429.6837
					TKPYIQVDIGGGQTK	1603.8926	1603.8569
					TFAPEEISAMVLTK	1535.8264	1535.7905
					KVTHAVVTVPAYFNDAQR	2015.0681	2015.0588
					DAGTIAGLNVMR	1216.6695	1216.6234
					IINEPTAAAIAYGLDKR	1815.0120	1814.9890
					VMEHFIK	902.4798	902.4684
					RALSSQHQAR	1152.6457	1152.6112
					AKFEELNMDLFR	1511.7843	1511.7442
					KSDIDEIVLVGGSTR	1587.8874	1587.8467
					EFFNGKEPSR	1209.6170	1209.5778
					VYEGERPLTK	1190.6711	1190.6295
					DNHLLGTFDLTGIPPAPR	1933.0238	1933.0057
					NKITITNDQNR	1315.7127	1315.6844
					ITITNDQNRLTPEEIER	2041.0551	2041.0440
					IDTRNELESYAYSLK	1800.9108	1800.8893
GRP75	6.02/70.9	5.87/73.9	42	P38646	ISASRAAAAR	972.5495	972.5464
					VLENAEGAR	957.5069	957.4879
					TTPSVVAFTADGER	1449.7553	1449.7099
					QAVTNPNNTFYATKR	1723.8924	1723.8641
					RYDDPEVQK	1148.5887	1148.5462
					ASNGDAWVEAHGK	1340.6623	1340.6109
					NAVITVPAYFNDSQR	1693.8750	1693.8423
					DAGQISGLNVLRVINEPTAAALAYGLDK	2868.3647	2868.5344
					STNGDTFLGGEDFDQALLR	2054.9547	2054.9545
					ETGVDLTKDNMALQR	1689.8679	1689.8355
					AQFEGIVTDLIRR	1516.8740	1516.8361
					AMQDAEVSKSDIGEVILVGGMTR	2405.1800	2405.1929
					SDIGEVILVGGMTR	1445.8009	1445.7547
					VQQTVQDLFGRAPSK	1672.9096	1672.8896
					SQVFSTAADGQTQVEIK	1807.9123	1807.8952
					VCQGER	747.3078	747.3333
					EQQIVIQSSGGLSKDDIENMVK	2417.1972	2417.2107
					YAEEDRR	937.4381	937.4253
					ERVEAVNMAEGIIHDTETK	2141.0647	2141.0422
					MRELLAR	887.5015	887.5011
					KDSETGENIR	1147.5712	1147.5469

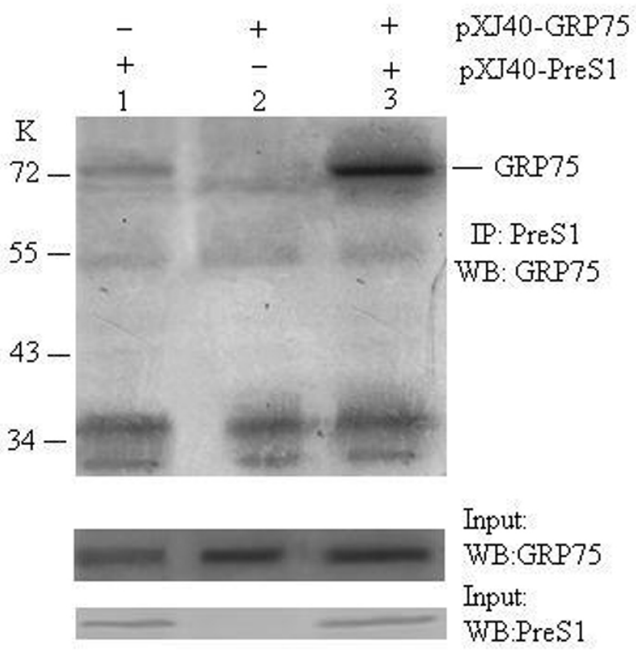

Figure 3. GRP75 binds to PreS1 in vivo. The COS7 cell lysates, co-transfected with pXJ40-PreS1 (lane 1) and pXJ40-GRP75 (lane 2) either alone or both (lane 3), were immunoprecipitated with anti-PreS1 antibody. Lysates from the transfected cell and the immunoprecipitates were subjected to Western blot analysis using the anti-GRP75 antibody

DISCUSSION

GST pull down combining with proteomic techniques provided a powerful tool for studying protein-protein interaction in vitro. By using these methods, some novel protein-protein interaction partners have been clarified, such as PRS1 associated with p300 (6), HBx protein of HBV binding to mitochondrial HSP60 and HSP70 (18), PKCβ and CtBP interacted with BMPR-II (5).

Both GRP78 and GRP75 belong to HSP70 family (HSP-70s) molecular chaperones which play critical roles in protein folding and trafficking (8). The major difference between GRP78 and GRP75 is the cellular localization. GRP78 almost localized in endoplasmic reticulum (ER) (8). While GRP75 mainly resided in mitochondria but also resided in ER and both of these protein are reported expressing in cell surface (15,17). HSP-70s are highly conserved and demonstrate a 60–78% base identity among eukaryotic cells. Biochemical studies have demonstrated that all HSP70s have N-terminal 44-kDa and 18-kDa domain, C-terminal 10-kDa fragment. N-terminal 44-kDa domain is ATPase domain. 18-kDa domain is peptide-binding domain. C-terminal 10-kDa fragment carries highly conserved EEVD terminal sequence (8). Therefore, it is likely that both GRP78 and GRP75 bound to PreS1 through N-terminal 18-kDa domain. GRP78 has been proved specifically bound to the PreS1 (1,4). Using the receptor binding assay they determined that amino acid residues 12 to 20 and 82 to 90 are essential for the binding of pre-S1 to GRP78. Which site of PreS1 mediates its attachment to GRP75 should be established.

Functional meaning of PreS1 interacting with both proteins may be explained in two ways. First, GRP78 and GRP75 mediate HBV virion binding to hepatocyte and internalization. The other is regulating proper folding of the L protein. The former possibility comes from the fact GRP78 bound specifically to the pre-S1 of native HBV particles. The association of GRP75 with the IL-1 receptor type was detected and proposed to play an important role in receptor internalization (16). GRP75 was also isolated as a FGF-1 binding protein by FGF-1 affinity chromatography and was shown to aid in its intracellular trafficking (12). In view of the in vivo biological relevance of GRP78 and GRP75, combined with the cell surface location, we postulate that one of role of GRP78 and GRP75 may be act as an adjunctive carrier in bridging HBV virion entry into the cell during viral infection. L protein are synthesized at ER. It has also been suggested that one of function of HSP-70s is to prevent transport of incompletely assembled, misfolded, or aggregated proteins from the ER (1,7,8). By analogy, we may therefore infer GRP78 and GRP75 may interact with the incompletely assembled L protein particles and retains them in the lumen of ER to prevent their secretion. Whether GRP78 and GRP75 involved in HBV morphogenesis by regulating proper folding of the L protein warrants further study.

In summary, we identified GRP75 as a novel PreS1 interacting protein besides GRP78, a protein previously

described to interact with preS1, using GST pull down combining with proteomic techniques. We postulate that GRP78 and GRP75 may play important roles in mediating HBV virion entering into hepatocyte and regulating proper folding of the L protein due to their critical functions in protein folding and trafficking.

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