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Hepatitis B virus interacts with albumin precursor in vivo

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A range of proteins has been recognized as mediators/hypothetical receptors for hepatitis B virus (HBV), but the results are conflicting and inconclusive especially regarding their biological significance. This study was aimed at identifying a novel HBV-interacting protein which would provide a better understanding of its transport in blood, attachment, fusion and entry into hepatocytes. Serum samples positive for HBV confirmed by PCR were subjected to ammonium sulfate fractionations at 50, 75, 100% saturation. PCR of each fraction demonstrated amplification of HBV in 100% fraction. Protein analysis by SDS PAGE of fractions showed one band of approximately 69 kDa protein, in 100% fraction. The 100% fraction band was excised from gel and sequence was determined by MALDI TOF which showed mass values from 705 to 3722. Mass spectrometry of trypsinized 69 kDa species revealed peptide sequences that covered 54% of the serum albumin precursor amino acid sequence, with pI of 5.9. Western blot, carried out using primary anti-albumin precursor antibody, further validated this protein. This study establishes that Hepatitis B Virus binds to albumin precursor, suggesting its role in the initiation of HBV infection and hence may offer new therapeutic strategies against HBV infection.

Key words: Albumin precursor, hepatitis B virus, mass spectrometry, western blotting.

INTRODUCTION

The Hepatitis B Virus (HBV) was discovered nearly 40 years back, yet the search for a receptor or a carrier protein is still going on. Despite considerable advances in the understanding of the nature of HBV disease, many of the steps in the virus life cycle remain unclear. Virus transport in blood, attachment to permissive cells, fusion and penetration through cell membranes and subsequent genome release, are largely an ambiguity. Current knowledge on the early steps of HBV life cycle has mostly evolved from molecular cloning and studies of the infection of duck hepatitis B virus (DHBV). However, considering the difference of composition and sequence between the surface protein of HBV and entry may be restricted (Lu and Block, 2004). Efforts to identify the cell surface receptor(s) for HBV have been hampered by the limitations of the in vitro models of HBV infection and non availability of in vivo models. A recent study (Deng et al., 2007), has suggested interaction (virus binding, virus capture, and cell attachment) of lipoprotein lipase (LPL), with PreS and HBV particles, but this too has been an in vitro study. Similarly, an array of proteins has been described as putative receptors for HBV; however, the biological significance of these molecules has not been confirmed (Barrera et al., 2005). This study was aimed at identifying a novel HBV-interacting protein which would provide a better understanding of its transport in blood, its attachment to hepatocytes, fusion and entry into the cells.

MATERIALS AND METHODS

All work was done at molecular biology laboratory at Aga Khan University, Karachi, Pakistan. Serum samples collected (by informed consent) from patients attending clinic at Ziauddin Medical University were stored at minus 70°C until analysed. Only HBV
positive sera detected by HBsAg (ELISA-MUREX kit by Abbot Laboratory) were included in this study. Ethical approval for this study was granted by the Ziauddin University ethical review committee.

**Protein precipitation and quantification**

Proteins from HBV positive serum were fractionated by using varying concentrations of NH₄(SO₄)₂ as described by Spadaro et al. (2003). Serum samples (200 μl each) of HBV infected high titer patients were subjected to ammonium sulphate precipitation and four fractions at 50, 75, 100 and supernatant of 100% were collected. The protein yield in each fraction was quantified by Bradford Method. A standard curve was drawn for this purpose, using solutions of bovine serum albumin (BSA) as standard, according to the method of Gornall et al. (1949).

**DNA extraction and PCR amplification**

In order to detect HBV associated protein fraction, DNA extraction was performed on all four fractions namely, 50, 75, 100% and the supernatant, using a DNA extraction kit (QIA AMP DNA mini kit 250 reactions Cat #51306).

PCR was performed to ascertain which fraction holds the activity of HBV, by using primers for HBV, P7F and P7R (from Gene Link NY, USA), for core protein. For reference HBV Clone of 620bp (reference strain; AF460235) was used as an appropriate positive control. Each precipitated fraction was washed with ammonium sulfate concentration to improve the protein yield in each fraction (Spadaro et al., 2003). Each precipitated fraction (75%, 100% and supernatant of 100%) were dissolved in sample solutions (10 μl), 10x buffer (50 mM KCl, 10 mM Tris pH 8.3), MgCl₂ (2 mM), dNTPs (200 μM), P7F and P7R (20 pmol each), H₂O (28.7 μl), Taq polymerase (2.5 U), with the Cycling as 94°C pre denaturation for 1 min, 94°C for 30 s, 55°C for 30 s, 72°C for 1 min for 35 cycles, 72°C for 7 min, and 4°C hold. To the positive control tube instead of DNA 1.0 μl of Clone template plus 9.0 μl of H₂O was added. For visualization of PCR product 2.5% agarose was prepared in 1x TBE buffer. The gel was run in 1x TBE buffer at a voltage of 90V. The amplified bands were visualized on Gel Doc system Quantity One (BioRad company, USA) with a medium or long wavelength (e.g. 300 nm) UV light and recorded in a TIFF file.

**Polyacrylamide Gel Electrophoresis (PAGE)**

Proteins fractionated by ammonium sulphate were analyzed by SDS-PAGE to determine the molecular size protein species present in each fraction (Spadaro et al., 2003). Each precipitated fraction was washed with ammonium sulphate solution at the respective salt concentration to improve the yield. Protein samples of fractions 50, 75, 100% and supernatant respectively were dissolved in sample diluting buffer in the ratio of 1:1 by boiling in a water bath for 3 min and resolved on 10% SDS PAGE. Electrophoresis was performed using tris-glyine buffer at constant voltage of 90V/plate of 10 mm length and 1 mm in thickness, for approximately 1 h; or till the dye migrated 95% of the gel length. The gels were stained with Coomassie Brilliant Blue-R (CBBR) and de-stained with glacial acetic acid (10%) to visualize the protein components. The 69 kDa band (separated out on 100% fraction) was extracted from the SDS-PAGE gel and digested with trypsin, and the peptide masses were examined by mass spectrophotometry.

**MALDI TOF**

Mass spectrophotometry of the excised band from gel of 10% SDS PAGE was done as described by Hong et al. (2004). Protein bands were excised manually from Coomassie Blue stained SDS PAGE gels and in-gel digested with trypsin using a Mass PREP robotic protein-handling system (Micromass). The samples were loaded onto the ZipTip by pipetting 20 times and washed using 10 μl 0.1% TFA twice. The tryptic fragments were eluted with 60% acetonitrile / 1% acetic acid. The tryptic fragments were mass analyzed by matrix-assisted laser desorption ionization (MALDI) mass spectrometry (Voyager DE-PRO; Applied Biosystems) and Q-TOF Ultima API (Micromass) was employed for sequence information. For LC-MS the material obtained after in-gel digestion was directly applied on a Q-TOF Ultima API (Micromass) instrument coupled with on-line CapLC (Waters). A column from Genetec, PepMap C18 3 μm, 100A, 75 μm x 150 mm was employed for separation of peptides prior to entry into mass spectrometer. For peptides separation, samples were applied on the column in buffer A containing 5% acetonitrile in water and 0.1% formic acid, eluted with buffer B, containing 95% acetonitrile in water and 0.1% formic acid. The (M+H)+ values for the peptide fragments produced by trypsin digestion were used for protein identification with MS-FIT program on Protein Prospector. Proteins were identified from peptide mass data by using BLAST search at http://www.ncbi.nlm.nih.gov/blast/blast.cgi

**Western blotting**

The protein fractions were solubilized in Laemlll sample buffer (Laemmli, 1970) and resolved on 10% SDS PAGE. Subsequently, the protein fractions were blotted to a nitrocellulose (Hybond™ ECL, GE Healthcare Life Science) membrane.

Nitrocellulose membrane was pre-wet with distilled water and then soaked in transfer buffer. The cassette was opened into a tray filled with transfer buffer. A foam sponge was placed on the open submerged cassette and pressed gently to expel any air bubble. Four sheets of blotting paper were placed on the sponge and the membrane was placed on the blotting paper. The gel was then placed on the membrane. All air bubbles were removed by gently rolling a glass pipette over the gel. The gel was then covered with four sheets of blotting paper and a sponge (6 mm). The cassette was then closed and inserted into the transfer unit containing transfer buffer. A cooling system was connected to the heat exchanger outlet of the tank and water was circulated at 10°C. A magnetic stirrer was placed in the buffer tank. Transfer was allowed at 250 mA for 4 h.

**Incubation of the nitrocellulose membrane with antibody**

The albumin precursor protein was detected with primary monoclonal antibody (Sigma-Aldrich, Saint Louis Missouri, USA), that is, anti-albumin precursor antibody (1:500) in conjugation with the horse radish peroxidase conjugated secondary antibody (1:10,000) and visualized using ECL detection kit (GE Healthcare) according to the manufacturers’ instructions.

**RESULTS**

**Amplification of HBV in 100% fraction**

A band of 140 bp length (nucleotide 2298 to 2436) of the C gene of HBV DNA was found more prominent in the PCR of 100% fraction signifying HBV activity along with the protein precipitated on 100% saturation (Figure 1). The first two fractions (50 and 75%) did not show any noticeable amplification. The positive control HBV clone used as the template amplified a 140 bp fragment insert.
Figure 1. PCR amplification to determine the HBV activity. Lane 1, DNA 100 bp ladder, lane 2, supernant of 100% ammonium sulfate fraction, lane 3, amplified product of 140bp was observed in 100% ammonium sulfate precipitated fraction, lane 4, ammonium sulfate 75% fraction, lane 5, ammonium sulfate 50% fraction and lane 6, HBV Clone was used as positive control.

Figure 2. Proteins fractionated by ammonium sulphate were analyzed by SDS-PAGE. Lane 1, shows SDS-PAGE low molecular weight standard, lane 2, ammonium sulfate 50% fraction, lane 3, ammonium sulfate 75% fraction and lane 4, ammonium sulfate 100% fraction.

The normal controls (HBsAg negative patients) did not give any results on PCR amplification (results not shown).

A single band of 69 kDa in 100% fraction on SDS

The first two fractions, namely 50 and 75% salt concentrations, revealed presence of two major groups of proteins: globulins and albumins respectively, as analyzed by SDS page. The third fraction (100%) presented a single band at 69 kDa position and the fourth fraction revealed no band (Figure 2). Thus PCR negative fractions establish that globulins and albumins do not carry HBV and it is the third fraction that grasped all the attention.

Mass Spectrophotometry identifies 69 kDa as albumin precursor

The peptide sequences of 69 kDa band, excised from the lane of 100% fraction on SDS-PAGE gel and digested with trypsin, were examined by mass spectrometry. The results, based on MALDI-TOF MS showed mass values from 705 to 3722 (Figure 3). Analysis of these masses through Protein Prospector revealed peptide sequences that covered 54% of the Serum Albumin Precursor amino acid sequence, with molecular weight of 69 kDa, pl of 5.9.

Western blotting confirms albumin precursor

Analysis shows a high expression of albumin precursor protein at position which coincides with the 69 kDa band of 3rd fraction (100%) on SDS PAGE, showing a protein-primary antibody-secondary antibody-enzyme complex, proving the presence of albumin precursor (Figure 4).

DISCUSSION

This study identifies albumin precursor as HBV binding protein which may be involved in interaction with the membrane receptor mediating the attachment to the liver cells. The data presented demonstrate the presence of HBV only in the fraction which was later identified to contain albumin precursor. It appears that albumin precursor of 69 kDa protein is similar to albumin yet has different characteristics in terms of molecular weight and no cross reactivity with antibody to albumin.

Since the discovery of HBV despite intensive efforts, a clear understanding about receptor or binding protein for HBV remains elusive. It is known that HBV, for its entry into the hepatocyte requires a binding protein and a cellular receptor (Lonberg and Philipson, 1981). Once it passes the attachment and entry steps of infection, HBV is able to replicate freely in hepatic cells. Many proteins have been identified as HBV envelope binding proteins with a proposed role as mediators in HBV attachment to the hepatocyte. At the same time, many theories have been proposed regarding HBV attachment and entry into hepatocytes but none of these theories or binding proteins have been thoroughly proved or investigated.

In this study, the albumin precursor precipitated along with the third fraction (100% ammonium sulfate saturation) of serum as was proved by analysis of this fraction by mass spectrometry and western blotting. It is important to note that this was the only fraction that turn out to be PCR positive for HBV, while all other fractions...
and albumin, but not with albumin precursor as revealed in this study. It is well established that human albumin is initially synthesized as a proprotein and subsequently transformed into serum albumin (Weigand and Alpert, 1981). The proalbumin, differs from circulating serum albumin only in containing a basic hexapeptide, Arg-Gly-Val-Phe-Arg-Arg, at its NH2 terminus (Peters, 1996). Repeatedly conflicting views have been published describing cooperation of so-called polymerized albumin with HBV. It has been demonstrated that pHSA binds to HBsAg particles (Pontisson et al., 1983) within the pre-S2 amino acid sequence (Machida et al., 1984). In 1989, Pontisso and his colleagues, using the membranes of surgically obtained human liver as a target, described the role of HBV preS2 region as a specific site that binds the poly-human albumin in vitro. They showed that pHSA binds necessary and sufficient for the attachment of pre-S2-containing M-rHBsAg particles to human liver plasma membranes. They also showed that pHSA enhances the L-rHBsAg particle binding to these membranes. Other investigators also reported similar findings with poly-human albumin in vitro (Machida et al., 1983) and the monomeric human albumin in vivo (Krone et al., 1990; Lu et al., 1988), but it was neither investigated further nor
confirmed. Other reports have demonstrated that native and glutaraldehyde-polymerized albumins bind to hepatocytes (Lenkei et al., 1977; Trevisan et al., 1982; Wright et al., 1987; Yu et al., 1987); however, presence of pHSA in vivo has not been established. It is therefore, not clear whether pHSA plays a role in HBV infection or not.

Preceding research did not report expression of albumin precursor as binding protein of Hepatitis B Virus. However, a number of proteins have been recognized as HBV envelope binding proteins with a proposed role as mediators in HBV attachment to the hepatocytes. These proteins have been identified to interact with various HBV domains. Thus, apolipoprotein H, (Mehdi et al., 1994) that binds to S domain; human serum albumin and a human soluble serum factor (Budkowska et al., 1993) and a 44 kDa protein (Falco et al., 2001) are to name a few in this regard. Neurath and his colleagues in 1994 discovered the binding site on pre-S1 domain from amino acid sequence 21 to 47 which is involved in the recognition of hepatocyte receptors. Recently, Blanchet and Sureau (2007) by mapping the entire pre-S domain for infectivity determinants demonstrated that the activity of the pre-S domain at viral entry solely depends on the integrity of only its first 75 amino acids. While De Falco et al. (2001) identified a 44 kDa protein by using the pre-S1 peptide originally shown to bind liver cells by Neurath et al. (1994). None of the candidate proteins have demonstrated biological activity in HBV infectivity. Secondly, the L protein is modified at the amino-terminal glycine of the pre-S1 domain with a myristate (Persing, 1987), which is required for infectivity (Bruss, 1996) and none of the above described proteins to date have been identified with a pre-S1 protein containing the amino-terminal myristate known to be essential for infectivity. These results are conflicting, and no conclusive and convincing biological data has been obtained to fully demonstrate the functional role of these proteins in HBV binding and internalization. Thus, the viral structures involved in attachment to the target cell were identified, but the binding protein for HBV could not be positively deter-mined and the biochemical events leading to infection remain unknown.

The binding of HBV with albumin precursor apparently shows the involvement of HBV with albumin precursor. Binding of HBV may be achieved through myristate to albumin precursor, which binds to receptor on the hepatocytes. Myristate has been found to have multiple (five) binding site on Albumin molecule (Curry et al., 1998). The very amino-terminal end of the pre-S1 domain, including the myristate group attached to it, is a putative primary receptor-binding domain (Blanchet and Sureau, 2006). Basically, two sites on its small HbsAg protein have been proposed by Cooper et al. (2003). One site is to attach to the binding protein we propose albumin precursor and a secondary attachment site that recognizes a distinct receptor on the cell membrane, supporting the hypothesis of a multivalent and cooperative mechanism of virus attachment to the cell surface, as occurs for many other viruses. Another factor that facilitates HBV binding to proteins is the presence of QLDPAF sequence within pre S1 region, which has been found in all HBV samples we studied.

Paran and his colleagues (2001) found that the QLDPAF sequence within this preS1 region was crucial for cell attachment. Further evidence to support this hypothesis is that analysis of the database revealed that this minimal epitope is shared by other viral, bacterial and cellular proteins that participate in cell adhesion, attachment and fusion. This suggests that the QLDPAF sequence or part of it may play a role in cell adherence and attachment.

**Conclusion**

Although many technical details remain to be explored, yet on the basis of evidence presented in this study elucidates that HBV binds to albumin precursor and it would not be unreasonable to propose that pre S region of HBV might be the most likely domain involved. At this point it is not clear yet how Human Albumin Precursor fulfills all of these different functions, however, assuming that this protein acts as a chaperone, thereby stabilizing HBV structures, a function in these varied processes might be envisaged.

**REFERENCES**


