A cellular protein which binds hepatitis B virus but not hepatitis B surface antigen

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The envelope of hepatitis B virus (HBV) consists of three related proteins known as the large (L), middle (M) and small (S) hepatitis B surface antigens (HBsAg). L-HBsAg has a 108–119 amino acid extension at the N terminus compared with M-HBsAg and contains the preS1 sequence of the HBV envelope. Previous research has identified this region as the likely virus attachment protein which is thought to interact with the cellular receptor for the virus. However, as the receptor has still not been identified unequivocally, we used the preS1 region of L-HBsAg to screen a human liver cDNA library by the yeast two-hybrid system. Several positive clones were isolated which encoded cellular proteins that interacted with the HBV preS1 protein. The specificity was examined in an independent manner in experiments in which baculovirus-derived glutathione S-transferase (GST)–preS1 was incubated with 35S-labelled protein expressed by in vitro translation from the positive clones. The intensity of the interactions using this alternative approach mirrored those observed in the yeast two-hybrid system and two proteins (an unidentified protein and a mitochondrial protein) were selected for further study. The specificity of the binding reaction between the preS1 protein and these two proteins was further confirmed in a competition assay; HBV purified from serum, but not purified HBsAg, was able to compete with preS1 and thus block GST–preS1 binding to the unidentified protein but not to the mitochondrial protein. The unidentified protein was then expressed as a fusion protein with GST and this was able to bind HBV virions in a direct manner.

Introduction

The Hepadnaviridae is a family of enveloped DNA viruses that produce persistent infection of hepatocytes, often resulting in the development of chronic hepatitis, liver failure and hepatocellular carcinoma (Ganem, 1996). Human hepatitis B virus (HBV) is the prototypic member of the hepadnavirus family. A characteristic feature of HBV and other hepadnaviral infections is strong species-specificity, and HBV can infect humans and chimpanzees but not baboons, lower primates or other mammals (Barker et al., 1975). This narrow host range, which has impeded in vitro studies of HBV, is thought to be governed at the level of virus entry into cells, as transfection with HBV DNA is able to circumvent this barrier and allow HBV replication in otherwise non-permissive human- (Sells et al., 1987; Sureau et al., 1986; Tsurimoto et al., 1987) or other mammalian-cell lines (Shih et al., 1990).

The S-open reading frame (ORF-S) in the HBV genome contains the S, preS2 and preS1 regions; these encode, respectively, the three related envelope proteins, S-HBsAg, M-HBsAg and L-HBsAg (Ganem, 1996; Heermann et al., 1984), which are expressed as glycosylated or non-glycosylated forms. The virus envelope proteins can be expected to interact with a cellular receptor and all three envelope proteins have been shown to interact directly or indirectly with a range of cellular proteins (De Meyer et al., 1997). The preS1 region binds to HepG2 cells and liver cell membranes (Neurath et al., 1986a; Pontisso et al., 1989; Qiao et al., 1994), the preS2 domain binds to HepG2 cells, T lymphocytes and liver cell membranes (Neurath et al., 1986b; Franco et al., 1992; Machida et al., 1983), and the S domain binds to hepatocytes, fibroblasts, mononuclear cells and Vero cells (Komai & Peeples, 1990;
Leenders et al., 1990). Many potential cellular receptors have been proposed (Budkowska et al., 1993, 1995; Dash et al., 1992; Franco et al., 1992; Hertogs et al., 1993; Machida et al., 1983; Mehdi et al., 1994; Neurath et al., 1992; Petit et al., 1992; Pontisso et al., 1992; Treichel et al., 1994) but it is still unclear whether the authentic receptor has been identified. HBV particles devoid of M-HBsAg are infectious (Sureau & Lanford, 1993) and the only direct evidence for the contribution of the various domains comes from a study of duck hepatitis B virus (DHBV) in which recombinant L-DHBsAg particles but not S-DHBsAg particles were able to inhibit DHBV replication in primary duck hepatocytes (Klingmuller & Schaller, 1993). (DHBsAg has no equivalent of the M-HBsAg domain.) Experiments by Sureau et al. (1993) showed that L-HBsAg is required for hepatitis delta virus infectivity and this is consistent with a role for the preS1 region in adsorption. Thus most workers agree that the preS1 sequence probably was required for hepatitis delta virus infectivity and this is consistent with a role for the preS1 region in adsorption. The preS1 region of the L-HBsAg gene was amplified by PCR using forward and reverse primers preS1F and preS1R, which contain an EcoRI and Fstl site respectively for ease of cloning. The sequences of the primers, with the restriction enzyme site underlined, are: preS1F, 5′ CTGCAGCTAGGCCTGAGGATGACTGTC 3′; preS1R, 5′ GCTGCTACCTAGGGCTGAGATGACTGTC 3′. These bind to nucleotide positions 2850 and 3174 on the HBV genome (Galibert et al., 1979) and will amplify the region encoding aa 1–108 of L-HBsAg. The template for this reaction was plasmid pKSBHV (Harvey et al., 1997), which contains a monomer of HBV DNA (type ayw). The 342 bp product was cloned into the respective restriction enzyme sites of plasmid pGBT9 (Clontech) and this plasmid was subsequently named pGBT9/preS1. A human liver cDNA library, fused with the GAL4 activation domain (AD) in the pGAD10 vector, was also obtained from Clontech.

The region representing the cellular gene insert in a number of reactive clones was amplified by PCR using primers pGAD10UP and pGAD10DOWN, which anneal to the GAL4-AD. The forward primer, pGAD10UP, contained a T7 RNA polymerase promoter region which was subsequently removed from the PCR product by digestion with the restriction enzyme SmaI. The sequences of the pGAD10UP and pGAD10DOWN primers are 5′ GCCGCTTAAACGG- ACTCCTATACCCCGGAGAAGCTACAATCACC 3′ and 5′ AAA GCGCCCGACAGTTGAAGTAATTTCCTTT 3′. These contain SmaI and NotI enzyme sites respectively (underlined). Eight reactive clones were amplified and the products, which contain 484 bp of GAL4-AD and the unique coding region, were subsequently inserted into the NotI and SmaI sites of pBluescript KS(−) (Stratagene). The inserts from two additional reactive clones were excised directly from the pGAD10 vector using the restriction enzyme HindIII and inserted into the same enzyme site of the pcDNA3 vector (Invitrogen).

Plasmid bacMP, which was used to construct the GST–preS1 recombinant baculovirus (bacMPV) for expression of the GST–preS1 fusion protein in Spodoptera frugiperda (Sf9) cells, was constructed by inserting a PCR cDNA fragment encoding the GST–preS1 fusion protein into the EcoRI site of pAcSG2T-tag (Wang et al., 1995), generated with primers preS1F and preS1R (5′ AAAGATCTAGGGGGAGATGACTGTC 3′) and preS1R (5′ AAAGATCTCATGGCCTGAGATGACTGTC 3′). As the forward primer contains a T7 RNA polymerase promoter (Sf9) cells, was constructed by inserting a PCR cDNA fragment encoding the GST–preS1 fusion protein into the EcoRI site of pAcSG2T-tag (Wang et al., 1995), generated with primers preS1F and preS1R (5′ AAAGATCTAGGGGGAGATGACTGTC 3′) and preS1R (5′ AAAGATCTCATGGCCTGAGATGACTGTC 3′). The forward primer contains a T7 RNA polymerase promoter and preS1BacR (5′ AAAGATCTAGGGGGAGATGACTGTC 3′) and preS1BacR (5′ AAAGATCTCATGGCCTGAGATGACTGTC 3′). The resulting plasmid was then digested with BamHI and inserted into the bacMPV vector.

**Screening of the liver cell cDNA library by the yeast two-hybrid system.** The screening procedure used was a modification of the method described by Chien et al. (1991); *Saccharomyces cerevisiae* HF7c (Clontech) was grown in YPD medium (1% yeast extract, 2% peptone, 2% dextrose) or synthetic minimal medium (0.67% yeast nitrogen base, 2% dextrose and appropriate auxotrophic supplements). This yeast strain carries a lacZ reporter gene under the control of GAL4-binding sites and was used to screen the liver cell cDNA library. Yeast cells were transformed with pGBT9/preS1 and the pGAD10/cDNA library using a modified version of the lithium acetate method previously published by Gietz et al. (1992) and Ito et al. (1983), and selected for histidine, leucine and tryptophan prototrophy. After 6–8 days at 30 °C, the colonies were assayed for β-galactosidase (β-Gal) activity by replica plating the yeast transformants onto filter paper. The filters were then washed with Tween 20 and incubated for 1–2 h at 30 °C in a buffer containing 4 mM X-Gal. Positive interactions were detected by the appearance of the blue colonies and were verified by isolation of these colonies, replating and retesting for β-Gal activity. The pGAD10 plasmids were isolated from positive yeast transformants by culture in leucine-deficient medium, which resulted in spontaneous loss of the pGBT9-derived plasmids.

**Methods**

**Plasmid construction.** The preS1 region of the L-HBsAg gene was amplified by PCR using as forward and reverse primers preS1F and preS1R, which contain an EcoRI and Fstl site respectively for ease of cloning. The sequences of the primers, with the restriction enzyme site underlined, are: preS1F, 5′ CTGCAGCTAGGCCTGAGGATGACTGTC 3′; preS1R, 5′ GCTGCTACCTAGGGCTGAGATGACTGTC 3′. These bind to nucleotide positions 2850 and 3174 on the HBV genome (Galibert et al., 1979) and will amplify the region encoding aa 1–108 of L-HBsAg. The template for this reaction was plasmid pKSBHV (Harvey et al., 1997), which contains a monomer of HBV DNA (type ayw). The 342 bp product was cloned into the respective restriction enzyme sites of plasmid pGBT9 (Clontech) and this plasmid was subsequently named pGBT9/preS1. A human liver cDNA library, fused with the GAL4 activation domain (AD) in the pGAD10 vector, was also obtained from Clontech.

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**Sequence analysis of pGAD10/cDNA.** The pGAD10/cDNA plasmid DNA was purified by CsCl gradient centrifugation to permit sequence analysis using the Matchmaker sequencing primer 2 (Clontech), which anneals to the GAL4-AD. Sequencing was performed by the dideoxynucleotide chain termination sequencing method (ABI PRISM, Perkin Elmer) and the resulting sequences were compared against the databases of EMBL/GenBank by the BLAST program via the Australian National Genome Information Service.

**In vitro transcription/translation of positive clones.** The inserts of clones from the cDNA library that were reactive in the yeast two-hybrid system were cloned into pbLauescript KS (+) and pcDNA3 as described above for use as templates for transcription of RNA. The pbLauescript KS and pcDNA3 constructs were linearized with SacI and NotI respectively, and then used in RNA transcription reactions according to the protocol of Rice et al. (1989). Transcribed RNA (1–10 µl) was denatured at 67 °C for 7 min and then added to the Promega rabbit reticulocyte lysate system. The translation mixture (as described by the manufacturer) was incubated at 30 °C for 90 min. The *in vitro* translated 35S-labelled proteins were dialysed against PBS for 5 h at 4 °C using a microdialysis system (GIBCO BRL) to remove any unincorporated radiolabel.

**Preparation of recombinant baculovirus and GST–preS1 and GST–protein6 expression.** For construction of recombinant baculoviruses, 1–2 µg of bacMP or bacRECO was mixed with 100–200 ng of Ac5-digested BacPak6 DNA (Clontech) in 30 µl of HBS buffer (20 mM HEPES pH 7,3, 150 mM NaCl) and added to 30 µl of HBS buffer containing 10 µl DOTAP (Boehringer). The transfection mixture was then added to a 35 mm Petri dish containing a monolayer of Sf9 cells. After 5–12 h incubation at 28 °C the transfection mix was replaced with 2 ml of TC100 tissue culture medium, supplemented with 3% foetal bovine serum (FBS, GIBCO) and the cells were incubated for a further 2 days at 28 °C. The tissue culture fluid (TCF) was used to infect fresh monolayers of Sf9 cells in 60 mm dishes that were then overlaid with TC100–5% FBS media containing 15% SeaPlaque agarose (FMS Bioproducts) and subsequently incubated for 4 days at 28 °C. Recombinant plaques (white) were identified by the addition of 1 ml of PBS containing 0.5 mg X-Gal (Promega) and incubated overnight at 28 °C in vitro. The X-Gal solution was replaced with 1 ml of PBS containing 2% neutral red (GIBCO) for 1 h at 28 °C; the neutral red was then removed and the dishes were inverted and stored at room temperature for 12–48 h until the plaques became visible. White plaques were picked, resuspended in 100 µl of sterile water and the virus amplified in Sf9 cells in 25 cm² tissue culture flasks. The TCF and cell lysates were harvested and GST–preS1 or GST–protein6 purified using glutathione–Sepharose 4B beads (Pharmacia) before analysis by SDS–PAGE. Recombinant baculovirus stocks for the expression of GST–preS1 or GST–protein6 were prepared and high titre recombinant virus stocks were used to infect Sf9 insect cells. Cell culture supernatants were harvested 4–5 days post-infection and the secreted GST–preS1 or GST–protein6 proteins adsorbed with 0.3–0.5 µl of glutathione–Sepharose 4B beads for 2–4 h at 4 °C.

**Purification of hepatitis B virions and HBsAg.** For the binding inhibition experiments (see below), highly purified hepatitis B virions were prepared from the serum of HBV-infected patients with high titres of HBV DNA (1 × 10^9–3 × 10^9 vge/ml) as described by Qiao et al. (1994). HBsAg was purified from HH1 cells, a mycoplasma-free cell line derived from PLC/PFR/S (Fowler et al., 1983) which stably expresses HBsAg. Briefly, the particles in the TCF were concentrated by pelleting through a 2 ml cushion of 20% sucrose [in 10 mM Tris–HCl pH 7,4, 100 mM NaCl, 1 mM EDTA (TNE)] in an SW41Ti rotor (Beckman) at 230000 g for 5 h at 4 °C. Each HBsAg pellet was resuspended overnight in 50 µl of TNE at 4 °C. The HBsAg preparations were then pooled and diluted to 2 ml with a CsCl solution to yield a final density of 1.2 g/cm³. The sample was overlaid on a discontinuous gradient containing 3 ml of 1.4 g/cm³ and 2 ml of 1.25 g/cm³ CsCl in TNE. The tube was then filled with 1.1 g/cm³ of CsCl in TNE, and centrifuged in an SW41Ti rotor at 270000 g for 40 h at 10 °C; 300 µl fractions were collected and assayed for HBsAg by ELISA. Positive fractions were pooled and recentrifuged through a sucrose cushion as described above. The pellet was resuspended in 200 µl of PBS. Both the HBV and HBsAg preparations were examined by SDS–PAGE and silver stain to confirm the sample purity and levels of HBsAg in each.

The HBV preparation used for interaction with GST–protein6 was only partially purified; 10 ml of HBV-infected patient serum was pelleted through a 2 ml cushion of 20% sucrose in TNE buffer as described above for HBsAg. The pellet was resuspended in a final volume of 500 µl of PBS and stored at 4 °C.

**GST–preS1 protein in vitro binding assay.** GST–preS1 protein was expressed and purified as described above. A recombinant baculovirus which expressed only GST (a gift from Alexander Khromykh, Sir Albert Sakzewski Virus Research Centre, Royal Children’s Hospital) was treated in the same manner for use as a negative control. Dialysed 35S-labelled proteins were dialysed against PBS for 5 h at 4 °C using a microdialysis system (GIBCO BRL) to remove any unincorporated radiolabel.

Additional assays were performed to examine the specificity of the interaction with HBV of proteins expressed from clones #1 and #6. Purified HBV and HBsAg, each containing 7.5 µg of p24 and gp27, were incubated with 2 × 10^5 c.p.m. of radiolabelled protein overnight at 4 °C prior to addition of GST–preS1 protein immobilized on beads, and then incubated for a further 4–6 h at 4 °C. The beads were then pelleted at low speed (1000 g), washed four times in RIPA buffer (50 mM Tris–HCl pH 7.5, 300 mM NaCl, 0.5% NP40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors. The pellets were solubilized in sample buffer (10% glycerol, 1% β-mercaptoethanol, 2% SDS, 50 mM Tris–HCl pH 6, 0.05% bromophenol blue) and boiled for 5 min. The samples were then separated by SDS–PAGE and analysed by phosphorimaging (Molecular Dynamics).

**Dose response curve.** To determine if HBV could compete with the interaction between protein #6 and GST–preS1 protein in a dose-dependent manner, the HBV competition assay described above was done using various dilutions of purified HBV, i.e. 1/2.5, 1/5, 1/10, 1/20, 1/50, 1/100. The dilutions were prepared relative to the original concentration of HBV used in the competition assay.

**GST–protein6 in vitro binding assay.** Concentrated HBV virions (5 × 10^9 vge/ml) were incubated with 1 µg of either GST or GST–protein6 immobilized on Sepharose 4B beads for 2 h at 4 °C. After incubation and washing, viral DNA was isolated from the virions bound to the beads and in the supernatant. This was done by digestion of the samples with 1 mg/ml proteinase K–2% SDS overnight at 55 °C, followed by phenol–chloroform extraction and ethanol precipitation. The HBV DNA present in each sample was detected by PCR using primers DAW1 and DAW2 (a gift from Ming Qiao, Institute of Medical and Veterinary Science, Adelaide, Australia), which are designed to amplify a 384 bp region from the core gene (Qiao et al., 1994). Plasmid pKSHBV (Harvey et al., 1997) was used as a positive control.
Results

Identification of cellular proteins which interact with the HBV preS1 protein in the yeast two-hybrid system

In an attempt to identify cellular proteins which interact with the preS1 component of L-HBsAg we used the yeast two-hybrid system. The HBV preS1 region, encoding aa 1–108, was fused to the GAL4 DNA-binding domain (BD) of pGBT9 and used to screen a cDNA library derived from human adult liver. This cDNA library was fused to the GAL4 activation domain in the pGAD10 vector. These two plasmids were cotransformed into strain HF7C of Saccharomyces cerevisiae. Of the 1 × 10⁶ transformants screened, 80 demonstrated β-Gal activity and grew in the absence of tryptophan, leucine and histidine. Fifty-six false positives were eliminated by culturing Leu⁺/Trp⁺ transformants in Leu⁻/SD medium. Under these conditions, pGAD10/cDNA (which carries the LEU2 gene) is maintained while the pGBT9/S1 plasmid is randomly lost. Of the remaining 24 Leu⁺ yeast transformants, only ten clones still expressed β-Gal activity when cotransformed with pGBT9/S1. Thus these ten clones were considered to express proteins which showed a genuine interaction with the preS1 protein of HBV.

DNA sequence analysis of these ten clones revealed high identity (80–99%) with a number of different genes in the GenBank database (Table 1), one of which was identified as apolipoprotein H, previously described as a candidate for the HBV cell-surface receptor (Mehdi et al., 1994). This suggested that the yeast two-hybrid system has the potential to detect previously acknowledged L-HBsAg–cell protein interactions.

A comparison of the ten cDNA fragments isolated using the yeast two-hybrid system and the corresponding gene listed in the GenBank database is shown in Table 2.

By macroscopic analysis, four of the ten clones displayed very intense β-gal activity, indicative of a strong interaction between the cellular protein and the preS1 protein, while the remaining six clones demonstrated only a moderate-to-weak interaction with the preS1 protein.

Confirmation of cellular protein–preS1 interactions

Although the yeast two-hybrid system can identify genuine protein–protein interactions, the system can also generate artefacts which appear to be positive. Thus, to confirm the above results in a more rigorous system, the specificity of the interaction of the preS1 region with the proteins expressed from the ten cDNA clones was examined in an in vitro binding assay using a GST–preS1 fusion protein.

Table 2. Comparison of the relative size of the pGAD10/cDNA fragments and the corresponding gene deposited in the EMBL and GenBank database

<table>
<thead>
<tr>
<th>Clone #</th>
<th>cDNA fragment size (bp)</th>
<th>GenBank sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>770</td>
<td>1044</td>
</tr>
<tr>
<td>2</td>
<td>620</td>
<td>1153</td>
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<td>3</td>
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<tr>
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<tr>
<td>9</td>
<td>1800</td>
<td>3761</td>
</tr>
<tr>
<td>10</td>
<td>1250</td>
<td>1490</td>
</tr>
</tbody>
</table>

In vitro transcription/translation of the ten cDNA partial sequences. The ten cDNA clones were excised from pGAD10

Table 1. Identity of the ten clones recognized using the yeast two-hybrid system

<table>
<thead>
<tr>
<th>Clone #</th>
<th>GenBank identity*</th>
<th>Relative intensity†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Human mitochondrial gene (GenBank acc. no. X62996)</td>
<td>++ + +</td>
</tr>
<tr>
<td>2</td>
<td>Human ApoH mRNA for β-glycoprotein I, apolipoprotein H</td>
<td>++ + +</td>
</tr>
<tr>
<td>3</td>
<td>Human hypoxanthine phosphoribosyltransferase (HPRT) gene</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Human thymosin β-4</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Human S-protein gene</td>
<td>++</td>
</tr>
<tr>
<td>6</td>
<td>Human partial cDNA sequence (GenBank acc. no. T74413)</td>
<td>++ + +</td>
</tr>
<tr>
<td>7</td>
<td>Human fibrinogen β-chain gene</td>
<td>+</td>
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<tr>
<td>8</td>
<td>Human X-box binding protein-1 mRNA</td>
<td>++</td>
</tr>
<tr>
<td>9</td>
<td>Human galactocerebrosidase mRNA</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>Human pigment epithelium-differentiation factor (PEDF) gene</td>
<td>+</td>
</tr>
</tbody>
</table>

* Sequences identified via GenBank.
† Based on the intensity of β-galactosidase activity.
Fig. 1. In vitro examination of the ten clones identified in the yeast two-hybrid system. (A) SDS–PAGE analysis of the ten [35S]methionine-labelled, in vitro-translated, cellular proteins. The radiolabelled proteins ranged in size from 23 to 35 kDa. The molecular mass markers were generated from a commercial standard (Bio-Rad). (B) SDS–PAGE analysis of the [35S]methionine-labelled in vitro-translated proteins bound to baculovirus-expressed recombinant GST–preS1-coated Sepharose beads. In both (A) and (B) lanes 1–10 represent the proteins derived from clones 1–10, respectively; in (B), lane M represents a binding reaction using a control in vitro translation mixture in which RNA was omitted. The bands in (B) were quantified with an ImageQuant (Molecular Dynamics); the results are shown numerically at the bottom of the panel. This value represents the total counts of the specific bands in each track.

and recloned into plasmids suitable for in vitro transcription of RNA (as described in Methods). This was then used for in vitro translation of radiolabelled protein and the products were examined by SDS–PAGE. The in vitro-translated proteins, which also contained 17.5 kDa of the GAL4-AD, ranged in size from 23 to 35 kDa (Fig. 1A).

Interaction of radiolabelled cellular proteins with GST–preS1 protein. The 35S-labelled proteins were then examined for their ability to bind a baculovirus-derived GST–preS1 fusion protein attached to Sepharose beads. All ten proteins synthesized by in vitro translation were incubated respectively with the immobilized GST–preS1. Bound proteins were detected by SDS–PAGE (Fig. 1B) and the relative intensities of the binding interaction was then quantified by phosphorimager analysis of the gel (Fig. 1B). Two proteins (#1 and #6) which showed strong binding in the yeast system also demonstrated strong binding to the GST–preS1 protein. In contrast proteins #2 and #8, which showed strong reactions in the yeast two-hybrid system, showed only moderate and weak binding respectively to the GST–preS1 protein. The remaining proteins which reacted weakly in the yeast two-hybrid system showed a similar weak binding reaction to the GST–preS1 protein (Fig. 1B). None of the radiolabelled proteins was able to bind to the GST protein (data not shown).

HBV competition assay

Next, we examined if the binding demonstrated by proteins #1 and #6 was physiologically relevant. Consequently, we examined the ability of purified HBV or HBsAg to compete with the binding between the cellular proteins and the GST–preS1 protein. The composition of the purified HBV preparation correlated with the data described by Heermann et al. (1984), i.e. bands for all forms of the HBV surface proteins, S (p24, gp27), M (gp33, gp36) and L (p39, gp42), were visualized by silver staining (Fig. 2, lane 2). The HBsAg preparation (Fig. 2, lane 1) contained intense bands at 24 and 27 kDa corresponding to the two forms of S-HBsAg (p24 and gp27). A number of higher molecular mass bands were observed in both preparations and one of these in the HBsAg preparation corresponded to the unglycosylated form of the L-HBsAg (p39). However, this band displayed a relatively weak intensity compared with that of p24 and gp27. Quantification by ImageQuant (Molecular Dynamics) of the polypeptide bands after normalization of the p24/gp27 levels showed that the purified virus preparation contained an approximately 17-fold higher level of L-HBsAg (47107 units to 814337 units). Hence, the HBsAg preparation was used as a control in the experiments described below that examined the specificity of binding of proteins #1 and #6 to the GST–preS1 protein.
Fig. 2. SDS–PAGE analysis of the polypeptide profile of the purified HBsAg (lane 1) and purified HBV (lane 2) preparations. After gel electrophoresis, the separated polypeptides were stained with silver. The molecular mass markers (lane M; kDa) were pre-stained low range standards (Bio-Rad).

Fig. 3. Analysis of the in vitro HBV competition assay. $^{[35]S}$methionine-labelled in vitro-translated proteins #1 and #6 were incubated respectively with HBV or HBsAg prior to incubation with GST–preS1-coated Sepharose beads. Radiolabelled protein bound to the beads was analysed by SDS–PAGE and examined by phosphorimaging.

Fig. 4. Analysis of the effect of dilution on competition between purified HBV and GST–preS1 protein for binding to protein #6. (A) SDS–PAGE analysis of the effect of different HBV concentrations on the interaction between protein #6 and GST–preS1 protein. (B) Quantification of the SDS–PAGE data using a logarithmic curve, generated with CA-Cricket graph III.

Radiolabelled proteins #1 and #6 were preincubated with purified HBV or HBsAg preparations (which contained similar levels of p24/gp27). After incubation, the mixture was added to the GST–preS1-coated beads. The results of this experiment showed that prior incubation of proteins #1 and #6 with protein binding buffer had no effect on binding to the GST–preS1-coated beads (compare Fig. 3, lanes 4 and 1, respectively, with Fig. 1B, lanes 1 and 6), and similar levels of binding to those demonstrated previously were noted. However, in a striking manner, HBV was able to compete with GST–preS1 for binding to protein #6 (Fig. 3, lane 3) whereas HBsAg was unable to do so (Fig. 3, compare lanes 1 and 2) and neither HBsAg nor HBV had any significant effect on the interaction between protein #1 and the GST–preS1 protein (lanes 5 and 6, respectively). Quantification of the level of binding between protein #6 and the GST–preS1 protein in the competition experiments (performed on two occasions) demonstrated that binding was reduced 4.5-fold by addition of purified HBV to the reaction as compared with the reaction containing HBsAg (64012 units to 14185 units). The possibility that the results were due to any direct effect by proteases which were present in the HBV preparation was eliminated by mixing radiolabelled protein with HBV alone; no degradation of either protein was observed (data not shown).

Dose-response curve

Having established that HBV could compete with the interaction between protein #6 and GST–preS1 protein, an experiment was performed to determine if this occurred in a dose-dependent manner. In this study, various dilutions of purified HBV were preincubated with $^{[35]S}$-labelled protein #6 prior to incubation of the protein with the GST–preS1-coated beads. The samples were then separated by SDS–PAGE and examined by phosphorimaging (Fig. 4A). The results demonstrated an increase in binding between protein #6 and GST–preS1 protein concomitant with decreased levels of HBV. The SDS–PAGE results were then quantified using ImageQuant, and the data were used to generate a dose-response curve (Fig. 4B). This shows that HBV competes for the interaction between protein #6 and GST–preS1-coated beads in a dose-dependent manner.
Discussion

GST–protein6 binds HBV in a direct manner

Additional studies, using a reciprocal approach, were carried out to further analyse the ability of protein #6 to interact with HBV virions by expressing the cellular protein as a fusion protein with GST by using the baculovirus expression system. GST or GST–protein6 immobilized on Sepharose beads were incubated with HBV concentrated from HBV-infected patient serum. After washing, the interaction between the virions and the immobilized proteins were examined by PCR and subsequent gel electrophoresis. An HBV DNA product of 384 bp was detected in DNA purified from GST–protein6, but not in the sample from GST protein alone (lanes 3 and 1 respectively). A PCR product of similar size was apparent in the positive control (lane 6), and in the supernatants of each reaction, suggesting that an excess of HBV virions was present.

Fig. 5. Analysis of the binding reaction between GST–protein6 and HBV by PCR and gel electrophoresis. GST– (lanes 1 and 2) or GST–protein6– (lanes 3 and 4) coated Sepharose beads were incubated with partially purified HBV virions. Hepatitis B virions bound to the beads after washing (lanes 1 and 3) and in the supernatant fluids prior to washing the beads (lanes 2 and 4) were then determined by PCR; a negative (no DNA) and positive control (pKSHBVA DNA) were examined in parallel and the results are shown in lanes 5 and 6 respectively. The numbers on the left-hand side refer to DNA fragment sizes derived from digestion of λ DNA with restriction enzyme PstI.

In a previous study, Kuroki et al. (1994) used the ability of DHBV to bind radiolabelled duck hepatocyte proteins to purify and clone a candidate cell-surface receptor. To confirm the authenticity of this interaction, they demonstrated binding of a GST–DHBV preS fusion protein to the 180 kDa receptor candidate, which was identified as a member of the carboxypeptidase gene family (Kuroki et al., 1995). These results were confirmed in independent experiments performed by others (Tong et al., 1995) who demonstrated that a 170 kDa cellular protein, subsequently identified as carboxypeptidase H, interacted with intact DHBV via a neutralizing epitope of the preS region. Thus, as the GST–DHBV preS fusion protein was successfully applied to identify the DHBV cell-surface receptor, we employed a similar strategy to confirm the specificity of the interaction of the HBV preS region with the ten proteins identified in the yeast two-hybrid system.

The results obtained with this assay demonstrated interactions similar to those observed in the yeast two-hybrid system, as proteins #1 and #6 displayed strong binding to the preS1 region. Sequence analysis of clones #1 and clone #6 revealed 80–99 % nucleotide identity with cDNA sequences present within the EMBL and GenBank databases, and clone #6 aligned with a partial cDNA sequence of hepatic origin. Further analysis demonstrated that purified HBV was able to compete with the binding of protein #6 to the GST–preS1 protein but had no effect on the interaction between protein #1, a mitochondrial protein, and preS1. This was interpreted to mean that HBV is able to interact directly with protein #6, a protein of hepatic origin, whereas protein #1 appears to have no direct interaction with whole virions. In contrast, purified HBsAg was unable to compete with protein #6 for binding to the GST–preS1-coated beads.

Since the actual level of L-HBsAg contained in the purified HBV preparation was determined to be 17-fold higher than the level of L-HBsAg in the HBsAg preparation these data are consistent with the interpretation that the L-HBsAg in the virus preparation was responsible for the interaction with protein #6. Nevertheless, this point has not been proved in a formal manner. However, assuming that this interpretation is correct, this suggests that the conformational structure of the protein-binding region in the GST–preS1 and the L-HBsAg of the virus is very similar. These data are consistent with theoretical perceptions of the HBV receptor that postulate that the preS1 region of the HBV L protein is the major attachment site of HBV (Neurath et al., 1986a) to ensure HBV infectivity despite overwhelming levels of 22 nm HBsAg particles. To our knowledge, we believe that this study has identified for the first time a protein which is able to bind HBV but not HBsAg.

Subsequent experiments using a baculovirus-derived GST–protein6 fusion protein support the above interpretation and showed that protein #6 is able to interact directly with HBV virions. In these experiments, HBV was bound to the GST–protein6 but not to the GST protein alone immobilized on Sepharose beads.
While the studies presented in the study are consistent with the identification of protein #6 as a potential candidate for the HBV cellular receptor, it remains a possibility that this protein is an intracellular protein which mediates the retention of L-HBsAg. Clarification of these points will await the isolation of the complete gene which, in turn, will allow tissue distribution and cellular localization studies to be performed. This work is currently under way.

The data presented in this paper describe a novel approach for the identification of virus cellular receptors. Thus, the yeast two-hybrid system may have application to the search for cell-surface receptors for a variety of virus families.

This work was supported by grants from the National Health and Medical Research Council of Australia and the Royal Children’s Hospital Foundation. Tracey Harvey received a scholarship from the University of Queensland. The phosphorimager was purchased from funds provided by the Clive and Vera Ramaciotti Foundation and the Royal Children’s Hospital Foundation. We also thank Dr Maxine Preston for construction of baculovirus expression plasmid bacMP.

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Received 17 August 1998; Accepted 30 October 1998