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Interaction between Duck Hepatitis B Virus and a 170-Kilodalton Cellular Protein Is Mediated through a Neutralizing Epitope of the Pre-S Region and Occurs during Viral Infection

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Identification of cell surface viral binding proteins is important for understanding viral attachment and internalization. We have fused the pre-S domain of the duck hepatitis B virus (DHBV) large envelope protein to glutathione *S*-transferase and demonstrated a 170-kDa binding protein (p170) in [^{35}S]methionine-labeled duck hepatocyte lysates. This glycoprotein was found abundantly in all extrahepatic tissues infectible with DHBV and in some noninfectible tissues, though it is not secreted into the blood. The interaction of pre-S fusion protein with p170 was competitively inhibited by wild-type DHBV in a dose-dependent manner. In addition, infection of hepatocytes with DHBV blocked the binding of pre-S fusion protein to p170, which suggests a biological role for p170 during natural infection. The p170 binding site was mapped to a conserved sequence of 16 amino acid residues (positions 87 to 102) by using 24 pre-S deletion mutants; this binding domain coincides with a major virus-neutralizing antibody epitope. Furthermore, site-directed mutagenesis revealed that an arginine residue at position 97 is critical for p170 binding. p170 was purified by a combination of ion-exchange and affinity chromatographies, and four peptide sequences were obtained. Two peptides showed significant similarities to human and animal carboxypeptidases H, M, and N. Taken together, these results raise the possibility that the p170 binding protein is important during the replication cycle of DHBV.

The human hepatitis B virus (HBV) and related animal viruses from woodchucks, ground squirrels, Pekin ducks, and herons form a group of hepatotropic DNA viruses in the family *Hepadnaviridae*. These viruses cause acute and chronic liver diseases and are associated with the development of hepatocellular carcinoma. The earliest event of infection, i.e., interaction between viral envelope protein and specific cellular receptor(s), is poorly understood. Hepadnaviruses express at least two coterminal envelope proteins from the single envelope gene through alternative use of in-frame AUG codons. In the case of duck hepatitis B virus (DHBV), the large envelope protein (pre-S/S protein) contains 161 to 163 amino acid residues at the amino terminus that constitute the pre-S domain and a carboxyl terminus of 167 residues called the S domain. The small envelope protein of the S domain is produced by translation from an internal AUG codon. The large envelope protein is myristylated and phosphorylated (8, 17, 22). Previous studies have suggested that the large envelope protein mediates the infectivity of DHBV and hepatitis delta virus, which uses the envelope proteins of HBV for entry into hepatocytes (5, 27, 28). Competition experiments have also revealed an inhibitory effect of yeast-derived large envelope protein (but not small envelope protein) on DHBV infectivity (12). Since the pre-S domain represents the sequence unique to the large envelope protein, it is believed to contain the hepatocyte receptor-binding site. Several cellular proteins which bind to the envelope proteins of HBV through the pre-S or S domain have recently been reported (1, 2, 10, 20, 21, 23), but it has been difficult to firmly establish that such proteins are components of the viral receptor.

We have employed DHBV as a model system to identify cellular proteins that may mediate hepadnavirus binding to the hepatocyte cell surface and allow viral penetration across the cell membrane. Compared with HBV, DHBV has only one pre-S-containing envelope protein, which is not glycosylated. Viral infections of ducklings and primary duck hepatocytes have been well established (25, 32). Thus, in this study, we used the DHBV pre-S domain fused to glutathione *S*-transferase (GST) to identify a 170-kDa duck glycoprotein (p170) which binds to a major neutralizing epitope of the pre-S domain. The interaction between p170 and pre-S fusion protein was specific and was inhibited in vitro by DHBV viremic sera and in vivo by endogenous replicating DHBV. Sequencing of the proteolytic fragments of p170 revealed some similarities to carboxypeptidases. These data suggest that p170 is a protein involved in the life cycle of DHBV and is similar to the 180-kDa glycoprotein recently described by Kuroki et al. (13).

MATERIALS AND METHODS

Cloning and expression of pre-S sequences in *Escherichia coli*. As a negative control, the pre-S region of HBV (31) was amplified by PCR and cloned between the *Bam*HI and *Eco*RI sites of vector pGEX 2TK (Pharmacia). DHBV DNA fragments covering the entire envelope gene (pre-S/S), the pre-S region, or part of the pre-S region were generated by PCR. A *Bgl*II or *Bam*HI restriction site was attached to the sense primer, and a translational termination codon and an *Eco*RI site were added to the antisense primer. Twenty cycles of amplification were carried out with 1 U of Vent DNA polymerase (New England Biolabs) and 1 to 10 ng of DHBV-16 DNA (18). The PCR products were cloned into the *Bam*HI-*Eco*RI sites of pGEX 2TK. Pre-S deletion mutants were designated by the positions of the first and last amino acid residues. For example, mutant 25-104 expressed amino acid residues 25 through 104 of DHBV envelope protein. Several pre-S deletion mutants with the 3′ ends of inserts located at the *Xho*I, *Sma*I, and *Hin*dIII sites of DHBV (corresponding to pre-S amino acids [aa] 138, 126, and 97, respectively) were constructed through double enzymatic digestion of recombinant 2TK plasmids. For example, mutant 25-126 was generated by removal of a short *Sma*I-*Eco*RI fragment from mutant 25-161, filling-in reaction, and blunt-end recircularization. Since a termination codon did not immediately follow the insert, fusion proteins expressed from these particular constructs would contain a few more amino acid residues at the carboxy terminus contributed by the vector sequence downstream of the *Eco*RI site.

To construct DHBV pre-S substitution mutants, a 1.4-kb *Eco*RI-*Bam*HI fragment covering the entire pre-S region was cloned into vector pALTER-II (Pro-

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mega). Mutagenesis was performed according to the supplier's protocol from single-stranded template DNA. Most mutations introduced or destroyed a restriction enzyme recognition site, thus facilitating rapid identification. Mutations were confirmed by DNA sequencing. The entire pre-S region of these mutants was amplified and subcloned into pGEX 2TK. The name of each substitution mutant consists of a single letter for the wild-type amino acid, the position of the amino acid, and a single letter for the mutant amino acid. The expression and purification of GST fusion proteins were based on the protocol provided by Pharmacia. Fusion proteins were expressed by induction with 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 1 h, and after sonication, they were purified with glutathione-Sepharose beads (at a ratio of about 1 μl of bead per ml of bacteria culture). For the expression of the entire pre-S/S fusion protein of DHBV, induction with IPTG lasted for 3 to 4 h. The size, purity, and yield of recombinant proteins were analyzed by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS–12% PAGE) and subsequently by Coomassie blue staining.

Preparation and labeling of primary duck hepatocytes. DHBV-free Pekin ducklings (less than 2 weeks of age) were perfused sequentially with 0.5 mM EGTA and 0.5 mg of collagenase per ml via the portal vein (25, 32). Hepatocytes were seeded in petri dishes at approximately 90% confluency by using F15 medium supplemented with 5% fetal calf serum. Subsequent cultures employed serum-free medium supplemented with 1 to 1.5% dimethyl sulfoxide (DMSO) (25). Cells were starved in methionine-free Dulbecco modified Eagle medium for 1 h and then incubated for 4 h with F15 medium supplemented with [^{35}S]methionine (Amersham) or Tran^{35}S Label (New England Nuclear) at a concentration of 0.1 mCi/ml. Cells from each 60-mm-diameter dish were treated with 2 ml of lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate) supplemented with the protease inhibitors aprotinin, leupeptin, and phenylmethylsulfonyl fluoride. Nuclei were removed by centrifugation, and lysates were stored at −80°C.

Detection of pre-S-binding proteins in labeled lysates. To reduce the levels of cellular proteins which bind to the GST component of the pre-S fusion protein or directly to Sepharose beads, labeled lysates were first preincubated twice at 4°C with a mixture of empty Sepharose beads and GST-bound beads. The beads were washed extensively and used as a negative control during SDS-PAGE. Precleared lysates were then incubated at 4°C for 6 to 16 h with the specific GST–pre-S fusion protein. After beads had been extensively washed four times with lysis buffer, bound proteins were eluted from beads by heating to 95°C for 5 min and separated by SDS–8% PAGE under denaturing conditions. Proteins retained in the second preclearing reaction were run in parallel. The gel was fixed with 10% acetic acid, treated with amplify solution (Amersham), dried, and exposed. Comparisons of the protein bands binding only to the pre-S fusion protein indicated the specificity of this interaction.

Detection of p170 in different duck tissues. For these experiments, the elution profile of p170 through an anion-exchange column was established. One milliliter of ^{35}S-labeled duck hepatocyte lysate was dialyzed overnight in 50 mM Tris-HCl (pH 8.3). Lysates were applied to a column packed with preswollen DEAE-cellulose (Sigma) equilibrated with 50 mM Tris-HCl (pH 8.3). Bound proteins were sequentially eluted with 100, 200, and 400 mM NaCl in 50 mM Tris-HCl (pH 8.3). The peak of radioactivity in each fraction was collected and dialyzed back against lysis buffer. After incubation with GST–pre-S fusion protein, bound proteins were revealed by SDS-PAGE and fluorography. To study the tissue distribution of p170, 0.6 to 1.2 g of frozen tissue was homogenized in 6 to 12 ml of lysis buffer; after overnight dialysis against 50 mM Tris-HCl (pH 8.3), insoluble materials were removed by centrifugation and subsequent filtration through a 0.45-μm-pore-size filter. The solution was passed through a column containing 8 g of preswollen DEAE-cellulose and eluted with 100 and 200 mM NaCl in 50 mM Tris-HCl (pH 8.3). The 200 mM NaCl eluent was dialyzed against the lysis buffer and precleared twice with 10 to 20 μl (bed volume) of Sepharose beads. The concentration of proteins in each sample was determined by the Bio-Rad protein assay, and 7 mg of protein from each sample was incubated with 4 to 8 μg of GST–pre-S fusion protein. After the separation of bound proteins by SDS–8% PAGE, protein bands were visualized by silver staining (Gelgold staining kit [Pierce]).

Protein microsequencing of p170. p170 was purified from 40 g of duck liver by the method described above, separated from GST–pre-S protein by SDS-PAGE, and transferred to polyvinylidene difluoride membranes. After being stained with ponceau S, strips of the membrane containing about 20 μg of p170 were obtained for sequencing analysis, which was carried out at the Harvard Microchemistry Facility. Briefly, p170 was digested with lyase C, and digests were separated by high-pressure liquid chromatography. Selected peptide peaks were sequenced by the Edman degradation method.

RESULTS

A 170-kDa glycoprotein binds to the DHBV pre-S region. The entire envelope protein of DHBV and its pre-S domain as well as the pre-S domain of HBV were cloned into vector pGEX 2TK and expressed as GST fusion proteins (Fig. 1A). In addition to a protein band of the expected size, all of the recombinant constructs displayed a band with mobility slightly faster than that of the intact GST protein, which was seen in all of the additional fusion protein constructs (see Fig. 4B and 6B) and most likely corresponded to a proteolytic cleavage product of the fusion proteins around the cloning site. Using DHBV pre-S/S protein fused to GST as a probe, we identified a 170-kDa binding protein in [^{35}S]methionine-labeled duck hepatocyte lysates (Fig. 1B, lane 3). Since this protein was not retained by GST alone (Fig. 1B, lane 1), it appeared to be specific for DHBV sequences. The p170 protein was also retained by the GST–pre-S fusion protein (Fig. 1B, lane 4), suggesting that the S domain was not essential for binding activity. The failure of a similar pre-S fusion protein derived from HBV to bind duck hepatocyte p170 (Fig. 1B, lane 2) as a control is consistent with species specificity of the p170–pre-S interaction. To determine if p170 is glycosylated, duck hepatocytes were labeled in the presence of tunicamycin (2 μg/ml). Under these conditions, a protein of around 145 kDa was detected (Fig. 1B, lane 5). These properties of p170 appear to be similar to those of p180, which was found by immunoprecipitation to be a binding protein for the DHBV pre-S domain (13).

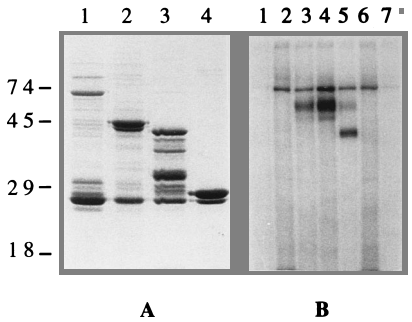

FIG. 1. A 170-kDa glycoprotein binds to the pre-S domain of the DHBV large envelope protein. (A) Expression and purification of GST fusion proteins of DHBV pre-S/S protein (lane 1), the pre-S domain (lane 2), and the HBV pre-S domain (lane 3). Lane 4 is the intact GST protein expressed from pGEX 2TK vector. The molecular sizes of protein markers (in kilodaltons) are shown on the left. (B) p170 is a glycoprotein interacting with the DHBV pre-S domain. ^{35}S-labeled hepatocyte lysates were precleared with Sepharose beads and GST-bound beads (lane 1) and then incubated with the following GST fusion proteins immobilized on Sepharose beads: DHBV pre-S/S protein (lane 3), its pre-S domain (lane 4), and the HBV pre-S domain (lane 2). Bound liver proteins were revealed by SDS-PAGE and fluorography. In a separate experiment, hepatocytes were metabolically labeled in the presence of tunicamycin. After preclearing (lane 7), lysates were reacted with GST fusion proteins of the DHBV pre-S domain (lane 5) or the HBV pre-S domain (lane 6).

Interaction of DHBV particles with p170 in vitro and in vivo. Viral envelope proteins expressed as fusion proteins in *E. coli* may not have the proper conformation and therefore may bind to irrelevant proteins. To address whether p170 could bind to native viral particles, a competition experiment was performed by adding highly viremic duck serum to the incubation reaction. The incubation of ^{35}S-labeled hepatocyte lysates with 5 μg of pre-S/S fusion protein gave rise to a strong band of p170 (Fig. 2A, lane 1). The addition of 60 (Fig. 2A, lane 2) or 200 μl (lane 3) of DHBV-free duck serum to the incubation mixture had little effect on the binding activity to p170. On the other hand, as little as 5 μl of DHBV-positive serum strongly inhibited p170 binding (Fig. 2A, lane 4). Increases in the volumes of DHBV-positive serum diminished the p170 band in a dose-dependent manner (Fig. 2A, lanes 5 to 7). With an incubation of 200 μl of viremic serum, virtually no p170 binding was observed (Fig. 2A, lane 7). A similar inhibitory effect was seen when DHBV particles purified through successive sucrose gra-

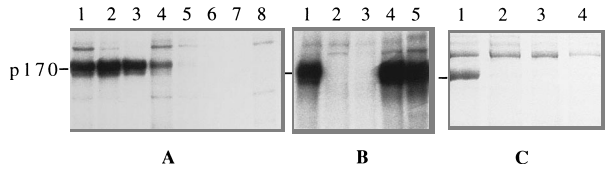

FIG. 2. Abrogation of interaction between p170 and GST–pre-S fusion protein by DHBV particles added in vitro or generated in vivo. (A) Inhibition of p170 binding by added DHBV particles. ^{35}S-labeled lysates (3 ml) were precleared (lane 8), and equal volumes of lysates were incubated with 5 μg of GST fusion protein of DHBV pre-S/S protein in the absence of duck serum (lane 1) or in the presence of 60 (lane 2) or 200 μl (lane 3) of DHBV-free duck serum or 5 (lane 4), 20 (lane 5), 60 (lane 6), or 200 μl (lane 7) of DHBV viremic serum. Bound proteins were revealed by SDS-PAGE and fluorography. (B) Reduced expression or inaccessibility of p170 in DHBV-infected hepatocytes. Three dishes of hepatocytes were obtained from a DHBV-free duckling, and two dishes of hepatocytes were infected with 30 or 300 μl of DHBV-positive serum. After 9 days, cells were metabolically labeled and precleared lysates were incubated with GST–pre-S fusion protein. Lane 1, noninfected cells; lane 2, cells infected with 30 μl of viremic serum; lane 3, cells infected with 300 μl of viremic serum; lanes 4 and 5, comparison of p170 expression in duck hepatocytes and fibroblast-like cells derived from duck hepatocytes, respectively. Hepatocytes were cultured in F15 medium supplemented with 1% DMSO (lane 4) or 5% fetal calf serum (lane 5) for 10 days and labeled with [^{35}S]methionine, and lysates were reacted with GST–pre-S fusion protein. (C) Reduced level of p170 detected by pre-S fusion protein in DHBV-infected liver tissue. Liver tissues from a DHBV-free duckling and a naturally infected duckling were homogenized in lysis buffer, precleared twice with Sepharose beads, and then incubated with GST–pre-S fusion protein immobilized on Sepharose beads. Bound proteins were electrophoresed by SDS-PAGE and stained with Coomassie blue. Lane 1, 2 g of DHBV-free liver tissue; lane 2, 2 g of infected liver tissue; lane 3, 2 g of DHBV-free liver tissue mixed with 1 ml of DHBV$^+$ serum from the infected duck; lane 4, 1 g of DHBV-free liver tissue mixed with 1 g of infected liver tissue. The position of p170 is indicated on the left of each panel.

dient centrifugations were applied as well (data not shown). These results suggest that p170 is recognized by virion particles, possibly through the pre-S region.

If DHBV particles bind p170 in vitro, they might also do so in vivo during natural wild-type viral infection. Three 100-mm-diameter dishes of primary duck hepatocytes were prepared from a DHBV-free duckling. One dish served as a control, while the other two dishes were infected overnight with 30 and 300 μl of DHBV viremic serum, respectively. Nine days postinfection, cells were metabolically labeled and lysed. While p170 was detected as a strong band in uninfected hepatocytes (Fig. 2B, lane 1), it was no longer detectable in infected hepatocytes from the other two dishes (lanes 2 and 3). Similar results were obtained with ^{125}I-labeled cell surface protein lysates (data not shown). Thus, either newly synthesized p170 was masked by binding to the endogenous DHBV envelope protein or de novo synthesis of p170 was severely inhibited during infection. To compare the steady-state levels of p170 in infected and noninfected duck livers, unlabeled liver tissues from 2-week-old ducklings were studied. One duckling was DHBV free, while the other was naturally infected. Liver tissues were homogenized in lysis buffer and precleared twice with empty Sepharose beads. After incubation with pre-S fusion protein immobilized to Sepharose beads, bound proteins were visualized by SDS-PAGE and Coomassie blue staining (Fig. 2C). When the same amounts of tissues were used, p170 was readily detected in uninfected liver tissue but barely visible in infected liver tissue (Fig. 2C, lanes 1 and 2). The addition of DHBV-positive duck serum or lysates of infected liver tissue masked p170 from the DHBV-free duckling (Fig. 2C, lanes 3 and 4). Thus, even if it had been present at normal levels, p170 would have been rendered undetectable by the large number of virus particles.

Tissue distribution of p170. To examine whether the expression of p170 is dependent on the differentiated status of duck hepatocytes, hepatocytes were cultured either in serum-free, DMSO-containing medium as described above or in F15 medium supplemented with 5% calf serum instead of DMSO. Incubation in the latter medium has been shown to cause rapid losses of both hepatocyte morphology and susceptibility to DHBV infection (25). After 10 days of culture, hepatocytes maintained with calf serum became largely elongated to become fibroblast-like. However, when cells were metabolically labeled and cell lysates were incubated with pre-S fusion protein, the intensity of the p170 band was virtually unaffected (Fig. 2B; compare lanes 4 and 5). Thus, the expression of p170 does not depend on the differentiated status of hepatocytes. To further study the tissue specificity of p170 expression, a two-step purification procedure involving an anion-exchange column and affinity chromatography was established. Pilot experiments with ^{35}S-labeled lysates established that p170 was eluted with 200 mM NaCl from a DEAE-cellulose column (Fig. 3A, lane 3). This fractionation procedure removed the majority of nonspecifically binding proteins. Tissue lysates or sera derived from DHBV-free ducklings were passed through a DEAE-cellulose column. The 200 mM NaCl eluents were precleared with Sepharose beads and then incubated with immobilized pre-S fusion protein. SDS-PAGE and silver staining revealed that p170 was highly expressed in the pancreas, liver, kidney, and spleen, where DHBV replication has previously been reported (Fig. 3B, lanes 13, 5, 10, and 7, respectively). p170 was also found in the lung and heart and to a lesser extent in the stomach and muscles (Fig. 3B, lanes 4, 3, 8, and 6, respectively). In the gall bladder, the major binding protein for the pre-S protein had a molecular size of around 180 kDa and was very abundant (Fig. 3B, lane 9). This 180-kDa band was not seen in the preclearing reaction (Fig. 3B, lane 15). Interestingly, p170 was not detected in serum (Fig. 3B, lane 11), even when a 10-fold-greater concentration of proteins was applied (lane 12). Therefore, p170 does not appear to be a secreted protein.

p170 binds to a major neutralizing epitope of the DHBV pre-S region. To define the region of the pre-S protein that is essential for binding to p170, nine progressive N-terminal and

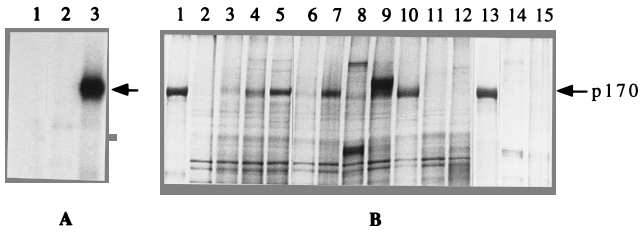

FIG. 3. (A) Elution of p170 through a DEAE-cellulose column. ^{35}S-labeled hepatocyte lysates were passed through a DEAE-cellulose column, and the flowthrough fraction (lane 1) and eluents at 100 (lane 2) and 200 mM (lane 3) concentrations of NaCl were incubated with GST–pre-S fusion protein. Bound proteins were revealed by SDS-PAGE and fluorography. The position of p170 is shown by an arrow on the right of each panel. (B) Tissue distribution of p170 in different duck tissues. Different tissues (0.6 to 1.2 g) were homogenized in lysis buffer, dialyzed, and passed through a DEAE-cellulose column. Proteins eluted at a 200 mM NaCl concentration were precleared, and 7 mg (7 and 70 mg for serum samples) was incubated with the specific GST–pre-S fusion protein immobilized on Sepharose beads. Bound proteins were separated by SDS–8% PAGE and revealed with a Gelgold silver staining kit. Lane 1, 2 μg of a 170-kDa molecular size marker (α_2-macroglobulin); lane 2, GST–pre-S fusion protein (the same amount used in purifying p170 from each tissue); lanes 3 to 13, purification and detection of p170 in heart, lung, liver, muscle, spleen, stomach, gall bladder, and kidney tissues; 7 and 70 mg of protein from serum; and pancreas tissues, respectively; lanes 14 and 15, the second preclearing reactions for stomach and gall bladder tissues, respectively. Lanes 13 to 15 were derived from a separate SDS-PAGE and staining experiment.

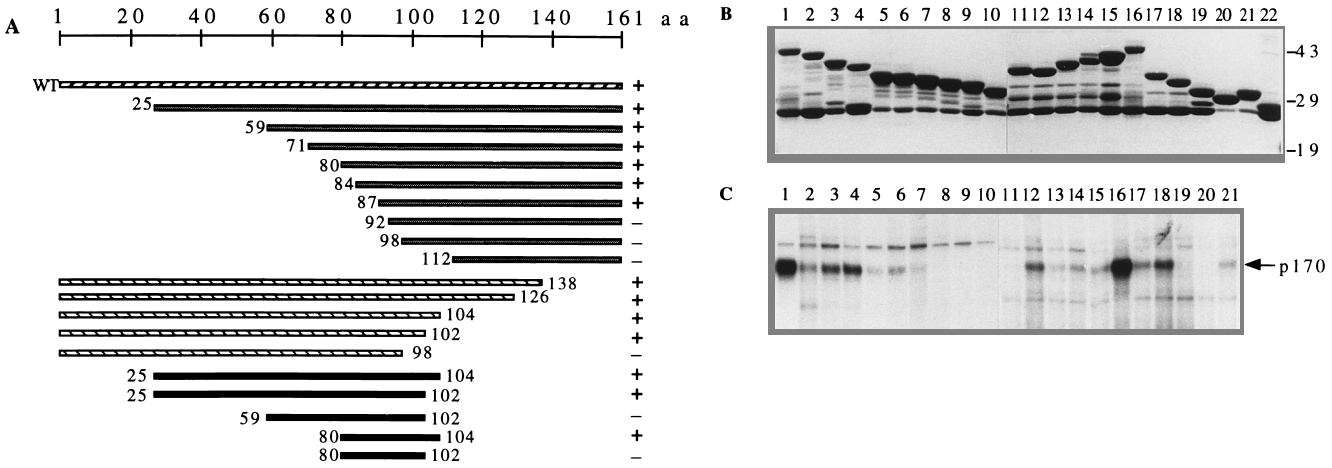

FIG. 4. Localization of the p170 binding site in the pre-S region. (A) Schematic representations of the pre-S deletion mutants and their binding results. All 161 aa of the DHBV pre-S region are shown to scale. The positions of the first amino acid residues in amino-terminal deletion mutants, of the last residues in carboxyl-terminal deletion mutants, and of both terminal residues in double-deletion mutants are given. The positive (+) or negative (−) binding result of each mutant with p170 is shown on the right. WT, wild type. (B) Expression and purification of GST–pre-S deletion mutants. The positions of molecular size markers (in kilodaltons) are shown on the right. (C) p170 binding results of the deletion mutants. (B and C) Lanes 1 through 21: intact pre-S; mutants 25-161, 59-161, 71-161, 80-161, 84-161, 87-161, 92-161, 98-161, 112-161, 1-98, 1-102, 1-104, 1-126, and 1-138; intact pre-S; and mutants 25-104, 25-102, 59-102, 80-102, and 80-104, respectively. Lane 22, intact GST protein.

five C-terminal deletion mutants were made and studied (Fig. 4A). All 14 deletion mutants expressed GST fusion proteins of the expected sizes, as judged by SDS-PAGE (Fig. 4B). The removal of up to 86 amino acid residues in the N terminus reduced but did not abolish binding (mutant 87-161; Fig. 4C, lane 7), while the deletion of 5 additional amino acid residues abolished binding (mutant 92-161; lane 8). A mutant with a C-terminal deletion of up to 59 residues retained strong binding capacity (mutant 1-102; Fig. 4C, lane 12), while a further deletion to aa 98 abolished binding (mutant 1-98; lane 11). Therefore, the pre-S sequence critical for p170 binding was localized to a 16-aa sequence (between residues 87 and 102). This sequence covers a known virus-neutralizing epitope (type II) that maps to aa 91 to 99 (33) and overlaps with two additional neutralizing epitopes located at aa 83 to 90 and 100 to 107 (see Fig. 8). To test if the minimum binding sequence can function independently, five double-deletion mutants were constructed (Fig. 4A and B). Of the three double-deletion mutants terminating at aa 102, only the one with a limited (24-aa) N-terminal deletion bound p170 (mutant 25-102; Fig. 4C, lane 18). On the other hand, both mutants terminating at aa 104 were able to bind p170 (Fig. 4C, lanes 17 and 21). The shortest double-deletion mutant capable of binding p170 was construct 80-104, which contained only a 25-aa sequence (Fig. 4C, lane 21).

The p170 binding site that we have mapped constitutes just a portion of the pre-S sequence reported to be essential for p180 binding (11). Coimmunoprecipitation of p180 was inhibited by type IV neutralizing antibody which binds to pre-S residues 58 to 66 (13, 33), and deletional analysis using the GST system mapped the p180 binding site to a large segment of the pre-S sequence between aa 43 and 108 (11). To further test the role of sequence around the type IV epitope in the p170 interaction, we constructed five additional double-deletion mutants, 25-126, 59-126, 71-126, 42-102, and 59-104 (Fig. 5A). For four mutants with an identical C terminus at residue 126 but different N-termini at residues 1, 25, 59, and 71, p170 retention was not reduced (but rather increased) by successive N-terminal truncations (Fig. 5B, lanes 2 to 5). For the three double-deletion mutants that terminate at aa 104, moving the amino end from aa 25 to aa 59 or 80 did not reduce p170 binding (Fig. 5B, lanes 9 to 11, respectively). Only for each mutant with a C terminus at aa 102 did a change in the N terminus from aa 25 to aa 42 greatly reduce p170 binding (Fig.

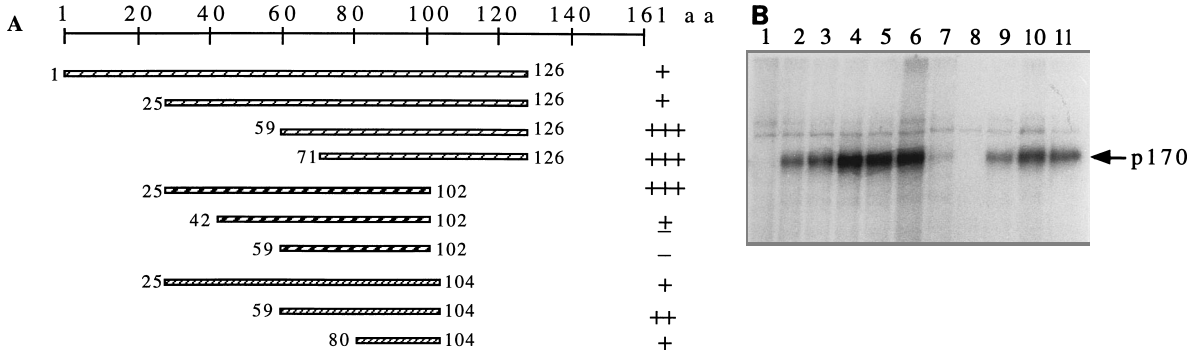

FIG. 5. The first 70 to 80 aa of the pre-S protein are dispensable for p170 binding. (A) Schematic representations of the pre-S deletion mutants and their relative binding capacities from very strong (+++) to negative (−). (B) Autoradiograph showing the retention of p170 by different mutants. Lane 1, preclearing reaction; lanes 2 to 5, mutants terminating at residue 126 (1-126, 25-126, 59-126, and 71-126, respectively); lanes 6 to 8, mutants terminating at residue 102 (25-102, 42-102, and 59-102, respectively); lanes 9 to 11, mutants terminating at residue 104 (25-104, 59-104, and 80-104, respectively). The position of p170 is indicated by an arrow on the right.

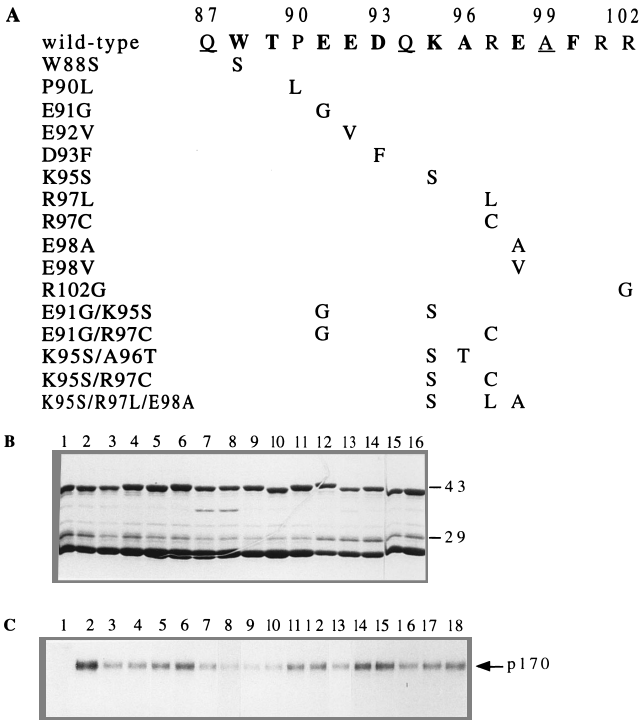

FIG. 6. Effects of individual amino acid substitutions in the p170 binding site on interaction with p170. (A) Locations and natures of amino acid changes in mutants. The amino acid sequence from positions 87 through 102 of the wild-type virus is given. Amino acid residues that are variable in a goose hepatitis B virus strain (25a) are underlined, while boldface letters denote residues conserved in heron hepatitis B virus (26). (B) Expression of GST–pre-S fusion proteins by mutants. Proteins purified from the same amounts of bacterial culture were applied to the 12% polyacrylamide gel. Mutants were arranged in the following order: E91G (lane 1), E92V (lane 2), D93F (lane 3), K95S (lane 4), R97L (lane 5), R97C (lane 6), E98A (lane 7), E98V (lane 8), W88S (lane 9), P90L (lane 10), R102G (lane 11), K95S/R97C (lane 12), K95S/R97L/E98A (lane 13), K95S/A96T (lane 14), E91G/K95S (lane 15), and E91G/R97C (lane 16). The positions of protein size markers are shown on the right. (C) Capacities of these mutants to bind to p170. Lanes 1 through 18: second preclearing reaction, wild-type pre-S fusion protein, W88S, P90L, E91G, E92V, D93F, K95S, R97L, R97C, E98A, E98V, R102G, E91G/K95S, E91G/R97C, K95S/A96T, K95S/R97C, and K95S/R97L/E98A, respectively.

5B, lanes 6 and 7). Moving the N terminus to aa 59 completely abolished binding (Fig. 5B, lane 8), as already shown in Fig. 4.

Effects of single-amino-acid changes in the major pre-S-neutralizing epitope region on p170 binding. To define the individual amino acid residues critical for p170 binding, site-directed mutagenesis experiments were carried out on aa 88 through 102 of the pre-S region. Eleven single-amino-acid substitution mutants involving nine residues were constructed, and their affinities for p170 binding were compared with that of the wild-type fusion protein (Fig. 6A and C). The levels of pre-S fusion proteins produced by the mutant constructs were similar (Fig. 6B), and the same amounts of fusion proteins were used for binding experiments. While all of the mutants exhibited reduced retention of p170, mutants R97L and R97C consistently showed the lowest binding activities in several independent experiments. The results from one experiment are shown in Fig. 6C (lanes 9 and 10). Mutant K95S also had a greatly reduced binding capacity (Fig. 6C, lane 8). These results suggest that these two adjacent basic amino acid residues are important in the interaction between the pre-S domain and p170. However, when different amino acid substitutions were combined to produce 2- or 3-aa changes (Fig. 6A and B), none showed further decreases in p170 binding, even in the levels for mutants of both residues 95 and 97 (K95S/R97C and K95S/R97L/E98A [Fig. 6C, lanes 17 and 18, respectively]).

p170 is structurally related to carboxypeptidases. To gain insight into the molecular identity of p170 and provide essential peptide sequence to clone p170 cDNA, we purified p170 from DHBV-free duck livers by the combination of ion-exchange chromatography and affinity chromatography. Purified proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The 170-kDa protein band was digested with lyase C, and selected peptides were sequenced. The four peptide sequences obtained are listed in Fig. 7A. Database searches for peptides 3 and 4 did not reveal overall sequence similarity to any known protein (except for small stretches of sequence). However, peptides 1 and 2 showed similarities to mammalian carboxypeptidases H, N, and M (Fig. 7B and C). Peptide 2 displayed moderate degrees of homology to all three carboxypeptidases (Fig. 7C), while peptide 1 had the highest degree of similarity to carboxypeptidase H, a moderate degree of similarity to carboxypeptidase N, and the lowest degree of similarity to carboxypeptidase M (Fig. 7B). Both of these peptides were found to have sequence similarities to the AEBP1 gene product, which is a novel eukaryotic transcriptional repressor with carboxypeptidase activity.

DISCUSSION

In this study, we have identified a 170-kDa duck hepatocyte-derived glycoprotein which specifically interacts with the pre-S region of DHBV envelope protein. A glycoprotein of similar size (180 kDa; gp180) was also recently found by immunoprecipitation experiments (13). The degree of glycosylation and the tissue distribution of the two proteins are similar. Like p170, gp180 was detected in fibroblasts and a variety of extrahepatic tissues (13). However, the binding sites of p170 determined in this study (positions 87 to 102) differed from those of gp180 as reported by Ishikawa et al. (positions 43 to 108 [11]).

```
A    peptide 1     SVELRELYVMEISDNPGVHEAGEPEFK

     peptide 2     LIDRTRIVIVPSLNPDGR-IA

     peptide 3     SLLSHEFQDETDTEEETLYSAK

     peptide 4     VEEGKVPVLNTPD


B    peptide 1     SVELRELYVMEISDNPGVHEAGEPEFK
     bovine CPH    .F.G...L.L.L........P......
     human CPH     .F.G...L.I.L........P......
     AEBP1 gene         KI.A........D..L.....R
     human CPN     ...G.H...L.F..H..I..PL...V.
     human CPM     ..KG.N.W.LVVGRF.KE.RI.I....


C    peptide 2     LIDRTRIVIVPSLNPDGR-IA
     AEBP1 gene    .VQD...HL........YEV.
     bovine CPH    ..HN...H.M.......
     human CPN     ..QD...H.L..M....YEV.
     human CPM     ..NS...H.M..M....
     human CPH     ..HS...H.M.......
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FIG. 7. Peptide sequences of p170 (A) and similarities of peptides 1 (B) and 2 (C) to carboxypeptidases. Dashes indicate undetermined residues. Dots denote residues identical to those in the peptide sequence. CPH, carboxypeptidase H; CPM, carboxypeptidase M; CPN, carboxypeptidase N; AEBP1 gene, a mouse transcriptional repressor with carboxypeptidase activity. The references for sequences are as follows: bovine CPH, 6; human CPH, 19; human CPM, 30; human CPN, 7; AEBP1 gene, GenBank accession no. X80478.

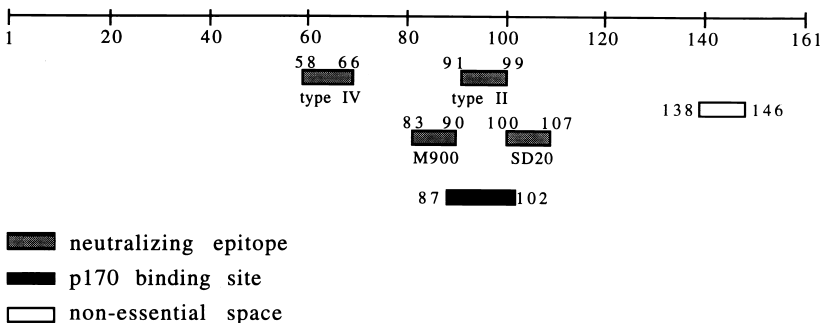

FIG. 8. The p170 binding site coincides with a neutralizing epitope of the pre-S region. The locations of the type II and IV epitopes are those of Yuasa et al. (33), and the locations of the M900 and SD20 epitopes are those of Chassot et al. (3). The location of a sequence nonessential for viral infectivity is that of Li et al. (16).

We found that pre-S residues 43 to 80 are not essential for p170 binding on the basis of the following observations. (i) In progressive N-terminal deletions to assess sequence requirements, moving the N terminus from residue 25 to residue 59 or 71 did not reduce p170 binding (Fig. 4C, lanes 2 to 4, respectively). (ii) For each double-deletion mutant with a C terminus at residue 126 or 104, the p170 reactivity was also not reduced (sometimes even increased) by removing these sequences (Fig. 5, lanes 2 to 5 and 9 to 11). The only exception was a series of mutants terminating at aa 102, which were highly sensitive to N-terminal deletions (Fig. 5, lanes 7 and 8). This finding may have been due to the fact that in these mutants, residue 102 (which is the exact C terminus of the p170 binding site) is also at the end of the fusion protein and that a major N-terminal deletion would greatly disturb protein conformation and could make the binding site inaccessible. In this regard, it is important that the binding of gp180 was inhibited by a neutralizing antibody against residues 57 to 65 (13). While this suggests the involvement of such sequences in gp180 binding, it could be equally explained by a conformational change that affects the binding site which is secondary to antibody binding.

A viral receptor protein should be expressed in tissues known to be susceptible to viral infection. We have demonstrated that p170 is expressed in the kidney, pancreas, gall bladder, and spleen. These tissues have been shown to support DHBV infection in vivo (9, 29). Whether the 180-kDa binding protein seen in the gall bladder is an isoform of p170 is presently under investigation. p170 was also detectable in the lung, heart, stomach, and muscles; however, no evidence of DHBV infection in these organs has been reported. This tissue distribution does not exclude p170 as a component of the DHBV receptor complex, since there may be additional unidentified components which are specific to liver and other infectible tissues (like the kidney, spleen, and pancreas). Alternatively, the organ specificity of DHBV infection could be controlled in part by liver-specific transcription factors necessary for viral replication. While p170 is barely detectable in the LMH chicken hepatoma cell line, it is clearly detectable (though less intensely) in ^{35}S-labeled Muscovy duck hepatocytes (30a). However, it is known that hepatocytes derived from this duck species support little DHBV replication in vitro (24). Nevertheless, one strong argument in favor of p170 as an important protein during DHBV infection is the observation that the interaction between p170 and the pre-S protein can be abrogated by DHBV particles added in vitro or generated during viral infection in vivo. The fact that 200 μl of viremic duck serum totally inhibited p170 binding to 5 μg of pre-S/S fusion protein implies that the large envelope protein in its native conformational state has a much stronger affinity for p170 than does the bacterially derived fusion protein. The lack of p170, as measured by binding to the GST–pre-S fusion protein, in experimentally infected hepatocytes and naturally infected liver tissue was possibly caused by the masking of p170 through binding sites occupied by DHBV virions (Fig. 2C, lanes 3 and 4), since immunoprecipitations of infected hepatocytes with a rabbit polyclonal antibody against the bacterially expressed pre-S domain revealed p170 (30a). Whether the absolute amount of p170 present was also reduced can be addressed only with the availability of p170 cDNA and antibodies and by Northern (RNA) blotting, immunoprecipitation, and Western blotting. Attempts to detect p170 (even in purified form in microgram quantities) with ^{32}P-labeled pre-S fusion protein on Far Western blots (immunoblots) were unsuccessful (30a).

Another line of evidence suggesting that p170 has biologic relevance to DHBV infection comes from the mapping experiments. Our results indicate that residues 87 to 102 were critical for interaction with p170. Since most of the deletion mutants had reduced binding capacities compared with that of the intact pre-S polypeptide (reductions varied among mutants and did not always correlate with the length of the deletion [Fig. 4C]), the entire pre-S sequence may be required to maintain an optimal conformation for binding. Interestingly, aa 87 to 102 belong to a stretch of highly conserved pre-S sequence among different DHBV strains, including the newly discovered goose hepatitis B virus (25a). As shown in Fig. 8, several virus-neutralizing epitopes have been found and mapped in the pre-S region; these include residues 58 to 66 (4, 33), 83 to 90 (3), 91 to 99 (4, 33), and 100 to 107 (3, 14). Thus, residues 83 to 107 define an important pre-S sequence, with a cluster of three neutralizing epitopes, and may well partly define the region responsible for binding to the hepatocyte cell surface. It is noteworthy that the pre-S binding site to p170 sits in the middle of this region and entirely covers the epitope defined by type II neutralizing antibodies (4, 33). Considering that moving the pre-S N terminus from residue 71 to residue 80, 84, or 87 greatly reduced p170 binding (Fig. 4C, compare lane 4 with lanes 5 to 7, respectively), the neutralizing epitope at residues 83 to 90 may play an important auxiliary role in this interaction. Site-directed mutagenesis experiments confirmed that residues 87 to 102 all contribute to p170 binding (Fig. 6). However, mutations at residue 97 and to a lesser degree at residue 95 had the most profound effect on p170 binding. Thus, these two basic amino acid residues located near the center of the type II epitope could be the critical contact points with p170. This is of particular interest since p170 show similarities to basic carboxypeptidases, which usually have acidic residues to contact the basic residues of the substrate. Finally, the p170 binding site lies outside the sequence near the carboxyl termi-

nus of the pre-S region, which can tolerate significant sequence changes for viral infectivity (Fig. 8) (15, 16).

In conclusion, characterization of the interaction between the DHBV pre-S domain and p170 raises the possibility that p170 is a component of the DHBV receptor complex on the hepatocyte cell surface. Protein microsequencing revealed some similarities between p170 and carboxypeptidases H, N, and M, all of which show substrate specificities toward basic residues, such as arginine or lysine. Carboxypeptidase H (or E) is found in secretory granules of the brain and pituitary gland and is involved in neuropeptide processing (6, 19). Carboxypeptidase N is synthesized in the liver and secreted into circulation to inactivate vasoactive and inflammatory peptides (7). Carboxypeptidase M is expressed in various tissues and found on cell surfaces (30). It may control interactions between peptide hormones and their receptors. It is important that p170 is much larger than any of these proteases (including the active subunit of carboxypeptidase N) and that peptides 3 and 4 did not have homology to these proteolytic enzymes. Whether DHBV attachment to and penetration of hepatocytes require proteolytic cleavage of the envelope protein by a combination of endo- and carboxypeptidases, including p170, warrants further studies.

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ADDENDUM

While the manuscript was under review, Kuroki and colleagues reported the cDNA sequence for gp180 (13a). With a minor difference of two amino acid positions, all four peptides of p170 could be found in the deduced amino acid sequence of gp180. This suggests that p170 and gp180 are the same protein or different splicing variants.

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