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Role of the Antigenic Loop of the Hepatitis B Virus Envelope Proteins in Infectivity of Hepatitis Delta Virus

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The infectious particles of hepatitis B virus (HBV) and hepatitis delta virus (HDV) are coated with the large, middle, and small envelope proteins encoded by HBV. While it is clear that the N-terminal pre-S1 domain of the large protein, which is exposed at the virion surface, is implicated in binding to a cellular receptor at viral entry, the role in infectivity of the envelope protein antigenic loop, also exposed to the virion surface and accessible to neutralizing antibodies, remains to be established. In the present study, mutations were created in the antigenic loop of the three envelope proteins, and the resulting mutants were evaluated for their capacity to assist in the maturation and infectivity of HDV. We observed that short internal combined deletions and insertions, affecting residues 109 to 133 in the antigenic loop, were tolerated for secretion of both subviral HBV particles and HDV virions. However, when assayed for infectivity on primary cultures of human hepatocytes or on the recently described HepaRG cell line, virions carrying deletions between residues 118 and 129 were defective. Single amino acid substitutions in this region revealed that Gly-119, Pro-120, Cys-121, Arg-122, and Cys-124 were instrumental in viral entry. These results demonstrate that in addition to a receptor-binding site previously identified in the pre-S1 domain of the L protein, a determinant of infectivity resides in the antigenic loop of HBV envelope proteins.

Hepatitis delta virus (HDV) is a satellite of hepatitis B virus (HBV), and it depends on the latter for the supply of envelope proteins that are essential for virion assembly and propagation (8). The HDV genome is a single-stranded circular RNA that encodes the small and large forms of the HDV antigen protein. These proteins can associate with each other and the viral genome to form a ribonucleoprotein (RNP) complex that constitutes the inner part of the HDV particle (27). Like that of HBV, the HDV envelope consists of a lipid membrane in which multiple copies of the three HBV surface proteins, designated large (L), middle (M), and small (S), are anchored (4, 13).

A single open reading frame on the HBV genome encodes the envelope proteins, which are translated from three different in-frame start codons to a common stop codon (7). The L protein contains three distinct regions: the N-terminal pre-S1, the central pre-S2, and the C-terminal S regions. The M protein includes the pre-S2 and S regions, whereas the S protein consists of the S domain only. S is also the most abundantly expressed, but the main characteristic of this protein lies in its ability to assemble empty subviral particles, which are secreted in large excess compared with the number of mature virions. Synthesis occurs at the endoplasmic reticulum membrane, and particles are formed after budding of envelope protein aggregates into the lumen of an intermediate compartment between the endoplasmic reticulum and the Golgi complex (14). Transport of the viral particles to the extracellular space is thought to follow the constitutive secretory pathway. In addition to their capacity for subviral particle formation, singly expressed S proteins can envelope the HDV RNPs, leading to the formation of particles that are structurally identical to mature HDV but noninfectious (31, 36). The infectivity of HDV, like that of HBV, is directly dependent on the L protein that is included in the viral envelope through lateral interactions with S.

Prior to this study, the main infectivity determinant on the L protein had been mapped to its N-terminal pre-S1 domain, which is likely to contain a primary receptor-binding site responsible for tissue and species specificity. Several lines of evidence, obtained with the HBV and HDV models, indicate that pre-S1 mediates binding to a cellular receptor: monoclonal antibodies specific to pre-S1 have been shown to prevent binding of virions to hepatocyte membrane preparations (20, 26), antibodies raised against pre-S1-specific peptides have demonstrated neutralizing activity in vivo an in vitro (9, 21, 33), HDV or HBV particles carrying chimeric L proteins with pre-S1 subdomains of the woolly monkey hepatitis B virus L protein exhibited altered host range specificity (3, 6), and synthetic peptides specific for the N-terminal 47 amino acids of pre-S1 were shown to be potent inhibitors of HBV infection in vitro (10). The pre-S1 domain of the L protein is also myristoylated at glycine residue in position 2, and this posttranslational modification is indispensable for infectivity (5, 11). However, HBV is unlikely to use a pre-S1-specific receptor at the hepatocyte surface as the sole component of viral entry, and the possibility exists that other surface-exposed domains of the HBV envelope proteins participate in viral entry.

In the HBV life cycle, L is a key element for both virion assembly and infectivity, whereas in the HDV life cycle, its role is limited to the entry event. Overall, the viral coats of HBV and HDV particles being identical, it is reasonable to assume

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that the early steps of viral entry, including receptor binding, are identical for both viruses. The in vitro HDV infection system can therefore be utilized as a surrogate model to study HBV envelope proteins functions at viral entry. With regard to the experimental approach, the HDV model presents also a few practical advantages: infection of susceptible cells leads to very high levels of replicating HDV RNA (up to 300 000 copies per cell) that are easily detectable by Northern blot hybridization as early as 6 days postinoculation (32), and infections being nonproductive in the absence of the helper HBV, the level of intracellular viral RNA that accumulates in an infected cell is directly proportional to the viral titer of the inoculum. We are therefore convinced that the HDV in vitro infectivity assay constitutes a robust model for analyzing the activity of the HBV envelope proteins at viral entry.

In the present study, we produced HDV particles coated with HBV envelope proteins carrying mutations in the antigenic loop, and we measured their infectivity in an in vitro model consisting of primary cultures of human hepatocytes or the recently described HepaRG cell line (12). Our results are in agreement with the presence of an infectivity determinant in the antigenic loop of the HBV envelope proteins.

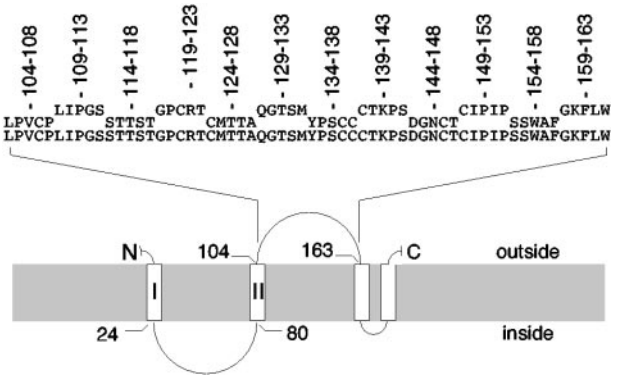

FIG. 1. Schematic representations of S protein mutants. A secondary-structure model for the S protein is represented. Open boxes represent hydrophobic transmembrane regions in the S protein, and the shaded area corresponds to the viral lipid membrane. Transmembrane signals I and II are indicated. Each mutant is designated by the positions of the first and last deleted amino acids (upper vertical letterings). The deleted sequences of each mutant are indicated (horizontal shifted letterings). Each five-residue deletion was replaced by a KL dipeptide. The numbers indicate the boundaries of the antigenic loop (residues 104 to 163) or the transmembrane domains. The antigenic loop is exposed at the surface of the viral particles (outside).

MATERIALS AND METHODS

Construction of plasmids. Plasmid pSVLD3 was used for expression of HDV RNPs, and plasmid p123 or pT7HB2.7 was used for expression of the S HBV envelope protein or coexpression of the S, M, and L proteins, respectively (30). Deletions and substitutions were carried out on plasmid p123 using the PCR technique with two complementary mutagenic oligonucleotides as described (17). The overlapping oligonucleotides contained a 15-nucleotide deletion corresponding to five amino acid residues in the S gene and a 6-nucleotide insertion for the HindIII cleavage site (corresponding to residues lysine and leucine) to provide easy detection and tracking of the mutation. The resulting S protein mutants thus contained a five-residue deletion that was replaced by insertion of the Lys-Leu sequence. They were designated by the numbers of the first and last residues of the deleted sequence.

For simultaneous expression of the mutations in the three HBV envelope proteins, an XhoI-NsiI (nucleotides 129 to 1070) fragment from the p123 mutants was substituted with its counterpart in the pT7HB2.7 plasmid. Single amino acid substitutions were carried out using the PCR overlap extension method on the pT7HB2.7 plasmid. The mutations were designated by the one-letter code of the wild-type amino acid followed by its position in S and the one-letter code for the substituted amino acid. All PCR-generated fragments were cloned in pT7HB2.7 and the resulting mutant plasmids were sequenced using the Big Dye Terminator sequencing protocol (Applied Biosystems). Clones containing the desired mutations within the PCR-generated fragments were selected and used for subsequent transfections.

Production of HDV particles in HuH-7 cells. HuH-7 cells were maintained in Williams' medium E supplemented with 10% fetal bovine serum. For production of HDV particles, cells were transfected with a mixture of plasmid pSVLD3 for the synthesis of HDV RNPs and an HBV recombinant plasmid (p123 or pT7HB2.7) or its derivatives for the supply of the wild-type or mutant envelope proteins, respectively. Transfection of 10^6 cells was carried out by use of the Fugene 6 reagent (Roche) as described (30), with 1 μg of pSVLD3 DNA and 1 μg of an HBV recombinant plasmid (p123 or pT7HB2.7) or its derivatives. Culture medium was harvested on days 5, 7, and 9 posttransfection and analyzed for the presence of viral particles by immunoblotting for detection of HBV envelope proteins and by Northern blotting for detection of HDV RNA.

Characterization of HDV particles produced in HuH-7 cells. Culture fluids harvested on days 5, 7, and 9 after transfection were clarified by centrifugation at 5,000 × g at 4°C for 30 min. Viral particles from the clarified medium were subjected to sedimentation by centrifugation for 2 h at 50,000 rpm in an SW50 rotor (Beckman) on 1 ml of a 30% sucrose cushion in 10 mM Tris-HCl (pH 7.4) containing 150 mM NaCl and 1 mM EDTA (TNE). After centrifugation, the particle-containing pellet was resuspended in TNE.

HBV envelope proteins were assayed as previously described (30). Briefly, particles were resuspended in protein disrupting buffer and proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transfer to polyvinylidene difluoride membranes, and immunodetection with rabbit R247 anti-S antibodies or a mixture of R247 anti-S and anti-pre-S2 antibodies (16). Immunoblots were developed by chemiluminescence and exposure to Kodak films for detection of light emission. For HDV RNA analysis, viral particles from the clarified medium were concentrated by precipitation in the presence of 10% polyethylene glycol. RNA extraction from the polyethylene glycol-precipitated particles or from transfected cells was carried out as described (30). Detection of viral or cellular HDV RNA was achieved after electrophoresis through a 1.2% agarose, 2.2 M formaldehyde gel, transfer to a nylon membrane (Roche), and hybridization to an HDV-specific RNA probe. A preparation of synthetic genomic HDV RNA standards was used to estimate the number of HDV molecules in each sample.

In vitro infection assays. For infection assays on primary human hepatocyte cultures, cells were isolated from residual human liver tissue that was not usable for liver transplant, and cultures were performed as described (30). HepaRG cells were maintained in Williams' medium E supplemented with 10% fetal bovine serum, 5 μg/ml insulin, and 5×10^{-5} M hydrocortisone hemisuccinate (12). Cells were treated with 2% dimethyl sulfoxide for 2 weeks prior to inoculation. For the preparation of the inocula, culture fluids collected from HuH-7 cells at days 5, 7, and 9 posttransfection were pooled and clarified by centrifugation at 5,000 × *g* for 30 min at 4°C, and viral particles were concentrated by precipitation in the presence of 10% polyethylene glycol. Primary hepatocytes or HepaRG cells (10^6 cells/well) were exposed to the inoculum for 16 h in a 35-mm-diameter well in the presence of 4% polyethylene glycol 8000. Cells were harvested at day 9 postexposure for measurement of intracellular genomic and antigenomic HDV RNA.

The antigenomic HDV RNA, a replication intermediate of the viral genome, which is absent in extracellular virions, served as a marker of infection. As described earlier, in the case of successful infection, the level of intracellular HDV RNA is undetectable prior to day 6 postinoculation and reaches a maximum level at day 9 (32). Infections being abortive in the absence of HBV, the intensity of the intracellular HDV RNA signal at day 9 is proportional to the infectivity of the inoculum.

RESULTS

Effects of combined S protein deletion and substitution mutations on secretion of subviral particle and HDV particles. To understand the function of the antigenic loop of the HBV envelope proteins in the morphogenesis of HDV particles, we constructed mutants consisting of a series of progressive dele-

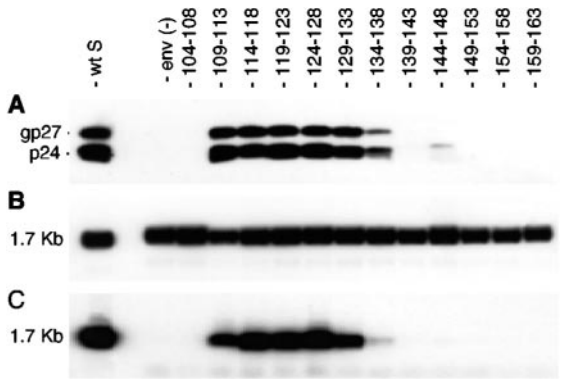

FIG. 2. Detection of HDV RNA-containing particles in culture fluids from cells cotransfected with pSVLD3 HDV plasmid DNA and wild-type or S protein mutant DNA. (A) Immunoblot analysis of S proteins extracted from culture fluids from 10^6 HuH-7 cells after transfection with a mixture of 1 μg of pSVLD3 plasmid DNA and 1 μg of wild-type, envelope-negative[env(-)], or mutant HBV DNA. Particles sedimented from 1 ml of culture medium were disrupted in Laemmli sample buffer and 2% β-mercaptoethanol. Proteins were separated on a 12% acrylamide gel, transferred to a polyvinylidene difluoride membrane, and probed with anti-S antibody (1:500). The molecular masses of the unglycosylated and glycosylated forms of the S envelope proteins are indicated at the left side of the panel in kilodaltons. (B) Cellular RNA was extracted from HuH-7 cells harvested at day 9 posttransfection. Five micrograms of total RNA was separated on an agarose gel and analyzed for the presence of HDV RNA after transfer to a nylon membrane and hybridization to a genomic strand-specific ^{32}P-labeled HDV RNA probe. Following hybridization, the filters were washed, dried, and subjected to autoradiography at −70°C for 16 h with an intensifying screen. The size, expressed in kilobases, of HDV genomic RNA is indicated. (C) RNA extracted from particles concentrated from 1 ml of culture medium was analyzed by agarose gel electrophoresis followed by transfer to a nylon membrane and hybridization to a genomic strand-specific ^{32}P-labeled HDV RNA probe as described for panel B.

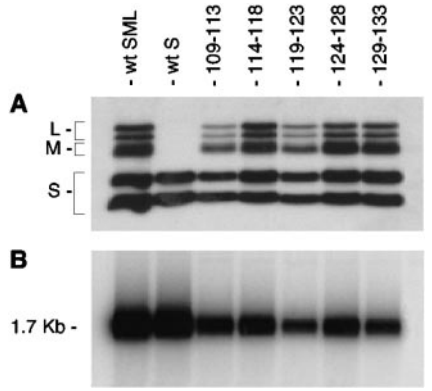

FIG. 3. Production of SML-HDV particles coated with mutant HBV envelope proteins. Culture fluids from HuH-7 cells were harvested on days 5, 7, and 9 after transfection of 10^6 cells with a mixture of 1 μg of HDV recombinant pSVLD3 plasmid DNA and 1 μg of pT7HB2.7 or mutant plasmid DNA coding for wild-type or mutant HBV envelope proteins, respectively. Particles from 1 ml of the culture fluids were concentrated and assayed for the presence of HBV envelope proteins (A) after electrophoresis on a 12% acrylamide gel, transfer to a polyvinylidene difluoride membrane, and immunodetection with a mixture of rabbit anti-S antibody (1:500 dilution) and rabbit anti-preS2 antibody (1:1,000). The S, M, and L HBV envelope proteins are indicated at the left side of each panel. Sedimented particles from the culture medium were also assayed for the presence of HDV RNA after RNA extraction, gel electrophoresis, and Northern blot hybridization by using a genomic strand-specific ^{32}P-labeled HDV RNA probe (B). The size of HDV genomic RNA is indicated in kilobases.

tions of five amino acids each between positions 104 and 163 of the S domain. Each deleted sequence was replaced with a Lys-Leu coding sequence as described in the Materials and Methods section. The mutant vectors were designated by the positions of the deleted residues in the S sequence, as indicated in Fig. 1.

Since S alone is sufficient for the packaging and release of HDV particles, mutations were created in a vector expressing the S protein only, and the mutant plasmid was introduced by transfection in the HuH-7 cells along with plasmid pSVLD3 for the supply of HDV RNP. Following transfection, culture supernatants were harvested on days 5, 7, and 9 posttransfection, and viral particles from 1 ml supernatant were analyzed for S protein by immunoblotting and for HDV RNA by agarose gel electrophoresis and blot hybridization. Cells were also harvested on day 9 posttransfection for analysis of intracellular HDV RNA. A control experiment was performed by cotransfection of HuH-7 cells with pSVLD3 and the Env-negative HBV plasmid pT7HB2.767.

As illustrated in Fig. 2, mutants 109–113, 114–118, 119–123, 124–128, and 129–133 were competent for subviral particle assembly and secretion. However, there was no evidence for the release of subviral particle after transfection with mutants 104–108, 139–143, 149–153, 154–158, and 159–163. Mutant 134–138 exhibited a reduced capacity for secretion of subviral particles compared to the wild type, and only trace amounts of unglycosylated S proteins (p24) were visible in the culture medium of cells transfected with mutant 144–148. Mutants 109–113, 114–118, 119–123, 124–128, and 129–133, which were competent for subviral particle release, retained the capacity for HDV production, as evidenced by the presence of HDV RNA in the sedimented particles. Therefore, the 109 to 133 domain of S does not contain a motif essential for morphogenesis, stability, or secretion of subviral particles or HDV virions.

Because the L envelope protein is required at the surface of HDV to confer infectivity, the mutations in the 109 to 133 sequence that were permissive for S-HDV particle assembly were introduced in a vector coding for the three HBV envelope proteins (S, M, and L) to produce SML-HDV particles amenable to in vitro infection assays.

Effects of small deletions in the envelope protein antigenic loop on infectivity of SML-HDV particles. For production of wild-type and mutant SML-HDV particles (109–113, 114–118, 119–123, 124–128, and 129–133), HuH-7 cells were cotransfected with pSVLD3 and pT7HB2,7 or its mutant derivatives. Culture supernatant was harvested on days 5, 7, and 9 posttransfection, and viral particles were sedimented before analysis for envelope proteins and HDV RNA. All deletions were tolerated for particle secretion and maturation of SML-HDV (Fig. 3). As shown in Fig. 3, the variation in the amount of viral RNA in the culture medium among the different mutants parallels that of the envelope proteins, indicating that the ratio of HDV virions to subviral particles is not affected by the mutation in the antigenic loop. Moreover, the relative amounts of L,

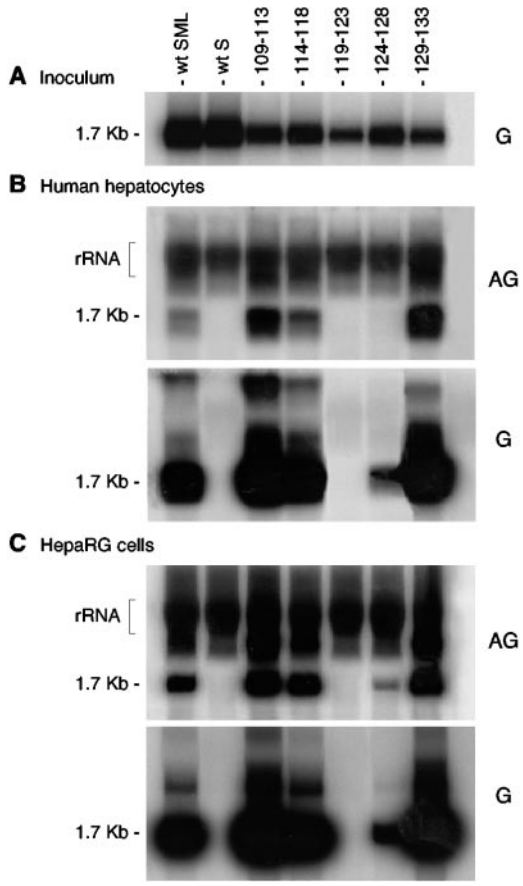

FIG. 4. Infectivity of HDV particles coated with HBV envelope proteins carrying deletions in the antigenic loop. Results are based on Northern blot analysis of HDV RNA extracted from primary human hepatocytes (B) or HepaRG cells (C) exposed to wild-type SML-HDV (wt SML), wild type S-HDV (wt S), and mutant SML-HDV particles. In this experiment, 10^6 cells were exposed to 2 ml supernatant from transfected HuH-7 cells as described for Fig. 3. Five micrograms of total cellular RNA extracted from 10^6 cells harvested 9 days after inoculation was analyzed for the presence of genomic (G) and antigenomic (AG) HDV RNA. RNA extracted from 1 ml of inoculum was analyzed under the same conditions (A). RNA was separated on a 1.2% agarose–2.2 M formaldehyde gel, transferred to nylon membranes, and hybridized to strand-specific ^{32}P-labeled HDV RNA probes. Following hybridization, the filters were washed, dried, and autoradiographed for 24 h. The size of HDV genomic and antigenomic RNA is indicated in kilobases.

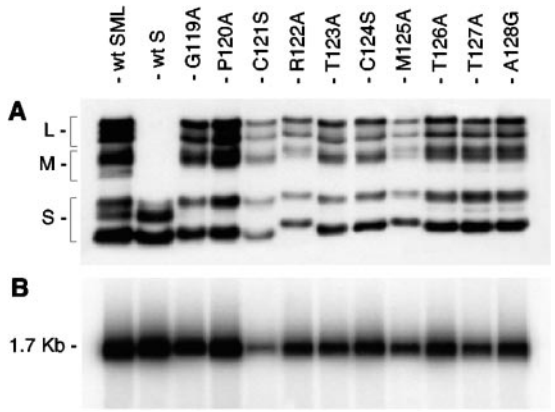

FIG. 5. Production of SML-HDV particles coated with HBV envelope proteins carrying point mutations in the antigenic loop. Culture fluids from HuH-7 cells were harvested on days 5, 7, and 9 after transfection of 10^6 cells with a mixture of 1 μg of HDV recombinant pSVLD3 plasmid DNA and 1 μg of pT7HB2.7 or mutant plasmid DNA coding for wild-type or HBV envelope protein mutants, respectively. Particles from 1 ml of culture fluids were concentrated and assayed for the presence of HBV envelope proteins (A) after electrophoresis on a 12% acrylamide gel, transfer to a polyvinylidene difluoride membrane, and immunodetection with a mixture of rabbit anti-S antibody (1:500 dilution) and rabbit anti-preS2 antibody (1:1,000). S, M, and L indicate the positions of the small, middle, and large HBV envelope proteins, respectively. Particles from 1 ml of culture medium were also assayed for the presence of HDV RNA after RNA extraction, gel electrophoresis, and Northern blot hybridization by using a genomic strand-specific ^{32}P-labeled HDV RNA probe (B). The size of HDV genomic RNA is indicated in kilobases.

M, and S in the envelope of wild-type and mutant virions were similar (Fig. 3A).

To assess the infectivity of the wild-type and mutant SML-HDV particles, primary cultures of human hepatocytes (30) and HepaRG cells (12) were used as susceptible cells (Fig. 4). Cells were exposed to 2-ml inocula that contained approximately 10^8 genome equivalents/ml for the S-HDV and SML-HDV inocula, 5×10^7 for mutants 109–113, 114–118, and 124–128, and 2.5×10^7 for mutants 119–123 and 129–133. S-HDV particles, which have been shown to be noninfectious in vitro, were used as a negative control (31). Nine days after exposure to the inoculum, cells were examined for the presence of intracellular genomic and antigenomic HDV RNA. Evidence of infection was observed in both primary hepatocytes and HepaRG cells exposed to wild-type and mutant SML-HDV carrying deletions 109–113, 114–118, and 129–133 (Fig. 4). In contrast, SML-HDV mutants 119–123 and 124–128 displayed a drastically reduced infectivity, as shown by the absence or the presence of only trace amounts of intracellular HDV RNA. As expected, particles coated with S only (S-HDV) were not infectious. Identical results were obtained using two separate preparations of HDV virions and two cultures of primary hepatocytes and HepaRG cells. Using twofold dilutions of SML-HDV virions, we verified that the level of intracellular HDV RNA at day 9 postinoculation was directly proportional to the viral titers of the inocula, ranging from 10^8 to10^6 genome equivalents/ml (data not shown).

In summary, our data demonstrate that a subdomain of the antigenic loop of the HBV envelope proteins, between amino acids 119 and 128, is critical to the in vitro infectivity of HDV. To further define this determinant, we conducted a mutagenesis of each amino acid of this sequence.

Effects of single-amino-acid substitutions in the envelope protein antigenic loop on infectivity of SML-HDV particles. Each residue from positions 119 to 128 in the antigenic loop was mutated to alanine except for Ala-128, which was converted to glycine, and the cysteine residues at positions 121 and 124, which were mutated to serine. Mutations were introduced in pT7HB2.7 for expression of S, M, and L mutant proteins, and wild-type and mutant SML-HDV particles were prepared as described. All mutations were tolerated for envelope protein secretion and for maturation of HDV (Fig. 5). As observed previously (2, 19), the C121S mutation was partially

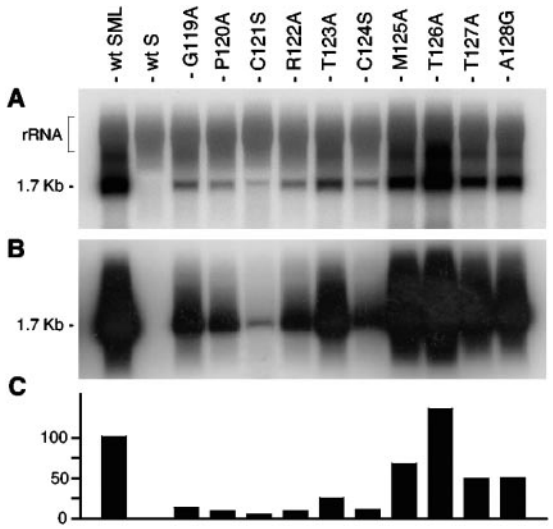

FIG. 6. Infectivity of HDV particles coated with HBV envelope proteins point mutations in the antigenic loop. Results are based on RNA blot hybridization analysis of HDV RNA extracted from HepaRG cells exposed to S-HDV (wt S), SML-HDV (wt SML), and mutant SML-HDV particles. In this experiment, 10^6 cells were exposed to 4×10^7 genome equivalents of wild-type or mutant HDV particles. Five micrograms of total cellular RNA extracted from 10^6 cells harvested 9 days after inoculation was analyzed for the presence of antigenomic (A) and genomic (B) HDV RNA. RNA was separated on a 1.2% agarose–2.2 M formaldehyde gel, transferred to nylon membranes, and hybridized to strand-specific ^{32}P-labeled HDV RNA probes. Following hybridization, the filters were washed, dried, and autoradiographed for 12 h. The size of HDV genomic and antigenomic RNA is indicated in kilobases. After exposure, signals were quantified precisely by excision of the radioactive spots on the nylon membrane and measurement of radioactivity by liquid scintillation counting using a Beckman LS600011C scintillation counter. (C) histogram showing the relative amount of cellular HDV RNA at day 9 postinoculation.

inhibitory to secretion of the envelope proteins and, therefore, to HDV assembly (Fig. 5A).

After adjustment of the inocula to 4×10^7 genome equivalents/ml, the infectivity of the mutant HDV particles was evaluated using the HepaRG cell line as target cells. Nine days after inoculation, cells were examined for the presence of intracellular genomic and antigenomic HDV RNA. For a precise evaluation of infectivity, we measured the ratio of intracellular genomic HDV RNA at 9 days postinoculation to genomic HDV RNA in the inoculum. Measurements of HDV RNA in the inocula or total cellular RNA were performed by Northern blot analysis in comparison to known amounts of synthetic RNA. After gel electrophoresis of the RNA samples, transfer to a nylon membrane, hybridization using an HDV-specific ^{32}P-labeled RNA probe, and autoradiography, signals were quantified by excision of the radioactive spots on the nylon membrane and measurement of radioactivity by liquid scintillation using a Beckman LS600011C scintillation counter. Evidence of infection was observed in hepatocytes exposed to the wild type and most of the SML-HDV mutants (Fig. 6). As summarized in Fig. 6C, substitutions of residues 119, 120, 121, 122, 123, and 124 had a pronounced detrimental effect on infectivity, estimated at 13.4%, 9.3%, 5.6%, 9.3%, 25.1%, and 11.0%, respectively, of that of the wild-type particles. Mutations of residues 125, 127, and 128 had a lesser effect (66.9%, 48.7%, and 49.9%, respectively, of wild-type HDV).

Overall, we noticed that single amino acid substitutions in the 119 to 128 domain, C121S in particular, significantly reduced infectivity but not to the extent of that observed with the 119 to 123 deletion/insertion.

DISCUSSION

This study shows that the 109 to 133 subdomain of the HBV glycoprotein antigenic loop, which tolerates mutations for subviral particle or HDV assembly, contains a determinant of infectivity in the 119 to 128 sequence. Prior to this report, our understanding of HBV entry was limited to a function of pre-S1 in the initial attachment of the virus to an unidentified primary receptor on the host cell membrane. Our results now raise the possibility that the antigenic loop is a major actor at viral entry, perhaps in binding to a secondary receptor at a postattachment step. In general, enveloped viruses mediate viral entry by fusion of their lipid membrane with that of the target cell, a process that is initiated by rearrangement of the viral envelope proteins after binding to a primary receptor at the plasma membrane or in endosomes at acid pH (29). Internalization can also occur in the absence of membrane fusion, following a conformational change of the envelope protein lattice to breach the cell membrane (25).

Because the HBV envelope is a protein-rich and compact structure that is likely to be stabilized by lateral envelope protein interactions, a prerequisite to cell virus membrane fusion might be disassembly of the envelope shell (37). Interestingly, the S protein contains 14 cysteine residues, eight of which are located in the antigenic loop and known to be engaged in disulfide bonds (18, 19). They are probably instrumental in the budding process and in maintaining the stability of the virion envelope. Therefore, if the envelope structure needs to be dismantled, disulfide bridges might have to be isomerized, and the binding of pre-S1 to its receptor could eventually trigger the reaction. Whether the HBV envelope has acquired an energy-rich structure, stabilized in part by intra- and intermolecular disulfide bridges, to facilitate disassembly is unknown.

Interestingly, the 119 to 128 domain that we have identified as instrumental in infectivity contains a CXXC motif (where C stands for cysteine and X for any amino acid) that is generally found on protein-disulfide isomerase-related proteins and, for instance, on the peripheral (SU) subunits of retroviral envelope proteins such as that of murine leukemia virus. In that model, the SU disulfide isomerase is activated at viral entry by a conformational change induced by binding of the envelope transmembrane (TM) subunit to the receptor, which leads to isomerization of SU-TM disulfide bonds and fusion activation (34). Whether such a mechanism is brought into play by the HBV envelope proteins is worth investigation, and experiments are in progress in our laboratory to look for disulfide bond isomerization or an eventual conformational change of the envelope proteins upon attachment of the virus to its receptor.

We made the choice to investigate the antigenic loop for its role in infectivity because it had been well known for many years that the main HBV-neutralizing epitopes lie within this

domain, which is exposed at the virion surface and known to bear a conserved determinant, referred to as the a determinant (22). The fact that monoclonal antibodies directed against epitopes of the antigenic loop have potent neutralizing activity both in vivo and in vitro (9, 15, 24, 28) may indicate that they interfere with receptor binding. Alternatively, antibodies with high affinity for S could neutralize infection by blocking the viral envelope disassembly that normally precedes internalization of the virion cargo.

How could mutations in the antigenic loop affect viral entry? The small combined insertions and deletions at 118 to 123 and 124 to 128 may have prevented infectivity by simply destroying an active motif, such as a secondary receptor-binding site, located at the site of deletion, or at a distal region by inducing a major conformational change of the loop polypeptide. Surprisingly, none of the single amino acid substitutions affecting the 119 to 128 region had the ability to reduce infectivity to the extent of that observed with mutant 119–123. A possible explanation is that the KL dipeptide substitution for GPCRT is responsible for this phenomenon, because the net result of the mutation appears to be the introduction of Leu and the removal of Gly-119, Pro-120, Cys-121, and Thr-123. The substitution of a lysine residue for arginine-122 is conservative, and it is observed in nature on the HBV envelope protein of the adw subtype (23). More importantly, the 119 to 128 region contains two cysteine residues in positions 121 and 124, which have been described as possibly engaged in a disulfide bond. As mentioned above, they constitute a disulfide bond isomerase motif (CXXC) that could eventually be activated following attachment of the virus to the host cell receptor (35). Mutations that remove either of the two cysteine residues might also exert their detrimental effect by inducing a change in the structure of the antigenic loop through the creation of illegitimate disulfide bonds that would be inhibitory to the envelope disassembly process.

In this study, we have presented evidence for the presence of a new infectivity determinant in the HBV envelope proteins, but the mechanism by which this motif participates in entry is as yet unclear. If it were a ligand to a secondary receptor, the identification of the latter and its precise role at the level of viral entry would obviously constitute the next step of our analysis. Alternatively, the antigenic loop might be active at viral entry without binding to a secondary receptor, for instance, by undergoing a postbinding rearrangement that renders the envelope permissive to the release of its cargo. Due to the compact nature of the virion envelope, a modification of the lateral protein-protein interactions in the envelope lattice is probably required, and the CRTC peptide would eventually serve as an internal switch motif for isomerization of the interprotein disulfide bonds. This could be addressed using inhibitors of thiol-disulfide interchange in in vitro infection assays.

In conclusion, our findings identify the antigenic loop of the HBV envelope proteins as a determinant of HBV infection that could represent a target of choice for new antiviral strategies. Molecules such as synthetic peptides specific for an antigenic loop motif, or its cellular ligand, could block a coreceptor-virus interaction, or it could interfere with enzymatic activities that are brought into play during viral uptake (1). Finally, it is worth noting that the emergence of HBV mutants bearing amino acid substitutions in the antigenic loop, in particular in the a determinant, represent a new challenge for diagnosis and vaccine procedures. Whether the infectivity of these naturally occurring mutants is altered could be investigated using our HDV-based in vitro infection protocol.

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