Mapping of the Hepatitis B Virus Pre-S1 Domain Involved in Receptor Recognition

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Hepatitis B virus (HBV) and woolly monkey hepatitis B virus (WMHBV) are primate hepadnaviruses that display restricted tissue and host tropisms. Hepatitis D virus (HDV) particles pseudotyped with HBV and WMHBV envelopes (HBV-HDV and WM-HDV) preferentially infect human and spider monkey hepatocytes, respectively, thereby confirming host range bias in vitro. The analysis of chimeric HBV and WMHBV large (L) envelope proteins suggests that the pre-S1 domain may comprise two regions that affect infectivity: one within the amino-terminal 40 amino acids of pre-S1 and one downstream of this region. In the present study, we further characterized the role of the amino terminus of pre-S1 in infectivity by examining the ability of synthetic peptides to competitively block HDV infection of primary human and spider monkey hepatocytes. A synthetic peptide representing the first 45 residues of the pre-S1 domain of the HBV L protein blocked infectivity of HBV-HDV and WM-HDV, with a requirement for myristylation of the amino terminal residue. Competition studies with truncated peptides suggested that pre-S1 residues 5 to 20 represent the minimal domain for inhibition of HDV infection and, thus, presumably represent the residues involved in virus-host receptor interaction. Recombinant pre-S1 proteins expressed in insect cells blocked infection with HBV-HDV and WM-HDV at a concentration of 1 nanomolar. The ability of short pre-S1 peptides to efficiently inhibit HDV infection suggests that they represent suitable ligands for identification of the HBV receptor and that a pre-S1 mimetic may represent a rational therapy for the treatment of HBV infection.

The hepatitis B virus (HBV) genome is a relaxed-circular, partially duplex DNA with a covalently attached polymerase that displays reverse transcriptase activities (40, 41). The viral glycoproteins contained in the HBV envelope are encoded by a single open reading frame (ORF) and are translated from different in-frame start codons to generate the small (S), middle (M), and large (L) proteins. All three proteins contain the surface domain (S), while the M protein has a 55-amino-acid (aa) extension designated the pre-S2 domain and the L protein contains an additional 108-aa pre-S1 domain. The L protein is modified at the amino-terminal glycine of the pre-S1 domain with a myristate (37), which is required for infectivity (4, 10, 32). The pre-S1 domain of the L glycoprotein has long been implicated in receptor binding and host range (5, 29, 43), yet, almost 40 years after the discovery of HBV, no receptor has been positively identified. Receptor candidates that are pre-S1-binding proteins have included the interleukin-6 receptor (35) and an immunoglobulin A-binding protein on hepatocytes (38). More recently, two groups have identified additional pre-S1-binding proteins. Ryu et al. identified an 80-kDa protein using a glutathione S-transferase-pre-S1 fusion protein (39), while De Falco et al. identified a 44-kDa protein (6) by using the pre-S1 peptide originally shown to bind liver cells by Neurath et al. (34). None of the candidate receptors have demonstrated biological activity in HBV infectivity, and none to date have been identified with a pre-S1 protein containing the amino-terminal myristate known to be essential for infectivity.

Hepadnaviruses characteristically display a hepatic tropism and a restricted host range that extends only to closely related species. HBV infects several species of Old World primates, including humans, great apes (gorillas, chimpanzees, and orangutans), and lesser apes (gibbons) (12, 25, 36, 48, 55); however, other Old World nonhuman primates, such as baboons (33), are not susceptible to HBV infection, presumably due to the inability of the virus to efficiently interact with the host cell surface receptor. However, our recent in vitro studies demonstrated that HBV glycoproteins interact with a cellular receptor on the surface of hepatocytes from a spider monkey, a New World monkey (1). Spider monkeys are permissive for infection with woolly monkey HBV (WMHBV), the only identified hepadnavirus that infects New World monkeys (22, 23).

Recently, in vitro studies with primary human hepatocytes and recombinant hepatitis B virions implicated the amino terminus of HBV pre-S1 in infectivity and host range (5, 29). In one study, HBV particles pseudotyped with a chimeric envelope comprising the WMHBV envelope with the first 30 amino acids of the HBV L protein were reported to have the same infectious capacity as wild-type HBV (5). However, studies using the hepatitis D virus (HDV) model indicated that residues 1 to 40 of HBV were not sufficient to provide the WMHBV envelope with the capacity to infect human hepatocytes at an efficiency comparable to that of HBV-HDV (1). This suggests that sequences downstream of residue 40 of the
L protein may influence infectivity and host range. These data seem logical in the context of accumulating evidence that pre-S1 consists of at least two critical domains involved in infectivity: one that is located at the amino terminus of the L protein and one that is downstream of this region. By using the duck HBV (DHBV) surrogate model of HBV, investigators have demonstrated that the pre-S domain can competitively inhibit infection of primary duck hepatocytes (30, 50, 51) and have identified receptor candidates that specifically bind to the pre-S domain (3, 7, 19, 20, 49, 52, 53). DHBV residues 2 to 41 of pre-S inhibit infection with DHBV (51), yet the gp180-receptor-binding domain mapped to residues 43 to 108 (14).

In this report, we examined the capacity of pre-S1 peptides and recombinant proteins to competitively inhibit infection of human and spider monkey hepatocytes with HDV pseudotyped with HBV and WMHBV envelopes. Competition assays revealed a requirement for the amino-terminal myristate and delineated the minimal sequence requirements for interference with HDV infection to pre-S1 residues 5 to 20, thus potentially defining the residues involved in receptor interaction.

### MATERIALS AND METHODS

**Hepatocytes.** Primary human hepatocytes were purchased from BD Gentest (BD Biosciences Discovery Labware, Bedford, Mass.) and incubated at 37°C and 10% CO₂. Both human and spider monkey primary hepatocytes were maintained in a simplified formulation of our original serum-free medium as described previously (24), and the medium was changed every 72 h. Primary hepatocytes were used 3 to 6 days postplating for HDV infection studies.

**Preparation of recombinant HDV particles.** The procedure for production of recombinant HDV particles has been previously described (1, 2, 47). Briefly, HepG2 cells were transfected with a mixture of cloned HDV cDNA, a gift from John Taylor (18), and the HBV or WMHBV envelope protein expression plasmid. Cells were transfected with 27 μg of the envelope protein expression plasmid and 3 μg of pSVD3 (HDV cDNA) by using 60 μg of TransIT-LT1 (Mirus Corp.). To remove residual DNA, transfected cultures were washed with phosphate-buffered saline (PBS) extensively on three separate occasions, days 0, 3, and 6 posttransfection, and the medium was changed every 3 days. Culture medium was harvested on days 9, 12, and 15 posttransfection, pooled, and clarified at 3,300 g for 30 min.

**Synthetic peptides.** The myristylated and unmyristylated synthetic peptides were purchased from Invitrogen Corp. (Carlsbad, Calif.) or Mimotopes Pty., Ltd. (San Diego, Calif.). Peptide sequences are described in Table 1.

### Table 1. HBV pre-S1 inhibiting peptides

<table>
<thead>
<tr>
<th>Sequence*</th>
<th>Peptide designation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myr- WGGSLSTSNPLGFFPDHQLDPAFRANTANP</td>
<td>Myr-1-45</td>
</tr>
<tr>
<td>Unmyr- WGGSLSTSNPLGFFPDHQLDPAFRANTANP</td>
<td>Unmyr-1-45</td>
</tr>
<tr>
<td>Myr- WGGSLSTSNPLGFFPDHQLDPAFRANTANP</td>
<td>Myr-1-40</td>
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</tr>
<tr>
<td>Myr- WGGSLSTSNPLGFFPDHQLDPAFRANTANP</td>
<td>WM-Myr-2-35</td>
</tr>
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* The amino acid sequence differences between HBV and WMHBV are underlined on WM-Myr 2–35. The boldface represents the minimal domain capable of inhibiting HDV infection.
RESULTS

Titration of HDV infectivity in primary hepatocyte cultures.
The in vitro HDV infection system has been valuable in the characterization of the roles of the envelope glycoproteins of HBV and WMHBV in viral infectivity (1, 15, 16, 42–47). In these studies, we examined a series of peptides and proteins that are based on the pre-S1 domain of the L envelope protein for the capacity to inhibit HDV infection of primary hepatocytes. Prior to initiating inhibition studies, the effect of viral dose on infectivity was examined. The doses of HDV RNA were expressed in picograms per culture and were derived by linear regression analysis of HDV RNA standards loaded on the same gel. Percent HDV RNA was derived from undiluted HBV-HDV-infected cultures as 100%. Human hepatocytes were inoculated with doses of HBV-HDV that were undiluted (1.8 × 10⁹ ge) or diluted 5-fold (3.6 × 10⁸ ge), 25-fold (7.2 × 10⁷ ge), and 125-fold (1.4 × 10⁶ ge). Cultures were harvested on day 11 postinoculation, and total cellular RNA was analyzed as described for panel A. A 270-pg HDV-RNA standard was run on the gel for comparison. HDV RNA extracted from the equivalent of 25% of the inoculum was analyzed under the same conditions (lane I). (D) The hybridized membrane from panel C was analyzed by a phosphorimager as described for panel B.

FIG. 1. Titration and saturation of HDV infectivity in human hepatocytes. (A) Human hepatocytes were inoculated with doses of HBV-HDV that were undiluted (4.7 × 10⁸ ge) or diluted 5-fold (9.4 × 10⁷ ge), 25-fold (1.9 × 10⁸ ge), and 125-fold (3.8 × 10⁶ ge). Cultures were harvested on day 12 postinoculation, and 5 μg of total cell RNA (approximately 15% of RNA from a 35-mm dish) was analyzed by Northern blot hybridization using a riboprobe for HDV genomic RNA. (B) The hybridized membrane from panel A was digitally scanned with a phosphorimager. The amount of HDV RNA is expressed in picograms per culture and was derived by linear regression analysis of HDV RNA standards loaded on the same gel. Percent HDV RNA was derived from undiluted HBV-HDV-infected cultures as 100%. (C) Human hepatocytes were inoculated with doses of HBV-HDV that were undiluted (1.8 × 10⁹ ge) or diluted 5-fold (3.6 × 10⁸ ge), 25-fold (7.2 × 10⁷ ge), and 125-fold (1.4 × 10⁶ ge). Cultures were harvested on day 11 postinoculation, and total cellular RNA was analyzed as described for panel A. A 270-pg HDV-RNA standard was run on the gel for comparison. HDV RNA extracted from the equivalent of 25% of the inoculum was analyzed under the same conditions (lane I). (D) The hybridized membrane from panel C was analyzed by a phosphorimager as described for panel B.

Northern hybridizations were analyzed by phosphorimaging to provide quantitative data. Standards of known amounts of synthetic HDV RNA were included on the same gel and were plotted to arrive at a linear regression model of total picograms of HDV RNA per infected culture. Cultures exposed to undiluted inoculum containing 4.7 × 10⁹ ge of HDV RNA yielded 27,700 pg of HDV RNA at harvest or approximately 2.8 × 10¹⁰ ge. The level of HDV RNA decreased linearly in proportion to the amount of inoculum, except at the highest dose of virus. A fivefold dilution of the starting inoculum decreased the signal to 58% of the value obtained with the undiluted inoculum, while further dilutions decreased the level of HDV RNA by approximately fivefold each (58%, 12.6%, 2.2% of undiluted values) (Fig. 1B). The lack of fivefold reduction in HDV RNA with the initial dilution may represent partial saturation of the receptor at high levels of virus. The undiluted inoculum represented a multiplicity of infection of approximately 247 ge per cell.

In an attempt to reach the saturation level of HBV-HDV for the cellular receptor, a second experiment was conducted that started with an inoculum containing approximately fourfold more HDV genome equivalents. Primary human hepatocytes
were inoculated with fivedfold decreasing doses of HBV-HDV, ranging from $1.8 \times 10^7$ to $1.4 \times 10^7$ ge. RNA levels reached a plateau by using inocula of $1.8 \times 10^9$ ge and $3.6 \times 10^8$ ge of HBV-HDV (undiluted and fivefold-diluted inocula), suggesting that saturation of a cellular receptor had been reached (Fig. 1C). Intracellular HDV RNA levels decreased to approximately 38.3% and 11.3% of undiluted HDV RNA levels when cultures were inoculated with 25- and 125-fold dilutions, respectively (Fig. 1D). One interpretation of these data is that saturation of the cell receptor(s) occurs under our current conditions at levels of virus exceeding 200 copies per cell. These estimates do not account for the level of L protein not associated with virus that is present in the inoculum and may occupy receptor sites.

We also examined the dose dependency of HDV infections in spider monkey hepatocytes by using recombinant HDV particles with wild-type primate hepadnavirus envelopes (HBV-HDV and WM-HDV) and chimeric envelopes (Hu40-HDV and WM40-HDV). In a previous study, we reported that HBV and WMHBV chimeric envelope L proteins with amino acids 1 to 40 exchanged generated HDV particles that retained infectivity for both human and spider monkey hepatocytes (1). Hu40 contains the WMHBV L protein backbone with the first 40 amino acids of pre-S1 derived from HBV, and WM40 contains the opposite configuration. Recombinant HDV particles were used to infect primary spider monkey hepatocytes at fivedfold-decreasing doses, from $6.4 \times 10^7$ ge to $2.6 \times 10^6$ ge, with the highest dose being in the linear range of infectivity determined in human hepatocytes. Cultures were harvested on day 12 postinoculation for detection of intracellular HDV RNA. Cellular HDV RNA yields resulting from inoculations with each virus displayed decreases consistent with dilutions of the viral inocula (Fig. 2A and B). RNA levels for HBV-HDV and WM-HDV infections appeared to be similar. Although one might anticipate greater infectivity for WM-HDV in spider monkey hepatocytes, our previous results demonstrated a similar level of infectivity for both HBV-HDV and WM-HDV in spider monkey hepatocytes (1). In agreement with our previous observations, Hu40-HDV infections exhibited an increased level of HDV RNA in comparison to both nonchimeric viruses, while WM40-HDV infections resulted in marginally lower levels of HDV RNA (Fig. 2A and B).

Collectively, these data demonstrate that HDV RNA replication levels following infection of primary hepatocytes with recombinant HDV particles is proportional to the titer of the
inoculum and, thus, should be an accurate indicator of the potency of inhibitors of infection.

**Inhibition of HDV infection with pre-S1 peptides.** To identify inhibitors of HDV infection, we examined an array of synthetic peptides for their ability to block infection of recombinant HDV particles in primary hepatocytes. The peptides were synthesized to span the pre-S1 region from residues 1 to 45 in order to determine domains that could significantly inhibit HDV infection (Table 1). Previous studies on the infectivity of hepadnaviruses revealed the requirement for the presence of myristate covalently attached to the glycine residue at the amino terminus of the L protein (4, 10, 32). The ability to synthesize peptides with or without amino-terminal myristate provided an opportunity to examine the significance of myristate for hepadnaviral infectivity by using the HDV model in competition studies.

Peptides representing HBV pre-S1 residues 1 to 45, which differ only in the addition of myristate (Table 1; Myr 1-45 and Unmyr 1-45), were used as competitive inhibitors of HDV infectivity in spider monkey hepatocytes. (Note that some of the peptides used in this study were erroneously synthesized with myristate on terminal methionine rather than on glycine. Later peptides were synthesized with myristate on the glycine [designated Myr 2- versus Myr 1-], and comparison of peptides with identical sequences made in both manners indicated that the change had no effect on inhibition potency [see Fig. 7]). Peptides were mixed with the inoculum, WM-HDV or HBV-HDV, and added to duplicate cultures for 2 h at 4°C. After the 2-h incubation, cells were washed vigorously to remove excess peptide and virus. Cultures were harvested on day 12 postinoculation, and total cellular RNA was analyzed as described in the legend for Fig. 1A. Lanes I, RNA extracted from the equivalent of 10% of the inocula. (B) Levels of HDV RNA from the same cultures analyzed in panel A were quantified by TaqMan RT-PCR and expressed as genomic equivalents per culture. Percent genome equivalents of HDV were derived from no-peptide control cultures as 100%.

![Graph](image.png)

**FIG. 3.** Inhibition of HDV infection with pre-S1 peptides. (A) Spider monkey hepatocytes were inoculated in duplicate with HBV-HDV or WM-HDV and competed with myristylated or unmyristylated HBV 1-45 peptides at 0.5 μM and 5 μM concentrations. Viruses were competed with peptides for 2 h at 4°C, and then cultures were washed to remove excess peptide and virus. Cultures were harvested on day 12 postinoculation, and total cellular RNA was analyzed as described in the legend for Fig. 1A. Lanes I, RNA extracted from the equivalent of 10% of the inocula. (B) Levels of HDV RNA from the same cultures analyzed in panel A were quantified by TaqMan RT-PCR and expressed as genomic equivalents per culture. Percent genome equivalents of HDV were derived from no-peptide control cultures as 100%.
pre-S1 peptide (Fig. 3B) and a 120-fold decrease when the peptide concentration was increased to 5 \( \mu \text{M} \) (Fig. 3B). The myristylated HBV pre-S1 peptide inhibited infection with WM-HDV by 3.3-fold and 51-fold at concentrations of 0.5 \( \mu \text{M} \) and 5 \( \mu \text{M} \), respectively. Addition of the unmyristylated HBV peptide to infected cultures resulted in an increase in HDV RNA levels; however, this increase was not observed in subsequent experiments (Fig. 4B; see Fig. 7).

These data demonstrate the requirement for myristate for peptide competition with HDV infectivity and the presence of a receptor-binding domain effective in competitive inhibition of infection in the first 45 amino acids of pre-S1. Furthermore, since the inhibition was conducted at 4°C for 2 h, the results imply that the peptide prevents binding of the virus to the cell receptor rather than preventing a later step in uptake or uncoating.

**Mapping the pre-S1 domain for inhibition of HDV infectivity.** To map the minimal domain of pre-S1 capable of competitive inhibition of HBV-HDV infectivity, we screened peptides of various lengths with deleted 5- or 10-amino-acid increments from the amino or carboxyl termini of the Myr 1-45 peptide (Table 1). All of the peptides were synthesized to contain myristate at the amino terminus, since it appears to be integrally involved with efficient peptide inhibition of HDV infectivity. Peptides were used in competitive inhibitions with HBV-HDV at concentrations of 0.5 \( \mu \text{M} \) or 5 \( \mu \text{M} \) by using cultures of primary human hepatocytes. Cells were exposed in duplicate to the inoculum/peptide mixtures for 16 h at 37°C and then washed to remove excess peptide and virus. The cells were harvested on day 9 postinoculation, and total cellular RNA was analyzed as described in the legend for Fig. 1A. Lane I, RNA extracted from the equivalent of 20% of the inocula. (B) Levels of HDV RNA from the same cultures analyzed in panel A were quantified by TaqMan RT-PCR and expressed as genomic equivalents per culture. Percent genome equivalents of HDV were derived from no-peptide control cultures as 100%.

![Image](http://jvi.asm.org/)
Competitive infections were also performed to confirm that the Myr 1-35 peptide could efficiently inhibit HDV infection in spider monkey hepatocytes inoculated with either HBV-HDV or WM-HDV. Cell cultures were exposed to inocula and 5 μM peptide (Myr 1-45 or Myr 1-35) for 16 h at 37°C and then washed to remove excess peptide and virus. The cells were harvested on day 7 postinoculation and total cellular RNA was analyzed for HDV RNA by Northern hybridization. Viral RNA was not detected from HBV-HDV infections and was only slightly visible for WM-HDV infections when either Myr 1-45 or Myr 1-35 was used for competition, indicating that the efficient inhibition of infection exhibited by these two peptides extends to spider monkey cells (Fig. 5A). In these studies, a control peptide was used for competition in place of the no-peptide control. The control peptide was based on the first 35 amino acids of the WMHBV pre-S1 sequence and was designated WM-Myr 1-35. This peptide was repeatedly shown to have no inhibitory effect on either HBV-HDV or WM-HDV infections in both human and spider monkey cells. This was unexpected, since a protein based on the full pre-S1 domain of WMHBV is an efficient inhibitor of HDV infectivity, although it was 25-fold less potent than the HBV counterpart (see Fig. 8 and 9). The peptide was synthesized a second time by a different company, this time with the myristate on glycine at position 2 (WM-Myr 2-35), and still the peptide exhibited no significant inhibition of HBV-HDV infection (see Fig. 7). In this experiment, the peptide served as a negative control, since it is a short myristylated peptide with similar properties as the HBV peptides. Quantitative RT-PCR revealed that both Myr 1-45 and Myr 1-35 inhibited both HBV-HDV and WM-HDV to less than 1% of the control cultures (Fig. 5B).

We further defined the minimal peptide domain capable of inhibiting infection by using peptides Myr 1-30 and Myr 1-25. As anticipated, Myr 1-35 and Myr 1-45 both inhibited infection to 2.2% and 2.9% of the no-peptide control. Myr 5-45 again exhibited partial inhibition at 10.1% of the control. Both Myr 1-30 and Myr 1-25 displayed inhibitor potency similar to Myr 1-45, with 4.7% and 4.8%, respectively, of the HDV RNA levels of the no-peptide control (Fig. 6A and B).

A final set of peptides was produced to further delineate the minimal domain capable of blocking HDV infection. Infections were conducted on spider monkey hepatocytes in duplicate by using the Hu40-HDV virus, which has a greater infectivity for spider monkey hepatocytes than either HBV-HDV or

FIG. 5. Peptide inhibition of HDV infection in spider monkey hepatocytes. (A) Spider monkey hepatocytes were inoculated in duplicate for HBV-HDV or in individual wells for WM-HDV and competed with myristylated peptides consisting of HBV 1-35 and 1-45 and WMHBV 1-35 at a 5 μM concentration. Viruses were competed with peptides for 16 h at 37°C, and then cultures were washed to remove excess peptide and virus. Cultures were harvested on day 7 postinoculation, and total cellular RNA was analyzed as described in the legend for Fig. 1A. Lanes I, RNA extracted from the equivalent of 20% of the inocula. (B) Levels of HDV RNA from the same cultures analyzed in panel A were quantified by TaqMan RT-PCR and expressed as genomic equivalents per culture. Percent genome equivalents of HDV were derived from WM-Myr 1-35-competed cultures as 100%.
Peptide inhibitors were used at a concentration of 5 μM. This set of peptides had myristate at glycine position 2. Myr 2-35 was a potent inhibitor of HBV-HDV infection, but was not as potent as Myr 1-45 and only marginally more potent than Myr 1-25, suggesting that having myristate attached to the methionine at position 1 did not significantly affect potency (Fig. 7A). There was a trend for longer peptides to have a slightly greater potency from Myr 1-45 to Myr 2-20 (0.3% and 6.5% of the no-peptide control, respectively), while further deletion to Myr 2-16 abolished inhibition (approximately twofold down in comparison to the no-peptide control) (Fig. 7B). We also synthesized a peptide with an internal deletion from residues 5 to 9, Myr 2-45 (Δ5-9), to determine whether the amino-terminal deletion of Myr 10-45 was responsible for the loss of activity rather than the placement of the myristate in a poor context (attached to proline at position 10). This peptide lacked activity. Newly synthesized WM-Myr 2-35 also lacked activity in this assay. Unmyristylated HBV peptide at concentrations of 25 and 75 μM also failed to exhibit any significant activity (75 μM was approximately twofold down from the no-peptide control). Finally, preincubation of cells for 2 h at 4°C with Myr 1-45, followed by removal of excess peptide prior to infection with HBV-HDV, still reduced infection to 4.5% of control levels, suggesting that the peptides directly interact with a cellular receptor (Fig. 7). Collectively, the peptide deletion studies mapped the minimal inhibition domain of pre-S1 to residues 5 to 20.

Inhibition of HDV infection with pre-S1 proteins purified from insect cells. Next, we examined whether inclusion of the entire pre-S1 domain would increase the affinity of the peptides for the receptor and, thus, increase the potency of the peptide as a competitor. To compare the efficiencies of longer peptides to inhibit infection, it was necessary to produce small proteins in cell culture, since it is difficult and expensive to synthesize very long peptides. We chose to produce the pre-S1 domain of both HBV and WMHBV in insect cells by using the baculovirus expression system, since we had previously demonstrated that insect cells myristylate the HBV L protein expressed (27). Pre-S1 proteins encompassed residues 1 to 108 and contained a 10-amino-acid FLAG sequence at the carboxyl terminus to facilitate affinity purification by using a monoclonal antibody to this sequence.

In the initial experiments, purified WMHBV pre-S1 protein (WM pre-S1) was used to inhibit HBV-HDV and WM-HDV infection of spider monkey hepatocytes. Since the pre-S1 proteins could not be produced and purified in the same quantities as synthetic peptides, they were used as competitors at 0.1 μM.
Competition studies were conducted in duplicate by incubating cultures with the pre-S1 protein/inoculum mixture for 2 h at 4°C. After the 2-h incubation, cultures were washed vigorously to remove unbound virus and harvested 12 days postinoculation. No HDV RNA was detected by Northern hybridization following infections with both HBV-HDV and WM-HDV competed with either 500 nM or 100 nM of pre-S1 protein (Fig. 8A). Analysis of the cultures by quantitative RT-PCR revealed that infections with both HBV-HDV and WM-HDV were decreased by over two logs at the 500 nM level. Control cultures with no competing protein possessed HDV RNA levels of 1.2 \times 10^8 and 1.6 \times 10^8 genome equivalents per culture for HBV-HDV- and WM-HDV-infected cultures, respectively. In WM-HDV-infected cultures, HDV RNA levels decreased to 2.8% and 0.4% of these levels with 100 nM and 500 nM of competing protein, respectively (Fig. 8B). The HDV RNA levels for HBV-HDV-infected cultures inhibited with 100 nM or 500 nM of WM pre-S1 decreased to 0.6% and 0.9%, respectively, of the control culture levels.

Since near total inhibition of infection occurred at 100 nM competitive pre-S1 protein, we performed a titration of both HBV and WM pre-S1 recombinant proteins in human hepatocytes infected with HBV-HDV. Human hepatocytes exposed to HBV-HDV were competed with HBV pre-S1, WM pre-S1, and HBV FTP199, a control protein. FTP199 comprises the first 199 residues encoded by the polymerase ORF with an amino-terminal FLAG epitope and was purified by the same methodology as the pre-S1 proteins. Cultures were exposed to inocula and competing proteins at concentrations ranging from 0.2 nM to 25 nM for 16 h at 37°C. Cells were washed vigorously to remove unbound virus and competing proteins and harvested on day 12 postinoculation. HDV RNA levels were reduced with HBV pre-S1 in a dose-dependent manner (Fig. 9A), while WM pre-S1 did not appreciably inhibit HBV-HDV infection at levels less than 25 nM.

Competition with increasing levels of HBV pre-S1 induced a progressive decrease in HDV RNA levels to 1 nM, which reduced HDV RNA to 26% of the no-protein control (Fig. 9B). Competition with WM pre-S1 only moderately decreased HDV RNA levels, with fluctuation from 40% to 44% and 50% of control levels when competed with 0.2 nM, 1 nM, and 5 nM, respectively, of WM pre-S1. This level of inhibition did not appear to be significant, since competition with the FTP199 control protein at 25 nM reduced HDV RNA levels to 39% of uncompeted control levels. Competition with 25 nM of WM pre-S1 reduced HDV RNA levels to 19% of the control level, which is only twofold less than FTP199 at the same protein level. Thus, in agreement with the data from two WMHBV synthetic peptides, the potency of the WM pre-S1 protein was reduced 25-fold in comparison to HBV pre-S1.
DISCUSSION

Although the receptors for a number of viruses have been identified in recent years, several decades of effort have not yielded the receptor for HBV. An array of proteins have been described as putative receptors for HBV; however, the biological significance of these molecules has not been confirmed. Most of our knowledge of hepadnaviral entry has been obtained from studies on DHBV and its cellular receptor carboxypeptidase D (3, 14, 19, 20, 49). Duck carboxypeptidase D was initially detected by immunoprecipitation of DHBV following incubation with labeled extracts of primary duck hepatocyte cultures (19). Only the pre-S domain of the DHBV L protein is required to bind to carboxypeptidase D (19). Upon transfection of a chicken hepatoma cell line (LMH) with cloned viral DNA, DHBV particles can be produced, indicating that these cells lack only the viral receptor or some other component of the DHBV entry-uncoating pathway (19). Yet carboxypeptidase D alone does not reconstitute susceptibility to infection in LMH cells.

Efforts to identify the cell surface receptor(s) for HBV have been hampered by the limitations of the in vitro models of HBV infection. Until recently, the only in vitro culture systems for HBV infection have been primary human and chimpanzee hepatocytes. Recently, primary tupaia hepatocytes have been shown to be permissive for HBV infection (8, 17, 54), while Gripon et al. reported a human hepatoma cell line susceptible to infection with HBV (11). The HDV in vitro model for HBV infection initially reported by Sureau et al. (43–45, 47) represents a unique approach to define the determinants of HBV host range and receptor interactions (1). The ability of HDV to exploit the HBV envelope proteins for assembly, secretion, and transmission suggests that both viruses use the same receptor. Due to the very high level of replication of HDV RNA, the HDV culture system represents the most sensitive assay of infection mediated by the HBV envelope proteins. Replication can be easily monitored by Northern hybridization, and the current use of real-time RT-PCR permitted the detection of infection even after several hundredfold inhibition. The validity of the HDV culture system was first demonstrated by the ability of HBV-neutralizing antibodies to block HDV infection (47) and was further confirmed by the observation that only particles containing the L protein were infectious (43). We recently reported the production of HDV psuedotypes with HBV and WMHBV envelope proteins, including L proteins with chimeric pre-S1 domains (1). We demonstrated the preferential infectivity of HBV-HDV and WM-HDV on human hepatocytes. Recently, primary tupaia hepatocytes have been shown to be permissive for HBV infection (8, 17, 54), while Gripon et al. reported a human hepatoma cell line susceptible to infection with HBV (11). The HDV in vitro model for HBV infection initially reported by Sureau et al. 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and spider monkey hepatocytes, respectively, which is in agreement with the in vivo host ranges of HBV and WMHBV, respectively. The HDV system should also provide a mechanism to explore the authenticity of receptor candidates.

In the present study, we demonstrated the requirement of myristate for HDV infectivity, as shown by HDV competition studies using myristylated and unmyristylated peptides. The myristylation of both DHBV and HBV L proteins is essential for infectivity. Replacement of the initial glycine of the DHBV pre-S with an alanine inactivated the myristylation signal and allowed the production of virions with unmyristylated pre-S protein, which were shown to be noninfectious in susceptible ducklings (32). In two reports analyzing mutant HBV particles containing a point mutation abolishing myristylation, infectivity of the particles on human hepatocytes was completely abrogated, indicating that myristylation of the L protein is necessary for HBV infectivity (4, 10). The role played by myristylation in the infectivity of hepadnaviruses is not known. However, the requirement of myristate by short peptides for competitive inhibition of infection at 4°C and the observation that preincubation of cultures with these peptides blocks subsequent infection in the absence of peptide suggest that myristate is directly involved in receptor interaction. The myristate could potentiate pre-S1 interaction with the cell membrane and, therefore, facilitate receptor interaction.

Here, we define a short domain of HBV pre-S1 spanning residues 5 to 20 that efficiently competed HDV infection. The inhibitory peptides used in the present study probably interact with one component of the HBV receptor that binds an amino-terminal site in pre-S1. A recombinant pre-S1 protein containing the complete pre-S1 region inhibited HDV infection more efficiently than the short, myristylated peptides. It is possible that the intact pre-S1 protein is locked into a more favorable conformation for receptor interaction by intramolecular interactions, while the short peptides are flexible and can assume multiple conformations. The transitional energy required to form the peptide into the most favorable structure is probably low, making it possible for peptides to assume the most favorable conformation after contact with the receptor and, thus, to increase the affinity of the interaction after binding. Alternatively, the entire pre-S1 domain may contain other regions involved in receptor interaction.

We previously speculated that the L protein may comprise two domains that affect infectivity (1). By exploring the host range differences of HBV and WMHBV chimeric L proteins that exchanged the amino-terminal 40 aa, several observations

FIG. 9. Inhibition of HDV infection with proteins purified from insect cells. (A) Human hepatocytes were inoculated with HBV-HDV and competed with HBV pre-S1, WM pre-S1, or FTP199 at 25 nM, 5 nM, 1 nM, and 0.2 nM concentrations. Virus was competed for 16 h at 37°C, and then cultures were washed to remove excess protein and virus. Cultures were harvested on day 9 postinoculation, and total cellular RNA was analyzed as described in the legend for Fig. 1A. Lane I, RNA extracted from the equivalent of 20% of the inocula. (B) Levels of HDV RNA from the same cultures analyzed in panel A were quantified by TaqMan RT-PCR and expressed as genomic equivalents per µg. Percent genome equivalents of HDV were derived from no-competing-protein control cultures as 100%.

![Image](http://jvi.asm.org/ on October 1, 2017 by guest)
were made. First, the amino-terminal 40 aa of the WMHBV L protein dramatically reduced the infectivity of chimeric HBV-HDV on human hepatocytes, suggesting that critical residues for receptor interaction reside within or are influenced by this domain. However, the HBV sequence from the same region, when placed in WMHBV, failed to increase the infectivity of WM-HDV on human hepatocytes. This led to the conclusion that, although necessary, the amino-terminal 40 aa are not sufficient for a high-affinity interaction with the human receptor, implying that sequences downstream of residue 40 of the L protein may influence host range. Furthermore, addition of the amino-terminal 40 pre-S1 residues of HBV onto the envelope of WMHBV (Hu40-HDV) increased infectivity on spider monkey hepatocytes. One possible interpretation of the data envisions the existence of at least two critical domains in pre-S1 for receptor interaction and infectivity: one that is located at the amino terminus of the L protein and one that is downstream of this region. It is possible that the amino terminus of the HBV-L protein exhibits a higher affinity for the spider monkey receptor(s) than the same sequence from WMHBV, while the downstream region of the WMHBV-L protein has the greatest affinity for the spider monkey receptor(s). It must be appreciated that infection of spider monkey hepatocytes represents a species jump for both HBV and WMHBV and that, although spider monkeys cannot be infected with HBV, infection with WMHBV results in a low-level infection in comparison to woolly monkeys (22, 23). Thus, the chimeric Hu40-HDV may contain the best of both viruses, with optimal sequences from both domains of pre-S1 for infectivity in spider monkeys. Data from the DHBV model support the hypothesis for two domains of pre-S1 involved in infectivity. A myristylated peptide from residues 2 to 41 blocks infection (13, 51), while a peptide from residues 80 to 104 (30) blocks infection and binds carboxypeptidase D. Collectively, these studies can be used to build a model in which the amino terminus of the L protein interacts with the receptor involved in tissue specificity, while a downstream region interacts with carboxypeptidase D. In the present study, myristylated peptides efficiently competed HDV infection during incubation at 4°C and blocked infection when used as a pretreatment for cultures. This suggests that the peptide inhibits a primary attachment step.

The week of submission of the manuscript, a report was published in this journal describing similar studies to those reported here but by using HBV particles and primary human hepatocytes or the HepaRG cell line (9). Observations from both reports are similar with regard to inhibition of infection with short, myristylated peptides. Gripón and coworkers also replaced the amino-terminal myristate with other hydrophobic moieties, demonstrating that inhibition potency could be increased or decreased depending on the acyl moiety present. While their studies demonstrated inhibition with a peptide spanning residues 2 to 28, we further defined the minimal inhibition domain to residues 5 to 20. Two significant differences between both reports were the inhibition of infection with unmyristylated HBV peptides and WM peptides. In our assay, the WM peptide, as well as the full-length WM pre-S1 protein, has substantially less potency than the HBV counterparts on both human and spider monkey hepatocytes. This is consistent with reduced replication of WMHBV in spider monkeys and the lack of significant replication of WMHBV in chimpanzees, despite equivalent replication potential of HBV- and WMHBV-transfected Huh7 cells (22, 31). We also observed very little to no inhibition with unmyristylated HBV peptides, even at very high levels (75 μM). Although it is difficult to compare significantly different in vitro models, one potential explanation for the differences is a higher affinity of HDV particles for the receptor. This would be consistent with the observation that some of the peptides in the Gripón report exhibited inhibition at lower levels than similar peptides in our studies (although our full-length pre-S1 protein was more potent than the HBV peptides). This is also consistent with our observations that HDV infects primary human, chimpanzee, and spider monkey hepatocytes more efficiently than HBV. A higher affinity of HDV for the receptor might also explain the ability of very low levels of HDV (the amount transmitted by intravenous drug users) to establish an infection in a host with preexisting HBV infection that involves virtually all hepatocytes and high levels of viremia. Resolution of these questions awaits identification and molecular cloning of the HBV receptor. The definition of small peptides capable of inhibiting infection provides the opportunity to use peptides as probes for the HBV receptor and possibly for the development of HBV therapeutics.

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REFERENCES


