Fine Mapping of Pre-S Sequence Requirements for Hepatitis B Virus Large Envelope Protein-Mediated Receptor Interaction[▽]

Andreas Schulze,[1] Alexa Schieck,[2] Yi Ni,[1] Walter Mier,[2] and Stephan Urban[1]*

Department of Infectious Diseases, Molecular Virology, Otto-Meyerhof-Zentrum, University Hospital Heidelberg, Im Neuenheimer Feld 350, 69120 Heidelberg, Germany,[1] and Department of Nuclear Medicine, University Hospital Heidelberg, Im Neuenheimer Feld 400, 69120 Heidelberg, Germany[2]

Previous studies showed that the N-terminal 75 amino acids of the pre-S1 domain of the hepatitis B virus (HBV) L protein are essential for HBV and hepatitis delta virus (HDV) infectivity. Consistently, synthetic lipopeptides encompassing this sequence or only parts of it efficiently block HBV and HDV infection, presumably through specific interference with a cellular receptor. Crucial for both virus infectivity and the inhibitory activity of the peptides are N-terminal myristoylation and a highly conserved motif within the N-terminal 48 amino acids. To refine the sequence requirements, we synthesized a series of HBV pre-S1 peptides containing deletions, point mutations, D-amino acid exchanges, or genotype-specific sequence permutations. Using the HepaRG cell line and a genotype D-derived virus, we determined the specific inhibitory activities of the peptides and found that (i) lipopeptides with an artificial consensus sequence inhibit HBV genotype D infection more potently than the corresponding genotype D peptides; (ii) point mutations, D-amino acid exchanges, or deletions introduced into the highly conserved part of the pre-S1 domain result in an almost complete loss of activity; and (iii) the flanking sequences comprising amino acids 2 to 8, 16 to 20, and, to a less pronounced extent, 34 to 48 gradually increase the inhibitory activity, while amino acids 21 to 33 behave indifferently. Taken together, our data suggest that HBV pre-S1-mediated receptor interference and, thus, HBV receptor recognition form a highly specific process. It requires an N-terminal acyl moiety and a highly conserved sequence that is present in primate but not rodent or avian hepadnaviruses, indicating different entry pathways for the different family members.

Hepatitis B viruses (HBVs) are small, enveloped DNA viruses that replicate their genome via reverse transcription of a pregenomic RNA transcript in the cytoplasm of infected hepatocytes (31). They are classified into the family *Hepadnaviridae* and are adapted to mammals (primates and rodents) and birds, where they cause acute and persistent infections. At present about 360 million people are chronically infected with the human HBV. Due to HBV-related progressive liver failure (cirrhosis or hepatocellular carcinoma) ≈650,000 people die each year (10). HBV, like duck hepatitis B virus (DHBV) or woodchuck hepatitis B virus (WHV), shows a pronounced species specificity and a remarkable liver tropism. These features have been assigned to specific early infection events, most likely receptor recognition. However, it cannot be excluded that other steps during or postentry contribute to the host restrictions of infection (13).

The HBV replication cycle has been deciphered in some detail. However, studies on the early infection events were hampered by the lack of appropriate *in vitro* infection systems. The establishment of the HBV-susceptible cell line HepaRG and systems based on primary human hepatocytes (PHH) and primary *Tupaia belangeri* hepatocytes resolved this issue and facilitated investigations on cellular and viral determinants involved in HBV entry. It became clear that infectivity can be assigned to different subdomains in two of the three viral envelope proteins (34). These three envelope proteins are termed large (L), middle (M), and small (S) proteins. They form a proteinaceous outer virus shell, which is embedded in an endoplasmic reticulum (ER)-derived lipid bilayer. Their coding mRNAs originate from one single open reading frame (4). Since their three start codons are in phase, L, M, and S proteins share the C-terminal 226 amino acids (termed the S domain in the context of the L and M proteins), which anchor the proteins via four putative transmembrane helices (TM domains) in the lipid bilayer. The S protein drives particle formation and accordingly serves an important function in virus assembly (4, 5). The S domain has further been shown to participate in virus entry since antibodies against the antigenic loop of S, located between TM domains 2 and 3, are neutralizing (11), and mutations introduced in this loop render assembled hepatitis delta virus (HDV) particles noninfectious (1, 20, 29). Linked to the N terminus of the S domain, the M and L proteins bear two hydrophilic extensions, one of 55 amino acids and, depending on genotype, one of 108 (genotype D), 118 (genotypes E and G), or 119 (genotypes A, B, C, F, H) amino acids called pre-S2 (M) and pre-S1 (L), respectively. The L proteins of all hepadnaviruses contain recognition motifs for *N*-myristoyltransferase (36, 37) and are accordingly subjected to this modification. The lack of a classical secretion signal in L results in an initial cytoplasmic orientation of its pre-S domains. After synthesis, myristoylation, and incorporation of the TM helices into the ER membrane, the pre-S domain of L traverses the lipid bilayer, allowing parts of it to become accessible at the particle exterior (7).

Crucial elements for virus attachment, specific receptor binding, or fusion have been identified within the L, M, and S proteins by two complementary approaches. One approach using HBV particles that were enveloped with mutated L proteins showed that myristoylation of L is mandatory for virus infectivity (6, 16). Successive deletions of 5 amino acids within the N-terminal pre-S1 sequence of residues 1 to 78 abrogated HBV infectivity (24). In contrast, most parts of the pre-S2 domain were dispensable for infection (23). Using HDV, these results were confirmed, indicating that both viruses share very similar entry modes (3). Moreover it became obvious that the S domain, either as part of the HBV L protein or as the S protein, being the major constituent of the viral envelope, is required (1, 20, 29). The second approach followed the observation that a synthetic myristoylated peptide consisting of the N-terminal amino acids 2 to 78 of the pre-S1 domain (HBVpreS/2-78^{myr}) prevents HBV infection of PHH and HepaRG cells even at nanomolar concentrations (17). Surprisingly, C-terminal shortening of this sequence up to amino acid 48 increases the inhibitory activity of the peptide (15). This indicates two separable and distinct functions within the pre-S1 infectivity determinant consisting of residues 1 to 78: one is related to the inhibitory activity of the peptide through interference with a hepatocyte-specific receptor, and the other, involving amino acids 49 to 78, is still unknown. The requirement of the N-terminal myristic acid moiety in the inhibitory activity is to some extent variable. Replacement of myristic acid by other hydrophobic saturated and unsaturated fatty acid moieties or even by cholesterol allows the modulation of specific activities according to the relative hydrophobicity (14, 15, 27). Thus, lipophilicity *per se* and not a specific lipid structure seems to play the key role for this part of the molecule.

Previous studies using synthetic peptides or recombinantly produced fusion proteins carrying internal deletions and point mutations identified a highly conserved pre-S1 sequence element (amino acids 9 to 18 in genotype D) that is very important for infection competition (2, 9, 14). Single amino acid exchanges in this region abrogate the antiviral activity of the peptides and render the respective recombinant hepatitis B virions noninfectious (9).

Our recent observation that HBV pre-S1-derived lipopeptides are also potent inhibitors of HBV infection *in vivo* (27) suggests that the lead substance of this group of entry inhibitors may be suitable for clinical applications in cases of acute and possibly chronic HBV and HDV infection in the future.

In this report, we examined the capacity of synthetic HBV pre-S1-derived peptides to interfere with the HBV infection of HepaRG cells. By using this approach we (i) further narrowed down the minimal amino acid sequence requirements and the sensitivity to amino acid exchanges for interference with the HBV infection, (ii) characterized in more detail the role of N- and C-terminal deletions, and (iii) defined the consensus sequence derived from an alignment of the different HBV genotypes as the most active peptide and the lead substance for clinical development.

MATERIALS AND METHODS

Cell lines. HepaRG cells were grown in Williams' medium E supplemented with 10% heat-inactivated fetal calf serum, 50 U of penicillin/ml, 50 μg of streptomycin/ml, 5 μg of insulin/ml, and 50 μM hydrocortisone hemisuccinate.

The cells were passaged every 2 weeks at a ratio of 1:5. Fourteen days before infection, cell differentiation was induced by adding 2% dimethyl sulfoxide to the maintenance medium (17). The medium was exchanged every 3 days.

Infection competition assays. As an infectious inoculum, HepAd38 (22) or HepG2.2.15 (32) cell culture supernatant, concentrated approximately 100-fold, was used. The inoculum was prepared from cell culture supernatants by precipitating viral particles with 6% polyethylene glycol 8000 (Sigma-Aldrich) for 12 to 16 h at 4°C. The precipitates were recovered by centrifugation (at 7,000 × *g* for 1 h at 4°C) and resuspended in phosphate-buffered saline (PBS) supplemented with 25% fetal calf serum. Aliquots were stored at −80°C. For infection, differentiated HepaRG cells (1 × 10⁶ cells/well of a 12-well plate) were incubated with a 20-fold dilution of the concentrated virus stock in medium (corresponding to ≈4 × 10¹⁰ genome equivalents) supplemented with 4% polyethylene glycol 8000 for 16 h at 37°C. At the end of the incubation, the cells were washed three times and further cultivated. Medium was changed every 3 days. Competition experiments were performed in 12-well plates. To ensure comparability within one concentration series, all assays with one peptide were done in a single 12-well plate. To guarantee the best comparability between different peptides, the respective tests of different concentration series were performed in plates of the same batch of cells. HepaRG cells were preincubated for 30 min at 37°C with the chemically synthesized acylated HBV pre-S1 peptides, followed by coincubation of cells with the peptides and virus for 16 h at 37°C. To quantify the infection, amounts of hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) secreted into the culture supernatant from day 7 to 11 postinfection (p.i.) were determined by enzyme-linked immunosorbent assay ([ELISA] AxSYM; Abbott). All competition experiments were performed in duplicate and repeated at least two times independently.

Quantification of cellular HBV binding. Attachment of HBV to HepaRG cells was determined by quantitative PCR to detect the viral DNA. Binding/uptake was performed for 4 h at 37°C in the absence or presence of 200 μg/ml heparin or 1 μM HBVpreS/2-48^{myr} (a synthetic myristoylated peptide consisting of N-terminal amino acids 2 to 48 of the pre-S1 domain). Unbound virus was removed by washing. Viral DNA was prepared from the cellular lysates with a NucleoSpin Blood Kit (Macherey-Nagel) according to the manufacturer's protocol. For the SYBR green-based real-time PCR (Invitrogen), the primer set Taq-HBV-F (5′-TCCCAGAGTGAGAGGCCTGTA-3′) and Taq-HBV-R (5′-ATCCTCGA GAAGATTGACGATAAGG-3′) was used. Quantitative PCR was performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). The reaction process started at 50°C for 2 min and 95°C for 15 min, followed by 45 cycles at 95°C for 15 s and 60°C for 1 min. All experiments were performed at least twice.

Peptide synthesis, purification, and analysis. All peptides were synthesized by standard solid-phase peptide synthesis employing the fluorenylmethoxycarbonyl/ *t*-butyl (Fmoc/tBu) strategy and purified by semipreparative reversed-phase high-performance liquid chromatography (HPLC) on a Cromolith SemiPrep column (Merck). The purity of all peptides was verified by analytical HPLC on a Performance Cromolith column (Merck). As solvents for analytical HPLC, 0.1% aqueous trifluoroacetic acid (solvent A) and acetonitrile containing 0.1% trifluoroacetic acid (solvent B) were used. The analysis was performed by applying a linear gradient (flow rate of 4 ml/min) from 0 to 100% solvent B within 5 min. The identity of the peptides was verified by mass spectrometry. The peptide WHVpreS/2-81^{myr} (2-GNNIKVTFNPDKIAAWWPAVGTYYTTTYPQNQSVF QPGIYQTTSLINPKNQQELDSVLINRYKQIDWNTWQGFPVDQKLP-81) was purchased from Peptide Specialty Laboratories GmbH (Heidelberg, Germany). For infection competition experiments, stock solutions of the peptides in dimethyl sulfoxide were prepared, prediluted in medium, and added to the cells at the concentrations indicated in the figures.

Immunofluorescence (IF) analyses. At 11 days p.i. the HepaRG cells were washed with PBS, fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, and permeabilized with 0.25% (vol/vol) Triton X-100 in PBS (30 min at room temperature). After a washing step, a hepatitis B core antigen (HBcAg)-specific polyclonal antibody (H363; provided by Heinz Schaller, ZMBH, University of Heidelberg, Germany) at a dilution of 1:1,000 in PBS containing 5% skim milk powder was added. Following overnight incubation at 4°C, cells were washed and incubated in the dark for 1 h at room temperature with a 1:500 dilution of an Alexa Fluor 488-conjugated secondary antibody (Invitrogen/Molecular Probes) in PBS containing 5% skim milk powder. After a washing step, cells were incubated for 30 min with a 1 μg/ml solution of 4′, 6′-diamidino-2′-phenylindole dihydrochloride (DAPI; Roche Applied Science). Cells were washed three times with PBS. Images were acquired with an inverted fluorescence microscope (Leica, Germany). All images were made under identical settings and afterwards handled identically. The images were analyzed using ImageJ software. The nucleus counter function of the Wright Cell Imaging

TABLE 1. Name, primary sequence, and *in vitro* inhibitory activity of the tested HBV peptides[a]

Peptide	Sequence	IC$_{50}$ [nM]
2-48^{stearoyl}	⌇⌇⌇⌇⌇⌇ GQNLSTSNPLGFFPDHQLDPAFRANTANPDWDFNPNKDTWPDANKVG	0.4
2-48scrambled^{stearoyl}	⌇⌇⌇⌇⌇⌇ TNNDPFKRTDLAWNGFDSVAQNDLLPNPPFNNWGDGSHQPADAKPFT	not active
1-48	MGQNLSTSNPLGFFPDHQLDPAFRANTANPDWDFNPNKDTWPDANKVG	not active
2-33^{stearoyl}	⌇⌇⌇⌇⌇⌇ GQNLSTSNPLGFFPDHQLDPAFRANTANPDWD	1-5
2-26^{stearoyl}	⌇⌇⌇⌇⌇⌇ GQNLSTSNPLGFFPDHQLDPAFRAN	4
2-21^{stearoyl}	⌇⌇⌇⌇⌇⌇ GQNLSTSNPLGFFPDHQLDP	3
2-15^{stearoyl}	⌇⌇⌇⌇⌇⌇ GQNLSTSNPLGFFP	400
2-48(P21A, F23A,N29A, P30A)^{stearoyl}	⌇⌇⌇⌇⌇⌇ GQNLSTSNPLGFFPDHQLDAAARANTAAADWDFNPNKDTWPDANKVG	12.5
5-48^{stearoyl}	⌇⌇⌇⌇⌇⌇ LSTSNPLGFFPDHQLDPAFRANTANPDWDFNPNKDTWPDANKVG	3
6-48^{stearoyl}	⌇⌇⌇⌇⌇⌇ STSNPLGFFPDHQLDPAFRANTANPDWDFNPNKDTWPDANKVG	80
7-48^{stearoyl}	⌇⌇⌇⌇⌇⌇ TSNPLGFFPDHQLDPAFRANTANPDWDFNPNKDTWPDANKVG	n.d.
9-48^{stearoyl}	⌇⌇⌇⌇⌇⌇ NPLGFFPDHQLDPAFRANTANPDWDFNPNKDTWPDANKVG	1000
2-48(A2-8)^{stearoyl}	⌇⌇⌇⌇⌇⌇ AAAAAAANPLGFFPDHQLDPAFRANTANPDWDFNPNKDTWPDANKVG	1000-4000
2-48(A2-9)^{stearoyl}	⌇⌇⌇⌇⌇⌇ AAAAAAAAPLGFFPDHQLDPAFRANTANPDWDFNPNKDTWPDANKVG	not active
5-33^{stearoyl}	⌇⌇⌇⌇⌇⌇ LSTSNPLGFFPDHQLDPAFRANTANPDWD	10
9-33^{stearoyl}	⌇⌇⌇⌇⌇⌇ NPLGFFPDHQLDPAFRANTANPDWD	500
2-48(Δ11-15)^{stearoyl}	⌇⌇⌇⌇⌇⌇ GQNLSTSNP-----DHQLDPAFRANTANPDWDFNPNKDTWPDANKVG	not active
2-48(A11-15)^{stearoyl}	⌇⌇⌇⌇⌇⌇ GQNLSTSNPAAAAADHQLDPAFRANTANPDWDFNPNKDTWPDANKVG	not active
2-48(D-11/13-15)^{stearoyl}	⌇⌇⌇⌇⌇⌇ GQNLSTSNPlGffpDHQLDPAFRANTANPDWDFNPNKDTWPDANKVG	not active
2-48(D-11/13)^{stearoyl}	⌇⌇⌇⌇⌇⌇ GQNLSTSNPlGfFPDHQLDPAFRANTANPDWDFNPNKDTWPDANKVG	not active
2-48(L11R)^{stearoyl}	⌇⌇⌇⌇⌇⌇ GQNLSTSNPRGFFPDHQLDPAFRANTANPDWDFNPNKDTWPDANKVG	not active
2-48(G12E)^{stearoyl}	⌇⌇⌇⌇⌇⌇ GQNLSTSNPLEFFPDHQLDPAFRANTANPDWDFNPNKDTWPDANKVG	500
2-48(F13S)^{stearoyl}	⌇⌇⌇⌇⌇⌇ GQNLSTSNPLGSFPDHQLDPAFRANTANPDWDFNPNKDTWPDANKVG	600-700
2-48cons^{stearoyl}	⌇⌇⌇⌇⌇⌇ GTNLSVPNPLGFFPDHQLDPAFRANSNNPDWDFNPNKDHWPEANKVG	0.08
2-48cons^{myr}	⌇⌇⌇⌇⌇⌇ GTNLSVPNPLGFFPDHQLDPAFRANSNNPDWDFNPNKDHWPEANKVG	0.14
(-11)-48cons^{stearoyl}	⌇⌇⌇⌇⌇⌇ GGWSSTPRKGMGTNLSVPNPLGFFPDHQLDPAFRANSNNPDWDFNPNKDHWPEANKVG	0.2
(-11)-48cons^{myr}	⌇⌇⌇⌇⌇⌇ GGWSSTPRKGMGTNLSVPNPLGFFPDHQLDPAFRANSNNPDWDFNPNKDHWPEANKVG	0.5

[a] To conserve space, all peptide names were shortened by deletion of the prefix "HBVpreS/." Sequence variations in comparison to HBVpreS/2-48^{stearoyl} (genotype D) are marked by gray boxes. IC$_{50}$s are given according to the corresponding HBsAg measurements. Not active, peptide is not active in the concentration range tested; ND, not determined in the concentration range tested.

Facility (WCIF) plug-in was used to count the number of labeled nuclei (DAPI). The cell counter plug-in was used to count the number of HBcAg-positive cells. For each peptide and concentration, four images were analyzed.

RESULTS

Sequence-specific and acylation-dependent inhibition of HBV infection by synthetic lipopeptides derived from the N-terminal pre-S1 domain of the HBV L protein. To substantiate the specificity of infection inhibition by HBV pre-S1-derived peptides, we infected differentiated HepaRG cells with HBV (genotype D) in the presence of increasing concentrations (1, 5, 25, 100, and 1,000 nM) of HBVpreS/2-48^{stearoyl}, the N-terminally stearoylated pre-S1-derived peptide of genotype D; HBVpreS/2-48scrambled^{stearoyl}, an N-terminally stearoylated peptide of the same amino acid composition but with randomized sequence; and HBVpreS/1-48, the nonstearoylated pre-S1 peptide carrying instead of a lipid moiety the initially biosynthesized methionine in position 1 (Table 1). Prior to the infection inhibition experiments, the purity of all synthesized peptides was controlled by HPLC, as shown for the three peptides in Fig. 1A. The peptides eluted in a single peak with no detectable side products or contaminations. A clear increase in the retention time (2.3 to 3.7 min) was observed for the stearoylated peptides compared to the nonacylated HBVpreS/2-48, indicating that nonacylated side products were completely removed.

Consistent with earlier results (15), HBVpreS/2-48^{stearoyl} inhibited HBV infection by more than 80% at a concentration of 1 nM (as measured by secreted HBsAg between days 7 to 11 p.i.) (Fig. 1B). Concentrations of >1 nM reduced HBsAg in the supernatant to almost the detection limit of the ELISA. In contrast, neither HBVpreS/2-48scrambled^{stearoyl} nor the nonstearoylated pre-S1 peptide was able to interfere with infection even at 1 μM. This confirms the specificity of the assay within this concentration range and excludes nonspecific effects caused by, for example, the acyl moiety. In a separate experiment performed under the same conditions but applying lower peptide concentrations, the half-maximal inhibitory concentration (IC$_{50}$) of HBVpreS/2-48^{stearoyl} was determined to be ≈400 pM (data not shown). Using a WHV-derived peptide (WHVpreS/2-81^{myr}) no interference with the *in vitro* HBV infection was observed while a parallel control experiment using 1 μM HBVpreS/2-48^{myr} completely abolished infection.

It has been shown that inoculation of HepaRG cells with acylated pre-S1 peptides prior to infection results in inhibition, indicating that the peptides address a cellular factor (15). In
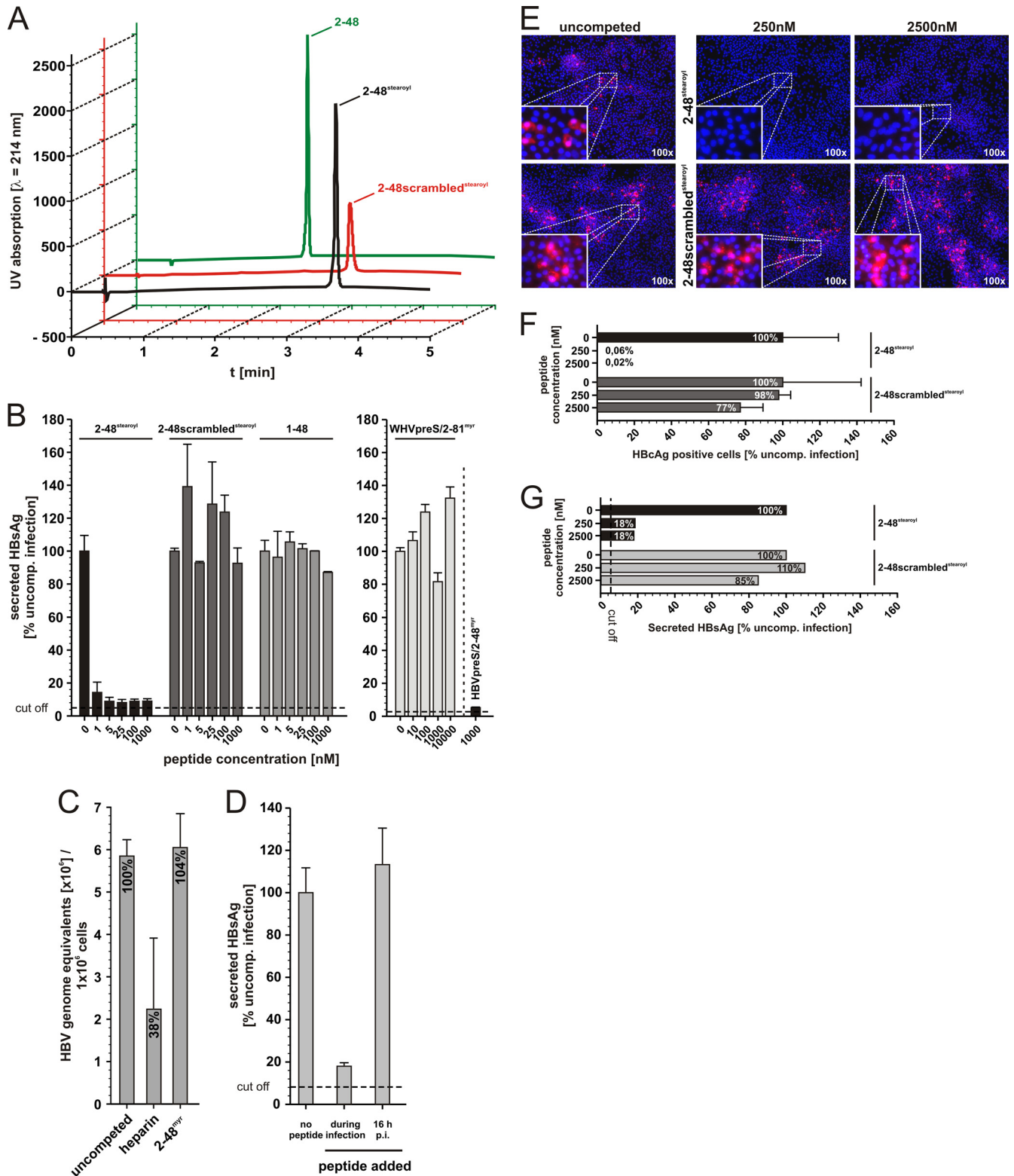
FIG. 1. HBVpreS/2-48[stearoyl] efficiently inhibits HBV infection of HepaRG cells, requires N-terminal acylation, and does not obstruct HBV attachment at concentrations up to 1 μM. (A) Elution profiles of analytical HPLC runs of HBVpreS/2-48[stearoyl], HBVpreS/2-48scrambled[stearoyl], and HBVpreS/2-48. Note that, due to size limitations, all peptide names have been shortened and are represented by their amino acid sequences (e.g., 2-48 for HBVpreS/2-48). Peptide bonds were detected by UV absorbance at 214 nm and are given as arbitrary units. (B) At left, data are shown for HBV infection inhibition using increasing concentrations (0 to 1,000 nM) of HBVpreS/2-48[stearoyl], HBVpreS/2-48scrambled[stearoyl], and HBVpreS/1-48. At right, data are shown for HBV infection inhibition using increasing concentrations (0 to 10,000 nM) of WHVpreS/2-81[myr]. As control, 1 μM HBVpreS/2-48[myr] was used in parallel. The amount of HBsAg secreted from days 7 to 11 p.i. was determined by ELISA and is given

addition, we along with others identified heparan sulfate proteoglycans (HSPG) as the primary cellular attachment sites of HBV (25, 30). To investigate if pre-S1-derived lipopeptides interfere with binding of the viral particles to cellular HSPG, we compared the HBV cell association in the presence of heparin or HBVpreS/2-48^{myr} (Fig. 1C). Addition of 200 µg/ml heparin led to a 62% reduction of cell-associated viral genome equivalents in comparison to the untreated control. In contrast, the presence of 1 µM HBVpreS/2-48^{myr}, a concentration that blocks infection, had no influence on HBV cell association, indicating that the HBV pre-S1 peptides do not affect primary attachment. To analyze whether HBV pre-S1 lipopeptides act at a postfusion step, we added HBVpreS/2-48^{stearoyl} 16 h after the beginning of virus inoculation (Fig. 1D). While coincubation with the virus led to a more than 80% reduction of infection, addition of the peptide at 16 h postinoculation was without effect. To verify the results of the infection inhibition experiments on a single-cell level, we performed HBcAg-specific IF analyses. While a subset of differentiated HepaRG cells stained positively for HBcAg in the untreated control infection, only sporadic infection events could be observed in the presence of 250 nM or 2.5 µM HBVpreS/2-48^{stearoyl} (Fig. 1E). As determined by cell counting, this corresponds to 0.06% (at 250 nM) and 0.02% (at 2.5 µM) of HBcAg-positive cells of the control infection (Fig. 1F). Under the same conditions HBVpreS/2-48scrambled^{stearoyl} did not lead to a significant reduction of HBcAg-expressing cells (98% at 250 nM and 77% at 2.5 µM). Parallel quantification of HBsAg secreted from days 7 to 11 p.i. showed a reduction to 18% of the control infection for 250 nM and 2.5 µM HBVpreS/2-48^{stearoyl} (Fig. 1G), indicating that some part of the observed HBsAg in the measurement is most likely due to remaining input virus. In accordance with the IF results, the addition of 250 nM or 2.5 µM HBVpreS/2-48scrambled^{stearoyl} had no effect on the HBV infection. HBcAg-specific IF was also performed for the subsequent experiments. These analyses confirmed the results obtained by the HBsAg/HBeAg measurements (data not shown).

Influence of C-terminal truncations of HBVpreS/2-48^{stearoyl}. It has been previously shown that successive C-terminal shortening of HBVpreS/2-48^{stearoyl} up to amino acid 18 results in a significant but not complete loss of the inhibitory activity of the peptide (2, 14). To refine the contribution of the sequence elements located within this segment and to specifically address the role of the pre-S1 amino acids 15 to 18, we chemically synthesized the peptides HBVpreS/2-33^{stearoyl}, HBVpreS/2-26^{stearoyl}, HBVpreS/2-21^{stearoyl}, and HBVpreS/2-15^{stearoyl} and quantified their HBV-inhibitory activity in concentrations ranging from 1 nM to 1 µM. As shown in Fig. 2, HBVpreS/2-

33^{stearoyl} exhibited about a 10-fold reduced activity compared to HBVpreS/2-48^{stearoyl}. Further truncations up to amino acid 21 had only a marginal influence on the respective IC$_{50}$s, indicating that this part of the pre-S1 sequence, although containing the two major neutralizing B-cell epitopes 20-DPAF-23 (19) and 26-NTANPDW-32 (28), does not contribute substantially to the inhibitory activity of the peptide. In contrast, removal of the next six adjacent amino acids increased the IC$_{50}$ by an extra factor of ≈100 to ≈400 nM. This demonstrates an important contribution of amino acids 16-DHQLD-20 and, to a lesser extent, of amino acids 34 to 48 to infection inhibition. To analyze the influence of the two aforementioned neutralizing B-cell epitopes in more detail, HBVpreS/2-48(P21A F23A N29A P30A)^{stearoyl}, a peptide comprising the four point mutations P21A, F23A, N29A, and P30A in this region, was tested. It exhibited about a 25-fold reduction in the IC$_{50}$ compared to HBVpreS/2-48^{stearoyl} (Fig. 2, right), supporting the notion that changes in these immunogenic epitopes have only moderate effects although the corresponding monoclonal antibodies block infection very efficiently (12, 26; also data not shown).

Effects of incremental N-terminal single amino acid deletions on infection inhibition. Using HDV as a surrogate system to study HBV-receptor interactions, Barrera et al. previously demonstrated that neither insertion of a methionine between residue G-2 and the myristic acid nor deletion of amino acids 2 to 4 and fusion of the hydrophobic moiety to residue L-5 results in abrogation of the inhibitory activity (2). This indicates that the N-terminal positioning of the lipid as well as the length of the lipid-bound amino acid sequence can be varied to some extent. To quantify the contribution of single amino acids within this very N-terminal part of the peptide, we performed comparative competition experiments using peptides with consecutive N-terminal truncations (HBVpreS/5-48^{stearoyl}, HBVpreS/6-48^{stearoyl}, HBVpreS/7-48^{stearoyl}, and HBVpreS/9-48^{stearoyl}) and peptides that carry polyalanine stretches of the respective length instead of the authentic sequence [HBVpreS/2-48^{stearoyl}(A2-8) and HBVpreS/2-48^{stearoyl}(A2-9), with alanines at residues 2 to 8 and 2 to 9, respectively].

As shown in Fig. 3A, removal of the three N-terminal amino acids 2-GQN-4 (corresponding to the peptide HBVpreS/5-48^{stearoyl}) reduced the IC$_{50}$ of the full-length peptide HBVpreS/2-48^{stearoyl} by a factor of ≈7.5 (IC$_{50}$ of ≈3 nM). Deletion of the next four amino acids (matching the peptide HBVpreS/9-48^{stearoyl}) resulted in a ≈2,500-fold reduced but still significant specific activity (IC$_{50}$ of ≈1,000 nM). This emphasizes an important contribution of the motif 5-LSTS-8 for the peptide activity. Incremental shortening from amino acid 5

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as a percentage of the control infection. The sensitivity of the ELISA (cutoff) is indicated by the dotted line. (C) Quantification of HBV binding/uptake (4 h at 37°C) to HepaRG cells in the absence (left) and presence of 200 µg/ml heparin (middle) or 1 µM HBVpreS/2-48^{myr} (right). Cell-associated HBV genome equivalents were quantified by real-time PCR. (D) Effect of 10 µM HBVpreS/2-48^{stearoyl} on HBV infection when added together with virus (middle) or 16 h after the start of virus inoculation (right). HBsAg amounts from days 7 to 11 p.i. were determined by ELISA. (E) Analysis of the inhibitory activities of HBVpreS/2-48^{stearoyl} (upper row) and HBVpreS/2-48scrambled^{stearoyl} (lower row) by HBcAg-specific IF. At 30 min prior to infection HepaRG cells were incubated with buffer (left) or 250 nM (middle) or 2.5 µM (right) of the respective peptides and subsequently infected with HBV. At 11 days p.i. cells were analyzed for HBcAg (red) and nuclei (DAPI; blue). (F) Quantification of the IF analyses shown in panel E. In total 61,304 DAPI-positive cells were counted for HBVpreS/2-48scrambled^{stearoyl}; for HBVpreS/2-48^{stearoyl} all HBcAg-positive cells in the corresponding well were counted. (G) Measurement of the secreted HBsAg (days 7 to 11 p.i.) of the infection shown in panel E. The results are depicted as a percentage of the control infection. The sensitivity of the assay (cutoff) is indicated by the dotted line.
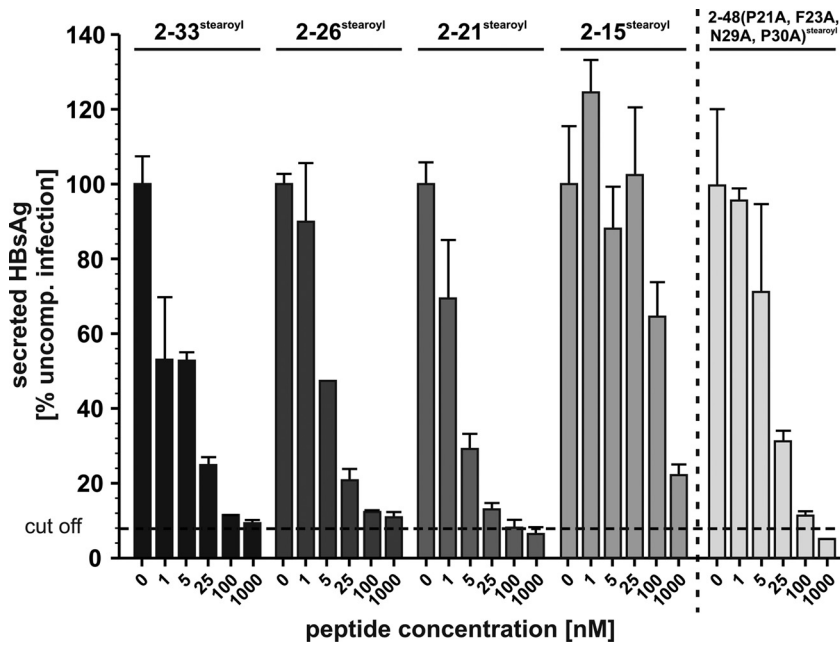
FIG. 2. Infection inhibition activities of C-terminally shortened and antigenically altered HBV pre-S1 lipopeptides. HBV infection competition experiments on HepaRG cells using increasing concentrations (0 to 1,000 nM) of the indicated peptides. All peptide names have been shortened due to size limitation. The amount of secreted HBsAg (days 7 to 11 p.i.) was determined by ELISA. Values are presented as a percentage of the control infection. The detection limit of the assay is indicated by the dotted line. Uncomp, uncompeted.

to 9 (corresponding to the peptides HBVpreS/5-48^{stearoyl}, HBVpreS/6-48^{stearoyl}, HBVpreS/7-48^{stearoyl}, and HBVpreS/9-48^{stearoyl}) led to steadily decreased IC$_{50}$s (Fig. 3B). HBVpreS/7-48^{stearoyl}, which includes residues S-8 and T-7, exhibited an ability to interfere with HBV infection that is comparable to that of HBVpreS/9-48^{stearoyl} while the presence of S-6 enhanced the activity by a factor of about 14.

To test whether the nine N-terminal amino acids serve a specific function or simply provide a spacer and/or myristoylation recognition sequence, we replaced amino acids 2 to 8 [HBVpreS/2-48(A2-8)^{stearoyl}] or 2 to 9 [HBVpreS/2-48(A2-9)^{stearoyl}] by alanine stretches and compared the activities of these peptides with activity of the deletion mutant HBVpreS/9-48^{stearoyl} (Fig. 3C). To better evaluate the specific activities, the maximal peptide concentration in these assays was raised to 10 μM. HBVpreS/9-48^{stearoyl} and HBVpreS/2-48(A2-8)^{stearoyl} displayed comparable activities (IC$_{50}$ of 1 to 4 μM; 80% inhibitory concentration [IC$_{80}$] of ≈10 μM), indicating that the N-terminal seven amino acids of the pre-S1 sequence contribute to the inhibitory activity in a sequence-specific manner. Notably, replacement of N-9 by alanine [HBVpreS/2-48(A2-9)^{stearoyl}] led to a complete abrogation of infection inhibition at concentrations up to 10 μM. We thus consider residue N-9 essential.

Combined N- and C-terminal truncations of HBVpreS/2-48^{stearoyl} do not cooperatively influence the infection inhibition. To determine whether the previously defined N- and C-terminal sequence elements cooperatively influence each other, we tested peptides with combined deletions of the N and C termini (HBVpreS/5-33^{stearoyl} and HBVpreS/9-33^{stearoyl}) and compared their infection inhibition activity with the previously determined IC$_{50}$s. As shown in Fig. 4, combination of the truncations did not further reduce the peptide activity.

HBVpreS/5-33^{stearoyl} displayed an IC$_{50}$ of approximately 10 nM while HBVpreS/9-33^{stearoyl} had an IC$_{50}$ of ≈500 nM.

The integrity of a highly conserved pre-S sequence of primate hepadnaviruses encompassing amino acids 11 to 15 is crucial for infection inhibition. Our previous studies using *Escherichia coli*-derived myristoylated HBV pre-S–glutathione *S*-transferase (GST) fusion proteins revealed that amino acids 11 to 15 play a profound role in both the inhibitory activity of the peptides (shown by deletions and point mutations in the pre-S1 part of the fusion proteins) and the infectivity of the corresponding HBV mutants (shown by infection assays using genetically altered viruses produced in hepatoma cells) (9). To more precisely analyze the role of this highly conserved sequence, we chemically synthesized peptides that either comprise a complete deletion of this sequence [HBVpreS/2-48(Δ11-15)^{stearoyl}], carry alanine substitutions for these five residues [HBVpreS/2-48(A11-15)^{stearoyl}], or have incorporated the respective D-enantiomeric amino acids at position 11, 13, 14, and 15 [HBVpreS/2-48(D-11/13-15)^{stearoyl}]. Consistent with our previous results, deletion of amino acids 11 to 15 resulted in a complete loss of the inhibitory activity even at concentrations of 1 μM (Fig. 5A) and 2.5 μM (data not shown). Replacement of 11-LGFFP-15 by alanines could not rescue the activity, confirming the sequence requirement. Interestingly, replacement of L-11, F-13, F-14, and P-15 with the respective D-amino acids also led to the complete inactivation of the peptide. The same held true when only two of these four D-amino acids, at positions 11 and 13, were exchanged (Fig. 5B). This not only points at the importance of these residues but also indicates a stereo-selective interaction of the peptide with its putative cellular receptor. To investigate the contribution of single amino acids within this region, we compared the inhib-
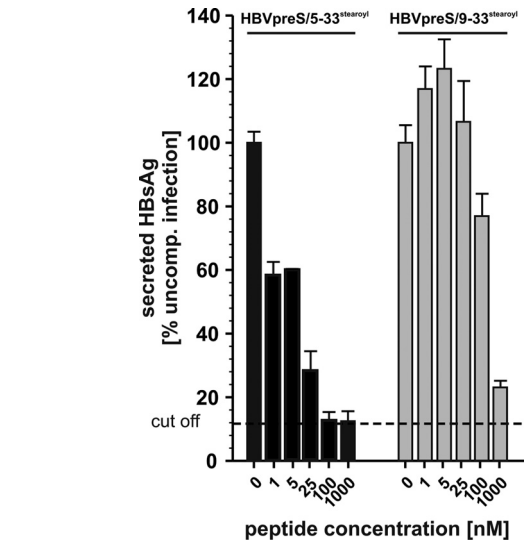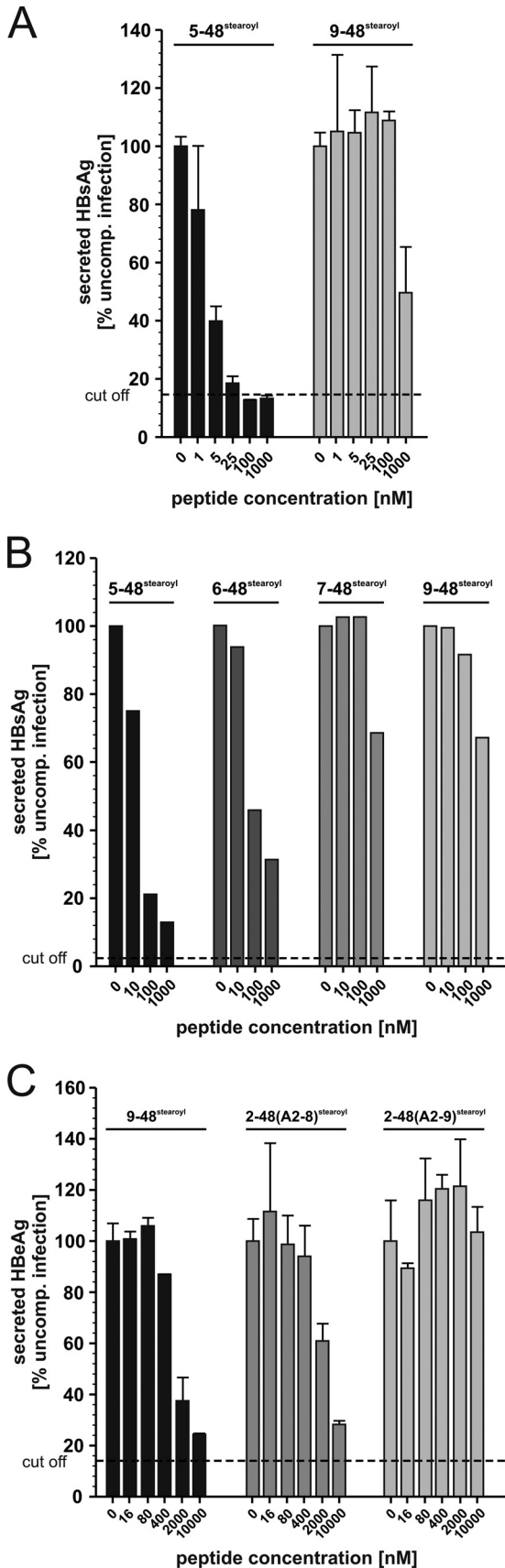
A

B

C

FIG. 4. Infection inhibition activity of combined N- and C-terminally deleted HBV pre-S1 lipopeptides. HBV infection competition using 0, 1, 5, 25, 100, and 1,000 nM HBVpreS/5-33[stearoyl] or HBVpreS/9-33[stearoyl]. All peptide names have been shortened due to size limitations. The amount of HBsAg secreted between days 7 to 11 p.i. was determined by ELISA. The sensitivity of the ELISA is indicated by the dotted line.

itory activity of peptides carrying the exchanges L11R, G12E, or F13S. They have been reported to render the respective virus noninfectious (9). While HBVpreS/2-48(G12E)[stearoyl] and HBVpreS/2-48(F13S)[stearoyl] showed a residual activity at 1,000 nM, HBVpreS/2-48(L11R)[stearoyl] had no effect on infection at this concentration (Fig. 5C). These data indicate that L-11 (similar to N-9) contributes with a high impact to the peptide activity. These results were supported by the IF analyses using 250 nM or 2.5 μM peptides (Fig. 5D and E).

Genotypic cross-inhibition with HBVpre-S1-derived lipopeptides encompassing the consensus sequences of genotypes A to H. We have previously shown that the IC_{50}s of pre-S1-derived peptides can be modulated by changing the length and, as a result, the hydrophobicity of the N-terminal acyl moiety (15). Through modification with palmitic or stearic acid, it was possible to generate inhibitors with about 10-fold increased specific activities compared to the authentically myristoylated peptides. Furthermore, preincubation experiments led us to the hypothesis that HBV pre-S1-derived lipopeptides address a cellular receptor and render it nonfunctional for supporting the subsequent virus entry (15). Assuming that the

FIG. 3. Infection inhibition activity of N-terminally shortened or modified HBVpre-S1 lipopeptides. (A to C) HBV infection inhibition using increasing concentrations (0 to 1 μM or 0 to 10 μM) of HBVpreS/5-48[stearoyl] and HBVpreS/9-48[stearoyl] (A); HBVpreS/5-48[stearoyl], HBVpreS/6-48[stearoyl], HBVpreS/7-48[stearoyl], and HBVpreS/9-48[stearoyl] (B); or HBVpreS/2-48(A2-8)[stearoyl], HBVpreS/2-48(A2-9)[stearoyl], and HBVpreS/9-48[stearoyl] (C). All peptide designations have been shortened due to size limitations. The amount of HBsAg or HBeAg secreted from days 7 to 11 p.i. was determined by ELISA. The values are given as a percentage of the untreated control infection. The sensitivity of the ELISA (cutoff) is indicated by a dotted line.
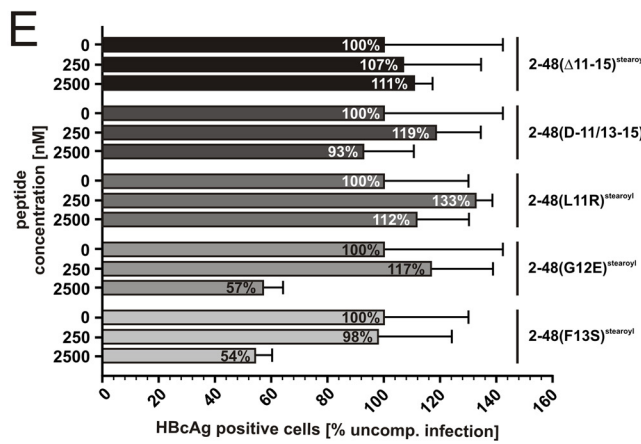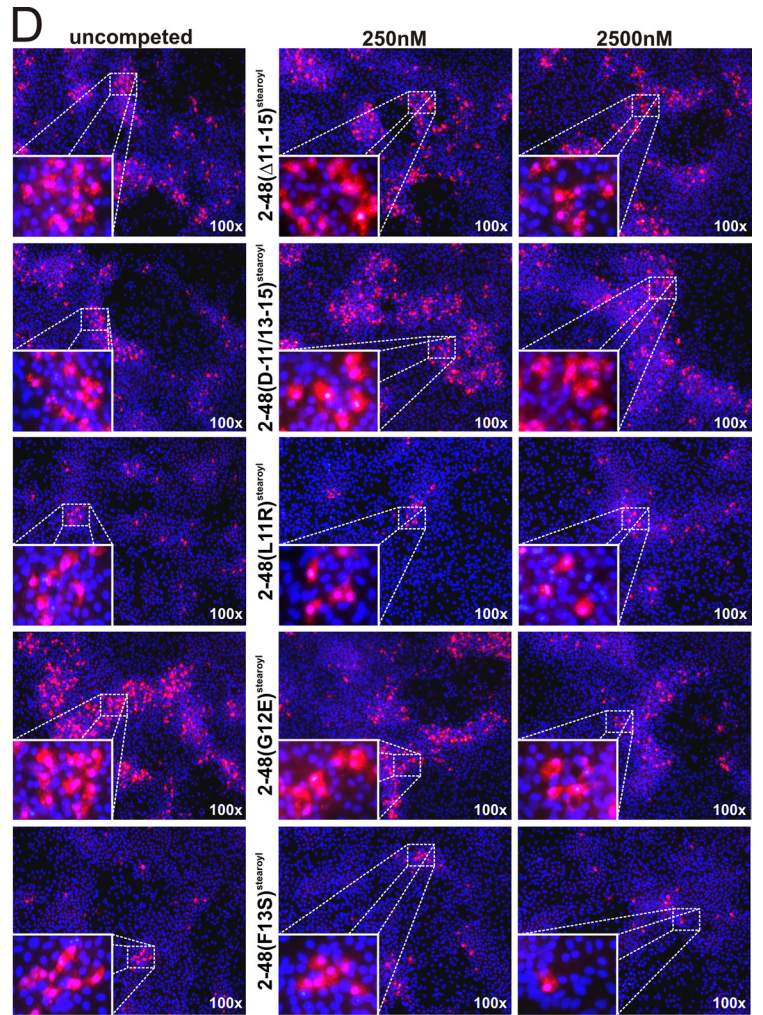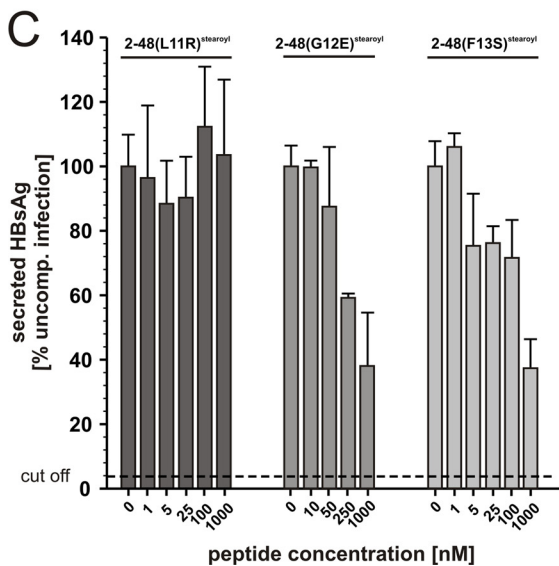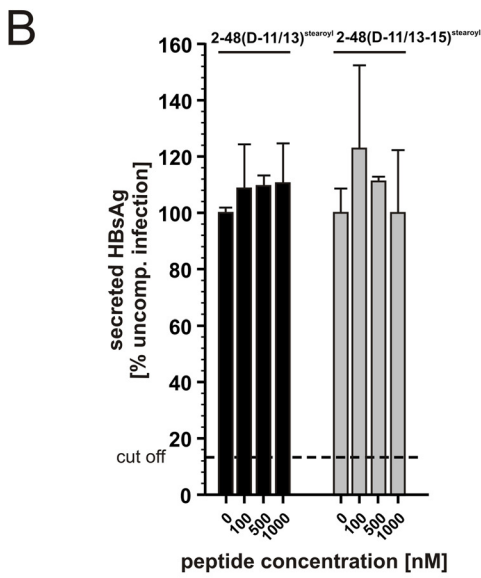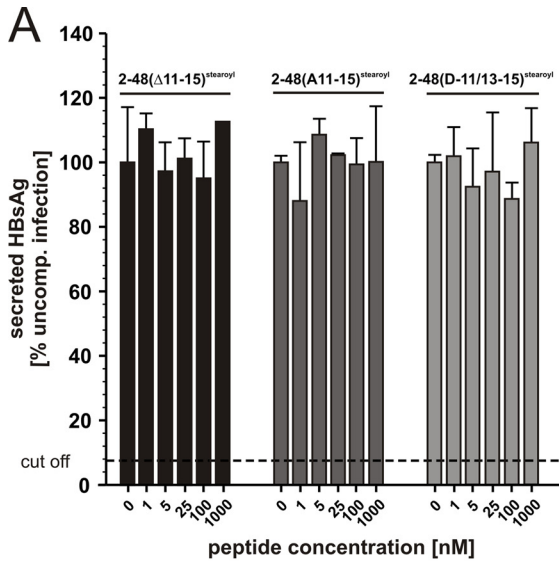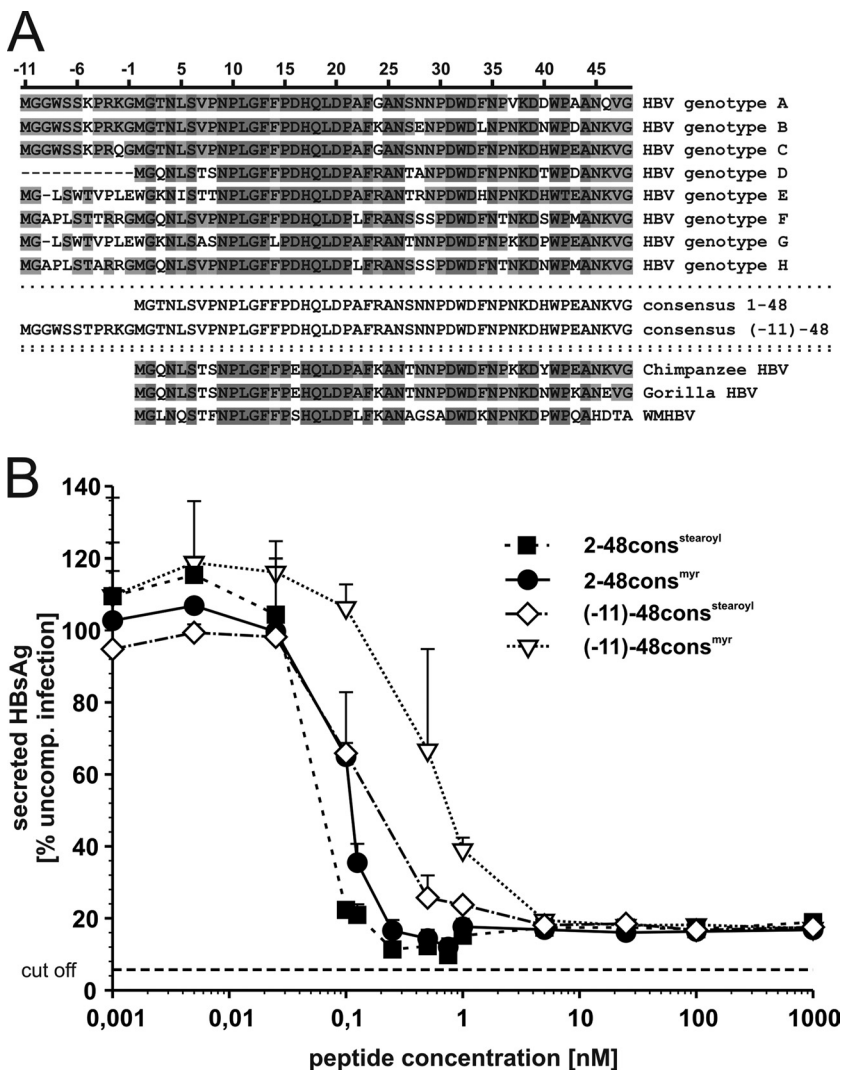
FIG. 6. Infection inhibition activity of myristoylated and stearoylated HBVpre-S1-derived peptides comprising the complete or a shortened consensus sequence. (A) Sequence alignment of the human HBV genotypes A to H and the chimpanzee, gorilla, and woolly monkey hepatitis B viruses. The short (residues 2 to 48) and long (residues −11 to 48) consensus sequences derived from HBV genotypes A to H are depicted in the middle. Identical amino acids are marked by dark-gray boxes. Nonidentical amino acids used for the consensus sequences are marked by light-gray boxes. (B) HBV infection competition using increasing concentrations (0 to 1,000 nM) of the aforementioned consensus peptides. All peptide names have been shortened due to size limitations. The amount of secreted HBsAg between days 7 to 11 p.i. was measured by ELISA and is presented as a percentage of the control infection.

different HBV genotypes (as well as other primate hepadnaviruses containing the aforementioned conserved pre-S sequence) address the same receptor, we would expect a peptide consisting of the consensus sequence of genotypes A to H to be inhibitory for a genotype D-derived virus.

The alignment of the HBV genotypes A to H (Fig. 6A) showed that, in comparison to genotype D, all other genotypes contain an additional 10 (E and G) or 11 (A, B, C, F, and H) N-terminal pre-S1 amino acids. Furthermore, all genotypes share the conserved region comprising amino acids 9 to 21.

FIG. 5. Infection inhibition activity of HBV pre-S1 lipopeptides carrying mutations in the genotypically highly conserved region 11-LGFFP-15. (A to C) HBV infection competition experiments using 0 to 1,000 nM HBVpreS/2-48(Δ11-15)^{stearoyl}, HBVpreS/2-48(A11-15)^{stearoyl}, HBVpreS/2-48(D-11/13-15)^{stearoyl} (A), HBVpreS/2-48(D-11/13-15)^{stearoyl} and HBVpreS/2-48(D-11/13)^{stearoyl} (B), or HBVpreS/2-48(L11R)^{stearoyl}, HBVpreS/2-48(G12E)^{stearoyl}, and HBVpreS/2-48(F13S)^{stearoyl} (C). All peptide names have been shortened due to size limitation. The amount of secreted HBsAg of days 7 to 11 p.i. was quantified by ELISA and is shown as a percentage of the control infection. (D) HBcAg-specific IF analyses (red) and nuclei staining (DAPI; blue) of infection competition experiments on HepaRG cells using a 250 nM or 2.5 μM concentration of the above-mentioned peptides. (E) Quantification of the IF analyses shown in panel D. In total, 143,678 DAPI-positive cells were counted. The number of HBcAg-positive cells is presented as a percentage of the untreated control.

This sequence is also present in the hepatitis B viruses isolated from chimpanzee, gorilla, and woolly monkey. Based on this, we synthesized four peptides which differ in length (58 versus 47 amino acids) and in their N-terminal modifications (stearoylation versus myristoylation). Since genotypes A, B, C, F, and H include an additional 11 amino acids and genotypes E and G have an additional 10 N-terminal pre-S1 amino acids in comparison to genotype D, the consensus peptide consists of 58 amino acids (starting at position -11 relative to genotype D sequence). This peptide, termed HBVpreS/(-11)-48cons, and a corresponding peptide which was truncated by this 11 additional amino acids (HBVpreS/2-48cons) were modified with stearic or myristic acid and tested for their inhibitory potential. HBVpreS/(-11)-48cons^{stearoyl} had an IC$_{50}$ (IC$_{50}$ ≈ 200 pM) similar to that of the stearoylated genotype-D derived peptide lacking the 11 amino acids (IC$_{50}$ of ≈ 400 pM) (Fig. 6B and data not shown). This indicates a genotype-spanning inhibitory activity and shows that insertion of additional amino acids at the amino terminus of pre-S1—in contrast to the deletions described above—is tolerated. Consistent with earlier findings (15), replacement of the authentic myristic acid by stearic acid resulted in about a 2.5-fold reduction of the IC$_{50}$ (200 to 500 pM). Interestingly, removal of the 11 N-terminal amino acids from the consensus peptides resulted in a significant (2.5- to 5-fold) increase of their activities, with IC$_{50}$s for HBVpreS/2-48cons^{myr} and HBVpreS/2-48cons^{stearoyl} of 140 pM and 80 pM, respectively. Thus, the artificially shortened consensus sequence-derived lipopeptides are more potent in inhibiting a genotype D HBV infection than the peptides with the respective authentic sequence. This again indicates that the peptides act via targeting a cellular factor. Moreover, our results define the consensus sequence-derived peptide as the most promising inhibitor for future preclinical and clinical development.

DISCUSSION

In this study we extended and refined previous investigations that characterized synthetic lipopeptides or recombinant fusion proteins derived from the N-terminal half of the pre-S1 domain of the L protein with respect to their inhibitory effect on HBV entry into hepatocytes (2, 9, 14, 15, 17, 27). Taking advantage of solid-phase peptide synthesis in contrast to production of recombinant fusion proteins as described previously (9), we were able to apply higher peptide concentrations and thus determine IC$_{50}$s of only weak inhibitors. Moreover, the opportunity to acylate at any position in the primary sequence allowed us to discriminate sequence requirements for recognition of N-myristoyltransferase from those that are important in receptor binding. Regarding the results of our mutational analyses, the N-terminal pre-S1 part could be dissected into different sequence elements. (i) Amino acids N-9, L-11, G-12, and F-13, located in a highly conserved part of the pre-S1 domain (amino acids 9 to 21), are essential for the peptide activity since deletions or amino acid exchanges lead to an abrogation of infection. Since the exchange of amino acids 11 and 13 or 11 and 13 to 15 by their respective D-enantiomers also resulted in a complete loss of activity, we have to assume a stereo-selective interaction of this "active site" of the peptide with a receptor on the hepatocyte surface. In addition to the previous data of Engelke et al., who demonstrated the impor-

tance of these amino acids using recombinant pre-S–GST fusion proteins, we could demonstrate by using higher peptide concentrations that L-11 has the highest impact on peptide activity. Following these observations, we narrowed down the active site of the peptide to the amino acid motif 9-NPLGFF P-15. (ii) The 8 amino acids following the myristoylation site in genotype D serve a specific role that exceeds the function of a simple spacer or recognition sequence for the N-myristoyltransferase. (iii) Two sequence elements (amino acids 16 to 20 and 34 to 48) fulfill accessory functions in inhibition activity while amino acids 21 to 33, containing the two major B-cell epitopes of the pre-S1 domain, serve a different role. The extent to which the two sequences with accessory function contribute to the HBV infection in the context of the pre-S1 domain of the HBV L protein in the viral particle remains to be determined. (iv) An artificial peptide consisting of the consensus sequence of all known primate genotypes is able to inhibit infection of genotype D virus with about a 5-fold increase in inhibitory activity. Besides a deeper understanding of the determinants for HBV receptor recognition, our results provide important clinical implications regarding the choice of the lead substance of an HBV entry inhibitor and the cross-genotype applicability of the substance.

Evidence for a highly specific interaction of the pre-S1 domain of the L protein with a hepatocyte receptor at some step during HBV entry comes from the observation that an HBV pre-S1 peptide with a scrambled amino acid sequence is unable to significantly interfere with infection at concentrations up to 2.5 μM (Fig. 1B and G). This excludes unspecific effects that could have been mediated by the interaction of the myristic acid with membranes. The observation that addition of the wild-type lipopeptide 16 h after the initiation of infection has no effect further confirmed that we specifically interfered with an early entry event, such as receptor recognition or membrane fusion.

The pre-S1 sequence identified here as the essential part of a more extended HBV receptor binding site is common in the different human HBV genotypes and the primate hepadnaviruses isolated from chimpanzee, woolly monkey, gorilla, and orangutan. It shows homology neither to the pre-S domain of other mammalian hepadnaviruses, e.g., from woodchucks and ground squirrels, nor to the pre-S domain of the avian hepadnaviruses. The absence of this motif in rodent and avian L proteins together with its requirement for infection (in the context of the viral L protein) and infection inhibition (as part of an inhibitory lipopeptide) leads to the hypothesis that avian, rodent, and mammalian hepadnaviruses address three different entry receptors and follow different entry pathways. For the avian viruses, it has been shown that carboxypeptidase D plays a crucial role in virus entry (21, 33). For HBV all attempts to assign a role of human carboxypeptidase D in entry were unsuccessful (S. Urban, unpublished results). The fact that myristoylated pre-S-derived peptides of duck and heron hepatitis B virus block DHBV infection of primary duck hepatocytes (35) but, on the other hand, do not substantially interfere with the human HBV infection (9, 14, 15) supports the aforementioned hypothesis. For WHV the situation has not been fully investigated. The lack of sequence homology to DHBV and HBV pre-S as well as the absence of inhibitory activity of WHVpreS/2-81^{myr} in HBV (Fig. 1B) and HDV (18) infections further

strengthens the hypothesis of different entry receptors for rodent and primate hepadnaviruses. However, taking into account that a WHV surface protein-enveloped human HDV can infect PHH (18) and HepaRG cells (unpublished results), we have to assume that these cells of human origin possess a pre-S receptor for both viruses. Since there is no report for the productive infection of PHH by WHV, we have to assume that additional restrictions besides entry are involved in the species-specific replication of hepadnaviruses. Support for this assumption comes from the additional observation that a woolly monkey hepatitis B virus (WMHBV)-derived pre-S peptide is able to inhibit HBV infection (15); also WMHBV cannot infect PHH (8). Thus, the pronounced host specificity of hepadnaviruses can only partially be explained by the presence or absence of a specific receptor. Other postentry steps may play important roles as well.

Although the precise mechanism of the inhibitory effect of the HBV pre-S1-derived peptides is not understood, there is accumulating evidence that the lipopeptides primarily address a specific target on differentiated hepatic cells. This is based on the observation that preincubation of PHH or HepaRG cells with HBVpreS/2-48^{myr} resulted in the abrogation of infection for several hours even when the peptide was absent during virus inoculation (15). Our observation that myristoylated and stearoylated peptides made up of an artificial consensus sequence show increased inhibitory activities compared to the respective peptides composed of the identical sequence present in the L protein of the infectious inoculum further supports this conclusion indirectly. Direct evidence for a sequence-specific and acyl moiety-dependent interaction with hepatocytes came from binding studies using fluorescently labeled lipopeptide conjugates (15). Using peptides with the point mutations defined in the infection inhibition experiments above, we could demonstrate specific binding of only the peptides with inhibitory activity to differentiated HepaRG cells and PHH and, surprisingly, also to primary hepatocytes of other non-HBV-susceptible species (unpublished results). It has been hypothesized that pre-S1-derived peptides block the primary attachment sites of HBV on the surface of hepatocytes since binding of L protein-enriched subviral particle preparations to primary *Tupaia* hepatocytes can be inhibited by HBVpreS/2-48^{myr} at micromolar concentrations (14). However, the lack of binding inhibition at the concentrations which are sufficient to block infection (Fig. 1C) and the observation that the IC$_{50}$s for heparin-mediated binding and infection competition are comparable (30) suggest a multistep entry process. This process may start with a low-affinity binding to cellular but not only hepatocyte-specific HSPG. It is followed by a high-affinity transfer to a hepatocyte-specific receptor that is blocked by acylated peptides in a complex manner (e.g., a dominant negative effect in the course of cooperative receptor binding). With regard to the viral particle, it is reasonable that the N-terminal pre-S sequence as part of the L protein and represented by the peptide is not freely accessible on the virus surface but may be released only after primary binding in the presence of the receptor.

Our previous observation that pre-S1-derived lipopeptides inhibit HBV infection of PHH-transplanted uPA/RAG-2 mice after subcutaneous application at very low dosing (27) opens a new therapeutic opportunity for the treatment of HBV- and HDV-infected patients. The demonstration that a peptide encompassing the consensus sequence of all known primate genotypes is able to inhibit infection of genotype D virus more potently than the respective autologous peptide provides important clinical implications regarding the choice of the lead substance of an HBV entry inhibitor. The observation that recombinant pseudotyped HBV with L proteins of genotypes B, C, E, and G are equally sensitive to inhibition with a consensus peptide (data not shown) indicates the cross-genotype applicability of the substance and strengthens the hypothesis that the peptide addresses a cellular receptor. Moreover, since the shortened versions of this sequence, HBVpreS/2-48cons^{myr} and HBVpreS/2-48cons^{stearoyl}, are the most active peptides characterized so far, they represent promising lead substances for preclinical studies and possible future clinical trials.

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