Full Paper

Inhibition of hepatitis B virus assembly with synthetic peptides derived from the viral surface and core antigens

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The long surface antigen (L-HBsAg) of hepatitis B virus (HBV) plays a central role in the production of infectious virions. During HBV morphogenesis, both the PreS and S domains of L-HBsAg form docking sites for the viral nucleocapsids. Thus, a compound that disrupts the interaction between the L-HBsAg and nucleocapsids could serve as a therapeutic agent against the virus based upon inhibition of morphogenesis. Synthetic peptides correspond to the binding sites in L-HBsAg inhibited the association of L-HBsAg with core antigen (HBcAg). A synthetic peptide carrying the epitope for a monoclonal antibody to the PreS1 domain competed weakly with L-HBsAg for HBcAg, but peptides corresponding to a linear sequence at the tip of the nucleocapsid spike did not, showing that the competing peptide does not resemble the tip of the spike.

Key Words——hepatitis B virus; inhibitors; protein-protein interaction; synthetic peptides

Introduction

Human hepatitis B virus (HBV) is the prototype of the family of *Hepadnaviridae*, which poses a major public health problem worldwide. It consists of an outer envelope derived from the host cell membrane. Embedded in the envelope are three distinct but related forms of surface antigen (HBsAg): S- (small), M- (middle) and L- (large) HBsAg. Internal to the envelope is the viral nucleocapsid. Within the nucleocapsid is the partially double stranded DNA genome of 3.2 kb which is covalently attached to the polymerase protein (P) (Ganem, 1991).

The L-HBsAg has the PreS1 domain of 108 or 119 amino acid residues (depending on virus subtype) followed by the PreS2 domain (55 residues) and the S domain (226 residues) (Heermann et al., 1984). Ostapchuk et al. (1994) showed that the PreS1, PreS2 and part of the N-terminus of the L-HBsAg are exposed on the cytosolic side of endoplasmic reticulum, suggesting an important role for these regions in virion assembly by forming docking sites for the nucleocapsids (Fig. 1). The nucleocapsid or core particle is made up of 180 or 240 subunits of core proteins (Crowther et al., 1994); serologically known as core antigen (HBcAg). Each subunit of HBcAg comprises 183 amino acids with a molecular mass of about 22 kDa, and two HBcAg subunits are linked by two intermolecular disulfide bonds (Zheng et al., 1992). Each dimer consists of a protruding spike that sticks out from the underlying shell domain (Fig. 1) (Crowther et al., 1994). Recently, Böttcher et al. (1998) showed that small peptides containing the core motif sequence LLGRMK, that bind at the tips of the core particles, block interaction with L-HBsAg in a cell-free system. Some related peptides also inhibit the production of HBV in hepatoma cells (Böttcher et al., 1998).

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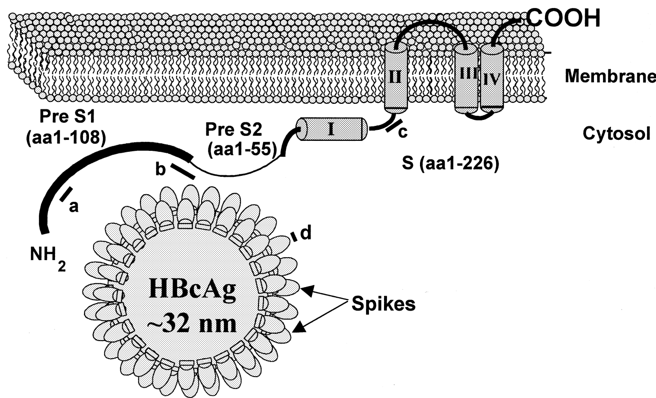

Fig. 1. Domain structure of L-HBsAg and its association with HBcAg.

a indicates the peptide sequence LDPAFR (residues 19–24 of PreS1 domain). b represents the peptide sequence PLSPPLRNTHPQAMQWNSTTF (13 C-terminal amino acids of PreS1 domain plus 8 N-terminal amino acids of PreS2 domain). c represents the peptide sequence PTSNHSPTSCPPTCPGYRWMCLRRF (residues 56–80 of S domain). d indicates the linear peptide sequence LEDPASR (residues 76–82 of subtype *adyw*) located at the tip of the spike of HBcAg. Four predicted hydrophobic transmembrane helices (Stirk et al., 1992) in the S domain are labeled I, II, III, and IV.

Earlier, Dyson and Murray (1995) showed that the synthetic peptide LDPAFR corresponding to amino acids 19–24 of the PreS1 (Fig. 1) and also the epitope for monoclonal antibody MA18/7 (Heermann et al., 1984) inhibit the association of L-HBsAg with HBcAg. Interestingly, residues LEDPASR (corresponding to residues 76–82 of subtype *adyw*) located at the tip of the HBcAg monomer (Fig. 1) show some similarities with the epitope sequence. Furthermore, this region constitutes a big portion of the immunodominant loop (residues 74–89) of HBcAg and was speculated to be involved in the interaction with L-HBsAg. It is, therefore, of interest to investigate whether the peptide LDPAFR resembles the tip, binds to a region on L-HBsAg and thus inhibits it from binding to HBcAg. In addition, two synthetic peptides corresponding to the binding regions (Fig. 1) in L-HBsAg (Poisson et al., 1997) were also synthesized and their ability to inhibit the interaction between L-HBsAg and HBcAg was studied in a cell-free system.

Materials and Methods

In vitro transcription and translation. Plasmid pMD-HBs3 (Tan et al., 1999) encoding full-length L-HBsAg was used as template in transcription reactions using the T7RNA polymerase (Promega, USA) as described (Tan and Dyson, 2000). In vitro translation was performed using the Flexi rabbit reticulocyte lysate system (Promega) in the presence of [^{35}S]-methionine as described (Tan and Dyson, 2000).

Preparation and purification of core particles. Expression of full-length HBcAg in *Escherichia coli* and purification of core particles with sucrose gradient centrifugation were performed as described previously (Tan and Dyson, 2000). Protein concentration was determined by the Bradford assay.

SDS-PAGE. In vitro translation product (1 µl) and purified HBcAg (1 mg/ml; 1 µl) were mixed with 1×loading buffer [Tris-HCl (62.5 mM, pH 6.8), SDS (2%), glycerol (10%), bromophenol blue (0.2%), and DTT (50 mM); 5 vol] and boiled for 5 min. The samples were then separated by SDS-15% PAGE at 30 mA. For the detection of [^{35}S]-L-HBsAg, the polyacrylamide gel was dried and visualized by autoradiography. For the analysis of HBcAg, the gel was stained with Coomassie brilliant blue (CBB) R-250.

Observation of core particles with electron microscopy. Purified HBcAg (0.1 µg/µl; 20 µl) was absorbed to a copper grid and stained with methylamine tungstate (2%, pH 6.5). The grid was viewed under the Hitachi H-7100 transmission electron microscope (TEM).

Inhibition of HBsAg binding to HBcAg by synthetic peptides. Purified HBcAg was diluted to 10 µg/ml with TBS buffer [Tris-HCl (10 mM, pH 8.0), EDTA (1 mM, pH 8.0), NaCl (150 mM)] and coated on U-shaped polystyrene wells (Costar's strip plates; 150 µl) by incubating them overnight at room temperature. The wells were washed three times with PBS [K_2HPO_4 (20 mM), KH_2PO_4 (5 mM), NaCl (150 mM)] and blocked with BSA (10 mg/ml; 200 µl) at 4°C for 2 h. [^{35}S]-L-HBsAg synthesized in rabbit reticulocytes was diluted 100-fold with NET-gel buffer [Tris-HCl (50 mM; pH 7.6), NaCl (150 mM), NP-40 (0.1% v/v), EDTA (1 mM), gelatin (0.25% w/v), NaN_3 (0.02% w/v), DTT (2 mM)] containing various concentrations of synthetic peptide (100 nM to 1 mM). An aliquot of these mixtures (100 µl, in triplicate) was incubated in the HBcAg coated wells for 24 h at 4°C. Then, wells were washed five times with NET-gel buffer with 10-min intervals and placed in scintillation vials for quantitation of radioactivity. Negative controls were carried out using the same conditions but the HBcAg used to coat the wells was

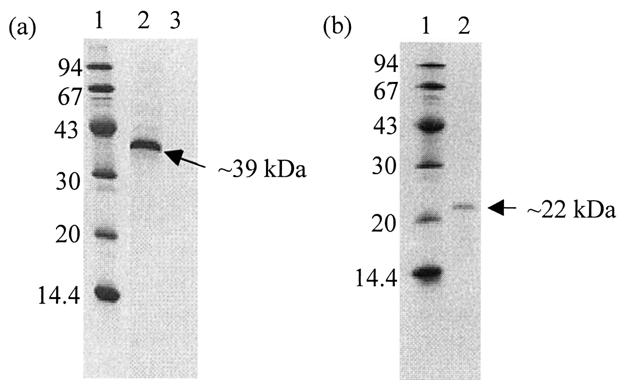

Fig. 2. SDS-PAGE of L-HBsAg and HBcAg.

(a) Lane 1: Molecular weight standards in kDa (stained with CBB). Lane 2: In vitro translation reaction mixture was separated by SDS-15% PAGE. The gel was dried and the product visualized by autoradiography after overnight exposure. The specific band (~39 kDa) is indicated by an arrow. Lane 3: Negative control was carried out with the same conditions as lane 2 but the in vitro translation reaction was performed in the absence of L-HBsAg mRNA. (b) Lane 1: Molecular weight standards in kDa. Lane 2: Purified HBcAg (1 μg) was analysed on SDS-15% PAGE and stained with CBB. The specific band (~22 kDa) is indicated by an arrow.

replaced with BSA (10 μg/ml). Peptides LDPAFR, EDPASR, LEDPASR, LLGRMKG, PLSPPLRNTHPQAMQWNSTTF and PTSNHSPTSCPPTCPGYRWMCLRRF were synthesized by ALBACHEM Limited (Edinburgh).

Results

In vitro translation of the L-HBsAg mRNA with rabbit reticulocyte lysates supplemented with [^{35}S]-methionine produced a band of about 39 kDa (Fig. 2a), which corresponds to the unglycosylated form of L-HBsAg. SDS-PAGE of the purified HBcAg gave rise to a sharp band at the expected apparent molecular mass of a monomer, about 22 kDa (Fig. 2b). Electron microscopic examination of negatively stained HBcAg revealed that the monomers associated to form particles with a diameter of 32±2 nm (Fig. 3).

Figure 4 shows the inhibition of [^{35}S]-L-HBsAg binding to HBcAg by synthetic peptides. Peptides EDPASR and LEDPASR did not inhibit the reaction. The concentrations of peptides required to inhibit 50% of the interaction (IC_{50}) are summarized in Table 1.

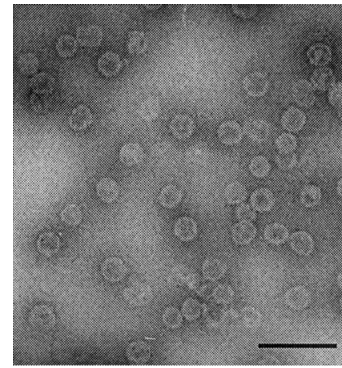

Fig. 3. An electron micrograph of the purified core particles.

HBcAg produced in *E. coli* assembled into particles. The bar on the micrograph represents 100 nm.

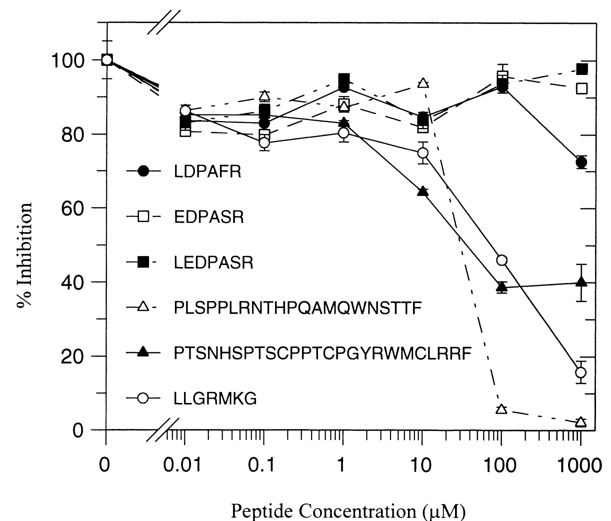

Fig. 4. Inhibition of L-HBsAg binding to HBcAg by synthetic peptides.

Constant concentration of [^{35}S]-L-HBsAg (100-fold dilution of a translation reaction) was mixed with serial dilutions of peptides ranging from 0 to 1 mM. The mixtures were then incubated on wells coated with HBcAg for 24 h, at 4°C. The wells were then washed and radioactivity determined by scintillation counting. The symbols representing the peptides are indicated in the graph. The points are arithmetic mean±standard deviation of triplicate determinations. Negative controls carried out with the equivalent reaction mixtures in BSA-coated wells showed negligible binding activities.

Discussion

HBV has a narrow host range, infecting only humans and other higher primates such as chimpanzees, but not baboons, lower primates or other mammals. Until recently, this virus could not be propagated in cell culture systems. Therefore, an in vitro assay established

Table 1. IC_{50} values of synthetic peptides that inhibit the association of L-HBsAg with HBcAg.

Peptide	IC_{50} (μM)	Description
LLGRMKG	78±5	Isolated from phage display library (Dyson and Murray, 1995). Binds to the tip of HBcAg spike (Böttcher et al., 1998).
LDPAFR	>1,000	Residues 19–24 of L-HBsAg and epitope for monoclonal antibody MA18/7 (Heermann et al., 1984).
EDPAFR	n.i.	Residues 77–82 of HBcAg. Located at the tip of HBcAg spike (Dyson and Murray, 1995).
LEDPASR	n.i.	Residues 76–82 of HBcAg. Located at the tip of HBcAg spike (Dyson and Murray, 1995).
PLSPPLRNTHPQAMQWNSTTF	30±1	13 C-terminal amino acids of PreS1 domain plus 8 N-terminal amino acids of PreS2 domain (Poisson et al., 1997).
PTSNHSPTSCPPTCPGYRWMCLRRF	35±4	Residues 56–80 of S domain (Poisson et al., 1997).

n.i. indicates no inhibition was observed at 1 mM of peptide concentration.

by Dyson and Murray (1995) provides an alternative means to study the viral protein-protein interactions (Tan et al., 1999; Tan and Dyson 2000) and to identify compounds that inhibit those interactions (Böttcher et al., 1998). In this assay, recombinant HBcAg was produced in *E. coli* because the synthesis of this protein does not require post-translational processing. HBcAg expressed in this system self-assembled into particles morphologically similar to authentic nucleocapsids isolated from infected liver. This finding accords with that reported by Cohen and Richmond (1982). Unfortunately, the other interacting component, L-HBsAg, could not be expressed efficiently in *E. coli* (Tan and Dyson, 2000); therefore the protein was produced in rabbit raticulocyte lysates and radiolabeled with [^{35}S]-methionine. Interestingly, L-HBsAg produced in this system, in the absence of microsomal membrane, was still able to interact with core particles (Dyson and Murray, 1995; Tan et al., 1999), indicating that the interaction does not require post-translational processing of the L-HBsAg such as membrane translocation (Eble et al., 1990), acylation (Persing et al., 1987) or glycosylation (Patzer et al., 1986), which occur naturally in vivo.

Electron cryomicroscopy and image reconstruction of core particles produced in *E. coli* revealed that the fundamental building block is a dimer, and each dimer consists of a 4-helix bundle, formed by the pairing of α-helical hairpins from both subunits (Böttcher et al., 1998; Conway et al., 1997). It appears that the immunodominant loop of amino acids 74–89 in the core protein (Argos and Fuller, 1988; Borisova et al., 1993; Salfeld et al., 1989) is exposed near the tip of these protruding domains. Since this region is nearest to the viral envelope, it is very likely to be involved in the interaction with the envelope proteins. Two peptides of variable lengths, EDPASR and LEDPASR that correspond to the residues at the tip of the spike were synthesized and used in the inhibition experiments, but neither the peptides inhibited the interaction even at 1 mM concentration. The peptide LLGRMKG, selected from the fusion phage display library against HBcAg (Dyson and Murray, 1995) which was included as a positive control, inhibited the interaction between L-HBsAg and HBcAg with 50% inhibition at a peptide concentration of 78±5 μM. However, peptide LDPAFR, which coincides with the epitope for monoclonal antibody MA18/7, did inhibit but with a small effect at 1 mM concentration and this amount was three-fold higher than that observed by Dyson and Murray (1995). This could be due to the fact that the reaction was incubated for 24 h rather than the 2.5 h used by them. Here, the incubation period was increased to 24 h because a time course study of the [^{35}S]-L-HBsAg binding to HBcAg showed that equilibrium was reached after 24 h at 4°C (Tan and Dyson, 2000). Clearly, the inhibition result rules out the speculation that peptide LDPAFR resembles the structure of the tip of HBcAg monomer, somehow associates with L-HBsAg and prevents it from interacting with HBcAg. It is likely that the peptide LDPAFR shares the same binding site with the sequences selected from a hexapeptide phage display library (LLGRMK, YLLRFR, LLGRFK, LLGRFR and other related sequences) (Dyson and Murray, 1995) because the first and the last two residues of the hexapeptide appear in the selected sequences. Moreover, it has been shown by Dyson and Murray (1995)

that the peptide LDPAFR inhibits not only L-HBsAg but also the phage-bearing amino acid sequence LLGRMK from binding to HBcAg.

The interactions between L-HBsAg and HBcAg involve a large area which is mediated by two interdependent binding sites; one located between residues 24 and 191, and the other between residues 191 and 322 of L-HBsAg. Separately, these sites bind with low affinities to their respective binding sites in HBcAg, but an intact complex consisting of these two sites binds to the HBcAg through high- and low-affinitiy interactions (Tan et al., 1999). In addition, point mutation studies show that the Arg residue at position 92 was particularly important for the interaction (Tan et al., 1999). Poisson et al. (1997) demonstrated that two synthetic peptides, corresponding to the 13 C-terminal amino acids of PreS1 plus the 8 N-terminal amino acids of PreS2, and amino acids 56 to 80 of the S domain bound directly to HBcAg. Again this suggests two distinct binding sites occur in L-HBsAg. Theoretically, binding of these peptides on HBcAg should disrupt the association of L-HBsAg with HBcAg. Indeed, both the 21- and 25-residue peptides showed a lower IC_{50} value compared to that of the peptide LLGRMKG. Although the length of these peptides is rather long, high resolution structural analysis of these peptides would furnish clues to the docking sites in L-HBsAg, which in turn may provide knowledge for designing therapeutic agents that block the assembly of HBV in vivo.

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