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Visualization of Hepatitis B Virus (HBV) Surface Protein Binding to HepG2 Cells

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Abstract: Viral surface proteins are known to play an essential role in attachment of the virus particle to the host cell membrane. In case of the hepatitis B virus (HBV) several reports have described potential receptors on the target cell side, but no definite receptor protein has been isolated yet. As for the viral side, it has been suggested that the preS region of the envelope protein, especially the preS1 region, is involved in binding of HBV to the host cell. In this study, preS1 region was recombinantly expressed in the form of a maltose binding protein (MBP) fusion protein and used to identify and visualize the expression of putative HBV receptor(s) on the host cell. Using laser scanned confocal microscopy and by FACS analysis, MBP-preS1 proteins were shown to bind to the human hepatoma cell line HepG2 in a receptor-ligand specific manner. The binding kinetic of MBP-preS1 to its cellular receptor was shown to be temperature and time dependent. In cells permeabilized with Triton X-100 and treated with the fusion protein, a specific staining of the nuclear membrane could be observed. To determine the precise location of the receptor binding site within the preS1 region, several short overlapping peptides from this region were synthesized and used in a competition assay. In this way the receptor binding epitope in preS1 was revealed to be amino acid residues 27 to 51, which is in agreement with previous reports. These results confirm the significance of the preS1 region in virus attachment in general, and suggest an internalization pathway mediated by direct attachment of the viral particle to the target cell membrane.

Key words: hepatitis B virus (HBV), HBsAg, PreS1, receptor.

Viruses are internalized into their host cells via some specific receptors expressed on the target cells (Tardieu *et al.*, 1982). The recognition of and attachment to these receptors are usually mediated by viral envelope proteins. Since this initial step is crucial for further endocytosis or membrane fusion, the attachment of the virus to the host cell usually determines viral tropism or pathogenesis (Szmuness *et al.*, 1980).

HBV associated viral particle has been described to bind to the human hepatoma cell line HepG2 (Hertogs *et al.*, 1993) as well as to purified human liver cell plasma membranes (Petit *et al.*, 1992). It is widely accepted that this interaction is mediated either by direct or indirect binding of the viral envelope proteins to some still unknown receptor on the host cell (Pontisso *et al.*, 1989). The envelope proteins of HBV are known as hepatitis B surface antigens (HBsAg) and consist of three kinds of related proteins in the same open reading frame, designated as the small (S), middle (M), and large (L) antigens. These are the translation products of 3 overlapping open reading frames with different initiation sites. The large L antigen, which is a minor component of the HBV envelope, has a 174 amino acid extension over the small S protein. This extended region includes the preS1 and preS2 components. Among these the preS1 domain was reported to possess a major HBV binding site, located between amino acid residues 21 and 47 (Szmuzness *et al.*, 1980; Tardieu *et al.*, 1982; Petit *et al.*, 1991). Until now no receptor for this viral ligand has been identified and isolated. To analyze the distribution and characterize the nature of the putative HBV-receptor, microscopic and fluorescence-activated cell analysis were carried out in order to visualize the binding property of preS1 on hepatic cells.

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Materials and Methods

Cell culture and preS1 ligand binding

The human hepatoma cell line HepG2 were kindly provided by C. J. Rhyu (Korea Research Institute of Bioscience and Biotechnology, Taejon, Korea). Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco). For performing binding assays, cells were harvested by trypsinization 2 days before analysis at 50% confluency of the cell culture and seeded on cover glasses to a concentration of 2×10^5 cells/well in 6 well tissue culture plates (Falcon). Analysis of MBP-preS1 binding was carried out with HepG2 cells adherent to the cover slips by incubation with MBP-preS1 fusion proteins or with MBP alone as a control. The recombinant *E. coli* transformed with the pMalpreS1-56, expressing the MBP-preS1 fusion protein was kindly provided by Dr. H. J. Hong (Korea Research Institute of Bioscience and Biotechnology, Taejon, Korea). Expression and purification of the recombinant protein as well as MBP was mainly performed as described previously (Rhyum *et al.*, 1994). Briefly, transformed cells were incubated in LBA medium and induced with 0.3 mM IPTG for 6 h. Cells were disrupted by sonication and the supernatant dialyzed against binding buffer (20 mM Tris-HCl, 1 mM EDTA, 25 mM NaCl, pH 7.4) and afterward purified by a DEAE-sepharose CL-6B column. After fractionation, the recombinant MBP-preS1 protein was identified by SDS-PAGE and the purity was determined by spectrophotometric analysis. Incubation of the ligands was carried out with differing concentrations of MBP-preS1 or MBP alone, in the presence of 2% gelatin, 1% normal rabbit serum (NRS) and 1% FBS.

Competition of preS1 binding by synthetic peptides

To characterize the actual receptor binding epitope within preS1, overlapping peptides from this region were designed and synthesized (Research Genetics, Inc., USA). These were purified using reverse phase HPLC and the purity was determined by photometric analysis. Each peptide was consisted of 15~16 amino acid residues, beginning from residue 12 of preS1 (*adr* type). Peptides were synthesized with an interval of 5 amino acid residues from the N-terminal end (Table 1). The anti-preS1, a.a. 21~47, monoclonal antibody, F35.25 (New England Biolabs, Beverly, USA) was used as positive control in the binding inhibition assays.

Laser scanning confocal microscopy of MBP-preS1 binding on HepG2 cells

Binding of MBP-preS1 or MBP ligands on HepG2 cells was carried out in the presence of 1% gelatin, 2.5% NRS and 10% FBS. Prior to this step, cells were optionally permeabilized with 2% Triton X-100, if the intracellular distribution of preS1 binding proteins was to be analyzed. Ater binding of ligands, cells were washed times in PBS and subsequently fixed by incubation in 3.7% paraformaldehyde for 30 min. Thereafter, rabbit anti-MBP antiserum (New England Biolabs, Beverly, USA) diluted 1 : 200 in PBS was added and the cells incubated for further 2 h on ice. Rabbit anti-MBP antiserum was then removed by washing three times with PBS and the probe was incubated with goat anti-rabbit-IgG-FITC for another 2 h on ice. Samples were examined by confocal laser scanning microscopy (Carl Zeiss LSM 410).

Table 1. Amino acid sequence of synthetic preS1 peptides

Peptide	Amino acid sequence
PreS(27~42)	D H Q L D P A F G A N S N N P D
PreS(32~46)	P A F G A N S N N P D W D F N
PreS(37~51)	N S N N P D W D F N P N K D Q

FACScan analysis

FACS staining were performed with each 2×10^5 cells, incubated with MBP-preS1 for different temperature or incubation times. In all cases incubation and washing were performed in the presence of 0.05% sodium-azide and 1% BSA. Binding of preS1 was visualized using rabbit anti-MBP antiserum followed by goat anti-rabbit-IgG-Texas red. Cells were analyzed using a FACScan (Becton Dickinson) and the software program LYSYS (Becton Dickinson).

Results and Discussion

Adhesion of the viral particle to the target cell membrane has been known as the initial step in internalization or penetration of the virus into the host cell. Regarding the attachment of the hepatitis B virus, there have been contradictory reports on the mechanism of cell adhesion, suggesting, on the one side, an involvement of intermediary molecules such as Interleukin-6 (Neurath *et al.*, 1992) or polymerized human serum albumin (Machida *et al.*, 1984; Yu *et al.*, 1985; Krone *et al.*, 1990; Dash *et al.*, 1991) leading to host cell attachment bridged by these mediator molecules, and on the other side, direct adhesion of the virus to the cell membrane via viral envelope proteins and some cellular receptors (Srikant *et al.*, 1992). To examine these two contradictory standpoints and thereby to evaluate the mechanism by which HBV enters the host cell, part of the HBV envelope protein was used to perform *in vitro* binding studies.

The preS1 region of the hepatitis B surface antigen

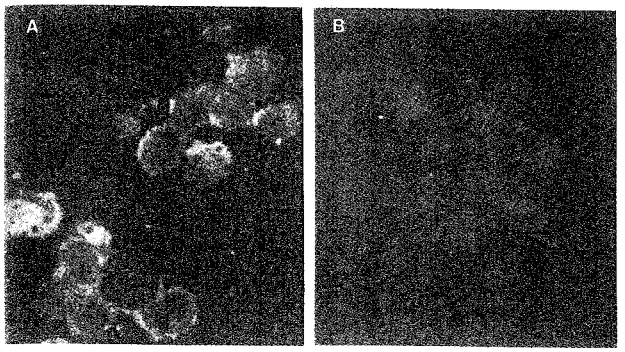

Fig. 1. Visualization of MBP-preS1 bound to HepG2 cell surface by laser scanning confocal microscopy. A : HepG2 cells incubated with MBP-preS1, B : HepG2 cells incubated with MBP alone.

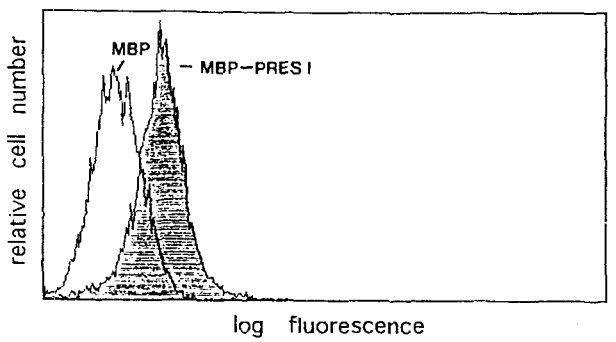

Fig. 2. FACScan analysis of MBP-preS1 binding on HepG2 cells. Histogram shows MBP-preS1 binding on HepG2 cells detected with rabbit anti-MBP antiserum and goat anti-rabbit-IgG-Texas red as described in materials and methods.

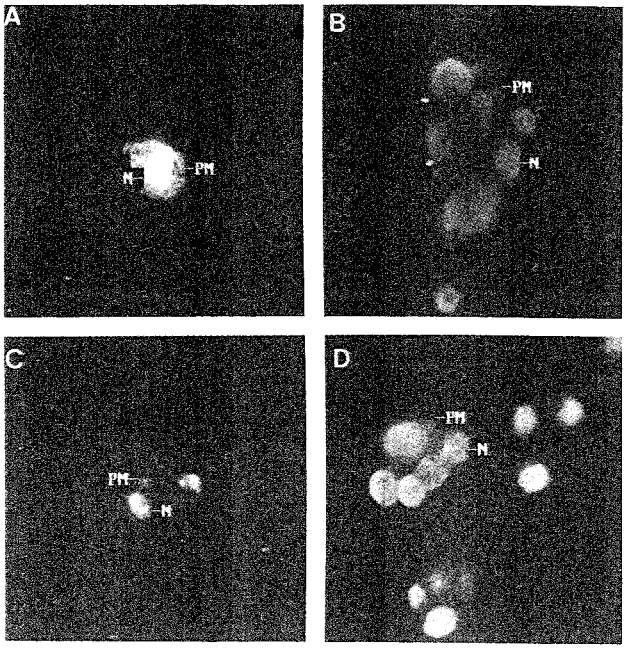

Fig. 3. Intracellular distribution of MBP-preS1 in HepG2 cells after permeabilization with Triton X-100. Position of the nucleus as well as the cell morphology for determination of the cell boundaries were analyzed by phase-contrast microscopy prior to fluorescence microscopy. A : HepG2 cells incubated with MBP-preS1 at 4℃ for 5 min, B : HepG2 cells incubated with MBP-preS1 at 4℃ for 5 min and thereafter shifted to 37℃ for 20 min, C : HepG2 cells incubated with MBP-preS1 at 4℃ for 15 min, D : HepG2 cells incubated with MBP-preS1 at 4℃ for 15 min and thereafter shifted to 37℃ for 20 min. PM : plasma membrane, N : nucleus.

(HBsAg), especially the a.a. 21~47 sequence, has been previously described to be the major part involved in HBV binding on hepatocytes (Neurath *et al.*, 1986). In order to visualize this specific interaction, in this study, the human hepatoma cell line HepG2 was used as target cells for preS1 binding. Part of the preS1 region containing the sequence 21~47 has been previously expressed as a recombinant protein fused to the maltose binding protein (MBP-preS1) (Rhyum *et al.*, 1994). In all assays performed, recombinant MBP from the same expression vector was used as negative controls. The specific binding of the preS1 region to HepG2 cells could be confirmed and visualized by confocal laser scanning microscopy as shown in Fig. 1. This binding was specific to HepG2 cells, since staining experiments under the same conditions but with the human fibroblast cell line, MRC5, showed no signals at all (data not shown). Since the binding conditions included neither interleukin-6 nor polymerized human serum albumin (see material and methods), it seems to be that this ligand binding is independent of additional intermediary molecules, indicating a binding mechanism of direct interaction between the virus and the host cell. Thus the major pathway for entry of HBV seems to be through a direct attachment rather than by mediatory molecules.

In order to analyze further the preS1 binding mechanism, HepG2 cells were incubated with the ligands as described above and the binding kinetics were carried out at different time points by FACS analysis. Fig. 2 shows that the time dependent binding behavior of preS1 on HepG2 cells reaches saturation after 6 h. Since incubation at the same conditions but at lower temperature (4℃) shows no saturation even after 6 h (data not shown), binding of preS1 is also dependent on incubation temperature.

To investigate the intracellular localization of MBP-preS1, at next, HepG2 cells were permeabilized with Triton X-100 and then incubated either with MBP-preS 1 or MBP alone. As shown in Fig. 3, MBP-preS1 could be detected in the cytoplasm as well as in the nuclei of these cells. When an additional incubation step was performed by transferring these cells from 4℃ to 37℃, there was a clear shift of the ligand binding site into the nuclei. These results demonstrate that preS1 binds to the nucleus and the rate of preS1 accumulation on the nuclear membrane could be accelerated by a temperature shift to 37℃. At this time it is not clear

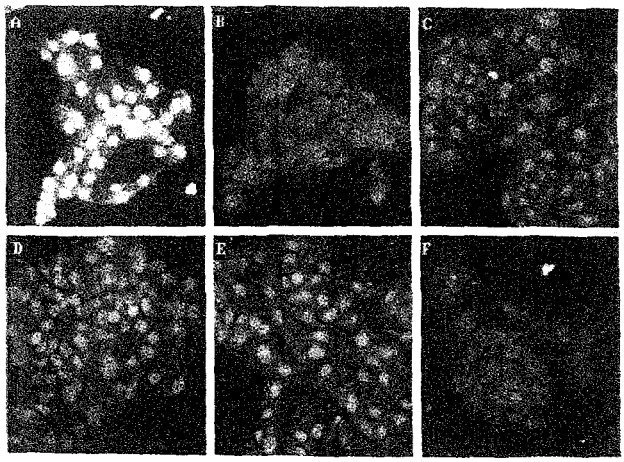

Fig. 4. Synthetic peptides of the preS1 region inhibit binding of MBP-preS1 on HepG2 cells. A: MBP-preS1 (1 μg/ml), B: MBP (1 μg/ml), C: MBP-preS1 (1 μg/ml)+preS1 (27~42) (100 μg/ml), D: MBP-preS1 (1 μg/ml)+preS1 (32~46) (100 μg/ml), E: MBP-preS1 (1 μg/ml)+preS1 (37~51) (100 μg/ml), F: MBP-preS1 (1 μg/ml)+F 35.25 monoclonal antibody (10 μg/ml).

if the receptor proteins on the nuclear membrane are the same as on the plasma membrane, but these data are in agreement with previous reports where a temperature dependency of internalization of the viral particle has been observed (Qiao *et al.*, 1994; Bruin *et al.*, 1995).

Since binding of the MBP-preS1 fusion protein could not be analyzed by a biological assay system, the specificity of the binding reaction as well as mapping of the precise binding site on preS1 by HepG2 cells had to be determined directly by competition, blockade, or saturation of the receptor. To do this, a range of short peptides corresponding to segments of the preS1 region were synthesized and used in a competition binding assay with MBP-preS1 ligands on HepG2 cells. Three peptides (see Table 1) which had a length of 15~16 amino acid residues and covered in an overlapping fashion amino acid residues 27 to 51 from the preS1 region were synthesized.

Fig. 4 shows the result of competitive binding between preS1 and each of the synthetic peptides. As in the case of competition with a monoclonal antibody against the preS1 21~47 region (F35.25), coincubation with each of these peptides resulted in a dramatical loss of fluorescence intensity. Since these peptides were also revealed to be the most effective preS1 binding inhibitors among a large set of peptides derived from the whole preS1 region in an independent binding inhibition assay (Lee *et al.*), it is obvious that the ligand binding site on the putative HBV receptor on HepG2 cells is masked by these peptides and therefore can inhibit the interaction of the virus. Taken together these data suggest that the preS1 region which interacts with the putative HBV receptor spans amino acid residues 27~51, confirming the results from previous reports (Neurath *et al.*, 1986; Pontisso *et al.*, 1989, 1990; Petit *et al.*, 1987, 1988, 1989, 1991), where amino acid residues 21~47 had been described to be the major binding site on preS1 (Petit *et al.*, 1992; Dash *et al.*, 1992).

As visualized here by confocal microscopy, cell attachment of HBV seems to be a process mediated by direct interaction of cell surface receptors and the preS1 region. It is therefore likely that the peptide sequences evaluated in this study could act as antagonist in HBV adhesion and thus can be used in *in vivo* experiments or in clinical application for prevention of further infection of cells by the virus.

The identity of the receptor proteins on HepG2 cells could not be solved in this study. But it could be concluded that there is a constant and uniform expression of preS1 receptors on HepG2 cell plasma membrane as well as on nuclear membranes. Attempts to isolate these proteins by immunoprecipitation are under way and isolation of these proteins and at least a partial amino acid sequence analysis will lead to further precise characterization of the mechanism of HBV infection.

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