A Cell-based Method to Monitor the Interaction between Hepatitis B Virus Capsid and Surface Proteins

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Interactions between the surface and capsid proteins of the hepatitis B virus (HBV) are critical for the assembly of virus particles. In this study, we developed a cell-based method to visualize the interactions between the capsid and surface proteins of HBV. Capsid-GFP, a capsid protein fused to a green fluorescence protein (GFP), forms nucleocapsid-like structures in the cytoplasm of mammalian cells. It relocates to the plasma membranes in cells expressing PH-PreS, a fusion protein consisting of the PreS region of the HBV surface protein and the PH domain of PLC-γ. Membrane localization of the capsid-GFP in these cells is prevented by an inhibitory peptide that blocks the interaction between the capsid and surface proteins. This dynamic localization of capsid-GFP is applicable for screening compounds that may potentially inhibit or prevent the assembly process of HBV particles.

Key Words: HBV, PreS, Capsid, Interaction

Introduction

HBV is small enveloped DNA virus that belongs to the hapadnavirus family, and its genome contains 4 genes that encode for the capsid protein, polymerase, surface protein and protein X. HBV capsid proteins self-assemble and form icosahedral nucleocapsid structures that harbor viral DNA and polymerases. The viral envelope, which consists of large (L), medium (M) and small (S) surface proteins translated from the single S gene, surrounds the nucleocapsid to form the mature HBV particle. The nucleocapsids assemble in the cytosol of infected cells, and the envelopment process of the nucleocapsid is initiated by an interaction between the nucleocapsid and the surface proteins expressed at the membrane of the host cell endoplasmic reticulum (ER). These processes are essential for the generation of mature infectious HBV particles, and proteins involved in this process have been considered as targets for the development of antiviral agents.[1] Among the three surface proteins, the L protein has been shown to be essential for the surface-capsid interaction, and the PreS region located at the N-terminus of the L protein is critically important for both the assembly process[2-4] and attachment to the host cell during infection.[5] Newly synthesized L proteins will localize to the membrane of the ER, and the PreS region will be oriented towards the cytoplasmic face of the membrane.[4,6] The envelopment process is initiated by the interactions between the nucleocapsids and the L protein in ER membrane,[7] and the enveloped virus particles are then exported through secretion pathway.[7] The interactions between the capsid and surface proteins have been previously studied using mutagenic analysis[8-11] and by directly measuring the interactions between the two proteins.[12-14] The central region of PreS was found to be critical for the envelopment process[9], and the surface protein interaction sites on the capsid protein have been mapped to the tip of the capsid dimer as well as to the basic residues at the base of the dimer.[15]

The assembly process of nucleocapsids and intact virus particles have been investigated with the goal of identifying novel antiviral agents. Antibodies against the HBV capsid protein,[16] capsid-specific aptamers,[17] immunosugars that prevent pregenomic RNA packaging,[18,19] and peptides that interfere with the envelopment of the nucleocapsid[20,21] have been reported. The assembly of HBV capsid proteins was effectively measured using a fluorescence-labeled capsid protein.[22] The envelopment of nucleocapsids was also investigated as a novel target site for anti-HBV agents. The surface proteins are critical for the assembly and export process of the virus[23], and blocking the interaction between the surface and capsid proteins was shown to abolish the production of an infectious virus.[20] Recombinant capsid protein and the PreS domain of the L protein have been used to characterize the interaction between the surface and capsid proteins,[12,14] and compounds that block these interactions were shown to inhibit the amplification of HBV particles in cells transformed with the HBV genomic DNA.[24]

Here, we developed a cell-based assay method to visualize the critical step in the envelopment process of HBV. The interaction between the surface and capsid proteins of HBV has been monitored using recombinant capsid-GFP and PH-PreS fusion proteins in mammalian cells. Localization of the cytosolic capsid-GFP to the plasma membrane by PH-PreS was readily detected by fluorescence microscopy.

Materials & Methods

Materials. *Escherichia coli* strain DH5α was used to amplify the plasmids. *E. coli* BL21(DE3) was purchased from Novagen (USA) and used to express Trx-PreS and HBcAg. Luria Broth medium and agar, obtained from Merck (Germany), was used as the growth media of *E. coli*. All restriction enzymes used for DNA digestion were obtained from New England Biolabs (USA). Isopropyl-β-D-thiogalactopyrano-

side (IPTG) was obtained from Sigma (USA). TAT-PS peptides were purchased from A&PEP Co. (Korea), and the molecular weight of the purified peptide was confirmed by mass analysis.

Plasmid construction. The DNA sequence used to construct the capsid and PreS expression vectors was previously reported.[14] The coding region of the capsid protein was amplified from the pHBcAg plasmid by the polymerase chain reaction and inserted into the *Sal*I and *Kpn* I restriction sites of a pEGFP-N1 expression vector (Clontech, USA) to generate pN1-HBcGFP. The DNA sequence encoding capsid-GFP was amplified from the pN1-HBcGFP, restricted by *Not*I and *Xho*I and inserted at the *Not*I-*Xho*I restriction sites in the pBud-CE 4.1 plasmid (Stratagene, USA) to generate the pHBcGFP plasmid. The DNA sequence encoding the PreS region of the HBV surface protein (amino acid 1-163) was amplified from pTrx-PreS[14] and fused at the N-terminus of the PH domain (amino acid 2-175) from mouse PLC-δ (Genebank ID:241276) by overlapping PCR. The DNA sequence encoding the PreS-PH fusion protein was restricted by *Hind*III and *Xba*I and inserted at *Hind*III-*Xba*I restriction sites in the pHBcGFP vector. The resulting plasmid, pHBsPH-HBcGFP, was designed to express capsid-GFP and PreS-PH fusion proteins under the control of the $P_{EF\text{-}1\alpha}$ and P_{CMV} promoters, respectively. Deletion of 26 amino acids located in the middle of the PreS region (Δ93~117 of PreS) in pHBsPH-HBcGFP was introduced to generate the pΔHBsPH-HBcGFP construct. The sequence of the constructed plasmids was verified by sequencing.

Cell culture, transfection and fluorescence microscopy. Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen, USA). HEK293T cells were transfected with plasmid DNA using the Lipofect AMINE and PLUS reagents (Invitrogen) according to the manufacturer's protocol. After transfection, the cells were incubated at 37 °C for about 48 h. Fluorescence images of the transfected 293T cells were acquired using a Deltavision microscope system (Applied Precision, USA), consisting of an Olympus IX70 inverted microscope with an Olympus PlanApo 60×, 1.40-numerical aperture oil immersion objective and Photometric CH300 charge-coupled device camera. Images were deconvoluted and analyzed off-line using Softworx acquisition and analysis software (Applied Precision).

Preparation of HBcAg and Trx-PreS and measurement of their interaction. Purification of Trx-PreS and HBcAg proteins and measurement of their interaction were previously described.[14] Briefly, 100 μl of the Trx-PreS protein (10 μg/mL) in buffer-A (50 mM sodium phosphate, 0.15M NaCl, pH 8.0) was incubated in a 96-well medium binding plate (Corning, USA) for 1 hr at room temperature. After blocking the plate with 5% (w/v) skim milk in buffer-A, HBcAg protein (final 0.4μM) were added to Trx-PreS coated 96-well plate. The plate was incubated with 100 μL of an anti-HBcAg antibody (1:2000, KOMA biotechnology), and then with an HRP-conjugated anti-rabbit IgG (1:2000, Sigma). Then the amount of bound HRP was measured by incubation with 100 μL of OPD (1mg/ml) in a stable peroxide substrate buffer (Pierce), and the absorbance at 490 nm was measured using a Spectra Max340 spectrophotometer (Molecular Devices Corp.).

Size exclusion chromatography. The size of capsid-GFP expressed in 293T cells was determined using size-exclusion chromatography. The 293T cells expressing core-GFP were harvested and lysed in 20 mM Tris-HCl, 150 mM NaCl, 1% NP-40 and 1 mM PMSF, pH 7.2. The lysate was loaded onto Superdex 6 10/300 GL column (Amersham, USA), and the proteins were eluted using a fast flow liquid chromatography system (Amersham, USA) in PBS buffer (100 mM Na-PO_4, pH 7.4, 150 mM NaCl). The fluorescence intensity of eluted fractions was measured using a multi plate leader Triad LT (DYNEX Technologies,USA).

Correlated fluorescence-atomic force microscopy (AFM) imaging of the capsid-GFP particles. The high-molecular weight fractions of capsid-GFP particles purified by size exclusion chromatography were analyzed using a spatially correlated fluorescence-AFM technique. Capsid particles deposited on cover glass in PBS buffer were first identified by observing the GFP fluorescence in the total internal reflection (TIR) mode (Melles Griot Ar^+ laser 488 nm, Olympus oil-immersion objective of NA 1.45, Kaiser super-notch filter), and subsequently their sizes were determined by using spatially correlated AFM (Veeco Bioscope/Multimode, USA) in tapping mode.

Results

Cytosolic capsid-GFP moves to plasma membrane in the presence of PreS-PH. Using the techniques outlined in "Materials and Methods" section, we successfully constructed mammalian vectors that can express either the capsid-GFP or both the capsid-GFP and the PreS-PH (Fig. 1). The coding sequences of capsid-GFP or PreS-PH in the expression vectors were under the control of strong mammalian promoters, P_{CMV} or $P_{EF\text{-}1\alpha}$, respectively. When HEK293T cells were transfected with the pHBcGFP plasmid, the expressed capsid-GFP was mainly found in cytoplasm (Fig. 2A). This localization of the capsid-GFP was expected since it did not contain any particular targeting signals. However, when the capsid-GFP was co-expressed with PH-PreS (pHBsPH-HBcGFP), the capsid-GFP was primarily localized in the plasma membrane (Fig. 2B). The PH domain has high affinity to inositol phosphate group of phosphatidylinositols.[25] and

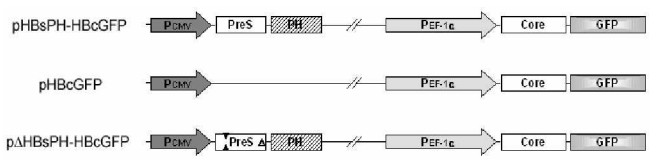

Figure 1. Schematic representation of the plasmid constructs. All of these plasmids have both strong CMV promoter (dark grey arrow) and weak EF-1α promoter sequences (light grey arrow). PreS and core were fused to the PH domain (hatched box) of PLC delta and GFP (shaded box), respectively. PreS-PH was inserted after the CMV promoter in order to express higher levels of this protein. In contrast, core-GFP was expressed using the EF-1α promoter. The deletion mutant of PreS (Δ93~117 of PreS: pΔHBsPH-HBcGFP) was used as a negative control, since it cannot interact with the core protein.

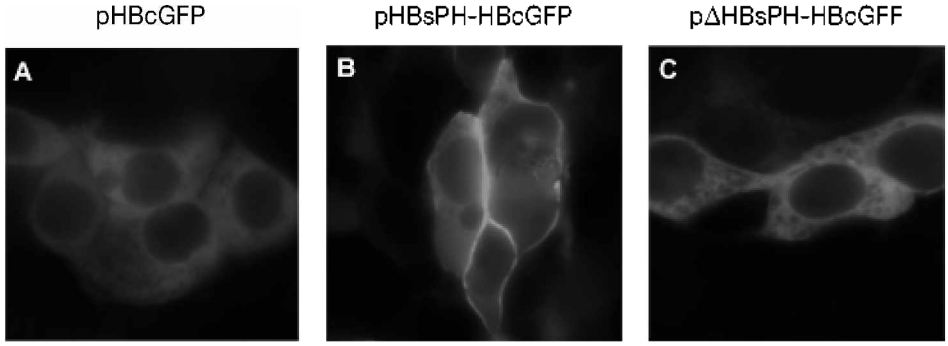

Figure 2. Localization of core-GFP protein in HEK293T cells. HEK293T cells were transfected with the pHBcGFP (A), pHBsPH- HBcGFP (B) and pΔHBsPH-HBcGFP (C) plasmids, and observed by fluorescence microscopy using a GFP filter (excitation/ emission 490/528 nm)

proteins containing PH domain are localized in plasma membrane.[26] These results indicate that the interaction between capsid-GFP and PH- PreS was sufficient to relocate the capsid-GFP to the plasma membrane. To verify that the membrane localization of capsid-GFP was due to interactions with the PreS-domain of PreS-PH, the localization of capsid-GFP was investigated in the presence of a mutant PreS-PH that had 26 residues critical for the interaction with the capsid protein deleted. In the cells transfected with pΔ HBsPH-HBcGFP (Fig. 1), capsid-GFP was detected mainly in the cytosolic space (Fig. 2C). These results indicate that the specific interaction between the capsid protein and the PreS region of L protein was sufficient to target capsid-GFP to the host cell membrane.

Capsid-GFP in HEK293T cell assembles into a high-molecular weight form. To examine whether the capsid-GFP expressed in HEK293T cell could form particle-like structure, the cell extract from HEK293T cell transfected with pHBc GFP was separated using gel-permeation chromatography, and the fluorescence of GFP from the elution fractions was monitored. As shown in Fig. 3A, a major fluorescence peak appeared near the exclusion limit of the column, which corresponded to a molecular weight of 3,000-5,000kDa, indicating that a majority of the capsid-GFPs expressed in HEK293T cells formed high-molecular weight assemblies. There were two minor peaks at the 100 and 700 kDa positions, and the fluorescent intensity of these minor peaks was less than 10% of the major peak at 3000-5000 kDa. These minor peaks are assumed to correspond to dimeric or oligomeric forms of capsid-GFP. These results indicate that majority of the expressed capsid-GFP in HEK293T cells may form nucleocapsid-like structure.

Capsid-GFP forms nucleocapsid like structure. To determine whether these high-molecular weight capsid-GFP assemblies have nucleocapsid-like structures, the size of these assemblies were examined using a spatially correlated TIR-AFM technique. Fluorescence spots on a cover glass containing the capsid-GFP assemblies were easily detected by 488 nm excitation in TIR mode (Fig. 3B). The fluorescence spectra of the individual spots showed the typical GFP fluorescence spectrum with a peak emission around 520 nm (Fig. 3C), indicating that these spots corresponded to the capsid-GFP assemblies. When the same surface was examined using spatially correlated AFM in tapping mode, various particles with heights that ranged from a few nm to ~50 nm were observed (Fig. 3D). Among them, only a few particles were spatially correlated with the GFP fluorescence spots observed in the TIR mode (Fig. 3B, D), and their heights ranged from 12 nm to 30 nm. The fluorescence-inactive particles may represent ribosomes or small vesicles that eluted in earlier fractions from gel-permeation chromatography. These results indicate that these spatially correlated particles are the nucleocapsid-like structure consisted of capsid-GFPs.

Inhibitory peptide of capsid-PreS interaction disrupts the membrane localization of capsid-GFP. To examine whether the localization of capsid-GFP to the host cell membrane in cells expressing PreS-PH could be disrupted by a peptide that

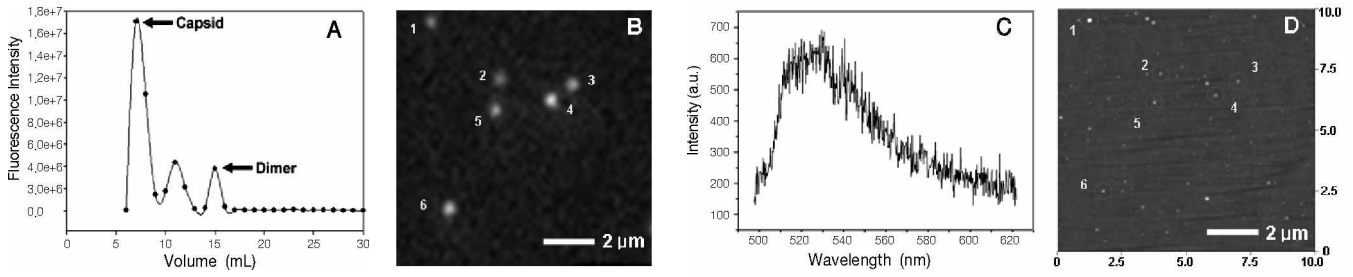

Figure 3. Size exclusion chromatogram and correlated fluorescence-AFM images of core-GFP expressed in HEK293T cells. (A) pHBc GFP was transiently transfected into HEK293T cells and expressed core-GFP was recovered from the cytosol fraction. Assembly of core-GFP was analyzed by size exclusion chromatography and fluorescence. The chromatogram peaks corresponding to the assembled capsid and dimeric form are indicated by the arrow; (B) TIR images of fluorescent core-GFP particles with 488 nm excitation; (C) Typical fluorescence spectrum of individual spots, indicating GFP fluorescence; and (D) Tapping mode AFM height image of the same area as in (B). The heights of six fluorescent particles, labeled with numbers 1 to 6, were 30.7, 9.0, 11.0, 9.9, 13.4, 13.0 nm, respectively.

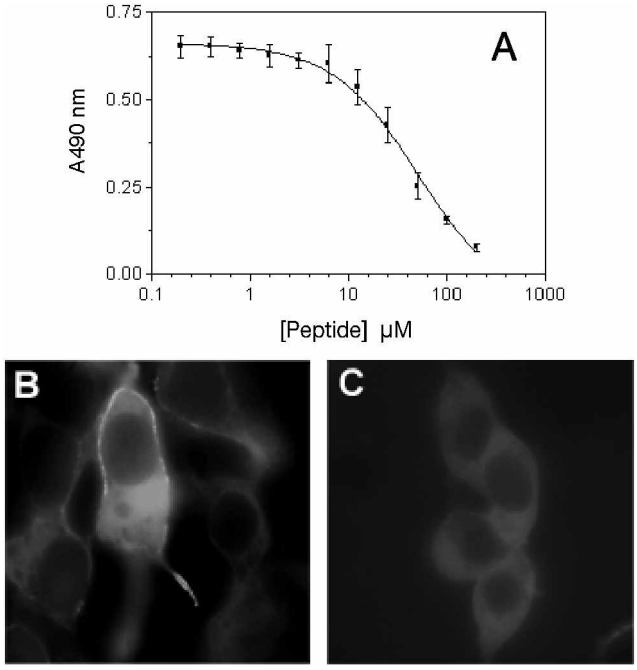

Figure 4. Inhibition of the interaction between PreS and core by TAT-PS peptide. (A) *In vitro* inhibition assay of TAT-PS peptide derived from PreS was performed by ELISA. PreS domain coated plates were incubated with the core domain and various concentrations of TAT-PS peptide. The interaction of PreS and the core domain was detected and measured using an anti-HBcAg antibody. (B, C) Cells expressing PreS-PH and core-GFP were incubated with (C) or without (B) 50 μM of TAT-PS peptide and observed by fluorescence microscopy using a GFP filter.

prevents the interaction between capsid and PreS domain, a synthetic peptide (TAT-PS), which consists of an arginine-rich membrane permeable sequence from the HIV-1 Tat protein[27] linked to the critical residues of PreS (amino acids 92-113) was prepared. Inhibition of the interaction between capsid and PreS by the TAT-PS peptide was examined using recombinant proteins. Previously, we showed that capsid particles bound specifically to PreS with a K_D value of 10 μM.[14] The amount of capsid protein that was bound to immobilized Trx-PreS decreased as the peptide concentration increased, and the IC_{50} value of the peptide was determined to be 51 μM (Fig. 4A). Then, the effect of TAT-PS peptide on the HEK293T cells transformed with pHBsPH-HBcGFP was examined. The capsid-GFPs are observed in the plasma membrane of the transformed HEK293T cells (Fig. 4B). When cells transformed with pHBsPH-HBcGFP were incubated with 50 μM of TAT-PS peptide, however, the expressed capsid-GFPs were dispersed in the cytoplasm in more than 60% of the HEK293T cells (Fig. 4C). Cellular images of capsid-GFP at higher concentrations of the peptide could not be obtained due to the cytotoxic effect of the peptide at concentration higher than 100 μM (data not shown). It is worth noting that the peptide concentration found to prevent membrane localization by more than 60% was in a similar range to the IC_{50} value against the interaction between capsid-GFP and PreS-PH in mammalian cells. These results indicate that the membrane localization of capsid-GFP in cells expressing PreS-PH could be disrupted by compounds that disrupt the interaction between the capsid and surface proteins or the envelopment process of HBV.

Discussion

The maturation of HBV nucleocapsid particles in infected cells is initiated by specific interaction between the capsid protein and the PreS region of L protein at the surface of ER membrane. In the present study, we have visualized the interaction of HBV proteins using capsid-GFP and PreS-PH expressed in mammalian cells. The capsid protein of HBV forms nucleocapsid-like structures when it expressed in *E. coli*[28] or mammalian cells.[29] Introduction of GFP in the protruding tip region of the capsid dimer generated nucleocapsid-like particle structures that were surrounded by the GFP motif.[30] The effect of inserting a foreign protein at the C-terminus of the capsid protein on the formation of nucleocapsid-like particle has not yet been reported. In this study, the capsid-GFP formed nucleocapsid-like structure in the transfected cells. The dimension of capsid-GFP was measured as 12 to 30 nm by AFM, which is slightly smaller than nucleocapsids of HBV.[29] It should be noted that the size of biomolecular particles in AFM measurement can be reduced by up to ~20% of the real size due to tip and surface interactions. The apparent size of high-molecular weight assembly of capsid-GFP expressed in mammalian cells indicated that it could forms nucleocapsid-like structure.

Specific interactions between the capsid and L proteins are essential for the assembly of HBV particles. The capsid protein of HBV expressed in *E. coli* formed nucleocapsid structure, and this structure specifically interacted with the PreS region of L protein.[14] Particularly, the central region of PreS (amino acid reside 92-113 of large surface protein) is critical for its interactions with the capsid protein, since the mutant L protein deleted in this region fails to interact with the capsid protein.[9] Furthermore, a synthetic peptide representing the central region of PreS was shown to inhibit the assembly of HBV particles and prevent HBV proliferation.[12] Likewise, capsid-GFP can interact with PreS-PH presented in the plasma membrane of transfected cells, and this interaction is sufficient to relocate the capsid-GFP in cytoplasm to plasma membrane (Fig. 2). In addition, the central region of PreS is critical for the interaction with capsid-GFP (Fig. 2C), and TAT-PS peptide, which representing the central region of PreS, effectively prevents the interaction between capsid and PreS protein (Fig. 4C).

In summary, we have visualized the interactions between HBV capsid proteins and the PreS region of HBV surface proteins using dynamic localization of capsid-GFP in mammalian cells expressing PreS-PH. The PreS-dependent distribution of capsid-GFP was readily detected using fluorescence microscopy, and this method could be exploited to screen anti-HBV agents.

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