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Microarray-Based Analysis for the Interaction between the Envelope Protein and the Core Protein of Human Hepatitis B Virus

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Abstract

In virion release of human hepatitis B virus (HBV) from hepatocytes, assembly of the viral particles involves the interaction of the preformed nucleocapsids with viral envelope proteins. As the core protein of nucleocapsids binds strongly to the pre-S region of the large envelope protein, the inhibition of such process is expected to prevent infectious viral production. In this study, we describe a protein microarray system for directly monitoring interactions between the core protein and the pre-S domain. Employing a fluorescently-labeled peptide derived from the pre-S region, we have developed a quantitative method based on fluorescence detection, which measures its interaction with the core protein. The applicability of the method has been demonstrated by performing competitive interaction studies with the pre-S protein. Therefore, the developed microarray-based interaction analysis would serve as a screening platform for developing anti-HBV agents.

Keywords: Microarray, HBV, Pre-S, Core protein, Fluorescently-labeled peptide, Protein-protein interaction, Competitive inhibition

Introduction

Hepatitis B virus (HBV) is a major viral agent that infects chronically over 350 million people throughout the world and causes acute and persistent liver diseases and hepatocellular carcinoma[1]. HBV consists of a partially double-stranded 3.2 kb circular genome encapsidated in a nucleocapsid, and a viral polymerase surrounded by a membraneous envelope[2]. The viral nucleocapsid is made up of approximately 100 copies of homodimers of the core protein (HBcAg), and the viral envelop contains three surface proteins known as long (L), medium (M), and short (S) envelop proteins. While the M envelop protein has an N-terminal extension termed pre-S2 following the 226 amino acid residues of the S protein, the additional pre-S1 domain is present at the N-terminus of the L envelop protein, which is thus constituted with Pre-S1, Pre-S2 and S regions[3,4]. Assembly of viral particles starts at the endoplasmic reticulum where the surface proteins are incorporated into the virion envelop[3-5]. In addition, virions bud into empty subviral particles in a nucleocapsid-independent manner. This complex and distinctive morphogenesis of HBV is not yet completely under-stood, but interactions between the core protein and the surface proteins of HBV are critical for virus formation[3]. Subsequent studies have also revealed that pre-S1, pre-S2 and cytosolic domains of the L envelope protein interact with the core particles[6,7]. Since the peptides that interfere with the interaction of the envelope protein with the core protein by directly binding to the core protein have been shown to prevent the production of HBV in cell cultures[8], the specific interaction between the HBV surface protein and the core protein would serve as a target for developing anti-HBV drugs[9]. The *in vitro* assessment of the interaction between viral proteins have also been accomplished by using an enzyme-linked immunosorbent assay (ELISA)[10], which has been successfully utilized for identification of inhibitory compounds[11].

Since the introduction of the idea of highly sensitive microarrays in the 1980s, microarray technology has become one of the principal platform technologies for the high-throughput analysis of biological systems[12]. Although DNA microarrays have changed the field of biomedical sciences over the past 10 years, antibody and other protein microarrays have been advanced at a much slower pace owing to the diversity and complexity of proteins. Only recently, protein microarrays have emerged as a powerful tool to complement DNA microarrays as rapid identifica-

tion of novel drug targets and increased assay throughput are required in the pharmaceutical industry. Microarray-based methods have several advantages including high detection sensitivity, high-throughput, low consumption of samples, and attractive manufacturing costs, thus allowing simultaneous, multi-parametric analysis of complex protein mixtures. Protein microarrays have tremendous potential as a tool for elucidation of interaction partners, modification sites and enzyme substrates as well as for rapid identification of protein binding to a particular drug[13]. Furthermore, protein microarrays have proven to be an efficient tool in profiling of diseases such as cancer[14]. They have also demonstrated the ability to measure the absolute concentration of small molecules, and to substitute the traditional assay method such as ELISA[13].

The present study was designed to devise a microarray as a screening platform for the development of anti-viral agents by targeting at the viral protein inter-

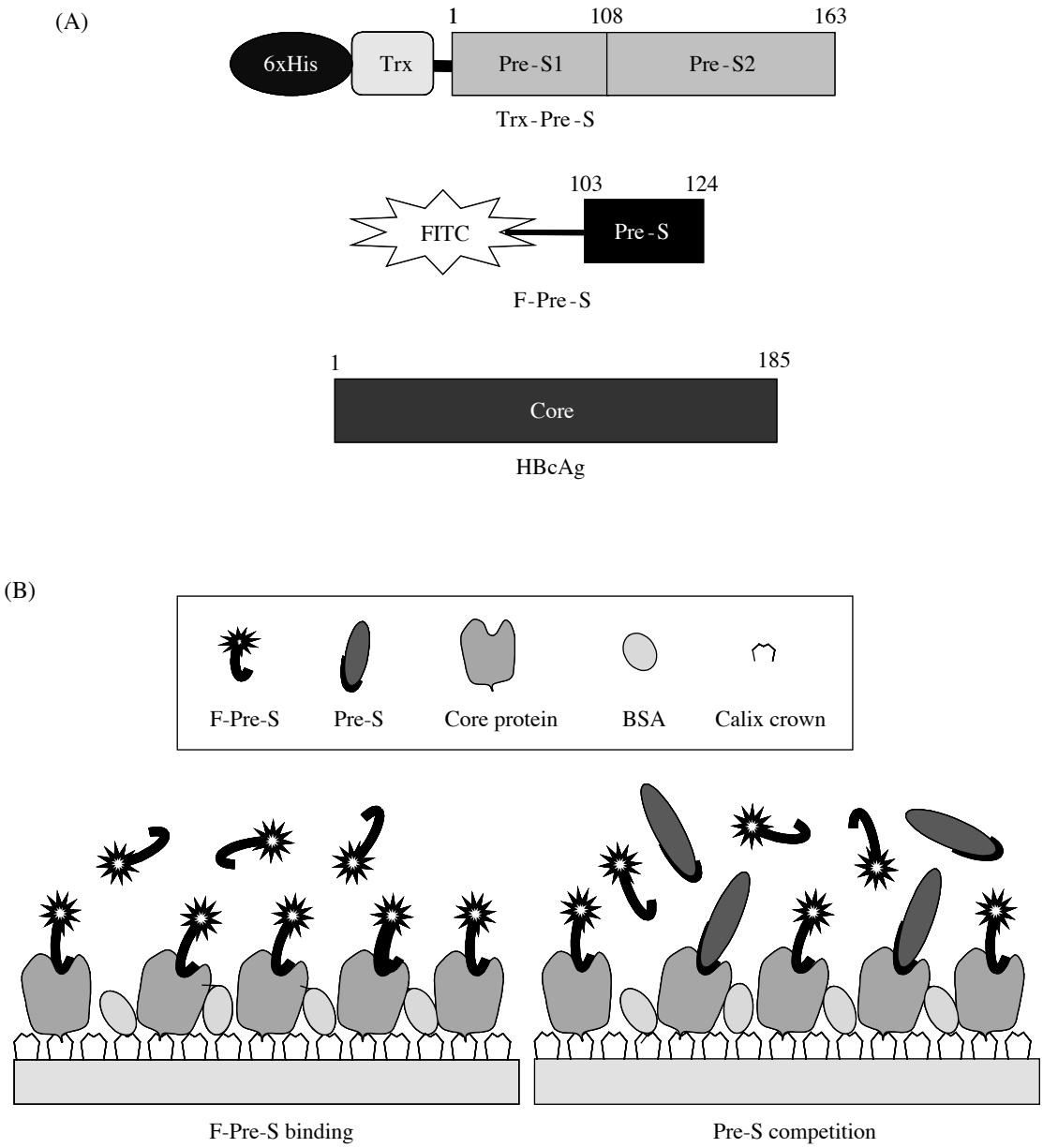

Figure 1. (A) Schematic representation of the constructed Pre-S domain of the HBV envelope protein in the Trx-fusion protein, fluorescein-labeled pre-S peptide F-Pre-S, and the core protein HBcAg. (B) Strategy for microarray-based analysis of the interaction of the immobilized core protein with the F-Pre-S peptide and competitive inhibition by the pre-S protein.

actions. Purified proteins and a fluorescently-labeled peptide were employed to demonstrate binding of the core protein to pre-S. Further, competitive inhibition studies were performed in a microarray format, which validated its utility in high-throughput screening.

Results and Discussion

The pre-S domains of the L-protein, which spans amino acids 1-163 was expressed as a histidine-tagged thioredoxin-fusion protein (Figure 1A), since the presence of thioredoxin domain was previously shown to increase the expression level and solubility of the protein[10]. While the pre-S domain expression in *E. coli* BL21 (DE3) yielded a high quantity of the purified protein, the expression level of the core protein containing amino acids 1-185 in the same strain of bacteria was very low. This was presumably due to the presence of 15 Arg codons rarely used in *E. coli*[15]. Accordingly, another strain of *E. coli* BL21-codonplus-RIL containing extra copies of tRNA for Arg was used to express the core protein. After a series of purification steps, both proteins were purified to at least 90% purity (data now shown).

For the analysis of interactions between the envelope and core proteins of HBV, we synthesized a fluorescently-labeled peptide probe F-Pre-S (Figure 1A). Since both pre-S1 and pre-S2 regions exhibit high affinity binding to the core protein, and the amino acid residues at the boundary of pre-S1 and pre-S2 regions are known to be critical for the interaction with the nucleocapsid[7,10,16], the peptide was designed to have the amino acid residues from 103 to 124. Our initial trial was made to set up a homogeneous binding assay based on fluorescence polarization as it has provided a convenient assay platform for analyzing interactions between peptides and proteins[17]. When the F-Pre-S peptide was incubated with the core protein, the formation of precipitates was observed with increasing protein concentrations, implying that the peptide may induce protein aggregation. Thus, further development of this method was not pursued due to incompatibility of fluorescence polarization measurements in the presence of precipitates.

Our next attempt was directed at designing a microarray-based analysis method for quantitation of the interaction between F-Pre-S and the core protein. As shown in Figure 1B, we designed the microarray-based assay in which the core protein is deposited and immobilized on the solid support using calixcrown self-assembled monolayers (SAMs) which act as excellent host molecules by recognizing ammonium ions in protein immobilization[18]. The F-Pre-S peptide is then spotted onto the protein arrays to analyze the interaction. If the solutions containing both the F-Pre-S peptide and the pre-S protein are applied, then competitive inhibition assays could be performed (Figure 1B).

The interaction of F-Pre-S with the core protein was first analyzed by applying varying concentrations of the peptide to the HBcAg-arrayed surface. As

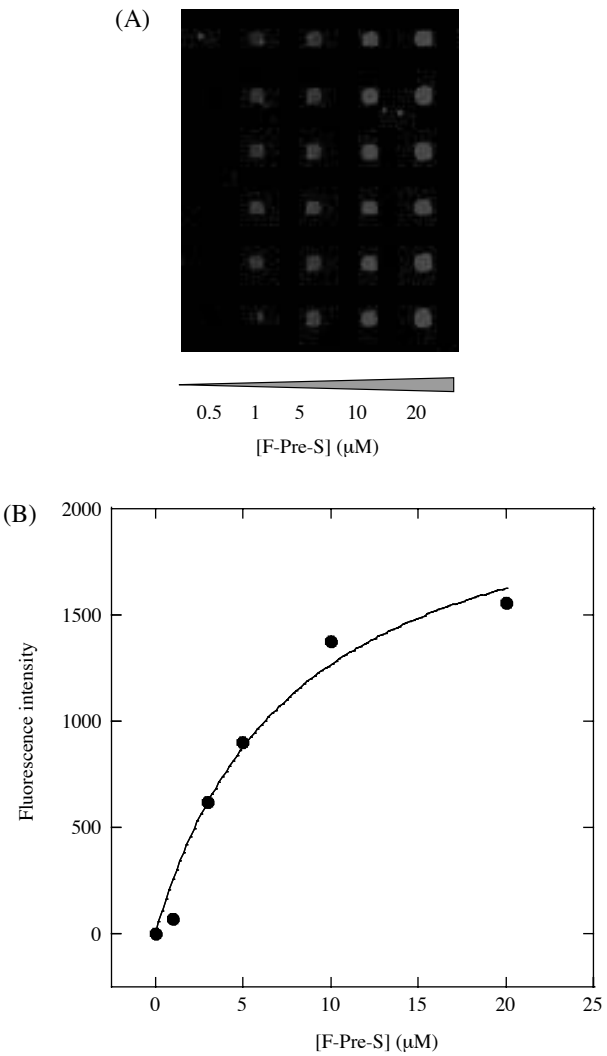

Figure 2. (A) Representative fluorescence images for the binding of F-Pre-S to the immobilized core protein. 10 μM of the core protein solution was spotted onto a calixcrown SAM -coated glass slide using a 120 μm pin. After blocking and washing, F-Pre-S at varying concentrations from 0.5 to 20 μM was re-spotted over the immobilized protein. (B) Dose-response curves for the interaction of F-Pre-S with the immobilized core protein. Fluorescence images from two separate experiments in sextuplicates were analyzed and represented by fluorescence intensity as a function of F-Pre-S concentration.

shown in Figure 2A, fluorescence signal increased with increasing peptide concentrations, indicating concentration-dependent peptide binding to the protein. These data were repeatedly reproduced to confirm the suitability of the designed microarray strategy for the intended interaction analysis. When the images were analyzed and plotted as a function of the concentration of F-Pre-S, a well-defined saturation curve was obtained (Figure 2B). The EC_{50} value from the plot was determined to be 8.0 ± 2.5 μM, which exhibited high affinity binding, similarly as reported previously[16,19,20]. It should be noted that the use of epoxy surfaces did not yield reproducible binding results (data not shown), implying that the capturing core protein is presumably not adequately oriented in cross-linking to the surface epoxy groups and thus may hamper proper interactions. Therefore, selection of appropriate surface chemistry is desired to achieve reliable interaction analysis.

To test whether the developed microarray system would be utilized in screening for inhibitors, we performed competitive binding assays using a mixture of F-Pre-S and the pre-S protein. At a fixed concentration of F-Pre-S, fluorescence signal decreased with increasing concentrations of pre-S (Figure 3A). The analyzed data showed an expected inhibitory curve (Figure 3B), indicating potent inhibition of the peptide-protein interaction by pre-S with an IC_{50} value of 14.9 ± 1.0 μM. These results demonstrated that the peptide used in the present microarray-based assay is appropriately designed to represent the specific interaction of pre-S with the core protein. Considering that the previously used ELISA assay[10,11] requires at least 100 μL of the 10 μM proteins per sample, which is more than enough to generate over a hundred protein spots on microarray, the microarray-based method provides a very economical and reliable screening platform for inhibitors of the interaction between HBV proteins.

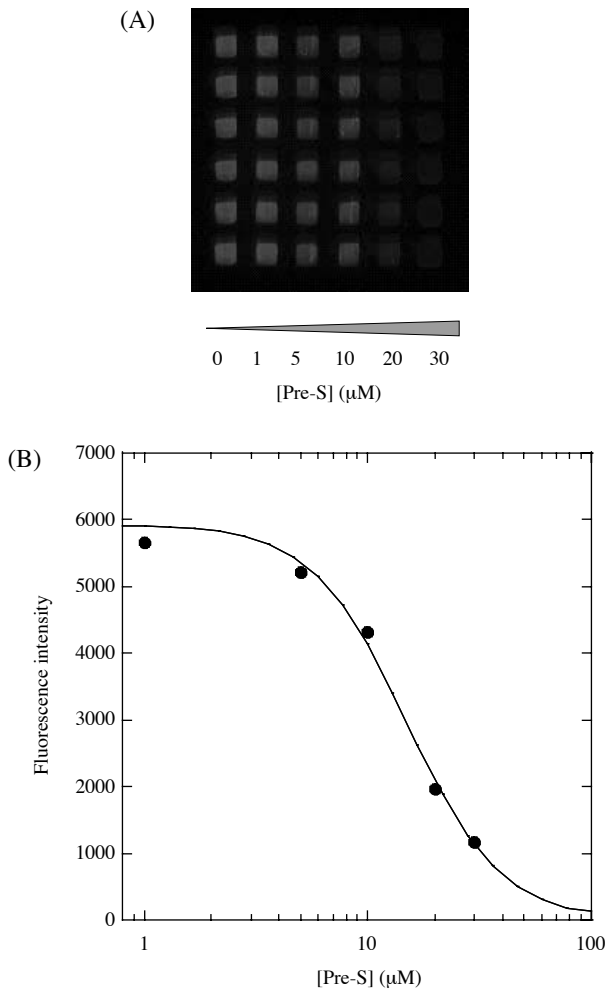

Figure 3. (A) Representative fluorescence images for the competitive binding of F-Pre-S and pre-S to the immobilized core protein. 10 μM of the core protein solution was spotted onto a calixcrown SAM-coated glass slide using a 300 μm pin. After blocking and washing, 20 μM of F-Pre-S mixed with varying concentrations of the pre-S protein ranging from 0 to 30 μM was re-spotted over the immobilized protein. (B) Competitive inhibition analyzed by fluorescence images from two separate experiments in sextuplicates. Fluorescence intensity is plotted as a function of pre-S concentration.

Conclusions

We presented the applicability of protein microarray for studies on the protein-protein interaction, which serves as a useful platform for miniaturized bioanalysis. As we also demonstrated that the protein microarray for interaction analysis could be used for inhibition studies, this *in vitro* system may be applied to screening compounds that have inhibitory activities against specific protein-protein interactions. While enzymes including viral DNA polymerase and proteases are routinely exploited as target proteins in developing anti-viral agents because they are essential for viral genome replication and viral protein processing, our microarray-based method used to evaluate the interaction between HBV proteins suggests that the interaction sites of the envelope and core proteins of HBV could provide an alternative target site for the development of anti-viral drugs, thereby extending the range of potential anti-viral therapeutics. Therefore, the developed microarray-based assay is being applied for high-throughput screening of inhibitors against the interaction of HBV.

Materials and Methods

Materials. A peptide containing amino acids 103-124 of the pre-S domain in the HBV envelope protein was prepared by AnyGen (Korea). Fluorescein was conjugated to the peptide with the N-terminal insertion of an aminocaproic acid (ACA) linker to synthesize the fluorescently-labeled Pre-S peptide (FITC-ACA-RQPTPISPPLRDSHPQAMQWNS-NH_2). The plasmids pTrx-Pre-S and pHBcAg were constructed by inserting into pET28a and pET21a (Novagen), respectively, as described previously[10]. The pre-S region of Trx-Pre-S contained amino acids 1-163 (GenBank accession number BAB17299) with substitutions of Thr87 to Ala and Ser177 to Asn. HBcAg contained amino acids 1-185 (GenBank accession number CAA96554). All other chemicals were of the highest grade commercially available.

Preparation of the recombinant proteins. The Pre-S protein subdomain and the core protein were expressed in *E. coli* strain BL21 (DE3) (Novagen, Germany) and BL21-CodonPlus-RIL (Stratagene), respectively. The recombinant proteins were induced by addition of 0.5 mM isopropyl β-D-thiogalactoside (IPTG) to the transformed bacteria grown in LB medium containing 50 μg/mL kanamycin for Trx-Pre-S, and 50 μg/mL ampicillin and 34 μg/mL chloramphenicol for HBcAg until optical density of the culture at 600 nm reached 0.7-0.8, followed by additional incubation for 3 hours at 37°C. Trx-Pre-S was purified from the harvested *E. coli* cells by centrifugation at 4,000 g for 15 minute at 4°C, followed by resuspension in buffer-A (50 mM sodium phosphate, pH 8.0, 0.3 M NaCl) containing protease inhibitor cocktail (Roche, USA), and by cell disruption through a French press at 16,000 psi. The crude cell extract was centrifuged at 10,000 g for 30 minutes at 4°C, and the supernatant was loaded onto a Ni^{2+}-charged Hi-Trap chelating HP column (GE Healthcare, USA) pre-equilibrated with buffer-A containing 20 mM imidazole. After the column was washed thoroughly with the same buffer, the bound proteins were eluted by applying a linear gradient of 20-500 mM imidazole in buffer-A. Fractions containing the target proteins were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), combined, dialyzed against buffer-B (20 mM Tris-HCl, pH 8.0), and applied to a Mono-Q HR column (GE Healthcare, USA) pre-equilibrated with buffer-B. The protein was then eluted with linear gradient of 0-0.5 M NaCl in buffer-B, collected, dialyzed against buffer-C (20 mM sodium phosphate, pH 8.0, 0.15 M NaCl), concentrated, and stored in 5% glycerol at −80°C until use. For the purification of HBcAg, the *E. coli* cells resuspended in 50 mM sodium phosphate (pH 7.4) containing 1% Triton X-100 and protease inhibitor cocktail were lysed in a French press at 16,000 psi. The crude cell extract was centrifuged at 10,000 g for 30 minutes at 4°C, and the supernatant was re-centrifuged at 140,000 g for 2 hours at 4°C. After the pellet fraction was re-suspended in 6 mL (per 1 L culture) of buffer-D (50 mM Tris-HCl, pH 7.8, 5 mM EDTA, 5% sucrose), insoluble proteins were removed by centrifugation at 10,000 g for 30 minutes. The supernatant was then applied to a Sephacryl S-400 (1.6 × 70 cm, GE Healthcare, USA) pre-equilibrated with buffer-D. HBcAg-containing fractions were combined, concentrated by ultrafiltration, and stored in 5% glycerol solution at −80°C. Purified proteins were analyzed by SDS-PAGE and quantified by BCA protein assay (Pierce, USA).

Interaction analysis by microarray. 10 μM of the HBcAg core protein in ArrayIt protein printing buffer (Telechem International, USA) was spotted on the ProteoChip (Proteogen, Korea) using a CM-1000 microarrayer (Proteogen, Korea) equipped with a stealth micro-spotting pin (SMP; Telechem International, USA) with 120 or 300 μm in diameter. After incubation of the chip slides for 3 hours in CM-1000 with humidity maintained at 70%, the slides were washed with PBST (PBS containing 0.5% Tween-20) for 10 minutes in High Throughput Wash Station (Telechem International, USA) with stirring. The slides were further blocked with PBS containing 3% BSA for 2 hours in an Array chamber (Genomictree, Inc, Korea), and washed in PBST for 10 minutes. The solutions containing F-Pre-S in the presence or absence of pre-S protein were then reprinted onto the spots printed with the core protein. After incubation for 1 hour in CM-1000, the slides were washed in PBST for 10 minutes and dried out completely by centrifugation using a ChipMate (Tomy Digital Biology, Japan). The resulting slides were scanned and analyzed using ArrayWorx (Applied Precision, USA) with excitation at 488 nm and emission at 520 nm.

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