Screening for PreS specific binding ligands with a phage displayed peptides library

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Abstract

AIM: To construct a random peptide phage display library and search for peptides that specifically bind to the PreS region of hepatitis B virus (HBV).

METHODS: A phage display vector, pFuse8, based on the gene 8 product (pVIII) of M13 phage was made and used to construct a random peptide library. E.coli derived thioredoxin-PreS was purified with Thio-bond beads, and exploited as the bait protein for library screening. Five rounds of bio-panning were performed. The PreS-binding specificities of enriched phages were characterized with phage ELISA assay.

RESULTS: A phage display vector was successfully constructed as demonstrated to present a pVIII fused HBV PreS1 epitope on the phage surface with a high efficiency. A cysteine confined random peptide library was constructed containing independent clones exceeding 5±10^8 clone forming unit (CFU). A pool of phages showing a PreS-binding specificity was obtained after the screening against thio-PreS with an enrichment of approximately 400 times. Five phages with high PreS-binding specificities were selected and characterized. Sequences of the peptides displayed on these phages were determined.

CONCLUSION: A phage library has been constructed, with random peptides displaying as pVIII-fusion proteins. Specific PreS-binding peptides have been obtained, which may be useful for developing antivirals against HBV infection.

Key words: Hepatitis B virus; Phage; Peptide

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INTRODUCTION

The attachment to hepatocytes by hepatitis B virus (HBV) during infection has long been proposed to be a potential target for antiviral intervention. It is thought that molecules specifically binding to HBV particles may interfere with viral attachment and hence reduce or block subsequent infection[1-3]. Ideally, these molecules should mimic the structural elements of a cell surface HBV receptor. Unfortunately, although a few host proteins have been demonstrated to interact with HBV particles or viral surface antigens, such a cell surface HBV receptor remains elusive[4-6]. Consequently, our knowledge on the molecular events leading to HBV attachment to hepatocytes is very limited. One longstanding notion is the role of HBV PreS region in mediating HBV attachment to the putative receptor on hepatocytes[7-9]. Specifically, the aa 21-47 segment within the PreS1 domain is believed to be essential for this process[10]. It is noteworthy that other important functions of the PreS region have also been reported, for example, in the assembly and budding of HBV virion[12,13]. Therefore, the PreS region has become a potential target for screening antivirals against HBV infection.

Strategies for ligands discovery are usually based on procedures for assembling a large number of compounds to produce a diverse array of molecules, followed by a screening process against targets of interest. Among various technologies for ligand discovery, phage display has evolved into one of the main approaches for obtaining lead molecules[14,15]. Filamentous phage M13 has a rather simple architecture composed of five different structure proteins. The product of gene 8 (pVIII) is a major capsid protein. There are approximately 2 700 pVIII molecules all over a phage particle, while other structural proteins are located at the tail of the phage particle with only a few copies of each[16]. Thus, by fusing with pVIII, hundreds or thousands of ligands can theoretically be displayed on the surface of the phage particle, which should help strengthen its interaction with a target[15,16]. Thus, pVIII fusion protein based phage display will offer a greater chance to find mildly binding ligands.

In this study, we have constructed a structurally constrained phage display library in which random peptides are displayed...
as N-terminal fusions to pVIII and confined in a loop by a pair of flanking cysteine residues\cite{19,20,21}. Recombinant thioredoxin-PreS protein was used as target in screening for the PreS-binding peptides. A pool of phages showing PreS-binding specificity was obtained with an enrichment factor of approximately 400. Phages with high PreS-binding specificities were selected and characterized. Sequences of the peptides displayed on five of PreS-binding phages were determined.

**MATERIALS AND METHODS**

**Materials**

The pCANTAB5E vector, T7 DNA sequencing kit, and HRP-conjugated M13 specific monoclonal antibody (anti-M13 mAb) are products of Amersham Pharmacia Biotech (Piscataway, USA). XL1-blue F' E. coli strain and helper phage VCSM13 were purchased from Stratagene (La Jolla, USA). PreS1 specific mAb 125E11 has been previously described\cite{20,21}. Maxisorp F16 module (Nunc, Denmark) was used for protein coating. ThiobondTM affinity resin and the thioredoxin specific mAb (anti-thio) were from Invitrogen (Carlsbad, USA). Recombinant enterokinase (rEK) was purchased from Novagen (Madison, USA).

**Construction of random peptide phage display library**

pCANTAB5E was utilized as the parental vector for constructing a pVIII fusion protein display vector. The coding sequence for M13 pVIII was amplified with specific primers (pVIII-forward: 5'-TAAGGATCCGGTGGTGCTGACGGGATCCATGATCCG-3', pVIII-reverse: 5'-AAGGATCCGGTGGTGCTGACGGGATCCATGATCCG-3'). The resulted product was digested with BamHI and EcoRI, and inserted into the same sites in pCANTAB5E, replacing the original gene 3. The resulted vector was named pFuse8 (Figure 1A). To construct pFuse8-E that encodes a PreS epitope fused with pVIII (Figure 1A), annealed oligonucleotides (sense: 5'-CAGCAATGGCAAGGGTGACGATCCCGC-3', antisense: 5'-AATTTCGGATCCGGGSGG-3') was annealed to the coding sequence in replace of gene 3; pFuse8-L, the coding sequence for random peptides are inserted into pFuse8; pFuse8-E, the vector harboring gene 8 coding sequence in replace of gene 3; pFuse8-L, the coding sequence for a specific epitope recognized by the 125E11 mAb is inserted into pFuse8; pFuse8-E, the coding sequences for random peptides are inserted into pFuse8-E; pFuse8-E, the coding sequence for random peptides are inserted into pFuse8-E.

**Figure 1** Diagram of library construction. A: a schematic representation of the steps in the construction of the phagemid encoding the pVIII fusion protein. pCANTAB5E, a pIII displaying vector; pFuse8, the vector harboring gene 8 coding sequence in replace of gene 3; pFuse8-E, the coding sequence for a specific epitope recognized by the 125E11 mAb is inserted into pFuse8; pFuse8-E, the coding sequences for random peptides are inserted into pFuse8-E; pFuse8-E, the coding sequence for random peptides are inserted into pFuse8-E.

**DNA sequencing**

Phagemid pool of randomly picked-up clones from the library was prepared with SV minipreps kit (Promega). DNA sequencing was performed with T7 DNA sequencing kit according to the manufacturer’s instruction.

**Preparation of PreS fusion protein**

Prokaryotic expression plasmid pThioHisA-PreS was constructed for the synthesis of thioredoxin fused PreS (thio-PreS) in E. coli\cite{22}. An affinity purification for the expression product was performed with ThiobondTM beads according to the manufacturer’s instruction.

**Library screening**

Total 1011 phages were mixed with 30 µL of the thio-PreS bound ThiobondTM beads and incubated on a rotating wheel for 10 min. After washing with PBS containing 0.05% Tween
20 for four times, the beads were treated with 1 unit of rEK at 30 °C for 6 h. Phages binding specifically to the PreS region were supposed to detach off the beads. Eluted phages in the collected supernatant were subject to propagation in XL1-blue F‘ cells, followed by VSCM13 co-infection to produce a phage pool for next round of panning. A total of five rounds of selection were performed.

Western blot

Western blot of thio-PreS was performed according to a standard method. Thio-PreS coupled on resins with or without rEK treatment were boiled and separated on SDS-PAGE, and transferred to nitrocellulose filters. Anti-thio mAb (1:1 000) was used as the primary antibody for the detection. Blots were developed using the ECL method with HRP-labeled rabbit anti-mouse Ig (1:2 000, DaKo, USA).

Phage ELISA

Phage ELISA was carried out as described previously. Purified thio-PreS proteins (1 µg/well) were immobilized on the microwells in PBS buffer overnight. Phages propagated from infected XL1-blue F‘ cells were precipitated by 0.1 volume of PEG8000/NaCl (20% PEG8000, 3 mol/L NaCl), resuspended in PBS buffer and applied to thio-PreS coated wells, followed by an incubation at room temperature for 1 hour. An HRP-labeled anti-M13 mAb (1:1 000) was used for the detection of bound phages.

Virus capture assay

For capturing HBV virions, each microwell was coated with 1011 phages in carbonate buffer (pH 9.5) at 4 °C overnight. After blocking with 1% BSA at 37 °C for 2 h, cultured medium of HepG2.2.15 that contains HBV particles was added and incubated for 37 °C, followed by a washing process with PBS buffer containing 0.05% Tween 20. An HRP-conjugated anti-HBs antibody was used to detect the captured HBV particles according to the manufacturer’s protocol (Sino-American Biotech, Shanghai).

RESULTS

Construction of pVIII based phage display vector

To construct the vector pFuse8, M13 gene 8 was amplified using the genomic DNA of VCSM13 phage as template. The PCR product was inserted into pCANTAB5E to replace the coding sequence of gene 3 and the E-tag (Figure 1A). The inserted gene 8 is located downstream to a leader signal sequence of gene 3, which is used to direct the pVIII fusion protein to the surface of the phage particle. In the phagemid pFuse8-E as shown in Figure 1B, a spacer between the leader signal and gene 8 encodes an in-frame peptide AAQPMAAG-SLNPSWD-GGGSGG. The first part of the peptide (AAQPMAAG) is a recognition sequence of the pelB signal peptidase. The 7-amino acids in the middle of this peptide represents an epitope within the HBV PreS1 region that is recognized by the 125E11 mAb (unpublished data), providing a molecular marker for detection of phage display. The ensuing glycine tether is used to link the peptide to pVIII, increasing the flexibility of the peptide.

Phages were generated by co-infection of the pFuse8-E transformed XL1-blue F‘ cells with helper phage VCSM13. The displayed 125E11 epitope was readily detected by phage ELISA in a dose dependent manner with the immobilized 125E11 mAb but not with an irrelevant mAb (anti-HBs) (Figure 2). As shown in Figure 2, the lac promoter employed in the vector was somewhat leaky so that pVIII-fused epitope could be easily detected even without IPTG induction.

Construction of structure-confined random peptide phage display library

Structure-confined random peptide library has the advantage to present peptides in a fixed loop so that their conformations are relatively rigid. In this study, random peptides fused with pVIII assume a form of CX-CX-CX, where X stands for any amino acids. Two cysteine residues flank the random amino acids in the middle. Under a non-reducing environment in the periplasm of E.coli, these cysteine residues may spontaneously form a disulfide bond that constrains the conformation of the displayed peptide. The oligonucleotides mixture coding for the random peptides was inserted into the pFuse8 vector (pFuse8-L, Figure 1A). The resulting library had the size of about 5.0×108 independent clones that represented a great complexity. The random property of the library was verified by DNA sequence analysis. As shown in Figure 3, in the region coding for the random peptides, all four kinds of nucleotides are equally distributed at the first two positions of the triplet codons, while T or G varies at the third position, indicating a sufficient diversity of the library.

Screening for PreS-binding peptides

The PreS region of HBV, when synthesized alone in E.coli, is vulnerable to degradation and largely insoluble. To overcome these problems, the PreS region was fused with the C-terminus of thioredoxin (Figure 4A), which has dramatically enhanced both the stability and the solubility of the PreS region (Figure 4B). An enterokinase site located at the junction between the PreS region and the thioredoxin tag (Figure 4A) facilitates the release of the PreS region by digestion with rEK, leaving the thioredoxin tag on the affinity matrix (Figure 4C). Thus, the PreS-binding phages could be
As shown in Table 1, the PreS-binding phages were greatly enriched as evidenced by a steadily rising enrichment factor (EF, phage eluted/phage applied). An approximately 400-fold enrichment of EF_{thio}/EF_{native} was achieved as estimated by the titer of the phages after the screening. The pool of phages from the final round of selection bound to the thio-PreS immobilized wells specifically in a dose-dependent manner, in sharp contrast to the original pool of phages before selection (Figure 5). A much weaker noise was noticed with the thioredoxin immobilized wells serving as a control, indicating that the thioredoxin-binding phages were also selected, though they might only be a small minority.

**Characterization of the PreS-binding phages**

The specificity of the phages with regard to PreS-binding was further characterized by virus capture assay. When coated on microplate wells, the pool of phages from each round of selection was tested for their abilities to capture HBV virions from the cultured medium of HepG2.2.15. The binding capacity to HBV virions increased greatly after the final round of selection (Figure 6), suggesting that the PreS-binding phages were truly selected. Phages from the final round of selection were picked for a further analysis. The specificities of these phages with regard to PreS-binding were analyzed with phage ELISA assay. Phages with a strong binding capacity to thioredoxin were considered nonspecific and discarded (data not shown). The corresponding phagemids of five of the PreS-binding phages were subjected to DNA sequencing. Amino acid sequences of the potential PreS-binding peptides were deduced (Figure 7).

**DISCUSSION**

The filamentous phage display system, whereby the expressed peptides are displayed as fusions to phage coat proteins, has been effective in the discovery of ligands [15,16]. Commonly exploited strategy is the surface display of N-terminus fusions to the minor coat protein pIII (product of phage gene 3). It represents a low-valent surface display, as only three to five copies of pIII are present at the tail of the phage particle, providing a selection platform for an interaction with a high affinity [15]. In our work, a pVIII fusion protein based vector was successfully constructed, and proved to work well in peptide displaying. Since pVIII is a major coat protein with thousands of molecules on a single phage, thus pVIII fusion protein based phage display will offer a greater chance to find mildly or weakly binding ligands. As PreS-binding peptides are concerned, the pVIII fusion protein based vector may be a better choice than the pIII fusion protein based system. It is supposed that the affinity of the HBV virion to its putative receptor on hepatocytes may not be strong, because an ensuing intracellular event of dissociation may occur closely after the penetration of the viral particle during the infection.

Linear oligopeptides likely present various solution conformations that may be quite different from the structure in the native or bound forms of a protein. It has been demonstrated that the structurally active region in native protein may be mimicked by cyclic amino acid sequences of constrained peptides displayed on the phage surface [15,16]. A pair of flanked cysteine residues was adopted to confine the random peptides in the middle by presumably forming...
an intra-molecular disulfide bond that provides a more defined scaffold when folded in the periplasmic environment of E.coli. In this study, we have constructed a conformation-constrained random peptide phage display library and performed the screening with this library, in the hope that potential peptides with more defined conformations may better fulfill the structural features required for PreS-binding. On the other hand, such a conformation-constrained library has its weaknesses. It is likely that during the interaction process between PreS and a potential peptide, both partners may exert their influences on each other so that the conformation of the potential peptide adjusts to better bind PreS. In such a situation, the peptide with a constrained conformation may be too rigid to accommodate itself upon binding PreS, which will probably result in a reduced binding affinity.

During the life cycle of HBV, the PreS region of the large surface antigen (LHBs) plays a crucial role in virion assembly and budding as well as in viral attachment to hepatocytes[8-12,28,29]. Given the pivotal role of the PreS region in HBV life cycle, the specific PreS-binding ligands are expected to be useful as inhibitors to block viral entry to hepatocytes, or to interfere with the process of virion assembly in HBV infected cells. Similar ideas have been pursued widely in the field of HIV research and proved to be a potential way for antiviral drug development[30,31]. Moreover, studies on the PreS-binding peptides will likely provide helpful information about the interaction between HBV virion and hepatocytes that may aid the identification of a yet-to-be-found receptor of HBV.

The binding capacity of the pool of phages to HBV virions increased greatly after the final round of selection. Thus, the thio-PreS fusion protein was proved to be an effective bait protein that might mimic the conformation adopted by the PreS region in the native virions. In addition, the affinity screening for the PreS-binding phages represents a promising approach for discovering HBV binding ligands. Several peptides encoded by the phagemids of five of the PreS-binding phages were determined. No consensus sequence or conserved amino acids were found in these five peptides. Therefore, these peptides likely bind to different fragments of the PreS region. On the other hand, sequences of more PreS-binding peptides need to be determined and the binding properties of the PreS-binding peptides await further investigations, which are currently on the way.

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