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Original Article

Selection of HBV preS1-binding penta-peptides by phage display

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Chronic hepatitis B virus (HBV) infection can lead to liver cirrhosis and hepatocellular carcinoma. Current therapies have a very limited efficacy in virus clearance. New antiviral targets and agents are urgently needed. The envelope of HBV virion contains three surface glycoproteins, namely the large (LHBs), middle (MHBs), and small (SHBs) proteins. LHBs has an amino terminal preS which is composed of the preS1 and preS2 domains. The amino half of preS1 which is myristoylated plays a pivotal role in HBV entry, which can be exploited as an antiviral target. A common motif of five amino acids had been previously discovered to bind preS1$_{1–65}$ and HBV particles. In this study, we used preS1$_{1–65}$ to screen a phage display library of random penta-peptides to select the penta-peptides possessing a high preS1-binding affinity. After nine rounds of panning, we obtained one peptide designated as A5 which could bind preS1 with a high affinity. By systematically substituting each residue of A5 with the other 19 amino acids, we identified a novel peptide with an increased preS1-binding affinity. Both peptides could inhibit HBV attachment to HepG2 cells, making them be potential candidates for HBV entry inhibitors.

Keywords HBV; preS1; PIII; phage display; peptide

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Introduction

More than 350 million people worldwide are chronically infected with hepatitis B virus (HBV) and at high risk of developing liver cirrhosis and hepatocellular carcinoma. Interferons and nucleos(t)ide reverse transcriptase inhibitors (NRTIs), which are the current therapies for chronic hepatitis B infection, have a very limited efficacy in virus clearance [1]. Moreover, the antiviral effect of long-term NRTI therapy is subdued by drug-resistant HBV mutants [2]. New antiviral targets and agents are highly needed.

HBV infection is a multi-step process that begins with viral entry via the interaction between viral envelope proteins and cellular receptors [3–5]. The virion envelope contains three related surface glycoproteins, namely the large (LHBs), middle (MHBs), and small (SHBs) proteins. They are translated from distinct initiation codons but share a common carboxyl domain (i.e. SHBs). Consequently, LHBs has an amino terminal (N-terminal) preS which is divided into the amino preS1 and carboxyl preS2 domains. MHBs contains the preS2 domain but not the preS1 domain. LHBs is mainly associated with virions and exists with smaller amount on rod-shaped subviral particles, but is missing in spherical subviral particles. PreS1 plays a pivotal role in HBV entry. In particular, the amino half of preS1 which is myristoylated is believed to be essential for viral attachment to hepatocytes.

Viral entry can be exploited as an antiviral target and the entry inhibitors against HIV infection are used in clinic [6–8]. The myristoyl polypeptide containing the residues 2–48 of preS1 (myr-preS1$_{2–48}$, genotype D) has been demonstrated to effectively inhibit HBV infection *in vitro* and *in vivo*, likely by competing with HBV for binding hepatocytes [9–13]. On the other hand, preS1-binding peptides might also be potential antiviral agents by 'wrapping' HBV virions to interfere with viral attachment to hepatocytes.

Since it was firstly described, phage display technology has evolved into one of the most powerful tools in ligand discovery [14]. We had previously used preS as a bait to screen a phage display library of random 12-amino acid peptides displayed as N-terminal fusions to protein eight (pVIII) of M13. Aligning the acquired preS-binding peptides, we discovered a common motif of five amino acids (WTXWW) that could bind preS1$_{1–65}$ and viral particles [15]. In this study, to select the penta-peptides with a high preS1-binding affinity, preS1$_{1–65}$ was used to screen a phage display library of random penta-peptides displayed as N-terminal fusions to protein three (pIII) of M13. After nine rounds of panning, one peptide designated as A5 with a high

preS1-binding affinity was obtained. Moreover, by systematically substituting each residue of A5 with the other 19 amino acids, a novel peptide was identified with an increased preS1-binding affinity. Both peptides could inhibit HBV attachment to HepG2 cells.

Materials and Methods

Construction of random penta-peptide phage display library

For the construction of random penta-peptide phage display library, an antisense oligonucleotide [5′-ACAAGCGGCC GCTCCACCACC(SNN)$_5$AAGGCCTGCTGCCATTGCTGG CT-3′; S : G or A; N : A, T, G, or C] was synthesized (Sangon, Shanghai, China). A complementary primer (5′-CAGCAATG GCAGCAGGC-3′) was annealed to the 3' end of the long oligonucleotide, followed by the fill-in reaction with Klenow polymerase to generate the double-stranded DNA. The DNA duplex was digested with *Sfi*I and *Not*I, and inserted into pCANTAB5E (Amersham Pharmacia, San Francisco, USA) digested with the same enzymes. The phagemid library was established by the transformation of the ligation mixture into XL1-Blue F′ competent cells (Stratagene, Cedar Creek, USA) via electroporation according to the manufacturer's instruction. Briefly, 10 μl of the transformed bacterial culture was serially diluted with 2YT medium and used to calculate the titer of the original library. The rest of bacterial culture was superinfected with the helper phage VCSM13 (multiplicity of infection, 10 : 1) and grown for 10 h to allow phage propagation. The phages were then precipitated with polyethylene glycol 8000 (PEG8000). The randomness of the peptide-coding sequences was verified by sequencing 50 randomly picked phagemid clones.

Preparation of MBP-preS1$_{1–65}$ fusion protein

The DNA fragment encoding the first 65 residues of preS1 (genotype B) was obtained by polymerase chain reaction (PCR) and inserted in pMAL-C2x (New England Biolabs, Massachusetts, USA) in frame to the maltose binding protein (MBP) cDNA. Expression of the MBP-PreS1$_{1–65}$ fusion protein or the control MBP protein was induced by 0.3 mM isopropyl-beta-D-thiogalactopyranoside for 4 h at room temperature. The proteins were purified with affinity chromatography using amylose beads (New England Biolabs) according to the manufacturer's instruction.

Library screening

MBP-preS1$_{1–65}$ protein (1 μg) in 0.1 M bicarbonate (pH 9.5) was added per microplate well (Nunc, Roskilde, Denmark) and incubated at 4°C overnight under moist condition. Afterwards, the well was blocked with 10% non-fat milk in phosphate-buffered saline (PBS) for 2 h at room temperature and washed with 0.05% PBST (PBS containing 0.05% Tween 20). A total of 1×10^{11} pfu phages pre-incubated with 200 μg MBP for 1 h at room temperature, were applied and incubated for 1 h at 37°C. After the well had been washed 10 times with 0.05% PBST, the captured phages were eluted with 200 μl elution buffer (0.2 M glycine-HCl, pH 2.2, 0.5% bovine serum albumin (BSA)), and the pH of the eluent was quickly neutralized with 0.1 volume of 20% PEG8000/3 M NaCl. The phages were amplified, titered, and used for the next round of panning. A total of nine rounds of panning were performed according to the same procedure as described above except that 1×10^9 pfu phages were used as input for rounds 2–9.

The preS1-binding affinity of the selected phages was assessed. A total of 1×10^{11} pfu of phages in 0.05% PBST were applied to the MBP-preS1$_{1–65}$-coated well and incubated for 1 h at room temperature with gentle agitation. After the well had been washed four times with 0.05% PBST, the captured phages were eluted and titered. The P18 phage from the previous study [15] (encoding peptide WTDM FTAWWSTP, the preS1-binding motif underlined) was assayed alongside for comparison, and the helper phage VSCM13 was used as negative control.

Peptide sequence analysis and synthesis

The penta-peptide sequences were deduced from the sequences of the inserts of randomly picked phagemids after the ninth round of panning.

The N-biotinylated wild-type and mutant A5 peptides (biotin-A5: N-biotin-SGSGLKKKWST, biotin-A5m: N-biotin-SGSGLKKKAST), N-biotinylated B10 (biotin-B10: biotin-SGSGLRNIRST), and N-myristoyl preS1$_{2–48}$ peptide (Myr-GTNLSVPNPLGFFPDHQLDPAFGANSNNPDWDF NPNKDHWPEANQVGK, genotype D) were chemically synthesized (GL Biochem, Shanghai, China).

Peptide mutagenesis, enzyme-linked immunosorbent assay, and virus capture assay

Each mutant peptide was made by replacing one of the five residues of A5 with one of the other 19 amino acids. Briefly, complementary oligonucleotides for a total of 95 mutant peptides were synthesized (Sangon). The complementary oligonucleotides for each mutant were annealed and inserted upstream of the FLAG (DYKDDDDK) tag in pcDNA3 (Invitrogen, Carlsbad, USA). FLAG peptides were produced with TNT T7 Quick Coupled Transcription/Translation System (Promega, Madison, USA) according to the manufacturer's instruction and the yields were estimated with enzyme-linked immunosorbent assay. Briefly, 100 μl of an anti-FLAG monoclonal antibody (mAb) (1 : 1000 dilution; Sigma, St Louis, USA) was added into the well coated with one of the mutant peptides. The plate was incubated for 4 h at 4°C, washed three times, and incubated with 100 μl horseradish peroxidase (HRP)-conjugated rabbit anti-mouse

antibody (1 : 1000 dilution; Dako, Produktionsvej, Demark) for 1 h at room temperature. The absorbance was measured at 450 nm.

The preS1-binding affinity of the mutant peptides was assessed similarly as described above except that 10 μg Myr-preS1$_{2–48}$ was added into each well coated with an equal amount of one of the mutant peptides. The captured preS1 peptide was detected by an anti-preS1 mAb (12E11; 1 : 1000 dilution; ALPHA Inc., Shenzhen, China).

In virus capture assays, 50 μl of PBS-diluted HBV serum sample (viral titer determined by real-time PCR) was mixed with 50 μl HRP-conjugated anti-HBs polyclonal antibody (SABC, Shanghai, China) and added into each well of a streptavidin-coated plate (Roche, Basel, Switzerland) immobilized with biotin-A5 or biotin-A5m. Captured viral particles were detected as described above.

Isothermal titration calorimetry

The affinity and thermodynamics of the interaction between myr-preS1$_{2–48}$ and A5 or B10 were determined using isothermal titration calorimetry (ITC). ITC analyses were carried out using a VP-ITC calorimeter (MicroCal, LLC, Northampton, USA) at 25°C with 0.1 mM myr-preS1$_{2–48}$ in the sample cell and 3 mM A5 or B10 peptide in the injecting syringe. Both samples were thoroughly degassed and then centrifuged to remove precipitates. With the exception of the first injection, 2 μl per injection was used for the subsequent injections. Consecutive injections were separated by an interval of 2 min to allow peak to return to baseline level. ITC data were analyzed with a single-site binding model using Origin 7.0 (Origin Lab Corp., Northampton, USA).

Immunofluorescence

HBV attachment to HepG2 cells was determined by immunofluorescence. HepG2 cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% FBS, L-glutamine (2 mM), penicillin (50 U/ml), and streptomycin (50 μg/ml). For HBV attachment, 1×10^6 cells were incubated with 10^7 genome equivalent HBV for 4 h at 37°C in the absence or presence of 1 μM of A5, B10, or A5m peptide. After being washed with ice-cold PBS, the cells were fixed with 3.7% paraformaldehyde in PBS (pH 7.4) for 15 min at room temperature. The cells were then permeabilized with 0.5% Triton X-100 in PBS for 30 min at room temperature, washed with PBS, blocked with 1% BSA in 0.05% PBST for 30 min, and incubated with an anti-HBsAg mAb (1 : 1000 dilution; Dako) overnight at 4°C. After being washed with PBST, the cells were incubated in dark for 1 h at room temperature with Alexa Fluor 488 donkey anti-mouse IgG (H + L) (1 : 500 dilution; Life Technologies, Carlsbad, USA) in 1% BSA in PBST. After being washed with PBST, the cells were stained with 4′,6′-diamidino-2-phenylindole (DAPI) (Sangon) in dark for 10 min, washed with PBS, and examined under a confocal microscope (Leica, Heidelberg, Germany).

Results

Selection of the phages encoding preS1-binding penta-peptides

A random penta-peptide phage display library was constructed for the selection of the penta-peptides with a high preS1-binding affinity. The penta-peptide was fused to pIII to facilitate the selection of strong peptide–bait interactions. The capacity of the original phagemid library (2.99×10^9 cfu/ml) was more than sufficient, since it far exceeded the theoretical number of all kinds of amino acid combination (3.2×10^6) for penta-peptide. We verified the diversity of the library by sequencing the inserts of 50 phagemid clones randomly picked from the original library.

The MBP-preS1$_{1–65}$ fusion protein was prepared and used as a bait. Input phages were pre-incubated with excess of MBP to maximally remove free MBP-binding phages. After nine rounds of panning, the MBP-preS1$_{1–65}$-binding phages were greatly enriched as evidenced by the steadily rising enrichment factor (enrichment factor (EF) = phage eluted/phage input) (**Table 1**). An ~6000 fold of enrichment (EF_{ninth}/EF_{first}) was achieved after the ninth round of panning.

Ninety-nine phage clones after the ninth round of panning were randomly picked and propagated. The peptide-coding sequences in these clones were determined and the corresponding peptide sequences were deduced. The results showed that these clones encode only five penta-peptides (**Table 2**). The A2 peptide (WTYTW) was similar to the preS1-binding motif described previously [15]. Surprisingly, the A5 peptide (LKKKW) occurred 66 times (66.7%), indicating that it had been significantly enriched. A5 was thus chosen for further characterization.

Table 1. Enrichment of the phages binding MBP-preS1$_{1–65}$ fusion protein

Round of panning	Phage input	Phage eluted	EF[a]
1	1.0×10^{11}	8.00×10^5	8.00×10^{-6}
2	1.0×10^9	4.38×10^4	4.38×10^{-5}
3	1.0×10^9	4.68×10^4	4.68×10^{-5}
4	1.0×10^9	9.00×10^4	9.00×10^{-5}
5	1.0×10^9	8.60×10^5	8.60×10^{-4}
6	1.0×10^9	8.60×10^5	8.60×10^{-4}
7	1.0×10^9	8.25×10^5	8.25×10^{-4}
8	1.0×10^9	6.08×10^6	6.08×10^{-3}
9	1.0×10^9	4.68×10^7	4.68×10^{-2}

[a]Enrichment factor (EF) = phage eluted/phage input.

Table 2. Frequencies of the peptides encoded by the phages enriched after the ninth round of panning

Clone	Frequency[a]
A1	14
A2	5
A3	1
A4	13
A5	66

[a]Occurrences in a total of 99 sequenced phage clones.

Table 3. Comparison of the preS1-binding activity between the A5 and P18 phages

Phage	Phage input	Phage eluted	EF[a]
A5	1.0×10^{11}	1.88×10^{9}	1.88×10^{-2}
P18	1.0×10^{11}	2.80×10^{6}	2.80×10^{-5}
Helper phage VCSM13	1.0×10^{11}	5.60×10^{4}	5.64×10^{-7}

[a]Enrichment factor (EF) = phage eluted/phage input.

The preS1-binding affinity of the A5-coding phage was assessed by measuring the EF after the phage had been incubated with the immobilized MBP-preS1$_{1–65}$ protein, which was then compared with the EF of the P18 phage derived from our previous study (**Table 3**). The helper phage VSCM13 was used as negative control. The EF of the A5 phage (1.88×10^{-2}) was 1000-fold greater than that of the P18 phage (2.80×10^{-5}), which might be an underestimate considering that the 12-amino acid peptide in the P18 phage is fused to pVIII, resulting in its display at more than two thousand copies.

Taken together, these results indicated that the selection has been successful and the A5-coding phage possesses a much higher preS1-binding affinity than previously selected P18 phage.

The A5 peptide could capture HBV particles

Virus capture assay was performed to investigate whether the A5 peptide was able to bind HBV particles. Biotin was conjugated to the amino terminus of the A5 peptide, separated by a flexible short spacer (S–G–S–G), to facilitate the immobilization of the peptide onto streptavidin-coated wells. A mutant A5 peptide was synthesized, in which the tryptophan residue was replaced by an alanine based on the previous finding [15]. As shown in **Fig. 1**, only the A5 peptide was capable of capturing viral particles.

Identification of a novel preS1-binding penta-peptide by mutagenesis of the A5 peptide

We performed a comprehensive mutagenetic analysis of the A5 peptide to further optimize its preS1-binding affinity.

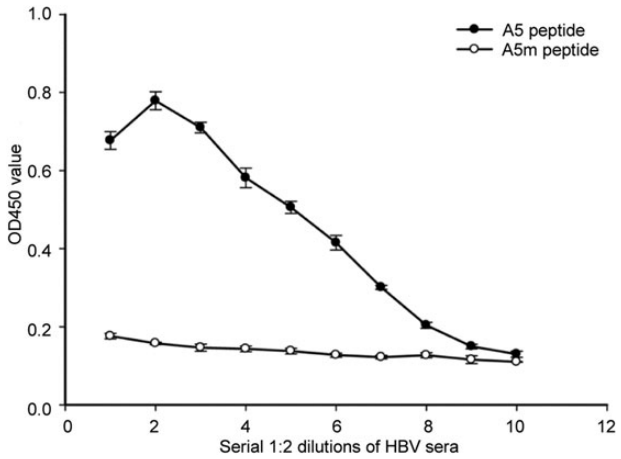

Figure 1. Capture of HBV particles by the A5 peptide Serial 1 : 2 dilutions of pooled HBV sera with an initial concentration of 10^6 viral DNA copies were applied to the wells coated with either the wild-type (A5) or mutant peptide (A5m). The captured viral particles were detected by the anti-HBs antibody. OD_{450}, optical density at 450 nm.

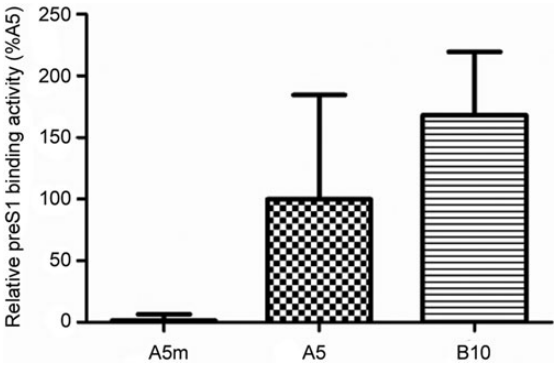

Figure 2. Comparison of the preS1-binding activity between the A5 and B10 peptides Myr-PreS1$_{2–48}$ peptides were added to the wells coated with one of the indicated N-biotinylated peptides. The captured Myr-preS1$_{2–48}$ peptides were detected by the anti-preS1 mAb 125E11. The data were presented as the relative preS1-binding activity to the value of A5 taken as 100%.

Table 4. Thermodynamic parameters of the interaction between myr-preS1$_{2–48}$ and A5 or B10 determined by ITC

	K_a (M^{-1})	K_d (μM)	ΔH (kJ/mol)	ΔS (kJ/mol)
A5 vs. myr-preS1$_{2–48}$	4.09×10^{3}	244	−63.5	−0.14
B10 vs. myr-preS1$_{2–48}$	5.48×10^{3}	182	−110	−0.29

N, stoichiometry; K_a, association constant; K_d, dissociation constant; ΔH, binding enthalpy; ΔS, binding entropic contribution.

Each residue of the A5 peptide was replaced by one of the other 19 amino acids and the preS1-binding affinity of each mutant peptide was assessed by its interaction with Myr-preS1$_{2–48}$. Compared with the A5 peptide, most mutant

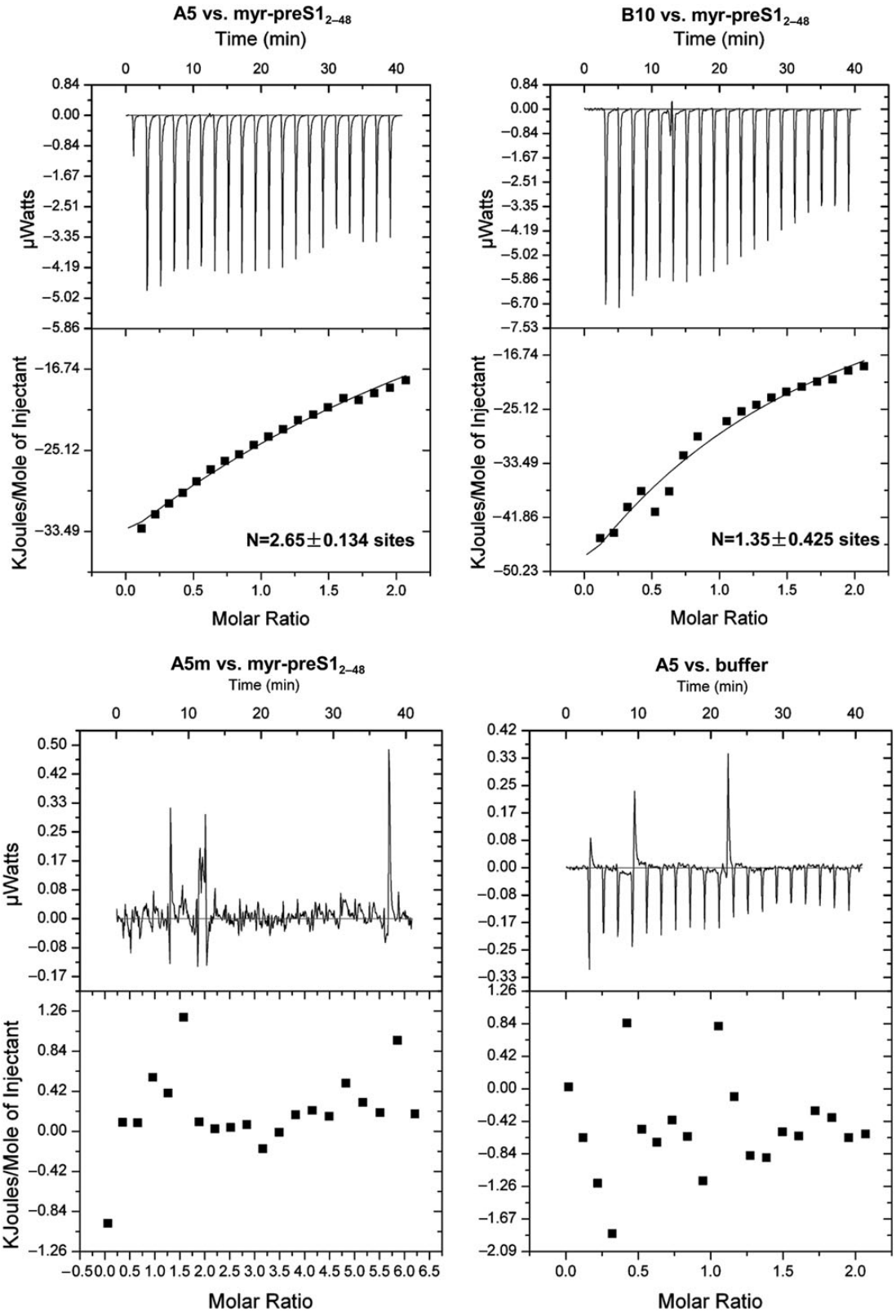

Figure 3. Interaction of A5 or B10 with myr-preS1$_{2–48}$ as determined by ITC Representative ITC graphs are shown. The upper parts of both panels show the thermogram (thermal power vs. time) after baseline correction, while the bottom parts are the binding isotherm (normalized heat vs. molar ration of reactions). (A) Calorimetric titration of A5. (B) Calorimetric titration of B10. (C) Calorimetric titration of A5m. (D) A5 vs. buffer.

peptides exhibited comparable or weaker preS1-binding affinity. However, one peptide, B10 (LRNIR), showed a moderately increased preS1-binding affinity (**Fig. 2**).

The ITC analyses further confirmed that both A5 and B10 could bind to myr-preS1$_{2–48}$ and the binding affinity of B10 is ~1.5-fold higher than that of A5 (**Table 4** and **Fig. 3**).

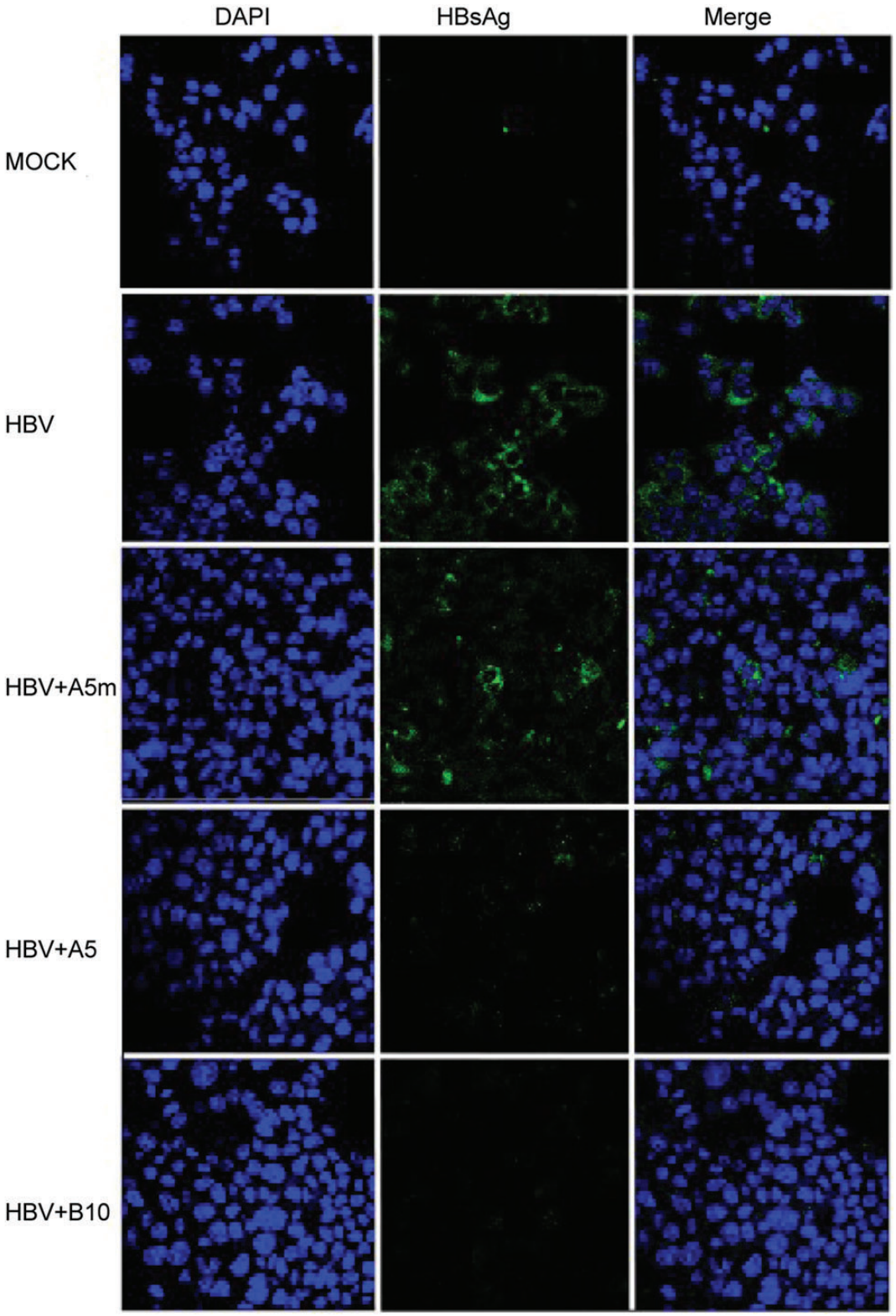

Figure 4. Inhibition of viral attachment by A5 and B10 Anti-HBsAg immunofluorescence (green) and nuclei staining (DAPI; blue) were performed on HepG2 cells incubated with HBV in the presence of 1 μM of one of the peptides (A5, B10, and A5m).

A5 and B10 peptides inhibits HBV attachment to HepG2 cells

HBV effectively attaches to hepatic cell line HepG2, which can be blocked by the preS1 antibodies [16]. Therefore, we chose HepG2 to investigate if the A5 or B10 peptide was capable of interfering with HBV attachment. As shown in **Fig. 4**, HBV attachment to HepG2 cells was significantly reduced in the presence of either the A5 or B10 peptide, but

not affected by the mutant A5 peptide, suggesting that both the A5 and B10 peptides can inhibit HBV attachment to HepG2 cells.

Discussion

Several preS1-derived peptides have been reported to exhibit inhibitory activities against HBV infection [9–13,17]. Those peptides likely act by competing with HBV to bind hepatocytes. Since the viral receptors may have other physiological functions, any side-effect that might accompany the long-term use of preS1-derived peptides remains to be evaluated. On the other hand, 'wrapping' HBV virions with preS1-binding peptides poses another strategy to prevent HBV attachment to hepatocytes. We have previously obtained several 12-amino acid peptides by screening a random peptide phage display library with HBV preS. Characterization of these peptides revealed one common preS1-binding motif (WTXWW) [15]. We hereby performed a new screening for the penta-peptides which might possess a high preS1-binding affinity.

Filamentous phage M13 is the most commonly used phage display system to search for short peptides. Random short peptides are displayed on the phage particles as partners fused either to the minor coat protein pIII or the major coat protein pVIII. One important difference between the two phage display strategies is the frequency of the displayed peptide. Fusion to pVIII results in a polyvalent display in which the peptide is displayed with each of the 2700 pVIII protein. In this case, the avidity effect is probably more significant than the affinity effect during selection. The pVIII-peptide phage display is thus more suitable for selection of ligands of mild or low affinity. On the contrary, because of the scarcity (five copies) of pIII on each M13 phage, the pIII-peptide phage display as used in this study is favorable for selection of ligands of high affinity.

Many studies have demonstrated that the residues 21–47 (corresponds to residues 10–36 in genotypes D, E, and G) of preS1 plays a crucial role in HBV infection [16,18,19]. Neurath *et al*. [20] reported that a synthetic $preS1_{21–47}$ peptide attached to HepG2 cells and inhibited HBV attachment. Le Seyec *et al*. [21] demonstrated that the residues 3–77 of preS1 were functionally required for HBV infection. Since preS1 produced in *Escherichia coli* is usually vulnerable to degradation and mainly insoluble, we chose the fragment $preS1_{1–65}$ which could be expressed in *E. coli* as a MBP fusion protein and purified in a stable and soluble form. $PreS1_{1–65}$ covers most of the known preS1 region that is required for HBV infectivity, making it be a suitable bait for screening peptides that may inhibit HBV attachment.

Five peptides out of 99 clones were obtained after the ninth round of panning. Only the A2 peptide, which occurred five times, is similar to the preS1-binding motif identified previously, suggesting that the preS1-binding affinity of this motif is weak. The other four peptides are rather different from the preS1-binding motif and also differ among themselves. Whether they interact with different regions of $preS1_{1–65}$ needs to be determined. Both the A5 and B10 peptides are rich in basic amino acids, suggesting that the ionic interaction between the peptide and preS1 might be important for their interaction. However, the A5m peptide, with all the basic amino acids intact, failed to bind $preS1_{1–65}$, suggesting that other mechanism also functions. Characterization of the interaction mechanism between these peptides and $preS1_{1–65}$ should facilitate the design of HBV entry inhibitors as well as shed light on the interaction mechanism between preS1 and the cellular receptor.

There are at least eight genotypes of HBV. We had used serum samples containing genotype B or C virus as well as culture supernatants of HBV-producing HepG2.2.15 cells containing genotype D virus in the peptide inhibition assays of viral attachment and observed similar effects as shown in **Fig. 4**. Therefore, A5 and B10 can at least interfere with cell attachment of genotype B, C, and D viruses. A more thorough investigation on the binding between A5 or B10 with different genotypes of HBV should be carried out in the future.

In conclusion, we have successfully identified several penta-peptides of high preS1-binding affinity by screening a random penta-peptide phage display library and mutagenetic analyses. The A5 and B10 peptides showed inhibitory effects on HBV attachment to HepG2 cells. The preS1-binding peptides can be useful candidates for HBV entry inhibitors. Further *in vitro* and *in vivo* studies are needed.

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