Journal of Peptide Science
J. Pept. Sci. 2008; **14**: 307–312
Published online 8 October 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.929

JOURNAL OF PEPTIDE SCIENCE

Identification of the critical regions in hepatitis B virus preS required for its stability

MIN LIAN,[a,b] XU ZHOU,[a] BIN CHEN,[a] CHAN LI,[a] XIAOCHENG GU,[a] MING LUO[a,c] and XIAOFENG ZHENG[a,b]*

[a] National Laboratory of Protein Engineering and Plant Genetic Engineering, Peking University, Beijing, 100871, China
[b] Department of Biochemistry and Molecular Biology, College of Life Sciences, Peking University, Beijing, 100871, China
[c] Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama, 35294, USA

Received 14 May 2007; Revised 24 July 2007; Accepted 26 July 2007

Abstract: As a hepatitis B virus (HBV) envelope domain, preS plays significant roles in receptor recognition and viral infection. However, the regions critical for maintaining a stable and functional conformation of preS are still unclear and require further investigation. In order to unravel these regions, serially truncated fragments of preS were constructed and expressed in *Escherichia coli*. Their solubility, stability, secondary structure, and affinity to polyclonal antibodies and hepatocytes were examined. The results showed that amino acids 31–36 were vital for its stable conformation, and the absence of 10–36 amino acids significantly reduced its binding to polyclonal antibodies as well as hepatocytes. The most stable fragment 1–120 (preS1 + *N*-terminal 12 amino acids of preS2), perhaps the core of preS, was discovered, which bound to HepG2 cells most tightly. Moreover, the availability of large amounts of well-folded and stable preS1-120 enables us to carry out further structural determination and mechanistic study on HBV infection. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: preS; critical region; stability; binding affinity; proper folding

INTRODUCTION

Hepatitis B virus (HBV) infection leads to acute and chronic hepatitis, which can result in serious liver diseases with a possible progression to cirrhosis or hepatocellular carcinoma [1–3]. The surface of HBV virions is composed of three proteins, termed small (S), middle (M), and large (L) proteins, which are anchored in the lipid bilayer [4]. These proteins share 226 amino acids (S protein). The M protein has 55 amino acids (preS2) added to the *N*-terminus of the S polypeptide, while the L protein has another extra 119 or 108 amino acids (preS1, depending on the genotype) added to the M protein at its *N*-terminus. The preS region is defined as preS2 and preS1 combined. By serological determinants, four HBV subtypes (*adw*, *adr*, *ayw*, and *ayr*) have been characterized [5]. Subsequently, eight HBV genotypes, A–H, have been classified [6]. The preS studied here belongs to genotype A and *adw* serological subtype.

According to previous reports, preS1 plays a significant role in receptor recognition, especially the region spanning amino acids 21–47, corresponding to amino acids 10–36 in the preS protein in this study (Figure 1(A)), which probably represents the major site for cell attachment [7]. A polypeptide covering the region 21–47 could inhibit virus–cell interactions, so does the antibody against this fragment [8,9]. A recent study further showed that amino acids 2–38 of preS1 is essential for attachment and infectivity of human HBV [10]. Further studies of Lanford *et al.* have suggested that amino acids downstream from residue 40 in the L protein may influence the infectivity and the host range of HBV [11,12]. It is likely that in addition to the region 21–47, there are other sites required for HBV attachment and the initiation of cell entry. Recent studies have shown that preS2 also plays an important part in translocation of the virus into host cells and the release of the virus into cytoplasm [13,14]. Meanwhile, without the *C*-terminus of preS1 and the *N*-terminus of preS2, hepatocytes transfected with an HBV genomic DNA were not able to secrete mature virions [15]. All these data suggest that the preS region is indispensable for HBV life cycle and is worthy of study.

However, the knowledge on the structure and function of the preS domains is still limited, which is mainly due to the difficulties in obtaining large amount of stable purified preS proteins. Here we investigated a series of segments truncated from the *N*-terminal and *C*-terminal ends of preS in order to find out the critical regions that sustain the proper conformation and to identify a stable fragment useful for further structural and biochemical studies.

MATERIALS AND METHODS

Construction of the preS Fragments

The preS fragments, 6–163, 11–163, 21–163, 31–163, 37–163, 41–163, 1–109, 1–120, and 1–146 (Figure 1(B)), were amplified from full-length preS of subtype *adw* (Accession

* Correspondence to: Xiaofeng Zheng, Department of Biochemistry and Molecular Biology, College of Life Sciences, Peking University, Beijing 100871, China; e-mail: xiaofengz@pku.edu.cn

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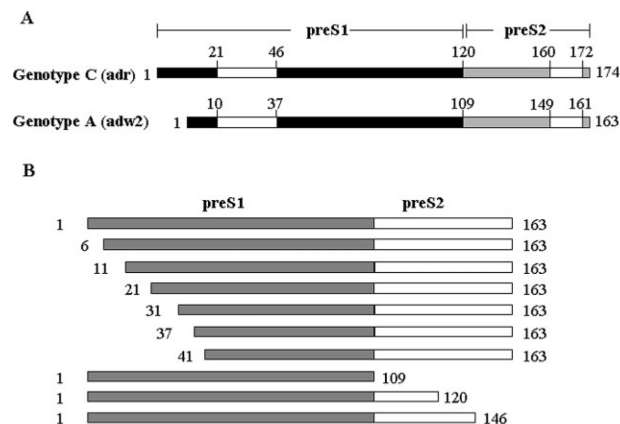

Figure 1 (A) The sketch of preS sequence between two different HBV genotypes. The amino acid residues crucial for viral recognition and infection spanning from 21 to 47 (genotype C, *adr*) or from 10 to 36 (genotype A, *adw2*) are indicated in blank boxes. The residues 41–52 in preS2, equivalent to 161–172 or 150–161 of preS, which are called the TLM motif, are indicated in blank boxes as well. (B) Different fragments of preS constructed for stability detection.

No. P03142) by PCR using the corresponding primers. The PCR products were inserted into pET28a vector with fusing his-tag at *N*-terminus after digestion with *Nde*I and *Xho*I. The resulting plasmids were verified by DNA sequencing and transformed into *Escherichia coli* expression strain BL21(DE3) for recombinant protein expression.

Purification of the preS Fragments

Protein purification was performed with a Ni-affinity column (Amersham Biosciences, Uppsala, Sweden). For each preS fragment, 1l bacteria were prepared in TN buffers (50 mM Tris–HCl, pH 8.0, 500 mM NaCl) supplemented with protease inhibitor cocktail (Roche, Germany), sonicated and then centrifuged at 15 000 rpm at 4 °C for 60 min. The supernatant was loaded on a 1-ml Ni-affinity column pre-equilibrated with TN buffer. Non-specifically bound proteins were washed away with the TN buffer containing 50 mM imidazole and the fusion proteins were eluted with a linear gradient (50–500 mM) of imidazole in the elution buffer (50 mM Tris–HCl, pH 4.0, and 500 mM NaCl). The purified proteins were collected and examined by SDS-PAGE. To increase protein homogeneity for circular dichroism spectroscopy analysis, the protein collected after Ni-affinity chromatography was concentrated and loaded on an S75 size-exclusion column (Amersham Biosciences, Uppsala, Sweden), equilibrated with the size-exclusion buffer (20 mM sodium acetate, pH 4.75). All fragments were purified following the same procedure as described above.

Stability Assay of preS Fragments by Degradation

Purified preS fragments were dissolved in the storage buffer (pH 4.0, 20 mM Na-citrate, and 1 mM EDTA) and incubated at 25 °C. Degradation was performed following a time course and determined by Western blot and *N*-terminal sequencing. All degradation assays were repeated three times to ensure the reliability of this experiment.

Circular Dichroism Spectroscopy and Secondary Structure Prediction

The concentration of the samples was 0.4 mg/ml diluted with the storage buffer. CD spectra were recorded at 25 °C on a Jasco J810 spectropolarimeter (Jasco, MD, USA) using a 0.1-cm path length quartz cell in the UV 190–250 nm wavelength range. Each spectrum was averaged by five scans after subtraction of the buffer baseline. The CD spectrum is reported as mean residue ellipticity, $[\theta]_{MRW}$, in deg cm^2 $dmol^{-1}$. The α-helical contents of the fragments were predicted by the k2d program available in the EMBL server (www.embl-heidelberg.de/~andrade/k2d/) [16].

ELISA Assays

Ninety-six well plates were coated with polyclonal anti-preS antibodies generated against the recombinant full-length preS at 4 °C overnight, followed by blocking with 3% BSA for 2 h. One hundred microliters of full-length and fragments of preS proteins (0.1 µmol) dissolved in PBS (pH 7.0) was added to the coated plates individually and the plates were incubated for 1 h. Specific binding was detected with an HRP-conjugated monoclonal anti-His-tag antibody and a chromogenic substrate containing 5 mM hydrogen peroxide. The reaction was stopped by adding 50 µl of 2 M H_2SO_4 and the absorbance was measured by a microplate reader at 450 nm (DNM-9602, Prolong, China). Each sample was measured in triplicate. All incubation steps were carried out at 37 °C and plates were washed with washing buffer (PBS pH 7.0) five times after each step unless noted.

Fluorescence-activated Cell Sorting (FACS) Analysis

HepG2 cells were harvested by centrifugation at 1000 rpm (Sigma 3K-15) for 5 min and then incubated with 0.1 mg/ml recombinant preS proteins at 25 °C for 1 h. Specific binding of preS fragments to cell surface was detected with the same polyclonal anti-preS antibodies and FITC-labeled goat anti-rabbit IgG antibodies. After each incubation at 25 °C for 45 min, cells were washed 3 times with PBS (pH 7.4, 0.1% Na-azide, 1 mM $CaCl_2$, and 0.5 mM $MgCl_2$). Cells were then scored using a BD FACS Calibur analyzer (Becton Dickinson, NJ, USA), and data were processed with CELL Quest software program (Becton Dickinson).

RESULTS

Cloning and Expression of preS Fragments

All constructs were successfully obtained through conventional cloning, and verified by DNA sequencing. Their expressions in *E. coli* BL21(DE3) were investigated. The results showed that the expressions of different preS fragments were distinct. preS6-163, preS11-163 and full-length preS showed similar expression levels (over 20 mg/l). preS37-163 and preS41-163 had the lowest expression level (1.1 mg/l), and was invisible on SDS-PAGE before being enriched by Ni-affinity chromatography. The expressions of recombinant proteins

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DOI: 10.1002/psc

were confirmed further by Western blot using polyclonal anti-preS antibody (data not shown).

Stability of preS Fragments

All the recombinant fragments were expressed in soluble forms. To determine the suitable temperature for examining the stability of the preS protein and its various fragments, we compared the stability of the full-length preS under different temperatures. Recombinant preS was most stable at −20 °C and fairly stable at 4 °C. As the protein began to degrade at 25 °C, we chose this temperature to perform the following degradation experiments. Besides, we examined the stability of the full-length preS at different pHs ranging from 3.6 to 8.0, and found that the recombinant protein aggregated and precipitated obviously above pH 7.0 but showed a better stability at pH 4.0; therefore the following experiments were performed at pH 4.0.

At 25 °C, the stabilities of preS37-163 and preS41-163 were extremely weakened (Figure 2(A)), which might cause the low expressions of these two polypeptides. The *N*-terminal-truncated fragments preS11–163, preS21–163 and preS31–163 behaved like the full-length preS. Interestingly, the fragment preS1–120, which contains preS1 and *N*-terminal 12 amino acids of preS2, was far more stable than any other fragments, while preS1–109 (preS1) was highly unstable (Figure 2(A)).

Western blot results showed that the cleavage of the recombinant preS protein occurred at a site near the *C*-terminus because all the degradation products could be stained by anti-His antibodies (Figure 2(B)), which was also confirmed by the *N*-terminal sequencing.

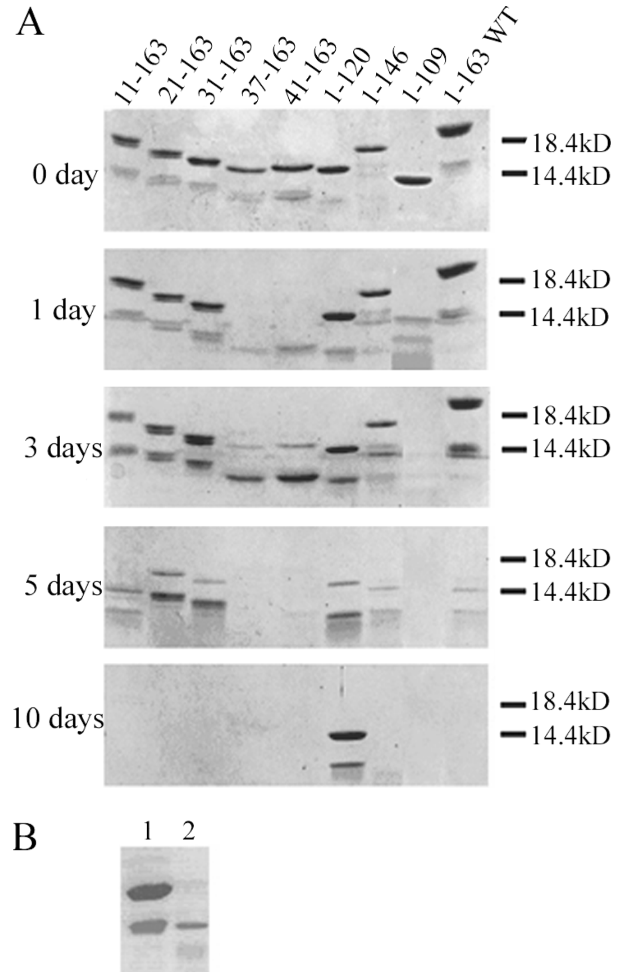

Figure 2 Stabilities of the recombinant preS fragments examined. (A) The stability of preS fragments detected at pH 4.0 at 25 °C. The samples of 0, 1, 3, 5, and 10 days under degradation were taken equally and tested by SDS-PAGE. (B) Western blot performed to demonstrate preS degraded from *C*-terminal. Lane 1 indicates full-length preS and lane 2 is the degraded fragment of preS. Anti-His was applied to examine whether the degradation occurred from the *N*-terminus.

Table 1 Analysis of CD spectra for α-helix secondary structural content of preS and the fragments by the k2d program

Polypeptides	α-Helix (%)	α-Helix (50% TFE) (%)	α-Helix (20 mm SDS) (%)
preS	15	28	28
preS41–163	6	28	27
preS1–120	27	28	28
preS1–146	27	29	28

Conformational Analysis of preS Fragments by CD Spectroscopy

Since CD spectroscopy is a sensitive method for determining the secondary structural contents of peptides and proteins [17], it was employed to analyze the conformational characteristics of the full-length preS and its fragments (Figure 3(A)). preS41–163 displayed minimal absorption at 208 and 222 nm with approximately 6% α-helix (Table 1), which was the least ordered structure among all fragments. The CD spectral patterns of preS1–120 and preS1–146 resembled each other with a weak negative absorption at 222 nm, and the two fragments were estimated to contain about 27% α-helix, 12% more than that of preS (Table 1). In all cases, approximate 30% sheet secondary element was observed.

TFE is known to stabilize and promote the formation of secondary structure elements in proteins, especially α-helices [18]. In our study, the negative maximum in the CD spectrum of the full-length preS was shifted from 200 to 205 nm and its α-helix secondary structure content increased from 15 to 28% when 50% TFE was added into the sample solution (Figure 3(B), Table 1). Meanwhile, in the presence of TFE, all the fragments

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DOI: 10.1002/psc

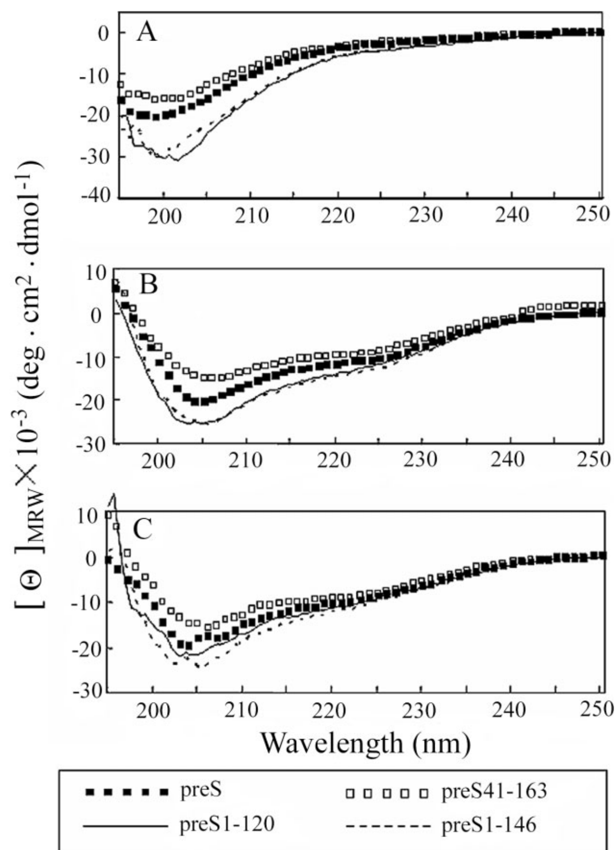

Figure 3 Circular dichroism spectra of the purified recombinant preS proteins. The protein samples are detected in the absence (A) or presence of 50% TFE (B), and in the presence of 20 mM SDS (C). The proteins were diluted in 20 mM sodium acetate, pH 4.75, to a final concentration of 0.4 mg/ml.

had a visible absorption at 222 nm in their CD spectra (Figure 3(B)), including preS41–163 which had 28% α-helical content, up from 6% in the absence of 50% TFE (Table 1). preS1–120 and preS1–146 still had the same pattern of CD spectra with or without 50% TFE, with a stronger absorption than that of the full-length preS (Figure 3(B)). Overall, the CD spectra suggested that there were no obvious differences in their secondary structure elements among all the fragments when TFE was used to stabilize the proteins.

In the envelope of HBV, preS may need to form a stable conformation and function in a lipid environment. To simulate the lipid environment, 20 mM SDS was added to the protein samples and the CD spectra were re-collected. The results showed that the CD spectra measured in the presence of 20 mM SDS were the same as those in the presence of 50% TFE (Figure 3(C), Table 1).

Affinity of preS Fragments to Polyclonal Antibodies

In binding affinity assays (Figure 4(A)), polyclonal anti-preS was verified by Western blot with recombinant preS protein and serum HBV virons (data not shown). Judging by the ELISA readings, the binding affinities of preS fragments were decreased as they were truncated further at the *N*-terminus. preS6–163 and preS11–163 showed almost the same binding affinity as the full-length preS, whereas preS31–163, preS37–163 and preS41–163 bound weakly to polyclonal anti-preS antibodies. These results indicate that a major epitope in preS may be located between residues 11–30, or these residues are required for presenting epitopes in preS. Moreover, residues between 1–10 at the *N*-terminus were unlikely to present any epitope to elicit a humoral immune response, nor were they required for stabilization of a proper protein conformation to present major epitopes in preS, except for the natural myristoylated site Glycine-2 which is essential for HBV infectivity [11,19,20]. Two fragments with *C*-terminal truncations, preS1–120 and preS1–146, showed similar binding affinity but both were lower than that of the full-length preS. preS1–109 presented a slightly weaker binding affinity than preS 1–120.

Binding to HepG2 Cells

Full-length preS, preS41–163, preS1–109, preS1–120 and preS1–146 were tested for their ability to attach to HepG2 cells. As shown in Figure 4(B), preS41–163 showed weak binding to HepG2 cells, whereas preS1–109, preS 1–120 and preS1–146 presented an enhanced affinity to HepG2 cells compared to the full-length preS.

DISCUSSION

During the last 20 years, numerous attempts have been made on unraveling the function of all three HBV surface proteins, which were well summarized by Glebe and Urban [21]. However, the information obtained on preS so far did not clearly report the regions that are critical for maintaining a stable and functional conformation of preS. Therefore, the aim of the present study was to further find out these regions by using a set of preS fragments of variable lengths.

The soluble expression of a stable protein usually suggests the proper folding of a recombinant protein [22]. In this study, preS37–163 and preS41–163 degraded quickly, whereas preS1–163, 11–163, 21–163 and 31–163 showed a similar stability as full-length preS (Figure 2(A)), indicating that amino acids 31–36 were pivotal for preS stability and therefore important for stabilization of the appropriate tertiary structure of preS. A similar result was drawn from the analysis by CD spectroscopy. The preS fragment lacking *N*-terminal 40 residues appeared to have few ordered secondary structure elements (Figure 3), which confirmed that the *N*-terminus of preS was essential for its folding and conformational stability.

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DOI: 10.1002/psc

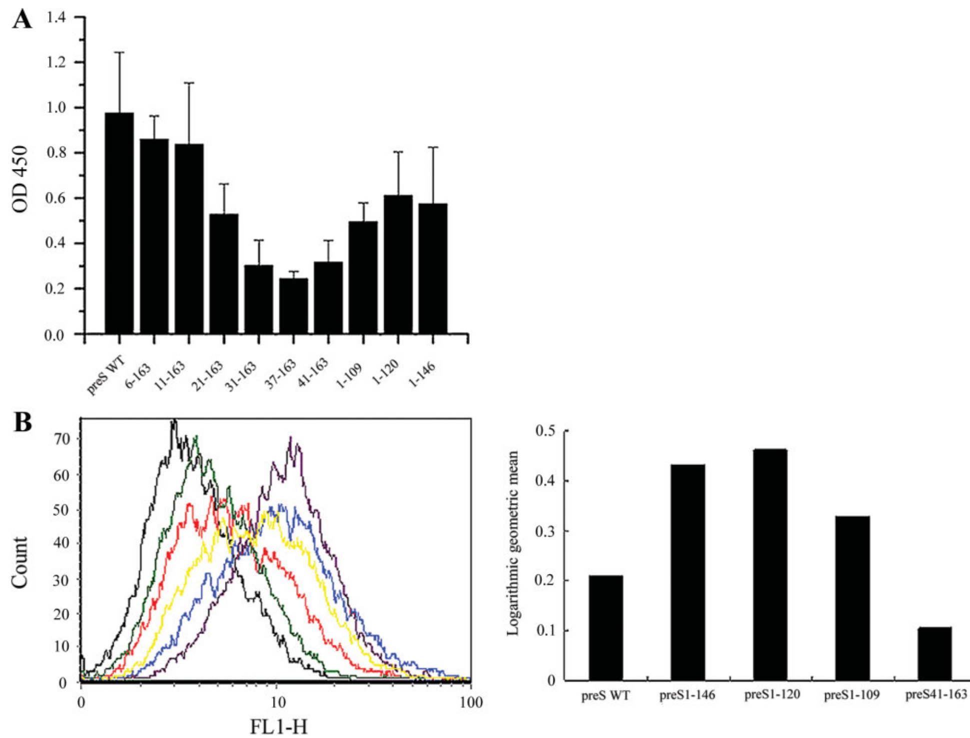

Figure 4 Binding affinity of various preS fragments to polyclonal antibodies and hepatocytes. (A) Binding of preS fragments to polyclonal antibody against full-length preS was detected using anti-His conjugated with HRP. (B) Flow cytometry analysis of recombinant preS proteins binding to HepG2 cells. (Left panel) Black line indicates mock control (treated with PBS). Purple, blue, yellow, red and green lines indicate recombinant preS1-120, preS1–146, preS1–109, preS WT and preS41–163. (Right panel) Quantitative analysis of data derived from flow cytometry by FCS Express Version 3 (De Novo software). The y-values for each protein are calculated on the basis of a statistics of 10^4 cells.

preS6–163 and preS11–163 bound to antibodies in a similar way as did the full-length preS, while preS31–163, preS37–163 and preS41–163 bound poorly to the polyclonal antibodies (Figure 4(A)). Furthermore, preS41–163 bound poorly to HepG2 as well (Figure 4(B)). These results suggested that the residues 11–30 are perhaps on the surface of the preS protein and are involved in epitope presentation and recognition of cell receptors. This inference is consistent with the results from previous studies [8,9,23,24]. Neurath and other groups have suggested that the region spanning amino acids 21–47 of preS (corresponding to amino acids 10–36 of the preS in this study) was essential for receptor recognition and viral infectivity [8,9,25,26].

It is interesting to note that the fragment preS1–120 was the most stable fragment with the molecular weight of 14 925 Da, close to that of the stable degraded fragment band of the full-length preS on SDS-PAGE (Figure 2). Together with the instability of preS1–109, our results suggested that preS1–120 comprising the preS1 region and the *N*-terminal part of the preS2 region was able to assume a more stable conformation than the full-length recombinant preS (Figure 2(A)). This inference was further confirmed by CD spectrum analyses, indicating that preS1–120 has 27% α-helix secondary structure content which was achieved in preS only in the presence of 50% TFE (Figure 3, Table 1). As shown in Figure 4, preS1–120 exhibited the strongest binding ability to HepG2. We therefore suppose that preS1–120 probably represents the core of the full-length preS and is able to maintain the stable conformation required for receptor attachment. preS does not exist as an independent protein in HBV virions since it is part of the L protein. A functional preS conformation may only be present in the context of the L protein. When produced as an isolated recombinant protein, additional sequences corresponding to the *C*-terminal part of preS2 appear to destabilize the conformation of the full-length preS, which may reduce its activities in terms of receptor recognition and epitope presentation.

In contrast to preS1–120, preS1–109 was extremely unstable and showed a weaker binding ability to polyclonal anti-preS antibodies and HepG2 cells (Figure 4), suggesting that deletion of amino acids 109–120 (the *N*-terminal 1–12 residues of preS2) might interrupt the core of preS, which might explain why the preS amino acid sequence between 91 and 113 preS (or 102 to 124, depending on the genotypes) was crucial for virus secretion, and lacking the initial five amino acids of preS2 would lead to a failure in virion assembly and secretion, according to Le Seyec's result [15].

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preS1–120 showed higher affinity with hepatocytes, but lower affinity with polyclonal antibodies than that of the full-length preS, suggesting that the *C*-terminus of preS was not directly involved in the cellular receptor recognition. However, this *C*-terminal region may be related to antibody recognition. Deletion of the region from residues 121 to 163, especially residues 147–163, decreased the binding affinity of preS to polyclonal antibody significantly (Figure 4(A)), which is in accordance with the earlier study that antibody recognition sites exist in the preS2 region [27].

Since full-length preS is unstable [22,28], identification of the crucial core region of preS and preparation of large amounts of stable and well-folded preS1–120 protein are important factors for determination of its tertiary structure and elucidation of the mechanism of HBV infection.

Acknowledgements

This work was supported by grants from International Centre for Genetic Engineering and Biotechnology (ICGEB) (Project No. CRP/CHN05-01) and the National Science Foundation of China (No. 30328006). The authors thank the Instrumental Analysis Fund of Peking University and Liying Du for her help in using the FACS Calibur analyzer.

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