Supporting Information

A Virus-like Particle of the Hepatitis B Virus preS Antigen Elicits Robust Neutralizing Antibodies and T Cell Responses in Mice

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Supplementary Materials and Methods

Plasmids and cells

The vector for expressing HBV preS (adw subtype, Accession Number AGW20902) in *E. coli* (pET28b-preS) was constructed previously (Lian et al., 2007). The His-tagged preS protein was expressed and purified as described previously (Lian et al., 2007). Recombinant preS was quantitated by the bicinchoninic acid assay (BCA assay). To prepared polyclonal rabbit anti-preS antibody, purified recombinant preS protein was mixed with Alum adjuvant and injected subcutaneously into New Zealand rabbits four times in two months, and the antisera was collected from the rabbits two weeks postimmunization and was subjected to affinity chromatography. The plasmids for expressing M1 protein (the matrix protein) of influenza virus A/sw/Spain/53207/04 and a preS-HA (HA=hemagglutinin) chimeric protein were constructed by inserting the coding sequence in pCAGGS. The amino acid 41 of M1 was mutated to Ala (pCAGGS-M1). The preS-HA has the sequence of HBV preS followed by aa521-566 of HA (pCAGGS-preS-HA). 293T cells were maintained in DMEM supplemented with 10% fetal bovine serum (Invitrogen). All the fetal bovine serum used in this study was inactivated at 56°C for 30 min.

Preparation and characterization of the virus-like particles

The pCAGGS-M1 and pCAGGS-preS-HA plasmids were transfected into 293T cells with polyethylenimine. 72 h after transfection, the culture medium was centrifuged at 6,000 rpm for 15 min at 4°C to remove cellular debris, followed by centrifugation at 22,000 rpm for 3 h at 4°C. The pellet was resuspended in PBS at 4°C overnight, and further purified through a 20%-60% sucrose gradient in a Beckman SW41Ti rotor at 30,000 rpm for 3 h at 4°C. The 40% sucrose fraction was harvested and diluted with

PBS by about 5 folds. After centrifugation at 22,000 rpm for 3 h at 4°C to remove the sucrose, the virus-like particles were resuspended in PBS at 4°C overnight. Quantitation of preS VLP total proteins was carried out by the bicinchoninic acid assay (BCA assay). A sample was applied to a 400mesh carbon-coated copper grid, and stained with 1% phosphotungstic acid (J&K Scientific). preS VLP was visualized on a Tecnai G^2 Spirit transmission election microscope operating at 120 kV.

Indirect immunofluorescence

293T cells were grown on glass coverslips and transfected with pCAGGS-M1 and pCAGGS-preS-HA. 48 h posttransfection, cells were fixed with 4% paraformaldehyde. Cells were classified into two groups. One was permeabilized with 0.2% Triton X-100 for 5 min, the other without permeabilization. After blocking for 1 h in PBS containing 5% goat serum, all cells were incubated with purified polyclonal rabbit anti-preS antibody at 4°C overnight. Cells were washed with PBS following incubation with Alexa Fluor® 488-Conjugated goat anti-rabbit secondary antibody (ZSGB-BIO, Beijing, China) for 1 h at 37°C. After washing, cells were stained with DAPI for 10 min, and then mounted onto microscope slides. Confocal slices were acquired with a 100× objective, using a Zeiss 510 confocal microscope with random sampling.

Western blot analysis

Western blot analysis was performed as described previously (Zheng et al., 2015). Briefly, samples were mixed with 4 × SDS-PAGE loading buffer followed by heating at 95°C for 10 min. The denatured samples were separated by electrophoresis on 12%-SDS-PAGE gel and transferred to a PVDF membrane. After blocking with a 5 % (wt/vol) skim milk solution, the membrane was incubated with purified polyclonal rabbit anti-preS antibody (1:1,000) and then with HRP-conjugated goat anti-rabbit IgG antibody (Abclonal). The chemiluminescent detection reaction was performed and monitored by ChemiDoc XRS System (Bio-Rad).

Glycosylation analysis

The glycosylation of preS-HA in VLP was analysed by western blot and LC-MS/MS. For western blot analysis, the sample from 40% sucrose fraction was treated with PNGase F and/or *O*-Glycosidase (New England Biolabs) following the manufacturer's instructions. Briefly, 2 μL of samples, 1 μL of Glycoprotein Denaturing Buffer (10×) and 7 μL of H_2O were combined in a total reaction volume of 10 μL, and samples were denatured by heating the reaction mixture at 100°C for 10 min followed by cooling to room temperature. 2 μL of GlycoBuffer 2 (10×) and 2 μL of 10% NP-40, 2 μL of Neuraminidase (only used for *O*-Glycosidase), H_2O, and 1 μL of *O*-Glycosidase and/or PNGase F were combined in a total reaction volume of 20 μL, followed by incubation at 37°C for 18 h. Samples were subsequently subjected to western blot analysis as described above.

For LC-MS/MS analysis, the sample from 40% sucrose fraction was subjected to electrophoresis on a 12%-SDS-PAGE gel, which was stained by coomassie R250. The bands corresponding to 25-30 kDa were cut out, subjected to reduction with 10 mM DTT at 56°C for 50 min, and subsequent alkylated with 55 mM iodoacetamide (IAA) for 30 min at room temperature in the dark. In-gel digestion was conducted with trypsin [Promega, enzyme: protein= 1:50 (wt/wt)] at 37°C for 16 h in 25 mM ammonium bicarbonate buffer, followed by lyophilization.

The lyophilized samples were re-dissolved in 2% acetonitrile, 0.1% formic acid, and loaded on a ChromXP C18 (3 μm, 120 Å) nanoLC trap column. The inline trapping, desalting procedure was carried out at a flow rate of 2 μL/min for 10 min with 100% solvent A (Solvent A: water/acetonitrile/formic acid = 98/2/0.1% solvent B: 2/98/0.1%). A 60-min gradient elution ranging from 5-35% acetonitrile (0.1% formic acid) was carried out onto an analytical column (75 μm x 15 cm C18- 3μm 120 Å, ChromXP Eksigent). LC-MS/MS analysis was performed with a Triple TOF 5600 System (AB SCIEX, Concord, ON) fitted with a Nanospray III source (AB SCIEX, Concord, ON). Data were acquired using an ion spray voltage of 2.5 kV, curtain gas of 30 PSI, nebulizer gas of 5 PSI, and an interface heater temperature of 150 °C. The MS instrument was operated as TOF-MS scans. For IDA, survey scans were acquired in 250 ms and as many as 25 product ion scans (90 ms) were collected if exceeding a threshold of 150 counts per second (counts/s) and with a +2 to +4 charge-state. A Rolling collision energy setting was applied to all precursor ions for collision-induced dissociation. Dynamic exclusion was set for ½ of peak width (~12 s).

For glycosylation analysis, the raw files were searched against the local database including the protein sequences for M1, preS-HA and decoys, using Byonic (version 2.11.0). All of the N-glycan and O-glycan databases were used for searching possible glycan modifications, with the following parameters: mass tolerances were 10 ppm and 40 ppm for the precursor and fragment ions, respectively; enzyme specificity was set as RK, with a maximum of two missed cleavages; carbamidomethylation of Cys residues were set as fixed modification (57.021464 Da); variable modifications including oxidation of Met residues, acetylation of protein N-terminals, and carbamidomethylation of N-terminal, His and Lys were allowed; the 1% False Discovery Rate Analysis was also engaged. Glycopeptides were filtered using the following criteria: Score≥300, Delta Mod≥10, |Log Probability|≥2.

Immunization and hydrodynamic transfection in Balb/c mice

All mouse experiments were conducted in accordance with the institutional guidelines following the experimental protocol reviewed and approved by the Institutional Animal Care and Use Committee of Peking University. Female Balb/c mice of 6-8 weeks old were immunized by injecting the antigen preparation in the hindlimb. A booster was given on day 22. Blood was collected on day 52 and 112, and neutralizing antibody

titers were determined by ELISA. On day 52, activated T cells in splenocytes were analyzed by ELISPOT and flow cytometry (FACS). The immunized mice were hydrodynamically transfected on day 70. 10 μg of pHBV1.3, a plasmid containing 1.3X genome length of HBV, genotype D, ayw subtype, was used in hydrodynamic injection to establish HBV replication as previously described (Yang et al., 2002). Blood samples were collected at different time points to measure HBV antigens. On day 77, mice were sacrificed and liver tissues were used for measuring antigens and RNA of HBV.

HBV infection and neutralization assays

Virus neutralization assays were conducted as described previously (Wang et al., 2016; Ye et al., 2016). HepAD38 cells were cultured in Dulbecco's modified Eagle's medium F12 (DMEM F12) (Invitrogen, Carlsbad, USA) containing 2 mM L-glutamine, 50 U/ml penicillin, and 10% fetal bovine serum (Invitrogen) at 37°C and 5% CO2. The virus-containing supernatant from HepAD38 culture was concentrated using Amicon Ultra-15 centrifugal filters (Millipore, Billerica, USA). HBV titers were quantified using a qRT-PCR HBV DNA detection kit (Qiagen, Hilden, Germany) and expressed as genome equivalents (geq). For infection experiments, 5×10^5 HepG2/hNTCP cells per well were cultured in 24-well plate and incubated overnight with the viral inoculum (M.O.I. of 500) alone or together with various dilutions of mouse anti-preS VLP sera or 1,000 fold dilution of hepatitis B immunoglobulin (HBIG) (from Chengdu Rongsheng Bioproduct Company, with a protein concentration of 144 mg/ml), with 4% PEG present during virus infection. Medium was changed every 2 or 3 days, and HBeAg was measured at 1 week post infection using Diagnostic ELISA Kit for Hepatitis B e antigen (Kehua Bio-engineering).

Isolation of splenocytes

For splenocyte isolation, splenocytes were gently grinded followed by passaging through 40 μm strainers and treating with ACK lysing buffer. After washing with PBS, cells were resuspended in DMEM supplemented with 10% fetal bovine serum (Invitrogen) and 1% Penicillin-Streptomycin–L-Glutamine.

Enzyme-linked immunospot assay

T cell responses were determined using an IFN-γ ELISPOT set (BD Biosciences) following the manufacturer’s protocol. Briefly, 96-well plates were coated with purified anti-mouse IFN-γ antibody (1:200) at 4°C overnight, and then were blocked for 2 h using DMEM supplemented with 10% fetal bovine serum (Invitrogen) and 1% Penicillin-Streptomycin– L-Glutamine. Splenocytes were seeded at 2×10^5/well. Peptides representing previously described epitopes present in preS (Table 1) or purified recombinant preS protein at a concentration of 20 μg/ml were used to stimulate cells for 36 h at 37°C in a 5% CO_2 and humidified incubator, with media and

phorbol myristate acetate (PMA)/ionomycin-treated cells used as negative and positive controls, respectively. After being washed, cells were incubated with biotinylated anti-mouse IFN-γ antibody (1:250) for 2 h at room temperature, and then incubated with streptavidin-horseradish peroxidase (HRP) (1:1,000) for 1 h. Following the final washes, 3-amino-9-ethylcarbazole (AEC) substrate (Alfa Aesar) was added to the wells and allowed to develop at room temperature for 25 to 35 min. The reaction was stopped with distilled water, and the plates were allowed to air dry before spot-forming cells were enumerated automatically with an ELISPOT plate reader.

Flow cytometry

Splenocytes were resuspended in DMEM supplemented with 10% fetal bovine serum (Invitrogen) and 1% Penicillin-Streptomycin– L-Glutamine, and then were seeded at 2×10^6 /well. The cells were then stimulated for 6 h with preS-specific peptides or purified recombinant preS protein diluted to a final concentration of 10 μg/ml in DMEM supplemented with 2 μg/ml brefeldin A (BD Biosciences). The cells were then washed in staining buffer (PBS containing 2% fetal bovine serum) and stained for CD8 and CD4 surface expression for 30 min at 4°C using fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD8 antibody (BD Biosciences) and peridinin chlorophyll protein(PerCP)-conjugated anti-mouse CD4 antibody (BD Biosciences). Then the cells were washed, fixed, and permeabilized using a commercially available Cytofix/Cytoperm kit (BD Biosciences). The cells were then stained for 30 min at 4°C for intracellular cytokine expression using phycoerythrin (PE)-conjugated anti-mouse IFN-γ antibody (BD Biosciences). After washing, cells were resuspended in staining buffer and analysed using a BD FACS Canto™ II flow cytometer (BD Biosciences) and FACSDiva Version 6.1.3. Results were generated from data gathered from 200,000 cells.

ELISA

Purified recombinant preS antigen (1 μg/ml) was absorbed to 96 well plates, blocked with 10% BSA, and then 50 μl of 1:100 dilution of sera was added and incubated for 30 min at 37°C. The plates were washed six times, and followed by incubation with a 1:5,000 dilution of HRP-conjugated anti-mouse IgG, IgG1 (Santa Cruz Biotechnology) or IgG2a (BD Biosciences) for 30 min at 37°C. After washing, the plates were incubated with TMB substrate for 10 minutes before stopping with 2 M H_2SO_4. Absorbance at 450 nm was measured by the 2104 EnVision® Multilabel Reader (PerkinElmer). Endpoint dilutions were performed (Fig. S5). In addition, serum samples were diluted 1:5 for HBsAg ang HBeAg detection (Kehua Bio-engineering).

ALT activity analysis

The level of serum alanine aminotransferase (ALT) was measured with a Hitachi 7600 Automatic Biochemistry Analyzer.

RT-PCR and qRT-PCR analyses

For detecting mRNA in 293T cells transfected with expression vectors, 48 h posttransfection, total RNAs from 293T cells were extracted using the QIAGEN RNeasy Mini Kit following the manufacturer's instructions. Total RNAs were stored at -20°C until being used. Any possible contaminating DNA was digested by DNase I (Takara). Total RNAs were quantified using the NanoDrop 2000 spectrophotometer (Thermo Scientific). 1 μg total RNAs were reverse transcribed into cDNA using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara) in a 20 μL reaction as described previously (Zheng et al., 2016). cDNA derived from 5 ng total RNAs was used as template for qRT-PCR amplification. Primers (preS-HA-FW: 5'-CCACCAATCGGCAGTC-3') and (preS-HA-RV: 5'-GCCACCAGCAGGAAGAT-3') were used for preS-HA transcripts; (M1-FW: 5'-TGACAACAACCAACCCACT-3') and (M1-RV: 5'-CTGCTGCTTGCTCACTCG-3') were for M1 transcripts; (β-actin-FW: 5'-TCATGAAGTGTGACGTGGACATC-3') and (β-actin-RV: 5'-CAGGAGGAGCAATGATCTTGATCT-3') were used for β-actin transcripts. qRT-PCR was performed with GoTaq qPCR Master Mix (Promega) following the manufacturer's protocol for a total reaction volume of 20 μL in the CFX96 Real-Time PCR Detection System (Bio-Rad). The reaction product was subjected to agarose gel electrophoresis.

For hydrodynamic transfection studies, total RNAs from the liver of HBV-transfected mice were isolated with Trizol reagent (Invitrogen) according to manufacturer's manual. Any possible contaminating DNA was digested by DNase I (Takara). Total RNAs were quantified using the NanoDrop 2000 spectrophotometer (Thermo Scientific). 1 μg total RNAs were reverse transcribed into cDNA using the PrimeScript RT Reagent Kit with gDNA Eraser (Takara) in a 20 μL reaction as previously described(Zheng et al., 2016). cDNA derived from 2 ng total RNAs was used as template for qRT-PCR amplification. Primers (HBV2270FW: 5'-GAGTGTGGATTCGCACTCC-3') and (HBV2392RV: 5'-GAGGCGAGGGAGTTCTTCT-3') were used for HBV RNA transcripts (Yan et al., 2012). qRT-PCR was conducted with GoTaq qPCR Master Mix (Promega) following the manufacturer's instructions for a total reaction volume of 20 μL in the CFX96 Real-Time PCR Detection System (Bio-Rad).

H&E staining and immunohistochemistry

Liver tissues were collected and fixed in 10% neutral formalin (Sigma). After paraffin embedding, liver sections were stained with hematoxylin and eosin (H&E). Immunohistochemistry was performed using polyclonal rabbit anti-HBcAg antibody (Dako) to detect HBV core antigen.

Immunization in HBV transgenic mice

All mouse experiments were performed in accordance with the institutional guidelines following the experimental protocol reviewed and approved by the Institutional Animal Care and Use Committee of Peking University. Six- to 8-week-old female HBV transgenic mice (ayw subtype) were obtained from Infectious Disease Center of No. 458 Hospital (Guangzhou, China). HBV transgenic mice were immunized intramuscularly with 20 μg of preS VLP in the hindlimb, and were boosted on days 22 and 43, respectively. Blood was collected on days 0, 30 and 70, and anti-preS antibody titers were determined by ELISA. On day 70, activated T cells in splenocytes were analyzed by ELISPOT and flow cytometry.

Statistical analysis

The data are represented as mean ± SEM. The statistical difference was determined by a two-tailed Student's t-test (*P<0.05; **P<0.01; ***P<0.001 vs. the mock group).

Table S1: Identification of glycosylated residues of preS-HA.

Peptide sequence	Glycans	|Log Prob|	Score	Delta Mod. Score
R.DSHPQAMQWN[+1257.44941]STAFHQALQDPR.V	HexNAc(3)Hex(4)	4.15	343	235.9
R.QPTPIS[+406.15875]PPLR.D	HexNAc(2)	6.66	528.6	92.2
R.QPTPIS[+203.07937]PPLR.D	HexNAc(1)	8.14	617	45.1
R.QPTPIS[+963.31794]PPLR.D	HexNAc(1)Hex(1)NeuGc(1)NeuAc(1)	4.98	354	39.3
R.QPTPIS[+730.26439]PPLR.D	HexNAc(2)Hex(2)	5.32	440.1	38.1
R.QPTPIS[+947.32303]PPLR.D	HexNAc(1)Hex(1)NeuAc(2)	7.32	514.1	38.1
R.QPTPIS[+568.21157]PPLR.D	HexNAc(2)Hex(1)	6.65	572.1	33.8
R.QPTPIS[+365.13220]PPLR.D	HexNAc(1)Hex(1)	8.14	619.8	32.4
R.QPTPIS[+656.22761]PPLR.D	HexNAc(1)Hex(1)NeuAc(1)	8.55	603.2	31.7
R.QPTPIS[+859.30699]PPLR.D	HexNAc(2)Hex(1)NeuAc(1)	4.43	421.9	24.1

R.QSGRQPTPIS[+656.22761]PPLR.D	HexNAc(1)Hex(1)NeuAc(1)	5.12	466.8	17.2

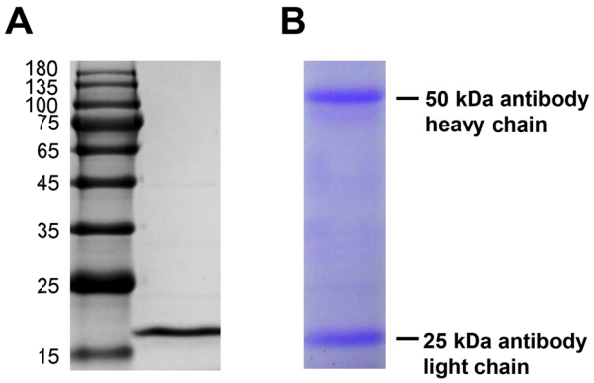

Fig.S1: SDS-PAGE analysis of purified recombinant preS protein (A) and polyclonal rabbit anti-preS antibody (B).

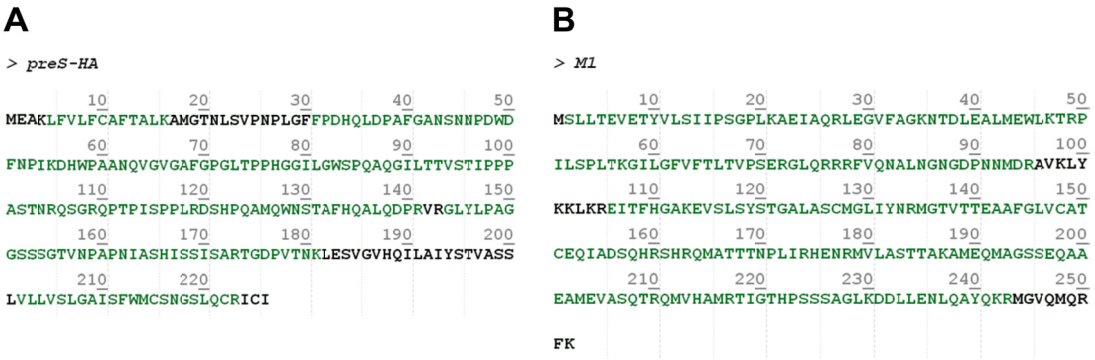

Fig.S2: LC-MS/MS identification of M1 and preS-HA in the sample from 40% sucrose fraction. The identified sequences are colored green.

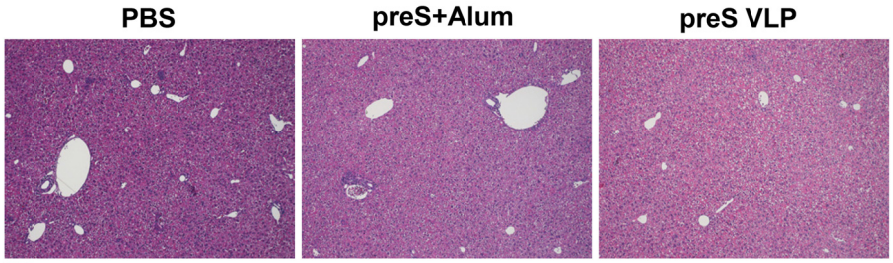

Fig.S3: Liver tissue sections of Balb/c mice from each group were stained with hematoxylin and eosin on day 7 after hydrodynamic transfection of HBV DNA. Magnification, 100×.

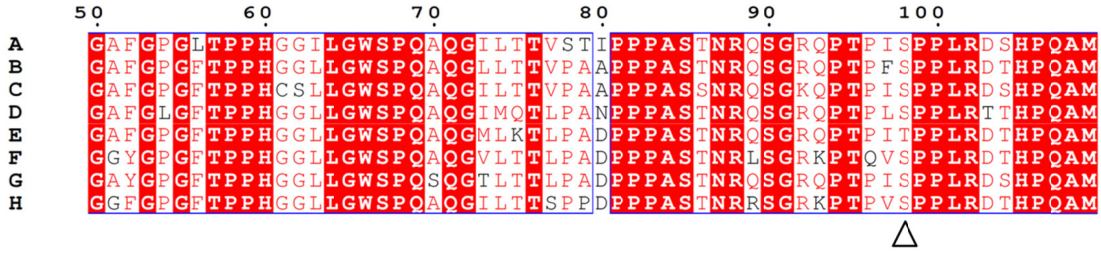

Fig.S4: Partial alignment of the primary sequences of preS from all HBV genotypes. preS in VLP is genotype A.

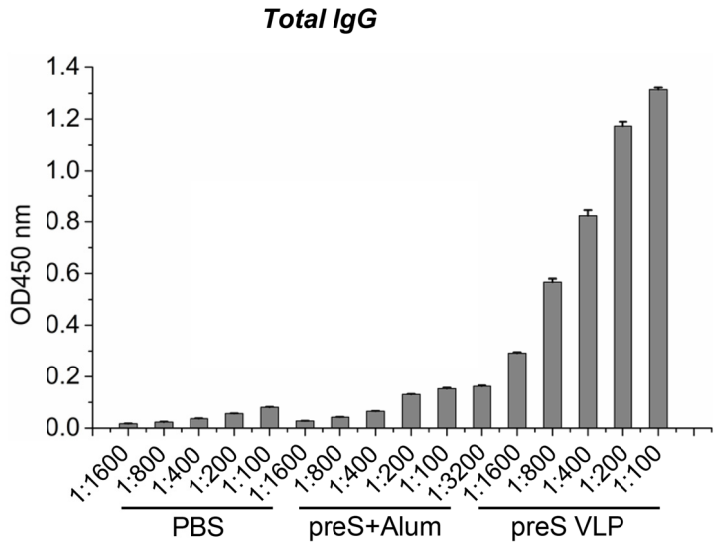

Fig.S5: Measurement of anti-preS antibodies by endpoint dilution assay. The serum anti-preS titers were determined by ELISA. The plates were coated with 1 μg/mL purified recombinant preS. The sera were diluted by various folds.

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