Hepatitis B Virus (HBV) Surface Antigen Interacts with and Promotes Cyclophilin A Secretion: Possible Link to Pathogenesis of HBV Infection^{∇‡}

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Cyclophilin A (CypA), predominantly located intracellularly, is a multifunctional protein. We previously reported decreased CypA levels in hepatocytes of transgenic mice expressing hepatitis B virus (HBV) surface antigen (HBsAg). In this study, we found that expression of HBV small surface protein (SHBs) in human hepatoma cell lines specifically triggered CypA secretion, whereas SHBs added extracellularly to culture medium did not. Moreover, CypA secretion was not promoted by the expression of a secretion deficient SHBs mutant, suggesting a close association between secretion of CypA and SHBs. Interaction between CypA and SHBs was observed by using coimmunoprecipitation and glutathione *S*-transferase pull-down assays. Hydrodynamic injection of the SHBs expression construct into C57BL/6J mice resulted in increased serum CypA levels and ALT/AST levels, as well as the infiltration of inflammatory cells surrounding SHBs-positive hepatocytes. The inflammatory response and serum ALT/AST level were reduced when the chemotactic effect of CypA was inhibited by cyclosporine and anti-CD147 antibody. Furthermore, higher serum CypA levels were detected in chronic hepatitis B patients than in healthy individuals. In HBV patients who had received liver transplantation, serum CypA levels declined dramatically after the loss of HBsAg as a consequence of liver transplantation. Taken together, these results indicate that expression and secretion of SHBs can promote CypA secretion, which may contribute to the pathogenesis of HBV infection.

Hepatitis B virus (HBV) infects more than 350 million people worldwide and is a major cause of chronic viral hepatitis and hepatocellular carcinoma (25). Three morphologically distinct forms of viral particles exist in the sera of HBV-infected patients, namely, the 22-nm-diameter spherical particles, tubular particles, and 42-nm-diameter virions (19). Strikingly, the subviral particles (spheres and tubules), containing only viral envelop glycoproteins and host-derived lipids, typically outnumber the virions by a factor of 1,000- to 10,000-fold (6, 11). There are three HBV envelop glycoproteins collectively known as HBV surface antigen (HBsAg), including the large (LHBs), middle (MHBs), and small (SHBs) surface proteins. Among them, SHBs is the most abundant viral envelop protein in virion and subviral particles. Although excess HBsAg subviral particles have been suggested to sequester the neutralizing antibody against HBV and contribute to a state of immune tolerance, thereby enabling the survival of infectious virions and leading to persistent infections (6, 27), the biological and

pathological significance of the overproduction of HBsAg subviral particles still remains elusive.

HBsAg has been proved extremely effective in inducing protective antibodies (anti-HBs) and thus has been used as the prophylactic vaccine. Thus far, most studies on HBsAg have focused on the development of hepatitis B vaccines (41), identification of HBsAg-interacting membrane proteins as potential host HBV receptors (9, 13), and characterization of the impact of naturally occurring HBsAg mutations on its antigenicity (12, 43). However, specific interactions between HBsAg and host intracellular factors have not been extensively studied.

To address this issue, SHBs-secreting cell lines and lineages of HBV transgenic mice persistently expressing HBsAg were used in our previous studies (28, 34, 44). We found that the level of cyclophilin A (CypA) decreased markedly in the livers of HBsAg transgenic mice but increased significantly in their sera (44). CypA is a multifunctional cellular protein. It is the major binding protein for the immunosuppressive drug cyclosporine (Cs) (14) and exhibits peptidyl-prolyl *cis-trans* isomerase activity (35). Recently, it was found CypA played important roles in regulating inflammatory responses and viral infections. Regarding these newly recognized physiological functions, CypA was speculated to be involved in HBV infection. In the present study, the mechanism and clinical implications of elevated secretion of CypA induced by SHBs were explored in detail, including studies in cell cultures, hydrodynamic injected

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mouse models, and chronic hepatitis B patients. Our findings indicate that expression and secretion of SHBs can trigger the secretion of CypA, which may induce liver inflammation and contribute to the pathogenesis of HBV infection.

MATERIALS AND METHODS

Plasmids. The SHBs gene (nucleotides [nt] 157 to 837) and the LHBs gene were amplified from a full-length genotype C HBV isolate C8 (GenBank accession no. AF461363) and cloned into the pcDNA3 vector (Invitrogen, Carlsbad, CA) with an N-terminal tag of hemagglutinin (HA) under the control of cytomegalovirus (CMV) promoter to construct the plasmid HA-SHBs and HA-LHBs. A secretion-deficient SHBs construct (N77) that contains R169P mutation and its corresponding wild-type SHBs expression construct (N65) were constructed as reported by Khan et al. (16). The HBV replicon plasmid C8-1.3 harboring 1.3 U of HBV genome was constructed in pUC19 vector as previously reported (36).

Cell culture and HBV transgenic mice. Huh7 cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 100 U of penicillin/ml, 100 µg of streptomycin/ml, 2 mM glutamine, 25 mM HEPES solution, and 1 mM sodium pyruvate. HepG2.2.15 cells were grown in the same complete medium supplemented with 250 µg of G418/ml. Cells were cultured at 37°C under 5% CO₂. All cell culture reagents were purchased from Gibco (Invitrogen). Yeast-derived recombinant HBsAg was purchased from Toca (Shanghai, China).

The generation of HBV transgenic mice has been described in our previous study (28). Twenty-four-week-old transgenic mice were used in the present study.

Transient transfections. Huh7 cells (2×10^5 per well) were seeded onto 24-well plates and cultured for 24 h. When cells were 90% confluent, plasmid DNA (0.75 µg per well) was transfected by using Lipofectamine 2000 reagent (Invitrogen). In all transfection experiments, pSEAP2-control vector (0.25 µg per well) was cotransfected to normalize transfection efficiency. Culture supernatants and cells were collected 48 h after transfection.

Protein expression and glutathione S-transferase (GST) pull-down assay. *Escherichia coli* BL21(DE3)/GST-CypA was cultured to mid-log phase in 200 ml of LB medium. IPTG (isopropyl-β-D-thiogalactopyranoside) was then added to the medium to a final concentration of 0.25 mM. Cells were harvested 12 h later at 25°C, suspended in ice-cold phosphate-buffered saline (pH 7.4), and homogenized by ultrasonication. The cell lysates were centrifuged at $10,000 \times g$ for 10 min at 4°C. The supernatants were applied to a column containing 0.1 ml of Sepharose 4B-glutathione (Amersham Biosciences, Pittsburgh, PA). An equal amount of either GST or GST-CypA fusion protein bound to glutathione-Sepharose 4B beads was mixed with SHBs protein which was *in vitro* transcribed and translated by TNT Quick-Coupled transcription/translation systems (Promega, Madison, WI) and incubated for 4 h at 4°C. Proteins bound to the beads were recovered by adding sodium dodecyl sulfate (SDS) loading buffer, boiled for 10 min, and then analyzed by SDS-PAGE and autoradiography.

Coimmunoprecipitation. Huh7 cells (2×10^6 cells) transfected with plasmid HA-SHBs were washed three times with ice-cold phosphate-buffered saline (PBS) and incubated at 4°C for 45 min with 0.5 ml of lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.5% Triton X-100, 5 mM EDTA, 15 mM MgCl₂, 60 mM β-glycerophosphate, 1 mM sodium orthovanadate, 20 mM NaF, 1× proteinase inhibitor cocktail). Detergent-insoluble materials were removed by centrifugation at $15,000 \times g$ for 10 min at 4°C. The whole-cell lysate was incubated at 4°C for 2 h with horse anti-HBsAg polyclonal antibody (Abcam, Cambridge, United Kingdom) or normal horse IgG (Santa Cruz, Santa Cruz, CA). Protein G-agarose beads (Roche, Basel, Switzerland) were pre-equilibrated by two washes with lysis buffer and then added, followed by an overnight incubation at 4°C. Beads were collected by centrifugation and were gently washed three times with the lysis buffer. The bound proteins were eluted by boiling in SDS sample buffer and resolved on a SDS-15% PAGE gel for Western blot analysis.

Hydrodynamic injection. As described (39), 10 µg of HA-SHBs plasmid and 5 µg of pSEAP2-control vector (Clontech, Mountain View, CA) were coinjected into the tail vein of 6- to 8-week-old C57BL/6J mice in a volume of saline equivalent to 10% of the body mass (e.g., 2.0 ml for a 20-mg mouse). The total volume was delivered within 5 to 8 s. Mice were sacrificed 4 days after injection. Sera and liver tissues were collected for further experiments.

In the Cs-treated group, concentrated Cs solution (Novartis Pharma, Schwiiz AG, Switzerland) was diluted in normal saline (0.9% NaCl) with a final concentration of 1.5 mg/ml. Diluted Cs solution was injected intraperitoneally into the C57BL/6J mice with a dosage of 15 mg/kg/day the day before hydrodynamic

injection and the following 4 days until the animals were sacrificed for further experiments. Mice intraperitoneally injected with normal saline were used as controls.

In anti-CD147 antibody (kindly provided by Zhinan Chen from the Fourth Military Medical University) treated group, 50 µg of anti-CD147 antibody was coinjected with HA-SHBs construct. Sera and liver tissues were collected for further experiments 4 days after injection. Isotype control antibody was used as controls.

Liver tissues of all hydrodynamically injected mice were immediately immersed in 4% formalin, fixed for 18 to 24 h, and paraffin-embedded. Hematoxylin and eosin (H&E) staining were performed on liver tissue sections for pathological analysis. HBsAg expression was detected by immunohistochemical staining using anti-HBsAg monoclonal antibody (Changdao Biotech, Shanghai, China). All sections were read under code by an independent pathologist.

Western blot and dot blot analyses. Protein samples were separated in SDS-15% PAGE gels and transferred onto the nitrocellulose membrane (0.2 µm; Schleicher & Schuell, Dassel, Germany). For dot blot analysis, 200 µl of culture supernatant or 10 µl of mouse or patient serum diluted 1:100 was loaded onto the nitrocellulose membrane under a vacuum. The membranes were incubated at room temperature for 2 h in blocking buffer (PBS containing 0.05% Tween 20 and 5% nonfat milk powder), followed by overnight incubation at 4°C with horse anti-HBsAg polyclonal antibody, anti-preS1 monoclonal antibody 125E11 (8, 9), or rabbit anti-CypA polyclonal antibody (Proteintech, Chicago, IL). After being washed with PBST (PBS containing 0.05% Tween 20), the membranes were incubated at room temperature for 2 h with corresponding horseradish peroxidase-conjugated secondary antibody. The target protein was revealed by using an ECL-Plus system (GE Healthcare, United Kingdom). A monoclonal antibody against GAPDH (glyceraldehyde-3-phosphate dehydrogenase; Sigma, St. Louis, MO) was used to normalize protein loading in a Western blot.

HBV-infected and control serum samples. Sera were collected from 115 chronic active hepatitis B patients admitted to Zhongshan Hospital or Huashan Hospital, Fudan University. All of the patients were serum HBsAg positive for at least 6 months, with elevated alanine aminotransferase (ALT). Patients coinfecting with hepatitis C virus, hepatitis D virus, or human immunodeficiency virus (HIV) were excluded. Sera from 23 healthy individuals without evidence of HBV infection and with normal ALT levels were included as controls. Consents for the use of their sera for this assay were acquired from all of the human subjects. In addition, paired serum samples were collected from 15 HBsAg-positive patients from the First Affiliated Hospital, Zhejiang University School of Medicine before and after liver transplantation (LT). These patients were given lamivudine (LAM) monotherapy before LT, and combination of LAM with hepatitis B immune globulin as prophylaxis post-LT as described previously (45). Maintenance immunosuppressive regimen consisted of FK506 in a dosage of 5 to 10 ng/ml. Samples were collected prior to LT, when patients were HBsAg positive, and within 3 months after LT, when all recipients became serum HBsAg negative. The relative amount of CypA was compared in these paired sera. All patients provided written informed consent prior to the study.

RESULTS

CypA secretion is promoted in the context of HBV replication. Previously, we noted elevated levels of CypA in the culture supernatant of Huh7 cells expressing SHBs and in the sera of HBsAg transgenic mice (44). However, it is unclear whether the increase in extracellular CypA occurs in the context of HBV replication. To clarify this issue, the levels of CypA in the culture supernatant and cell lysate of HepG2.2.15 cells were determined and compared to HepG2 cells. HepG2.2.15 is a HepG2 cell line stably transfected with a replication-competent HBV genome and thus produces HBV virions and subviral particles (29). The level of CypA in the culture supernatant of HepG2.2.15 cells was much higher than that of HepG2 cells, whereas the level of CypA in the cell lysate of HepG2.2.15 cells was lower (Fig. 1A). Furthermore, transient transfection of Huh7 cells with a construct containing a terminally redundant replication-competent HBV genome (C8-1.3) also led to a marked increase in extracellular CypA and a decrease in intracellular CypA (Fig. 1B) compared to cells transfected with

FIG. 1. Increased secretion of CypA in HBV replicating cells. (A) Comparison of CypA levels in the cell lysate and culture supernatant between HepG2.2.15 cells and HepG2 cells. The left panel shows Western blot analysis of CypA within cells. GAPDH was used for normalization of sample loading. The right panel shows Western blot analysis of CypA in supernatant. (B) Comparison of CypA protein levels in the cell lysate and culture supernatant between Huh7 cells transiently transfected with HBV replicating construct C8-1.3 and pUC19 vector. The left panel shows Western blot analysis of CypA within cells. The right panel shows Western blot analysis of CypA in supernatant.

the vector. Therefore, CypA secretion is enhanced in cells undergoing HBV replication.

CypA secretion is specifically promoted by the expression of SHBs. The enhanced secretion of CypA might be due to a general induction of cellular secretory function rather than a specific induction by SHBs. To examine this possibility, alpha fetal protein (AFP) which is secreted by embryonic hepatocytes and hepatocarcinoma cells was quantified in the culture supernatant of Huh7 cells transfected with either HA-SHBs expression plasmid or the vector. No obvious increase in AFP secretion was observed in SHBs-expressing Huh7 cells (Fig. 2A). In addition, in HBsAg transgenic mice, serum levels of complement component 3 (C3) and albumin, two plasma proteins secreted by the liver (18), were similar to those in the control C57BL/6J mice (Fig. 2B). To find out whether the induction of CypA secretion was SHBs specific, other two HBV proteins, namely, e antigen (HBeAg) and large surface protein (LHBs), were expressed in Huh7 cells. In both cases, no obvious change in CypA secretion was observed compared to vector transfected cells (Fig. 2C). Taken together, CypA secretion in Huh7 cells is specifically enhanced by the expression of SHBs.

CypA secretion is not promoted by extracellular SHBs. Because SHBs is secreted by Huh7 cells transfected with HA-SHBs, it is possible that extracellular SHBs *per se* may induce CypA secretion. To examine this possibility, yeast-derived recombinant SHBs (150 ng/ml), comparable to the concentration of SHBs in the supernatant of transfected cell, was added to the culture supernatant of Huh7 cells. After a 4-day incubation,

FIG. 2. CypA secretion was specifically promoted by the expression of SHBs. (A) Supernatants of HA-SHBs or vector transfected Huh7 cells were collected 48 h after transfection, and AFP levels were determined by enzyme-immunoassay. Mock, mock-transfected cells. Media, fresh culture medium. (B) Serum albumin and complement component 3 (C3) level in serum samples of HBsAg expressing transgenic mice (Tg) and C57BL/6J control mice (C57). (C) CypA secretion was not affected by expression of HBeAg and LHBs. Supernatants and cells were harvested 48 h after transfection. Extracellular and intracellular CypA were analyzed by Western blotting. LHBs expression was analyzed by Western blotting with anti-preS1 antibody. GAPDH was used for normalization of sample loading. HBsAg and HBeAg in the supernatant were determined by enzyme-linked immunosorbent assay (ELISA) and are shown in the right panel.

no apparent increase in CypA secretion was observed (Fig. 3), indicating that CypA secretion is not induced by extracellular SHBs.

Enhanced CypA secretion is associated with SHBs secretion. Although CypA secretion is not enhanced by extracellular SHBs, it is uncertain whether enhanced CypA secretion is dependent on the secretion of SHBs. To solve this uncertainty, a secretion-deficient SHBs construct (N77) that contains the R169P mutation (16) was transfected into Huh7 cells. The intracellular and extracellular levels of CypA were then determined and compared to Huh7 cells transfected with the wild-type SHBs construct (N65) or the vector. The deficiency in SHBs secretion resulted in a dramatic reduction of CypA secretion to the level similar to that of vector transfected cells (Fig. 4), while the intracellular level of CypA in cells expressing the secretion-deficient SHBs was not decreased. These results suggest that CypA secretion is closely associated with SHBs secretion.

CypA interacts with SHBs. To explore the molecular mechanism whereby SHBs induced CypA secretion, potential interaction between SHBs and CypA was investigated by GST pull-down assay and coimmunoprecipitation experiment. GST pull-down assay was performed using GST-CypA fusion protein and *in vitro* translated HA-SHBs protein. The result showed that SHBs was pulled down by GST-CypA fusion protein, which indicates a specific binding between these two proteins (Fig. 5A).

FIG. 3. Impact on CypA secretion by SHBs added to culture medium of Huh7 cells. Supernatants and cells were collected 2 days after yeast-derived recombinant SHBs (150 ng/ml) was added to the medium of Huh7 cells. (A) Western blot analysis of intracellular CypA. Actin was used for normalization of sample loading. The results are represented as means $\pm$ the standard deviations (SD) in the right panel. (B) Western blot analysis of CypA in supernatant. The results are represented as means $\pm$ the SD in the right panel.

To examine the interaction of SHBs and CypA in cell cultures, SHBs was precipitated by anti-HBsAg antibody from the cell lysate and culture supernatant of SHBs expressing Huh7 cells, respectively, and bound CypA protein was detected by Western blot analysis with anti-CypA antibody. The results clearly demonstrated coprecipitations of CypA from both the cell lysate and culture supernatant (Fig. 5B). Further studies revealed that CypA protein also coprecipitated with the secretion-deficient SHBs mutant (N77) and large surface antigen (LHBs) (see Fig. S1A and B in the supplemental material),

FIG. 4. Induction of CypA secretion was dependent on SHBs secretion. Secretion-deficient SHBs construct (N77) and wild-type SHBs construct (N65) were transfected into Huh7 cells. The left panel shows Western blot analysis of CypA in supernatant and intracellular expression of CypA and SHBs. GAPDH was used for normalization of sample loading. The right panel shows the ELISA results of SHBs secretion in supernatants.

FIG. 5. Interaction between SHBs and CypA. (A) *In vitro*-translated SHBs was incubated with an equal amount of GST or GST-CypA fusion protein. SHBs bound to GST or GST-CypA was detected by Western blotting. (B) SHBs was immunoprecipitated from the cell lysate and supernatant of HA-SHBs-transfected Huh7 cells by a horse anti-HBsAg polyclonal antibody. Precipitated HA-SHBs was detected by Western blotting with anti-HA antibody, while coprecipitated CypA was determined by anti-CypA antibody. Horse normal IgG was used as a negative control for the immunoprecipitation assay. (C) Immunofluorescence assay of SHBs (red) and CypA (green) was performed in the SHBs-overexpressing cells (SHBs cell) and the vector control cells (vector cell). DAPI (4',6'-diamidino-2-phenylindole) was used to stain the nucleus.

although neither N77 mutant nor LHBs could be secreted and enhance the secretion of CypA.

To further confirm the interaction between CypA and SHBs, cellular distribution of CypA and SHBs were studied in SHBs overexpressing Huh7 cells by immunofluorescent staining. CypA protein colocalized with SHBs in cytoplasm, while CypA was distributed both in the nucleus and in the cytoplasm (Fig. 5C). Taken together, these data suggest that CypA specifically interacts with SHBs.

Enhanced CypA secretion is associated with infiltration of inflammatory cells in mouse liver temporarily expressing SHBs. Significant increase in serum CypA was found in all 13 HBsAg-positive transgenic mice compared to 10 C57BL/6J control mice (Fig. 6) ($P < 0.001$, Student *t* test). However, HBsAg transgenic mice did not show liver inflammation due to immune tolerance. To study the potential biological significance of secreted CypA, HA-SHBs plasmid and pcDNA3-HA control plasmid were respectively injected into C57BL/6J mice by hydrodynamic injection. Serum CypA increased dramatically in mice injected with HA-SHBs, compared to mice receiving the vector (Fig. 7A and B). To monitor the injury of liver tissues, serum ALT/AST levels were determined. In SHBs expression mice group, ALT/AST levels were significantly el-

FIG. 6. Increased serum CypA levels in HBsAg expressing transgenic mice. (A) Higher serum CypA levels in HBsAg transgenic mice ($n = 13$) than in C57BL/6J control mice ($n = 10$) ($P < 0.001$, Student t test). The left panel shows a representative Western blot image of serum CypA. The right panel shows the relative density of Western blot images. (B) Real-time PCR analysis of CypA mRNA levels in liver tissues of HBsAg transgenic mice and C57BL/6J control mice. GAPDH was used as an internal control for normalization.

evated compared to those of control group (Fig. 7C). Liver tissues were serially sectioned and examined by both H&E staining and immunohistochemical staining with anti-HBsAg antibody. The coexistence of spotty necrosis foci and HBsAg-positive hepatocytes was observed in liver sections of HA-SHBs-injected mice. HBsAg-positive hepatocytes were found surrounded by inflammatory cells in HA-SHBs injected mice (Fig. 7D and E), whereas neither HBsAg positive cells nor foci of infiltration of inflammatory cells were observed in the control mice (Fig. 7F and G). To determine whether this inflammatory effect was due to the hydrodynamic injection and the expression of a foreign protein, vesicular stomatitis virus G protein (VSV-G) expression construct was injected into mice hydrodynamically as a control. Serum ALT/AST levels remained normal (Fig. 7C), and no inflammation foci were observed in the control mice, which indicated a specific induction of inflammation by SHBs expression in liver tissue (Fig. 7H and I).

To verify the association of inflammatory responses in liver tissues with enhanced CypA secretion, Cs was injected intraperitoneally into HA-SHBs-injected mice to counteract the chemotactic effect of CypA. Although Cs injection did not affect serum HBsAg or CypA levels (Fig. 8A and B), it did markedly reduce the serum ALT/AST levels (Fig. 8C) and infiltration foci in liver tissues (Fig. 8F and G) compared to injection with normal saline (NS) (Fig. 8D and E). These results were further confirmed in anti-CD147 antibody treatment group. The inflammatory responses (Fig. 8J and K) and serum ALT/AST levels (Fig. 8C) were reduced in anti-CD147 antibody treated mice, when the chemotactic effect of CypA

FIG. 7. Serum HBsAg, CypA, ALT/AST, and infiltration of inflammatory cells in the mouse liver after hydrodynamic injection with HA-SHBs plasmid. (A) Serum HBsAg levels in C57BL/6J mice 4 days after hydrodynamic injection with HA-SHBs plasmid or vector. (B and C) Serum CypA (B) and ALT/AST levels (C) in SHBs expression mice, VSV-G expression mice, and vector control mice. Serum CypA and ALT/AST levels were increased significantly in HA-SHBs injected mice (*, $P < 0.05$ [Student t test]). (D and E) Immunohistochemical staining of HBsAg in serial liver tissue sections (400 $\times$ magnification) of HA-SHBs-injected C57BL/6J mice (D) and H&E staining (E). Infiltration of inflammatory cells was observed around HBsAg-positive hepatocytes. (F and G) Immunohistochemical staining of HBsAg (F) and H&E staining (G) of serial liver tissue sections from vector injected control mice. (H and I) Immunohistochemical staining of VSV-G protein (H) and H&E staining (I) of serial liver tissue sections from VSV-G-injected control mice.

FIG. 8. Impact of Cs or anti-CD147 antibody treatment on serum HBsAg, CypA, and ALT/AST levels and infiltration of inflammatory cells in the mouse liver hydrodynamically injected with HA-SHBs plasmid. (A) Serum HBsAg levels of mice injected with Cs or anti-CD147 antibody 4 days after hydrodynamic injection of HA-SHBs plasmid or vector. (B and C) Serum CypA (B) and ALT/AST (C) levels in Cs- or anti-CD147 antibody-treated C57BL/6J mice with hydrodynamic injection of HA-SHBs or vector. (D and E) Immunohistochemical staining and H&E staining of liver tissue sections (400× magnification) of mice receiving NS and HA-SHBs. Infiltration of inflammatory cells is shown around HBsAg-positive hepatocytes. (F and G) Immunohistochemical staining of HBsAg (F) and H&E staining (G) of liver tissue sections of Cs and HA-SHBs-injected C57BL/6J mice. (H and I) Immunohistochemical staining of HBsAg (H) and H&E staining (I) of liver tissue sections of mice receiving isotype control antibody and HA-SHBs. (J and K) Immunohistochemical staining of HBsAg (J) and H&E staining (K) of liver tissue sections of anti-CD147 antibody and HA-SHBs injected C57BL/6J mice. (L and M) Immunohistochemical staining (L) and H&E staining (M) of liver tissue sections from mice receiving vector DNA which were used as negative control. Serial sections were used in the present study. Cs, cyclosporine.

was inhibited by blocking its receptor CD147. These findings demonstrate that inflammatory responses in HBsAg-positive liver tissues of mice were most likely mediated by elevated circulating CypA.

Elevated serum CypA level in chronic hepatitis B patients. To determine whether serum CypA level is elevated in natural HBV infection, CypA in human blood samples was assayed by Western blotting. The serum CypA level was significantly higher in chronic active hepatitis B patients ($P < 0.01$, t test) than in HBV-negative healthy individuals (Fig. 9A). The association of enhanced CypA secretion with HBV infection was further confirmed in 15 hepatitis B patients who underwent liver transplantation. All patients were HBsAg positive before transplantation and negative after transplantation for at least 6 months. The results of dot blot showed that except for two patients (P5 and P8), serum CypA level decreased markedly after liver transplantation (Fig. 9B).

DISCUSSION

We demonstrate here that CypA secretion can be specifically induced by SHBs expression and secretion in cells and in mice. Since both SHBs and CypA are secreted via the vesicular secretion pathway (26, 33), the interaction between SHBs and CypA, either by direct interaction or indirectly bridged by some cellular components as revealed by GST pull-down and coimmunoprecipitation assays provides a reasonable mechanism for SHBs-induced CypA secretion. It is likely that CypA binds to SHBs and is secreted along with HBsAg particles. However, whether CypA is incorporated into the viral particles or merely bound with HBsAg or both is an interesting question that ought to be explored in the future. It is noteworthy, however, that given the large number of cellular proteins participating in the cellular secretion machinery (3), other cellular factors might also be involved in the interaction between CypA

FIG. 9. Increased serum CypA levels in natural HBV infections. (A) Serum CypA levels were compared among chronic hepatitis B patients and healthy individuals. Serum CypA was increased significantly in chronic hepatitis B patients ($P < 0.01$, Student t test). The left panel shows a representative Western blot image of serum CypA. The right panel shows the semiquantification of the relative densities of dot blot images. (B) Dot blot analysis of serum CypA levels pre- and posttransplantation in HBV-infected patients who underwent liver transplantation. In 13 of 15 patients, the serum CypA level was decreased markedly after liver transplantation.

and SHBs. Details in the cosecretion of CypA-HBsAg complex remain to be further investigated.

CypA is a ubiquitously and abundantly expressed cellular protein belonging to the immunophilin family (40). It was originally identified as the receptor for immunosuppressive drug Cs (14) and was later considered an important cellular chaperone molecule (35). The CypA-Cs complex inhibits the protein phosphatase activity of calcineurin and subsequently inhibits signal transduction pathway for initiating T-lymphocyte activation (21, 22, 40). CypA was recently found secreted from cells in response to oxidative stress (15, 33) and inflammatory stimulations (2, 17). Our results indicate that viral protein expression such as SHBs is a novel mode of triggering CypA secretion.

Secreted CypA can act as a potent chemoattractant to inflammatory cells such as T cells (1), monocytes (30), eosinophils, and neutrophils (38). In the present study, we used a hydrodynamic injection mouse model to investigate the potential role of CypA played in HBsAg-related liver inflammation. Our results showed that SHBs expression in mice increased serum CypA levels, elevated serum ALT/AST levels, and caused infiltration of lymphocytes surrounding hepatocytes that expressed SHBs. However, mice injected with the empty vector or VSV-G expression construct were HBsAg negative in the serum and liver, were low in serum CypA and ALT/AST levels, and showed no evidence of inflammatory cell infiltration, which was consistent with previous reports that exogenous genes could be efficiently delivered and expressed in the liver by hydrodynamic injection without obvious inflammation responses and liver histopathological changes after 1 day postinjection (20). Taken together, our results demonstrate that the inflammatory effect observed was associated with SHBs expression and secretion but not due to a general induction by the expression of an irrelevant foreign protein.

Further studies revealed that pretreatment with Cs, which is known to block the chemotactic function of CypA (2), reduced the serum ALT/AST to the normal levels and prevented the infiltration of lymphocytes in the liver. Since Cs is regarded as an immunosuppressive drug, it is possible that a decrease in infiltration foci is caused by its general suppression of the immune system rather than its interference with the chemotactic function of CypA. To further confirm the regulatory effect of CypA in SHBs associated liver inflammation, anti-CD147 antibody was used to inhibit chemotactic function of CypA. CD147 was identified as a receptor protein responsible for CypA-induced signaling and chemotactic activities (42). Arora et al. reported that blocking CypA-CD147 interactions significantly, though not completely, reduces lipopolysaccharide-induced lung inflammation in a mouse model (2). Similarly, our results show that the anti-CD147 antibody treatment decreased the serum ALT level and inflammatory foci in the liver. It is possible that the anti-CD147 might not have completely blocked the CypA chemotactic activity *in vivo* or a CypA activity not inhibited by the antibody might also be involved in the inflammatory response. In addition, other factors aside from CypA might be involved in inducing inflammatory responses in the studied mouse models which could not be blocked by anti-CD147. Nonetheless, elevated serum CypA seems to be associated with the inflammatory response in mouse liver temporarily expressing SHBs.

In addition, serum CypA appears to be higher in chronic hepatitis B patients than in healthy subjects, and the majority of patients after liver transplantation exhibit a reduction in serum CypA. These observations collectively suggest that CypA secretion induced by SHBs might contribute to the pathogenesis of HBV infection.

Besides induction of inflammatory responses, other important roles of CypA in viral infections have been recognized.

CypA is incorporated into HIV-1 capsid through direct binding with viral Gag protein (10, 24). Disruption of CypA incorporation leads to an attenuation of HIV-1 infectivity and decrease in HIV-1 replication (4, 31, 32). Cyclophilins are also involved in HCV replication. CypA and CypB interact with the nonstructural viral protein NS5B and stimulate its RNA-binding activity (37). Furthermore, CypA has been reported to regulate life cycles and pathogenesis of several viruses, including influenza A virus, severe acute respiratory syndrome coronavirus, and vaccinia virus (5, 7, 23).

In conclusion, our discovery indicates that expression and secretion of SHBs is a novel mode of inducing secretion of CypA, which may play a role in the pathogenesis of HBV infection. Further study on the molecular details of SHBs-induced CypA secretion will likely provide new opportunities for developing novel antiviral therapeutics.

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