A Putative Amphipathic Alpha Helix in Hepatitis B Virus Small Envelope Protein Plays a Critical Role in the Morphogenesis of Subviral Particles

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ABSTRACT Hepatitis B virus (HBV) small (S) envelope protein has the intrinsic ability to direct the formation of small spherical subviral particles (SVPs) in eukaryotic cells. However, the molecular mechanism underlying the morphogenesis of SVPs from the monomeric S protein initially synthesized at the endoplasmic reticulum (ER) membrane remains largely elusive. Structure prediction and extensive mutagenesis analysis suggested that the amino acid residues spanning W156 to R169 of S protein form an amphipathic alpha helix and play essential roles in SVP production and S protein metabolic stability. Further biochemical analyses showed that the putative amphipathic alpha helix was not required for the disulfide-linked S protein oligomerization but was essential for SVP morphogenesis. Pharmacological disruption of vesicle trafficking between the ER and Golgi complex in SVP-producing cells supported the hypothesis that S protein-directed SVP morphogenesis takes place at the ER-Golgi intermediate compartment (ERGIC). Moreover, it was demonstrated that S protein is degraded in hepatocytes via a 20S proteasome-dependent but ubiquitination-independent nonclassic ER-associated degradation pathway. Taken together, the results reported here favor a model in which the amphipathic alpha helix at the antigenic loop of S protein attaches to the lumen leaflet to facilitate SVP budding from the ERGIC, whereas the failure of the budding process may result in S protein degradation by 20S proteasome in a ubiquitination-independent manner.

IMPORTANCE SVPs are the predominant viral product produced by HBV-infected hepatocytes. Their levels exceed those of virion particles by 10,000- to 100,000-fold in the blood of HBV-infected individuals. The high levels of SVPs, or HBV surface antigen (HBsAg), in the circulation induce immune tolerance and contribute to the establishment of persistent HBV infection. The loss of HBsAg, often accompanied by the appearance of anti-HBsAg antibodies, is the hallmark of durable immune control of HBV infection. Therapeutic induction of HBsAg loss is thus considered to be essential for the restoration of the host antiviral immune response and functional cure of chronic hepatitis B. Our findings on the mechanism of SVP morphogenesis and S protein metabolism will facilitate the rational discovery and development of antiviral drugs to achieve this therapeutic goal.

KEYWORDS functional cure, HBV surface antigen, subviral particle morphogenesis, subviral particles, hepatitis B virus

Hepatitis B virus (HBV)-infected hepatocytes produce not only virions containing nucleocapsids with a relaxed circular, partially double-stranded DNA genome (1), i.e., complete virions, but also virion-like particles with nucleocapsids containing viral

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FIG 1 HBV S protein membrane topology. Important residues, structural regions, and primary sequence boundaries are denoted. See text for details.

pregenomic RNA at the various stages of reverse transcription (2–4) or with “empty” capsids devoid of viral RNA/DNA (5), i.e., RNA-containing virions and genome-free virions, respectively (6). However, the most abundant viral product made by HBV-infected hepatocytes is not the virions or virion-like particles but subviral particles (SVPs) consisting of only small (S), middle (M), and large (L) envelope (surface) proteins. The SVPs are spherical or filamentous lipoprotein particles with a diameter of 22 nm and exceed the virion particles by 10,000- to 100,000-fold in the blood of HBV carriers (6). S protein alone is sufficient to form spherical SVPs with an octahedral symmetry in eukaryotic cells (7). A spherical SVP consists of nontypical bilayer lipid and approximately 100 copies of S protein. However, assembly of the filamentous SVPs requires the coexpression of L and S proteins (8, 9).

As the predominant viral product in the blood of HBV-infected individuals, the biological function of SVPs remains to be firmly determined. While a study with duck hepatitis B virus (DHBV) suggested that SVPs may facilitate virion infection of hepatocytes via an unknown mechanism (10), another study in chimpanzees indicated that SVPs may prime a CD4⁺ T cell response that determines the outcomes of HBV infection (11). Resolution of an acute HBV infection is associated with the induction of a vigorous polyclonal cytotoxic T cell response against viral proteins and the appearance of antibodies against S protein (HBV surface antigen [HBsAg]) (12). However, chronic HBV infection is associated with the functional exhaustion of viral antigen-specific T cells (13, 14) and atypical HBsAg-specific memory B cells deficient in the production of antibodies (15, 16). It has been speculated that the high levels of intrahepatic and circulating HBsAg play a critical role in the induction of immune tolerance and the establishment of persistent HBV infection (17–19). Clinically, the loss of HBsAg and the emergence of anti-HBsAg antibodies indicate the durable immune control of HBV infection or the functional cure (20, 21). Accordingly, therapeutic induction of HBsAg loss is considered to be essential for restoration of the host antiviral immune response and functional cure of chronic hepatitis B (22, 23). Apparently, knowledge on the mechanism of HBV envelope protein metabolism and SVP morphogenesis is important for the rational discovery and development of antiviral drugs to achieve this therapeutic goal.

S protein is a 226-amino-acid transmembrane protein that is cotranslationally integrated into the membrane of the endoplasmic reticulum (ER) with a complex topology (9, 24). As illustrated in Fig. 1, transmembrane domain 1 (TM1), located between residue 8 and residue 22, is the first translocation signal that directs the N terminus of S protein into the ER lumen. TM2, located between residue 80 and residue 98, is a type II

translocation signal that directs its C-terminal polypeptide into the ER lumen. Two transmembrane alpha helices, TM3 and TM4, spanning residues 170 to 192 and 202 to 224, respectively, direct the topogenesis of the hydrophobic C-terminal domain of S protein (25). The residues between TM1 and TM2 form cytosolic loop 1 (CYL-1), which plays important roles in S protein intracellular trafficking (26) and virion and SVP morphogenesis (9, 27). The residues between TM2 and TM3 form the antigenic loop (AGL) containing a single N-glycosylation site (N146) that is essential for the production of virions but not SVPs (28). In addition, the eight conserved cysteine residues in the AGL are crucial for HBsAg antigenicity (29), hepatitis D virus (HDV) infectivity (30), and SVP and virion production (29). While TM1 and TM2 are essential for S protein-directed SVP production (31, 32), the role of the hydrophobic C-terminal region in SVP morphogenesis remains controversial (32–34).

Biogenesis of spherical SVPs relies on extensive intermolecular interaction of S proteins. Such interactions begin at the dimerization of S proteins in the ER membrane through the homotypic interaction between the TM2 domains or CYL-1 (34). The disulfide-linked S protein dimerization and oligomerization are also required for SVP production (29, 34). However, the cysteine residues involved, as well as the cellular compartment where disulfide-linked oligomerization occurs, remain to be determined (29). The S protein exits the ER by using the coat protein complex II (COPII) vesicle transport machinery via Sec24A recognition of the S protein R78/R79 dipeptide signal in CYL-1 (26). Although it is generally considered that the SVPs are budded in the ER-Golgi intermediate compartment (ERGIC) (35, 36), some studies suggested that it might occur in the ER (37–40). The host cellular factors essential for SVP morphogenesis have not been identified. Upon budding, the SVPs are transported into the Golgi apparatus, where the N-linked glycan is processed from the high-mannose type to the complex type and subsequently secreted from hepatocytes via exocytosis (36, 41).

In an effort to investigate the molecular mechanism of S protein-directed SVP morphogenesis, we found that a putative amphipathic alpha helix formed by residues 156 to 169 in the C-terminal region of AGL played a crucial role in S protein metabolic stability and SVP morphogenesis. The mechanistic insights from this study shed light on the development of therapeutics that can selectively destabilize HBV S protein and disrupt SVP production, which should facilitate the restoration of the antiviral immune response against HBV and achieve the functional cure of chronic hepatitis B.

RESULTS

Residue A157 of S protein plays a critical role in the production of SVPs. In our study of HBV genome variations in chronic hepatitis B patients, an A157D substitution in S protein was identified in the majority of HBV DNA clones derived from a patient with low HBsAg antigenemia. In order to investigate its role in SVP production, the A157D substitution was introduced into plasmids expressing an N-terminally hemagglutinin (HA)-tagged S protein of genotype A, B, C, or D. Transfection of the plasmids expressing either wild-type (WT) or mutant S protein into HepG2 cells demonstrated that A157D substitution resulted in undetectable levels of secreted S protein or HBsAg in the genetic background of all four HBV genotypes examined (Fig. 2A and B) and significantly reduced the levels of intracellular S protein (Fig. 2B). The similar levels of mRNA specifying WT and mutant S proteins in the transfected cells indicated that the reduced levels of intracellular and secreted S protein in the cells transfected with the plasmids expressing S protein with the A157D substitution were due to the reduced metabolic stability and dysfunction of the mutant S protein in SVP assembly and/or secretion and not the instability of viral RNA (Fig. 2C).

To further investigate the role and mechanism of residue A157 in S protein metabolic stability and SVP production, we performed a complete spectrum of single-amino-acid substitutions of this residue in a genotype B genetic background. Intracellular and secreted S protein from the transfected HepG2 cells were analyzed by Western blotting and enzyme-linked immunosorbent assays (ELISAs). The

FIG 2 Characterization of A157D mutant HBsAg in genotype A to D genetic backgrounds. HepG2 cells were transfected with the plasmid expressing either WT or A157D mutant HBsAg in the genotype A to D genetic backgrounds (GT-A to GT-D). Culture medium and cells were harvested at 48 h posttransfection. (A) HBsAg/SVPs in the culture medium were determined by ELISA (Kehua, China), and results are presented as the average and standard deviation of optical density (OD) values ($n=2$). (B) Secreted (upper panel) and intracellular (middle panel) HBsAg were determined by Western blotting with a monoclonal antibody against the HA tag. β -Actin served as a loading control (lower panel). (C) HBsAg mRNA was determined by a Northern blot assay with an [α - 32 P]UTP-labeled full-length HBV minus-strand RNA probe. Ribosomal 18S and 28S RNA served as loading controls.

consistent detection of HBsAg and S protein by ELISAs and Western blot assays, respectively, in the culture medium of the transfected cells indicated that the substitutions at residue A157 did not alter the antigenicity of S protein (Fig. 3A). This finding is consistent with the fact that A157 is outside the “a” determinant of HBsAg, which is located between residue 124 and residue 147 of S protein (42). Based on the comparative analysis of intracellular and secreted S protein levels for cells expressing WT or mutant S proteins, the phenotype of A157 substitutions could be categorized into two groups (Fig. 3B). While A157S, A157P, and A157G substitutions only slightly reduced the levels of secreted HBsAg, all other substitutions resulted in drastically reduced amounts of secreted HBsAg with apparently reduced (such as A157D, A157E, A157V, and A157Y) or slightly reduced (such as A157K and A157R) amounts of intracellular S protein. These results indicated that replacement of A157 by amino acids with a short side chain, such as S, P or G, did not significantly compromise S protein metabolic stability and SVP morphogenesis/secretion. However, replacement with other amino acids, particularly with negatively charged, aromatic, or hydrophobic side chains, reduced the metabolic stability of S protein and impaired SVP production. These results strongly imply that residue A157 plays a critical role in S protein metabolic stability and SVP morphogenesis/secretion.

A putative amphipathic alpha helix between residue 156 and residue 169 of S protein is critical for SVP production. As illustrated in Fig. 1, Bruss and colleagues recently predicted that amino acid residues 156 to 169 in the C-terminal region of the AGL may form an amphipathic alpha helix and attach to the inner leaflet of the ER membrane (34). Because the attachment of an amphipathic alpha helix usually induces changes of membrane fluidity and/or curvature and plays an important role in membrane fusion and vesicle budding (43, 44), we speculated that the critical role of S

FIG 3 Effects of residue A157 replacements with other amino acid residues on S protein stability and SVP production in HepG2 cells. HepG2 cells were transfected with a plasmid expressing WT S protein or S protein with A157 substitutions. Culture medium and cells were harvested at 48 h posttransfection. (A) HBsAg/SVPs in the culture medium were determined by ELISA, and results are presented as the average and standard deviation of OD values ($n=2$). (B) Intracellular and secreted S protein were determined by Western blotting with a monoclonal antibody against the HA tag. Sup, supernatant. Tubulin served as a loading control. The amounts of S protein were quantified by ImageJ.

protein A157 in SVP morphogenesis may take place in the context of this putative amphipathic α helix. Interestingly, an α helix structure of residues W156 to R169 can indeed be predicted by several protein structure analysis programs (Fig. 4A, left), and three polar amino acid residues appear on one side of the helix (Fig. 4A, right). Next, two mutagenesis approaches were applied to probe the existence and to investigate the function of the putative amphipathic α helix. First, insertion of a single amino acid residue, such as alanine, into an α helix rotates the position of all of the downstream amino acid residues by 100° , which alters the position of polar amino acid residues in the α helix and thus changes its amphipathic properties (32). Accordingly, an alanine residue was inserted immediately after the indicated amino acid residue between residue 142 and residue 172 of S protein (Fig. 4B). Analysis of intracellular and secreted S protein in the transfected HepG2 cells by ELISAs and Western blot assays indicated that, while the alanine insertion immediately after residues 142, 147, and 149, as well as residues 170 and 172, only modestly reduced the level of S protein in culture medium, alanine insertion at any position between residue 151 and residue 169 significantly compromised the secretion of S protein, with variable effects on the steady-state level of intracellular S proteins (Fig. 4B). Particularly, S protein (or HBsAg) secretion was completely abolished by an alanine insertion immediately after residues 155, 156, 157, and 162. As anticipated, alanine insertion at residue 147 abrogated the N-linked glycosylation signal and production of gp27 but did not prevent the secretion of unglycosylated S protein (p25) (Fig. 4B), which is consistent with previous reports demonstrating that glycosylation of S protein is not required for SVP production (45, 46). However, this mutation did impair the antigenicity of HBsAg, as indicated by the significant reduction of ELISA signals (Fig. 4B).

The second mutagenesis strategy is to validate the function of amino acid residues forming the hydrophilic and hydrophobic surfaces of the amphipathic α helix. First, replacement of the three residues (K160, E164, and S167) on the hydrophilic surface with leucine, which contains an isobutyl side chain, significantly reduced

A

AA156-169:WAFKFLWEWASVR

B

FIG 4 Validation of the putative amphipathic alpha helix between residue 156 and residue 169 of S protein by single-amino-acid insertion mutagenesis analysis. (A) Predicted amphipathic alpha helix structures of residues 156 to 169 of S protein are presented as space-filling model representations by Schrödinger (left) and helical wheel (right) projections. (B) HepG2 cells were transfected with a plasmid expressing WT HBsAg or the indicated mutant HBsAg. An alanine residue was inserted immediately after the indicated amino acid residue. Culture medium and cells were harvested at 48 h posttransfection. HBsAg/SVP levels in the culture medium were determined by ELISA, and results are presented as the average and standard deviation of OD values ($n=2$). Intracellular and extracellular S protein levels were determined by Western blotting with a monoclonal anti-HA antibody. Sup, supernatant. Tubulin served as a loading control. The amounts of HBsAg were quantified by ImageJ.

intracellular and secreted S protein levels. However, replacement of these residues by amino acids with hydrophilic side chains was generally tolerated (Fig. 5, upper). Interestingly, while replacement of K160 with negative charged residue E significantly reduced both intracellular and secreted S protein levels, replacement of E164 with either negatively or positively charged residues (D or R) apparently did not alter the levels of intracellular and secreted S protein. This later observation implies that the positive charge at residue 160 is important for S protein stability and/or SVP production. At the hydrophobic surface, replacement of residues F158, A159, F161, L162, W163, W165, and A166 by residues with charged side chains (K or D) significantly reduced both intracellular and secreted S protein levels (Fig. 5, middle and lower), with only a few exceptions (W163K and W165K). As expected, replacement of these hydrophobic residues with A or T was generally well tolerated, except for F161T, which significantly

FIG 5 Validation of the putative amphipathic alpha helix between residue 156 and residue 169 of S protein by single-amino-acid substitution mutagenesis analysis. HepG2 cells were transfected with a plasmid expressing WT S protein or an indicated mutant S protein. X represents a triple mutant S protein with K160F, E164W, and S167L substitutions. Culture medium and cells were harvested at 48 h posttransfection. HBsAg/SVPs in culture medium were determined by ELISA, and results are presented as a percentage of the WT OD value ($n=2$). Intracellular and extracellular S protein levels were determined by Western blotting with a monoclonal anti-HA antibody. Sup, supernatant. Tubulin served as a loading control. The amounts of S protein were quantified by ImageJ.

reduced S protein secretion. Interestingly, the two amino acid residues at the N and C termini (W156 and R169, respectively) of the putative alpha helix demonstrated unique properties. As shown in Fig. 4A, right, W156 and A157 are located at the boundary of the hydrophobic and hydrophilic surfaces. While residue 157 favors amino acids with

smaller side chains (A, G, S, and P) (Fig. 3), replacement of W156 by amino acids with larger hydrophobic side chains, such as Y and L, only slightly reduced the secretion of SVPs (Fig. 5, middle). However, replacement of W156 by amino acids with smaller hydrophobic side chains or hydrophilic amino acids resulted in reduced intracellular accumulation and secretion of S protein. In agreement with a previous report (28), the residue R169 appears to be critical for SVP production, because only replacement by lysine (K) did not significantly reduce intracellular accumulation and secretion of S protein (Fig. 5, lower).

Taken together, the results presented above are consistent with the predicted amphipathic alpha helix structure between residue 156 and residue 169. In addition, replacement of the positively charged K160 with oppositely charged residues significantly reduced HBsAg secretion (Fig. 5, upper), suggesting that the charge of this residue is critical for SVP morphogenesis, probably by mediating interaction with charged structure motifs of partner proteins in the lumen of the ER or ERGIC. Moreover, the strict constraint on the structure of residues 156 and 157, which define the boundary of the hydrophilic and hydrophobic surfaces at the N terminus of the alpha helix, strongly indicates that these amino acid residues may also be involved in specific molecular interactions with host cellular proteins critical for SVP morphogenesis.

Disruption of the putative amphipathic alpha helix destabilizes S protein and/or compromises SVP morphogenesis. S protein is cotranslationally inserted into the ER membrane and initially produced as a 25-kDa protein (p25); approximately one-half of the total p25 is glycosylated at N146, yielding a 27-kDa species (gp27). Both p25 and gp27 are transported to a not-yet-defined membrane-bound compartment, presumably the ERGIC, where SVP budding takes place. The SVPs are subsequently transported into the Golgi apparatus and secreted from the cells via exocytosis. An interesting observation in the mutagenesis studies presented in Fig. 2 to 5 is the striking discrepancy between the secreted and intracellular levels of S protein in the cultures expressing many different mutant S proteins, i.e., many single-amino-acid substitutions in the putative amphipathic alpha helix region drastically reduced the amounts of secreted S protein but did not reduce or only modestly reduced the amounts of intracellular S protein. These results imply that the amphipathic alpha helix may play an important role in SVP morphogenesis and/or secretion. To determine the role and mechanism of the putative amphipathic alpha helix in each step of S protein-directed SVP production and secretion, we first intended to separate intracellular membrane-integrated S protein from intracellular SVPs by membrane flotation centrifugation (47). Briefly, HepG2 cells transfected with a plasmid expressing WT S protein were physically lysed in a detergent-free hypotonic buffer. After the removal of cell nuclei by a low-speed centrifugation ($900 \times g$), the supernatant of the cell lysate (S900) as well as concentrated SVPs from the culture medium of transfected cells were subjected to membrane flotation centrifugation analyses in a noncontinuous sucrose gradient. S protein levels in each fraction were measured by Western blotting for detection of total S protein and by native agarose gel electrophoresis-based particle gel assay for detection of SVPs. Calnexin, an ER transmembrane protein, was used in Western blot assays to serve as a marker of the ER membrane. As shown in Fig. 6A to C, the ER membrane floated to the top fractions (fractions 1 and 2) of the gradient, whereas the relatively dense SVPs floated in fractions 5 to 10 and peaked at fractions 6 to 8. As anticipated, all of the secreted S protein in the culture medium existed in the form of SVPs and floated to fractions 5 to 10 (Fig. 6A and B), but intracellular S protein could be separated into membrane-integrated forms in fractions 1 and 2 and SVPs in fractions 5 to 10 (Fig. 6A and C). Moreover, small spherical particles with 22-nm diameters, presumably SVPs, were observed in culture medium and fractions 5 to 10 of cellular lysates (S900) by electron microscopy (Fig. 6D and E). These results indicated that the membrane flotation assay can efficiently separate the membrane-integrated S protein and intracellular SVPs. Interestingly, analysis of the cytoplasmic lysates (S900) of HepG2 cells transfected with a plasmid expressing A157K or A157D mutant S protein with the membrane flotation assay revealed that the mutant S proteins either primarily (A157K) or exclusively

FIG 6 Analysis of cellular membrane-integrated S protein and SVPs in HepG2 cells expressing WT and mutant S proteins. HepG2 cells were transfected with a plasmid expressing either WT or A157 mutant HBsAg. Culture medium and cells were harvested at 48 h posttransfection. Five milliliters of culture medium were concentrated by ultracentrifugation, and the pellet was dissolved in 400 μ l of hypotonic buffer. The cells were physically lysed in hypotonic buffer. The cell lysate was centrifuged at $900 \times g$ for 5 min. The supernatant (Sup) was harvested, designated S900, and subjected to the membrane flotation assay. (A) S protein levels in the fractions of sucrose gradients loaded with the concentrated culture supernatant (purple) or cell lysate S900 (orange) were determined by ELISA. The sucrose concentration (green) of each fraction was plotted. (B and C) Total S protein and SVPs in each of the fractions from the supernatant (B) and cell lysate (S900) (C) of cells expressing WT S protein were determined by Western blot (WB) and particle gel assays. (D and E) SVPs in the supernatant (D) and cell lysate (fraction 5 to 9) (E) of cells expressing WT S protein were detected by transmission electron microscopy after negative staining. White arrowheads in selected regions indicate particles with a diameter of roughly 22 nm, consistent with the known size of HBV SVPs. Scale bar, 100 nm. (F and G) Total S protein and SVPs in each of the fractions from cell lysates (S900) of cells expressing A157K (F) and A157D (G) mutant S proteins were determined by Western blotting and particle gel assays. Calnexin (CNX) served as an ER marker.

FIG 7 Secreted S proteins are quantitatively correlated with the intracellular SVPs but not total intracellular S protein. HepG2 cells were transfected with a plasmid expressing WT S protein or an indicated mutant S protein. The culture medium and cells were harvested at 48 h posttransfection. Two milliliters of the culture medium were concentrated by ultracentrifugation and resuspended in 120 μ l of hypotonic buffer. The cells were physically lysed in hypotonic buffer, and the lysate was centrifuged at $900 \times g$ for 5 min. The supernatant (Sup) was designated the S900 fraction, whereas the pellet was resuspended in an equal volume of hypotonic buffer and designated the P900 fraction. S protein and/or SVPs in each of the samples were analyzed by Western blot (WB) and particle gel assays. Tubulin served as a loading control.

(A157D) presented in ER membrane fractions (fractions 1 and 2) and intracellular SVPs were significantly reduced (A157K) or undetectable (A157D) (Fig. 6F and G, compared with Fig. 6C). These results indicated that the A157D mutation reduced the metabolic stability of S protein, whereas both A157D and A157K mutations impaired intracellular SVP production (or morphogenesis).

Because the membrane-associated S protein could not be detected by the particle gel assay (Fig. 6C, F, and G), we reasoned that intracellular SVPs in the cytoplasmic lysates (S900) could be directly detected by the particle gel assay without membrane flotation centrifugation. Indeed, examination of S protein and SVPs in culture medium (Fig. 7), supernatant (S900), and pellet (P900) of hypotonic lysates of HepG2 cells expressing WT or mutant S proteins with variable phenotypes demonstrated that intracellular SVPs were exclusively in the S900 fraction. Interestingly, the amounts of secreted S protein were perfectly correlated with the amounts of intracellular SVPs but not total intracellular S protein. These results reinforce the notion that the selected single-amino-acid substitutions in the putative amphipathic α helix region compromised either the intracellular trafficking of S protein to the site of SVP budding or the budding process itself but not the secretion (exocytosis) of SVPs.

SVP budding is a post-ER but pre-Golgi complex event. To investigate the molecular mechanism of S protein intracellular trafficking and SVP budding, we established a HepG2-derived stable cell line supporting doxycycline (Dox)-inducible expression of S protein, designated HepG2-3SG11. Addition of Dox to the culture medium induces the transcription of mRNA specifying S protein. HBsAg became detectable by ELISA in culture medium at 2 h post Dox addition and increased continuously in the following 8 h. To determine the role of COPII vesicle trafficking from the ER to the ERGIC and Golgi complex in SVP morphogenesis and secretion, the cells were treated with H-89 or

FIG 8 Effects of vesicle budding and trafficking inhibitors on SVP production and secretion. (A) A cartoon depicts the vesicle trafficking between the ER, ERGIC, and Golgi complex, as well as the anticipated targets and effects of H-89 and BFA on the dynamics of intracellular membrane system. See text for details. (B) Schematic presentation of the experimental schedule. HepG2-3SG11 cells were cultured in the absence of Dox until confluence. Dox was then added to the culture medium to activate HBsAg mRNA transcription. At 4 h after Dox addition, the cells were mock treated with dimethyl sulfoxide (DMSO) or treated with 40 μ M BFA and/or 100 μ M H-89. The culture medium and cells were harvested at the indicated times. (C) HBsAg in the supernatant of each sample was detected by ELISA, and results are presented as the average and standard deviation of OD values ($n=2$). ***, $P < 0.001$. (D and E) Intracellular SVPs were detected by particle gel assay (D). The amounts of SVPs were determined by ImageJ and plotted as fold changes relative to the amount of the corresponding SVP at time zero (E). Means and standard deviations from two biological replicates are presented. ***, $P < 0.001$. (F and G) SVPs in fractions 5 to 9 of membrane flotation centrifugation from the culture medium of mock-treated HepG2-3SG11 cells (F) or the lysate of HepG2-3SG11 cells treated with BFA for 4 h (G) were detected by electron microscopy. Scale bar, 100 nm.

brefeldin A (BFA) to disrupt COPII vesicle budding or to deplete the Golgi complex, respectively. As illustrated in Fig. 8A, H-89 is a protein kinase A inhibitor that blocks COPII-coated vesicle assembly by preventing the binding of small COPII coat GTPase SAR1 to the ER membrane (48, 49). It was reported that treatment of mouse

embryonic fibroblasts with a high concentration of H-89 (100 μ M) led to the dispersal of the ERGIC53 but not the *cis*-Golgi (50). BFA is a fungal metabolite that inhibits the activation of ADP-ribosylation factor 1 (ARF1), which prevents association of the COPI coat and ultimately induces fusion of the Golgi complex into neighboring ER and ERGIC membranes (50, 51).

As depicted in Fig. 8B, HepG2-3SG11 cells were cultured in the presence of Dox to induce S protein expression followed by treatment with H-89 and BFA, alone or in combination. Culture medium and cells were harvested at the indicated time points. Interestingly, treatment of HepG2-3SG11 cells with H-89 and/or BFA completely abolished the secretion of HBsAg into the culture medium (Fig. 8C). Analyses of intracellular SVPs in the cell lysate by a particle gel assay indicated that, due to the efficient secretion of SVPs, the mock-treated cells did not accumulate significant amounts of SVPs. However, BFA treatment induced a drastic intracellular accumulation of SVPs (approximately 14-fold at 4 h of treatment), but H-89 treatment prevented BFA-induced intracellular SVP accumulation (Fig. 8D and E), suggesting that H-89 inhibited SVP production but BFA inhibited SVP secretion. The existence of secreted SVPs in mock-treated cells and intracellular SVPs in the lysates of BFA-treated cells was further confirmed by electron microscopic imaging (Fig. 8F and G). Based on the mode of action of these compounds, the results are consistent with the notion that the ER and Golgi complex are not directly involved in SVP budding and a cellular compartment between the ER and Golgi complex, most likely the ERGIC, is the site of SVP morphogenesis. In support of this notion, a recent report demonstrated that R79K mutant S protein is deficient in SVP morphogenesis, due to its failure to exit the ER (26). As anticipated, the Golgi complex plays an essential role in SVP secretion.

Mutant S proteins deficient in SVP formation have a dominant negative effect on WT S protein-directed SVP morphogenesis. It can be speculated that extensive interactions between S proteins must occur during SVP morphogenesis and the deficiency of mutant S proteins in SVP production could be due to abnormal interactions between S proteins. To test this hypothesis, we determined the effects of coexpression of WT and mutant S proteins in hepatoma cells on SVP production. In agreement with the results presented above (Fig. 7), expression of A157D, I_n157, A157K, or R79K S protein alone in HepG2 cells resulted in undetectable or significantly reduced (A157K) amounts of intracellular SVPs and undetectable HBsAg/SVP in the culture medium (Fig. 9A). However, cotransfection of a plasmid expressing WT S protein with a plasmid expressing the indicated mutant S protein at a 1:1 molar ratio resulted in a significant reduction of intracellular SVPs and more dramatic reduction of secreted SVPs (Fig. 9B). The observed “dominant negative” effects of mutant S proteins on SVP production strongly support the notion that the interaction between S proteins is important for SVP morphogenesis.

Concerning the physical nature of S protein interactions for oligomerization, it may occur through either noncovalent interactions, such as hydrophobic interactions between its TMs, or formation of intermolecular disulfide bonds between the cysteines in the AGL of S proteins. While the hydrophobic interactions of TM2 and noncovalent interactions of CYL-1 of S proteins were detected recently by a fluorescence-activated cell sorting (FACS)-based Förster resonance energy transfer (FRET) assay and demonstrated to be essential for S protein dimerization in the ER membrane (34), the covalent interactions via intermolecular disulfide bonds between the AGL of S proteins can be reliably detected by nonreducing SDS-PAGE and Western blot assays (29, 35). As shown in Fig. 10A, a nonreducing SDS-PAGE analysis demonstrated that S protein existed as monomers, dimers, or oligomers in intracellular membranes (predominantly ER membrane) and intracellular or secreted SVPs. This result suggests that extensive disulfide-linked S protein oligomerization may occur before SVP budding. In order to investigate whether the deficiency of the mutant S proteins in SVP production is due to the abnormality in disulfide-linked oligomerization, the cellular membrane-integrated S proteins were purified from HepG2 cells transfected with a plasmid expressing WT S protein or the indicated mutant S protein by membrane flotation centrifugation. As shown in

FIG 9 Mutant S proteins deficient in SVP production inhibited WT S protein-directed SVP morphogenesis. HepG2 cells were transfected with a plasmid expressing WT or an indicated mutant S protein alone (A) or cotransfected with WT S protein and an indicated mutant S protein expression plasmid at the molar ratio of 1:1 (B). The culture medium and cells were harvested at 48 h posttransfection. Two milliliters of culture medium were concentrated by ultracentrifugation and resuspended in 120 μ l of hypotonic buffer. The cells were physically lysed in hypotonic buffer. S protein and/or SVPs in each of the samples were analyzed by Western blotting and particle gel assays. Sup, supernatant. Tubulin served as a loading control. The amounts of S protein and/or SVPs were quantified by ImageJ.

Fig. 10B, nonreducing SDS-PAGE analysis indicated that, similar to WT S protein, all of the mutant S proteins examined also existed in monomer, dimer, and oligomer forms in cellular membranes. However, the mutant S proteins accumulated more oligomers but fewer dimers, in comparison with WT S protein. The increased accumulation of oligomers by the mutant S proteins could be due to their failure to form SVPs. Taken together, these results suggest that R79K and A157 mutant S proteins were not deficient in disulfide-linked oligomerization but most likely failed to mediate the critical interactions of S protein with host cellular factors essential for S protein trafficking and/or SVP budding.

S protein is degraded by 20S proteasomes in a ubiquitination-independent manner. The results presented above showed that A157D and many other single-amino-acid substitutions in the putative amphipathic α helix region not only compromised SVP morphogenesis but also reduced the steady-state levels of intracellular S protein. To ascertain the mutations accelerating the decay of S protein, we first determined the decay kinetics of WT S protein and A157D S protein in HepG2 cells. As anticipated, upon the inhibition of protein synthesis by cycloheximide, the level of A157A mutant S protein declined faster than that of WT S protein (Fig. 11A and B).

In mammalian cells, one-third of the cellular proteins are processed into mature forms in the ER through proteolytic cleavage and/or posttranslational modifications (52). These cellular processes are monitored by the ER protein quality control system via selective degradation of misfolded proteins through two distinct pathways, i.e., autophagy-mediated lysosomal degradation and ER-associated degradation (ERAD) by proteasomes (53, 54). In order to determine the cellular pathway(s) responsible for the degradation of WT and mutant S proteins, HepG2 cells transfected with a plasmid expressing WT S protein or the indicated mutant S protein were mock treated or treated with chloroquine, a compound that inhibits autophagy-mediated lysosomal

FIG 10 Mutations in the putative amphipathic alpha helix do not disrupt the disulfide-linked S protein oligomerization. HepG2 cells were transfected with a plasmid expressing WT S protein or an indicated mutant S protein. Culture medium and cells were harvested at 48 h posttransfection. (A) S proteins integrated in cellular membranes (mHBs) or associated with intracellular SVPs (In-SVP) or secreted SVPs in supernatant (Sup-SVP) were resolved by nonreducing SDS-PAGE without or with treatment of the samples with BME. S proteins were detected by Western blotting with a monoclonal antibody against the HA tag. (B) S proteins integrated in cellular membranes (mHBs) prepared from the cells transfected with a plasmid expressing WT or indicated mutant S proteins by membrane flotation centrifugation were subjected to nonreducing SDS-PAGE as described above. S protein monomers (p25 and gp27) are indicated by arrows. Disulfide-linked S protein dimer and oligomers were indicated by asterisks. Calnexin (CNX) served as a loading control.

degradation (55), or MG-132, a 20S and 26S proteasome inhibitor (56). The results demonstrated that, although the chloroquine treatment efficiently inhibited autophagic flux, as indicated by the increased amount of LC3A/B in treated cells, the compound did not alter the intracellular levels of S protein (Fig. 11C). In contrast, MG-132 treatment not only inhibited the degradation of A157D and W156A S proteins but also increased the levels of WT and A157K S proteins (Fig. 11D). These results indicate that both WT and mutant S proteins are predominantly degraded by a proteasome-dependent pathway.

ERAD of misfolded ER proteins depends on the detection of substructures within proteins such as exposed hydrophobic regions and immature glycans by cellular chaperones and glycan recognition proteins and then translocation to the cytoplasm by valosin-containing protein p97 (VCP/p97), a transitional ER ATPase (57). To investigate the role of the ERAD pathway in S protein degradation, HepG2 cells transfected with a plasmid expressing WT S protein or the indicated mutant S protein were treated with CB-5083, a VCP/p97 ATPase inhibitor that inhibits Sec61-mediated protein translocation at the ER (58). As anticipated, inhibition of VCP/p97 can inhibit the degradation of C/EBP homologous protein (CHOP) in the ER through the classic ERAD pathway (58). However, the compound treatment did not alter the levels of intracellular WT or mutant S proteins (Fig. 11E), suggesting that S protein degradation most likely does not depend on the classic ERAD pathway.

To investigate whether S protein polyubiquitination is required for proteasomal degradation, all four lysine (K) residues of S protein were replaced with alanine (A) in WT S protein or A157D S protein to prevent ubiquitination. As shown in Fig. 11F, comparing to the parental WT and A157D S proteins, alanine substitutions significantly reduced the basal levels of S proteins. However, MG-132 treatment significantly increased the levels of both parental S protein and S protein with alanine substitution, suggesting that the proteasomal degradation of S protein occurs in a ubiquitination-independent manner.

To distinguish the role of 20S and 26S proteasomes in S protein degradation, HepG2 cells transfected with a plasmid expressing WT S protein or A157D mutant S

FIG 11 S protein is degraded via a ubiquitin-independent, 20S proteasome-dependent pathway. (A and B) HepG2 cells were transfected with a plasmid expressing either WT S protein or A157D S protein. Transfected cells were treated with 50 μ g/ml cycloheximide (CHX), starting at 40 h posttransfection, for the indicated times. Intracellular S protein was determined by Western blot assay (A) and gray value analysis (B). (C to E) HepG2 cells were transfected with a plasmid expressing either WT S protein or an indicated mutant S protein. The cells were mock treated or treated with 50 μ M chloroquine (C) or 20 μ M MG-132 (D), starting at 36 h posttransfection, for 12 h or were treated with 20 μ M CB-5083 (E), starting at 40 h posttransfection, for 8 h. Intracellular S protein was detected by Western blotting. LC3A/B and CHOP served as markers for the inhibition of autophagy flux and VCP/p97 ATPase activity, respectively. (F) HepG2 cells transfected with a plasmid expressing WT S protein or the indicated mutant S protein were mock treated or treated with 20 μ M MG-132, starting at 36 h posttransfection, for 12 h. Intracellular S protein was detected by Western blotting. (G) HepG2 cells transfected with a plasmid expressing WT S protein or an indicated mutant S protein were mock treated or treated with 10 μ M RA190 for 12 h, starting at 36 h posttransfection. Intracellular S protein was detected by Western blotting assay. Hsp70 served as a positive control for inhibition of 26S proteasome activity. Tubulin served as a loading control. (H) *In vitro* 20S degradation of HA-tagged S protein. HepG2 cells transfected with a plasmid expressing WT and A157D mutant S protein, HA-tagged S protein were pulled down by anti-HA beads and then incubated with 20S proteasomes at 37°C for the indicated times before immunoblotting analysis.

protein were treated with RA190, an inhibitor of RNP13 protein in the 19S regulatory particle of 26S proteasome (59). Although RA190 efficiently rescued the degradation of Hsp70, which is degraded by 26S proteasomes (60), the treatment did not alter the levels of intracellular WT or mutant S proteins in HepG2 cells (Fig. 11G). However, treatment of immunoaffinity-purified WT or A157D S with 20S proteasomes *in vitro* significantly reduced the amounts of S proteins (Fig. 11H). Taken together, the results

presented in Fig. 11 are consistent with the hypothesis that S protein is degraded via a 20S proteasome-dependent but ubiquitination-independent nonclassic ERAD pathway.

DISCUSSION

HBV S envelope protein has an intrinsic ability to direct the morphogenesis of small spherical SVPs. Previous studies have demonstrated the critical role of TM1 and TM2, as well as cysteine residues at the cytosolic loop and AGL, in S protein-directed SVP morphogenesis (27, 31, 32, 34). In this study, we obtained several lines of evidence suggesting that amino acid residues 156 to 169 of S protein may form an amphipathic alpha helix that is essential for SVP morphogenesis and regulates intracellular S protein metabolism (Fig. 4 and 5). In order to understand the molecular events leading the monomeric transmembrane S protein in the ER membrane to the production of SVPs in an intracellular membrane compartment, presumably the ERGIC, we first developed methods to distinguish intracellular membrane-associated S protein and SVPs (Fig. 6 and 7). We then took advantage of the various mutant S proteins deficient in SVP morphogenesis to map the molecular events important for S protein oligomerization and SVP budding (Fig. 8 to 10). These studies reveal the following important findings.

First, extensive disulfide-linked S protein oligomerization occurs before SVP budding. The disulfide-linked S protein oligomers are the predominant forms of S proteins detected in both cellular membrane-integrated and SVP-associated S proteins (Fig. 10). Because R78/R79 dipeptide was recently identified to mediate S protein interaction with Sec24A, a component of the COPII, the R79K mutant S protein was thus deficient in COPII vesicle-mediated S protein exit from the ER (26). While the failure of R79K mutant S protein to form SVPs is consistent with the notion that SVP budding occurs in a post-ER compartment (Fig. 9), the accumulation of disulfide-linked R79K mutant S protein oligomers in the transfected HepG2 cells implies that the disulfide-linked S protein oligomerization takes place in the ER membrane (Fig. 10B). However, a legitimate argument is that the presence of high levels of protein disulfide isomerase in the ER lumen may resolve the disulfide-linked S protein oligomers into dimers. Huovila and colleagues thus proposed that disulfide-linked S protein dimers quickly formed in the ER membrane upon synthesis and were subsequently sorted into a post-ER, pre-Golgi compartment without protein disulfide isomerase, where the disulfide-linked S protein oligomerization extensively occurred (35). Apparently, resolution of these contradictory interpretations requires development of technologies for more accurate detection of S protein intracellular trafficking and subcellular localization of the disulfide-linked S protein oligomers. Nevertheless, in agreement with the lack of cysteines in the putative amphipathic alpha helix region, the A157 mutations did not alter the disulfide-linked S protein oligomerization (Fig. 10B).

Second, the putative amphipathic alpha helix plays an essential role in SVP budding. The subcellular compartment where SVP assembly and budding take place remains to be firmly determined. On one hand, an electron microscopic study showed that HBsAg self-assembly initially forms filamentous SVPs within the perinuclear space, presumably a part of the ER. The filaments are subsequently clustered, packed into crystal-like structures, and transferred by ER-derived vesicles to the rough ERGIC, where the filamentous SVPs are converted to spherical SVPs (38, 39). On the other hand, Huovila and colleagues provided biochemical and cell biological evidence through analyses of disulfide-linked S protein oligomerization to support SVP morphogenesis in the ERGIC (35). Our observations that BFA treatment induced extensive intracellular accumulation of SVPs and that H-89 treatment abolished the BFA-induced SVP accumulation strongly support the notion that SVP budding takes place at a post-ER but pre-Golgi compartment and that the Golgi complex is essential for SVP secretion but not production (Fig. 8). The fact that many single-amino-acid substitutions or insertions in the putative amphipathic alpha helix region significantly compromised intracellular SVP production indicates the essential role of the region in SVP budding but not S protein oligomerization. It is possible that this amphipathic alpha helix embeds in the

lumen leaflet of ER and ERGIC membranes, which may alter the membrane curvature and/or lipid fluidity and consequentially facilitate SVP budding. This interpretation is based on the findings that the membrane-attached amphipathic alpha helices in other proteins play important roles in membrane fusion and vesicle budding. For example, ArfGAP1, a GTPase-activating protein for ARF1, triggers rapid dissociation of ARF1 and coatamer from COPI vesicles, depending on its amphipathic lipid packing sensor (ALPS) motif (61). Another example is the critical role of amphipathic alpha helix of interferon-induced transmembrane proteins in modulating viral envelope fusion with cellular membranes (44, 62).

Third, S protein is degraded by 20S proteasomes in a ubiquitination-independent manner. The efficient rescue of WT and mutant S protein degradation by MG-132, which inhibits both 20S and 26S proteasomes, but not RA190, which inhibits only the activity of 26S proteasomes, implies that S protein is most likely degraded by 20S proteasomes. The ubiquitination-independent degradation of S protein further favors a role of 20S, but not 26S, proteasomes. The biochemical study clearly demonstrated that 20S proteasomes can indeed degrade purified WT and mutant S proteins. However, it remains to be determined how 20S proteasomes recognize nonubiquitinated S proteins in ER or ERGIC membranes for degradation. Because all of the mutant S proteins with reduced intracellular accumulation are also deficient in SVP production, we speculate that the accelerated degradation of mutant S proteins may occur due to the failure of SVP budding at the ERGIC but not the misfolding or accumulation of S protein in the ER. The finding that the classic ERAD inhibitors failed to restore the stability of S proteins support this hypothesis.

An important implication of our studies is that intracellular S protein metabolism and SVP morphogenesis may be pharmacologically modulated by specifically targeting the putative amphipathic alpha helix of the AGL, which may result in the degradation of S protein by 20S proteasomes. Compared to reducing the production of S protein by inhibiting covalently closed circular DNA (cccDNA) transcription (63, 64) or promoting viral mRNA decay (65, 66), not only does pharmacological induction of S protein degradation reduce HBsAg antigenemia but also the accelerated degradation of S protein may facilitate the activation of HBV-specific T-cell immune responses to control viral infection (67, 68) and to increase the rate of functional cure of chronic hepatitis B (69).

MATERIALS AND METHODS

Cell culture. The HepG2 cell line was purchased from the ATCC (number HB-8065). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin.

Establishment of the HepG2-3SG11 cell line. The HepG2-3SG11 cell line was established by cotransfection of HepG2 cells with plasmids pCMV-Tet3G (catalog number 631168; ClonTech) and pTRE3G-SG, in a ratio of 1:10, followed by puromycin selection. Plasmid pTRE3G-SG supported the expression of bicistronic genes of HBV S protein and green fluorescent protein (GFP) in a *tet-on*-controlled manner. The cell line was maintained in DMEM/F12 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin without Dox. To activate HBsAg mRNA transcription, Dox (1 µg/ml) was added to the culture medium.

Plasmid construction. The plasmids expressing N-terminally HA-epitope-tagged HBV S protein were constructed by PCR-mediated cloning of the S protein-coding region of genotype A (GenBank accession number [AP007263](#)), B (GenBank accession number [AY220698](#)), C (GenBank accession number [AF461363](#)), or D (GenBank accession number [AB267090](#)) genomes into the EcoRI and PstI sites of plasmid pXF3H (70). A commercial point mutagenesis kit (catalog number SMK-101; Toyobo) was used to introduce single-amino-acid substitution or insertions into S protein by following the instructions of the manufacturer. All mutagenesis was confirmed by DNA sequencing.

HepG2 cell transfection. Cells grown in 12-well plates were transiently transfected with 1 µg of plasmid per well using Lipofectamine 2000 (Invitrogen) according to the instructions of the manufacturer. Six hours after transfection, the culture medium was removed, and 2 ml of fresh medium was added to the cells. Two days posttransfection, the culture medium was harvested and centrifuged for 10 min at 12,000 × *g* to remove cell debris and to harvest the supernatant and cells in the plate. The cells were lysed as specified below for Western blot, membrane flotation, or particle gel assays.

Chemical reagents. Proteasome inhibitor MG-132 (catalog number 474790), autophagy inhibitor chloroquine (catalog number C6628), BFA (catalog number B6542), H-89 dihydrochloride hydrate (catalog number B1427), and protein synthesis inhibitor cycloheximide (catalog number C7698) were

purchased from Sigma. RA190 (catalog number HY-100739) and CB-5083 (catalog number HY-12861) were purchased from MCE. Anti-HA beads (catalog number 88836) was purchased from Thermo Fisher Scientific. The purified human 20S proteasome (catalog number BML-PW8720-0050) was purchased from Enzo Biochem Inc., USA.

Western blotting. To detect secreted S protein, 2 ml of culture medium was overlaid on a 3-ml cushion of 20% (wt/wt) sucrose dissolved in TNE buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, and 1 mM EDTA) and ultracentrifuged at 46,000 rpm for 3.5 h at 4°C with a Beckman SW55 rotor. The pellet was dissolved in 120 μ l 1 $\times$ LDS buffer (Invitrogen) supplemented with 2.5% β -mercaptoethanol (BME) (Sigma). To detect intracellular S protein, cells in 12-well plates were lysed with 200 μ l per well of 1 $\times$ LDS buffer containing 2.5% BME. An aliquot of each sample was subjected to denaturing gel electrophoresis with a Novex 14% Tris-glycine gel and Novex Tris-glycine SDS running buffer (Thermo Fisher Scientific). Proteins were transferred from the gel onto a polyvinylidene difluoride (PVDF) membrane using an iBlot 2 dry blotting system (Thermo Fisher Scientific). The membrane was blocked for 1 h at room temperature with 5% nonfat milk in TBST (Tris-buffered saline containing 0.1% Tween 20) and was incubated overnight at 4°C with the indicated antibody. Anti-HA (2367; Cell Signaling Technology), anticalnexin (2679; Cell Signaling Technology), anti-Hsp70 (4872; Cell Signaling Technology), anti- α / β -tubulin (2148; Cell Signaling Technology), and anti- β -actin (3700; Cell Signaling Technology) antibodies were used as primary antibodies. The membrane was extensively washed with TBST and followed by incubation with Li-Cor IRDye secondary antibodies or horseradish peroxidase (HRP)-linked secondary antibodies. Membranes were again washed with TBST and imaged with an Odyssey system (Li-Cor Biotechnology) or ChemiDoc MP Imaging System (Bio-Rad).

ELISA. HBsAg in culture medium was quantified by using a commercial HBsAg ELISA kit (Kehua, China) and chemiluminescence immunoassay kit (Autobio, China) following the manufacturers' instruction. Fifty microliters of culture medium was used for the assay.

Membrane flotation assay. Cells in a 6-cm dish were lysed with 500 μ l hypotonic buffer (10 mM Tris-HCl [pH 7.5], 10 mM KCl, and 5 mM MgCl₂) supplemented with 5 μ l of a 100 $\times$ cocktail of protease inhibitors (Sigma). The cell lysate was passed through a 26-gauge needle 25 times. Nuclei and other cell debris were removed by centrifugation at 900 $\times$ g for 5 min at 4°C, and the supernatant was designated S900. Four hundred microliters of S900 or whole-cell lysates were mixed with 2 ml of 72% (wt/wt) sucrose solution in low-salt buffer (LSB) (50 mM Tris-HCl [pH 7.5], 25 mM KCl, and 5 mM MgCl₂) and overlaid with 2 ml of 55% (wt/wt) sucrose solution in LSB, followed by the addition of 0.6 ml of 10% (wt/wt) sucrose solution in LSB as the top layer. The sucrose gradient was centrifuged at 46,000 rpm for 15 h at 4°C in a Beckman SW55Ti rotor. After centrifugation, 500- μ l fractions were collected from the top of the gradient, and 300 μ l of each fraction was precipitated with 4 volumes of methanol and centrifuged at 12,000 $\times$ g for 10 min at 4°C. The pellet was dissolved in 50 μ l of 1 $\times$ LDS buffer containing 2.5% BME and was subjected to Western blot analysis. Fifty microliters of each fraction was used for ELISA detection of HBsAg or detection of SVPs by particle gel assay. The sucrose concentration of each fraction was determined by a fractionator.

Particle gel analysis. Intracellular or secreted SVPs were detected by a native agarose gel electrophoresis-based particle gel assay as reported previously, with modifications (71). Briefly, cells in 12-well plates were lysed with 200 μ l per well hypotonic buffer and were passed through a 26-gauge needle 25 times. The S900 fractions were subjected to electrophoresis in 1.3% agarose gel with 1 $\times$ TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) as the running buffer. SVPs were transferred to nitrocellulose membranes with capillary transfer in TNE buffer. The membrane was sequentially fixed with 2.5% paraformaldehyde (diluted in PBS) for 10 min and with 50% methanol (diluted in PBS) for 20 min at room temperature. The membrane was then blocked with 5% nonfat milk in TBST for 1 h at room temperature, followed by incubation with the indicated antibody overnight at 4°C. Antibody against the HA tag or HBsAg (10-H05H; Fitzgerald) was used as the primary antibody, as indicated in the figure legends. The membrane was washed with TBST, followed by incubation with Li-Cor IRDye secondary antibodies or HRP-linked secondary antibodies, and SVPs were visualized with a Li-Cor Odyssey system (Li-Cor Biotechnology) or ChemiDoc MP Imaging System (Bio-Rad).

Negative staining and transmission electron microscopy. Cells in a 10-cm dish were lysed with 1 ml of hypotonic buffer (10 mM Tris-HCl [pH 7.5], 10 mM KCl, and 5 mM MgCl₂) supplemented with a 1 $\times$ cocktail of protease inhibitors. The cell lysate was subjected to the membrane flotation assay, and gradient fractions 5 to 9 were pooled and dialyzed overnight with a 10-kDa-cutoff Slide-A-Lyzer dialysis cassette (Thermo Fisher Scientific) to remove the sucrose. After concentration with a 100-kDa-cutoff Amicon ultrafiltration unit (Millipore), particles were negatively stained with 2% phosphotungstic acid (Electron Microscopy Sciences) and observed in a Tecnai T12 transmission electron microscope (FEI). For detection of secreted SVPs, the cells were cultured in serum-free medium for 24 h, and the supernatant was centrifuged at 10,000 $\times$ g for 20 min to remove the cell debris, concentrated with 100-kDa-cutoff Amicon ultrafiltration units, and subjected to negative staining and transmission electron microscopy.

Nonreducing SDS-PAGE assay. To prepare the membrane-integrated S protein, cells in a 6-cm dish were lysed with 500 μ l hypotonic buffer with protease inhibitors, and the whole-cell lysate was subjected to membrane flotation centrifugation as described above. Collecting from the top of the gradient, the first two fractions were collected as membrane-integrated S protein. The intracellular and secreted SVPs were prepared by overlaying 0.5 ml of S900 fraction or 2 ml of culture medium and ultracentrifugation through a 20% sucrose cushion at 46,000 rpm for 3.5 h at 4°C with a Beckman SW55 rotor. The pellet was dissolved with 120 μ l hypotonic buffer. The samples were mixed with 5 $\times$ nonreducing sample buffer (Thermo Fisher Scientific) without 2.5% BME (nonreducing conditions) or with 2.5% BME (reducing conditions). After denaturation at 100°C for 10 min, the samples were subjected to denaturing gel

electrophoresis with a Novex 4% to 20% Tris-glycine gel and Novex Tris-glycine SDS running buffer. S protein was detected by Western blotting as described above.

Northern blotting. Total cellular RNA was extracted with TRIzol reagent (Invitrogen) following the manufacturer's directions. Four micrograms of total RNA from each of the samples was fractionated by electrophoresis in a 1.5% agarose gel with 6% formaldehyde and 1× MOPS (morpholinepropanesulfonic acid) running buffer (Sigma). RNA was transferred to a Hybond-XL membrane (GE Healthcare) by capillary transfer in 20× SSC (3 M NaCl, 0.3 M sodium citrate). The membrane was cross-linked in a UV cross-linking chamber. HBV RNA was detected by hybridization with an [α -³²P]UTP-labeled full-length minus-strand RNA probe.

Immunoaffinity purification of S protein and 20S proteasome digestion. HepG2 cells were transfected with a plasmid expressing WT or A157D mutant S protein and lysed at 48 h posttransfection with 1 ml of buffer (25 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% NP-40, 5% glycerol, and 1× protease inhibitor cocktail) at 4°C for 1 h. The cell lysates were cleared by centrifugation at 12,000 × g for 20 min at 4°C. The supernatants were mixed with anti-HA beads (30 μ l) for 1 h at room temperature. Five washes were performed with NP-40 lysis buffer, followed by elution with elution buffer (pH 2.8). Eluted samples were concentrated with 10-kDa-cutoff centrifugal filter units (Millipore) and were resuspended in 20S proteasome reaction buffer (25 mM HEPES [pH 7.4], 0.5 mM EDTA [pH 8.0], 0.03% SDS), followed by incubation with 20S proteasomes (0.5 μ g) in the reaction buffer at 37°C for the indicated times. The reactions were terminated by the addition of 4× LDS sample buffer, followed by SDS-PAGE and immunoblotting analysis.

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