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REVIEW

Host factors involved in hepatitis B virus maturation, assembly, and egress

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Abstract Hepatitis B virus (HBV) is a major cause of liver disease. Due to the tiny size of its genome, HBV depends on the critical interplay between viral and host factors for the generation of new viral particles from infected cells. Recent work has illuminated a multiplicity of spatially and temporally coordinated virus-host interactions that accompany HBV particle genesis. These interactions include the requirement of cellular chaperones for the maturation of the three viral envelope proteins, the cellular factors involved in dynamic modification, maturation, and intracellular trafficking of the nucleocapsids, and the host components of the multivesicular body (MVB) pathway enabling virion budding at intracellular compartments. Beside infectious virions, HBV produces at least two other types of particles, subviral empty envelope particles and subviral naked capsid particles, likely as a result of the engagement of different host factors by the viral structural proteins. Accordingly, HBV exploits distinct cellular pathways to release its particle types. Here, I review recent progress in these areas of the cell biology of HBV genesis.

Keywords Dual membrane topology · Host chaperones · Empty envelope particles · Naked capsid particles · Virus assembly · ESCRT machinery

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Introduction

HBV is a global human pathogen responsible for acute and chronic liver disease. Worldwide an estimated 350 million individuals are chronically infected and potentially develop liver cirrhosis or liver cell carcinoma that accounts for more than 1 million deaths per year. Despite the availability and use of an effective vaccine, HBV infection remains an important public health problem, as current therapeutics for chronic carriers have shown only limited success. Understanding the HBV life cycle and host cell protein interactions involved is thus a vital prerequisite for the development of new antiviral concepts. As the prototype member of the hepadnavirus family, HBV is an enveloped, DNA-containing pararetrovirus that replicates by reverse transcription. With about 3 kb, the HBV genome is one of the smallest viral genomes known. Hence, it shows an extremely compact organization and encompasses four overlapping open reading frames encoding the multifunctional polymerase/reverse transcriptase (RT), the capsid-forming core protein, the three closely related envelope proteins, and the regulatory X protein.

In the following, a brief overview of the HBV life cycle is presented (for references, see below). The reader interested in more in-depth information on the as yet poorly understood attachment and entry process, the hepadnaviral reverse transcription strategy, and HBV pathogenesis is referred to recent reviews [1–3]. The HBV virion is a double-shelled sphere, 42 nm in diameter, with an inner nucleocapsid and an outer lipoprotein envelope. Upon envelope-mediated attachment to hepatocytes via largely unknown receptors, the virion probably enters the host cell via endocytosis. Following virus disassembly, the nucleocapsids are delivered to the nucleus where the partially double-stranded DNA (dsDNA) genome is converted to the

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covalently closed circular DNA (cccDNA). The episomal cccDNA serves as a template for the transcription of the pregenomic (pg) RNA and the other viral mRNAs by the host RNA polymerase II. After nuclear export and protein synthesis, the assembly of progeny virions begins with the formation of icosahedral nucleocapsids that package the viral pgRNA together with the viral polymerase. Inside the capsids, composed of 180 or 240 copies of the single core protein, the pgRNA is reverse transcribed to the dsDNA genome via a single-stranded DNA intermediate. Mature nucleocapsids, formed in the cytoplasm, can then be enclosed by the viral envelope composed of cellular lipids and three viral glycoproteins, the small S, middle M, and large L envelope protein that originate at the endoplasmic reticulum (ER) membrane. Subsequent budding of HBV is thought to occur at intracellular membranes. Because HBV is viewed as a non-cytopathic virus, its final transport to the extracellular space must occur in a non-lytic manner, but as yet only limited data are available on this theme. The production of infectious particles is accompanied by the formation of empty envelope particles, also referred to as subviral particles (SVP), which greatly outnumber mature virions. Since the non-infectious SVPs share the antigenic features of the virion envelope, they are used as HBV vaccines for decades. In the in vivo situation, they presumably act as decoys for the immune system. In addition, envelope protein-independent core particle budding and egress have been reported for HBV-replicating cells, but the functional implications and pathways of release of naked capsids remain enigmatic.

In regard to its tiny genome and its restricted number of protein entities, HBV depends particularly on the critical interplay with host factors. Below I will review recent progress in understanding the virus-host interactions at the level of the infected cell with special focus on the viral structural proteins. The engagements of diverse host interactors may enable the virus to produce its distinct types of particles. New insights into morphogenesis pathways of viral, subviral envelope, and subviral core particles will be discussed.

The HBV envelope proteins: Structure, function, and host interaction partners

The S protein

The envelope proteins S, M, and L provide a striking example of hepadnaviral economy, as they are expressed from a single open reading frame of the viral genome by means of three different start codons that are spaced at intervals of 108 (or 119, depending on genotype) and 55 codons. Accordingly, the 226-amino acid (aa) sequence of S is repeated at the C-termini of M and L that carry the additional preS2 domain or preS2 and preS1 domains, respectively (Fig. 1a). All three proteins are cotranslationally integrated into the endoplasmic reticulum (ER) membrane, directed by the action of an uncleaved signal-anchor and a stop-transfer sequence encoded within the first and second transmembrane (TM) segments (TM1,

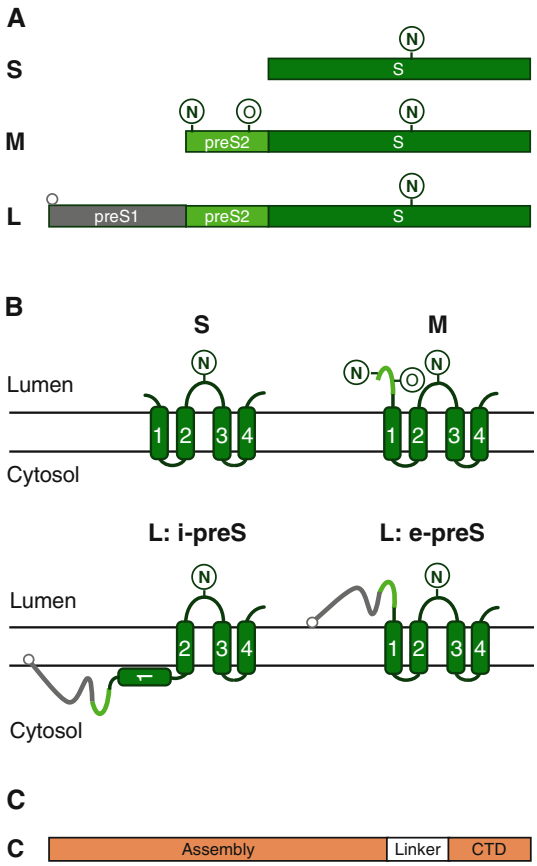

Fig. 1 The HBV structural proteins. **a** Domain structures of the S, M, and L envelope proteins. Due to the usage of a single open reading protein, all three envelope proteins contain the S domain (*dark green*) consisting of 226 aa. The M protein is N-terminally extended for the preS2 domain (*bright green*) encoding for 55 aa. The L protein carries in addition the N-terminal preS1 domain (*gray*) consisting of 108 or 119 aa, depending on genotype. N-glycans linked to the S and preS2 domains are indicated by a circled N, while the circled O refers to O-glycans attached to the preS2 domain of M. The myristic acid attached to the N-terminus of L is indicated by an open circle. **b.** The predicted four TM segments of the S protein project its N- and C-terminus into the ER lumen thereby generating two cytosolic and one luminal loop. The M protein exhibits a topology similar to S with protruding its N-terminal preS2 domain into the ER lumen. The L protein displays a split mixed topology. Upon cotranslational membrane insertion, the preS1 and preS2 regions of L protein are initially located on the cytosolic side of the ER membrane with TM1 being not inserted in the membrane (L: i-preS). During maturation, about half of the L molecules posttranslationally translocate the preS region to the luminal space (L: e-preS). **c** Linear map of the core (C) protein. The N-terminal assembly domain is separated from the C-terminal arginine-rich CTD region by a short linker, as diagrammed

TM2) of their S domains [4]. Two further membrane-spanning segments are predicted in the C-terminal third of the S domain, although it has not been established whether these segments contain topogenic information. Computer models of the secondary structure of S thus suggest four hydrophobic integral domains that are separated by hydrophilic loops and direct the N- and C-terminus into the lumen (Fig. 1b) [5, 6]. Consistent with these structure models is the usage of the N-glycosylation site located in the predicted luminal loop (Asn-146) [7]. Moreover, this loop contains the major conformational hepatitis B surface antigen (HBsAg) epitope. After budding, this region is exposed on the surface of the secreted envelope [8].

The S protein is the major constituent of both the viral and subviral envelope. Beyond its scaffolding role, S contributes to HBV entry and exit in such that it assists the L envelope protein in virus attachment to liver cells and nucleocapsid envelopment. This will be discussed in more detail below. The folding of the related M and L proteins depends on cellular chaperones, like the cognate heat shock protein Hsc70 and the ER resident BiP and calnexin chaperones [9–14]. However, S does not verifiably interact with these host factors [10–13]. Rather, the maturation of S involves the ER luminal protein disulfide isomerase (PDI) that appears to monitor proper disulfide cross-linking of S chains during the formation of the envelope scaffold [15]. In addition, cyclophilin A (CypA) was recently identified as an S-specific binding protein [16]. Transgenic mice and human liver cells stably expressing S displayed decreased intracellular levels of CypA concomitant with increased amounts of this chaperone in the extracellular milieu [16], implicating that S triggers the secretion of CypA. Although the functional role of the S/CypA complex formation and cosecretion remains to be established, it is tempting to speculate that CypA may assist in envelope assembly and release, possibly by binding to the several proline residues present in the first cytosolic loop of S. Apart from chaperones, S has been recently shown in association with LC3, an autophagosomal membrane protein [17]. To account for this interaction, a subversion of the cellular autophagy machinery by HBV for viral envelopment has been suggested [17].

The M envelope protein

The topogenesis of the M protein is highly similar to that of S. Because the hydrophilic preS2 domain lacks any signal sequence activity, M is translocated into the ER membrane by the proximal TM1 and TM2 signals of the S domain that simultaneously govern ER import of the preS2 region [18]. Thereby, the preS2 domain of M protein is N-glycosylated at Asn-4. In addition to N-glycans, the preS2 domain of many HBV genotypes contains O-glycans at Thr-37 (Fig. 1b) [10, 19]. Interestingly, the secretion of subviral and viral particles depends on the preS2-linked N-glycans that mediate a productive interaction with calnexin, a key component of the ER quality control machinery [10, 20, 21]. However, the impact of this interaction remains unclear, because M is dispensable for viral morphogenesis and particle functionality [22]. Moreover, M is not essential for infectivity of HBV or hepatitis delta virus, a viroid that requires the helper function of the HBV envelope [23, 24]. Nonetheless, the conservation of M among orthohepadnaviruses reinforces its function in virus infectivity and pathogenesis. Putative regulatory roles assigned to M are beyond the scope of this review and will not be discussed further.

The L envelope protein

Owing to the small size of their genome, viruses have evolved by packing a maximum of information in a minimum of polypeptide sequence. As a consequence, many of the proteins or protein domains encoded by viruses are multifunctional. The L envelope protein of HBV is an extreme example of such multifunctionality, as it mediates attachment of HBV to liver cells, envelopment of viral capsids, release of (sub) viral particles, regulation of cccDNA amplification, and transcriptional transactivation. The multifunctional nature of L is related to its unusual dual topology, enabling it to dispose its N-terminal preS1 and preS2 (together preS) domain to both the cytosolic (i-preS) and luminal (e-preS) side of membranes [25–27]. Upon synthesis, L is cotranslationally translocated into the ER membrane by the proximal topogenic signals of its S domain. Thereby, its preS domain fails to be translocated and initially remains cytosolic. After biosynthesis, approximately half of the L molecules posttranslationally translocate the preS region across the membrane, thereby generating a dual topology that is also existent in the secreted virion envelope (Fig. 1b) [25–27]. The posttranslational mode of preS translocation is substantiated by the lack of cotranslational modification of the preS-specific N-glycan acceptor sites.

The formation of the dual topology is an intrinsic property of L and does not require any other of the virus-encoded products [27, 28]. Known *cis*-acting determinants responsible for the split topology include a C-terminal region of the preS1 domain, as the deletion of residues 70–94 leads to cotranslational preS translocation and a uniform L topology [11]. This region, referred to as cytosolic anchorage determinant (CAD), is even capable to prevent cotranslational protein translocation of a reporter protein. Follow-up studies showed that CAD specifically interacts with the cytosolic Hsc70 chaperone, likely to control L topogenesis [11]. The mechanism(s) by which

preS traverses the lipid bilayer is less understood, but seemingly does not involve an HBV-specific membrane channel [28]. Formation of such a channel has been proposed to depend on dimerization of S, M, and/or L chains, concomitant with lateral interactions between their three amphipathic TM1, TM3, and TM4 segments that pack together, thereby shielding their hydrophilic faces from the lipid [6]. Mutational analyses, however, demonstrated that neither of the components implicated in channel formation is required for posttranslational preS translocation. Rather, the TM2 segment of L, which is unlikely to form an autonomous translocation channel, was mapped as another critical *cis*-acting determinant of the topological reorientation of preS [28, 29].

The uncommon structural heterogeneity of L suggests the involvement of regulatory mechanisms. Indeed, several studies demonstrated that L interacts with host chaperones, like the cytosolic Hsc70/Hsp40 complex and the ER luminal BiP [11–14]. Hsc70 binds to the CAD sequence of i-preS in a productive manner, as the modulation of its in vivo activity interferes with L topogenesis. Hyper-active Hsc70 suppresses preS posttranslocation, while hypo-active Hsc70 has opposite effects, with the consequences that the improper topological reorientation of L leads to defects in virus formation [12, 30]. RNA interference studies yielded similar results, since the knock-down of Hsc70 inhibits HBV replication [30]. Of note, however, Hsc70/Hsp70 not only regulates the proper folding of L, but also activates the hepadnaviral polymerase [31]. Hence, its manipulation may have pleiotropic effects in the viral life cycle. Within the ER, the BiP chaperone interacts with e-preS [12–14], likely to provide the driving force to pull on the preS chains into the ER lumen, until L has adopted its final dual topology. Translocational regulation of L topogenesis is further suggested by the identification of NACA (nascent polypeptide-associated complex alpha polypeptide) as a preS1-specific binding partner [32]. The heterodimeric NAC complex has been shown to bind several nascent polypeptide chains as they emerge from the ribosome, presumably to protect them from interactions with inappropriate cytosolic factors [33]. In the case of L, a polypeptide of about 200 aa (i.e., preS) must be synthesized until its TM1 signal-anchor sequence emerges from the ribosome. Its association with NACA may thus provide a protective environment, until preS is handed over to the Hsc70/Hsp40 complex. Another cellular protein specifically interacting with the i-preS form of L is γ2-adaptin, which was discovered in a yeast two-hybrid screen using the preS1 domain as bait [34]. γ2-Adaptin, however, does not assist in L topogenesis, but is involved in subsequent steps of virus maturation and egress [35, 36].

Apart from host proteins, cellular membrane lipids, such as cholesterol, also influence L topogenesis. A role for cholesterol in the HBV entry process has been well documented, but investigation of virus release from cells treated with cholesterol-lowering agents led to controversial results. By using lovastatin, an inhibitor of cholesterin and isoprenoid synthesis, Lin et al. observed no effect on virion secretion [37]. In contrast, a significant reduction of virion secretion was detected by Bremer et al. [38] upon treatment of HBV-producing cells with NB598, a drug that only targets the cholesterin pathway. To exclude unwanted drug effects, Dorobantu et al. [39] used a non-toxic approach to deplete cholesterol by preventing its cellular uptake. Under these conditions, HBV release was strongly decreased. Interestingly, the detailed analysis of distinct steps of virus maturation revealed that the loss of cholesterol enhances posttranslational preS translocation of L across the ER membrane, possibly due to a decrease in membrane rigidity [39]. As a consequence of this, less i-preS is available for capsid envelopment and hence virus production.

The topological isoforms of L play manifold roles in the viral life cycle. In the early steps of infection, the e-preS domain is functionally important for hepatocyte binding and intracellular uptake of virions via largely unknown receptors. Reverse genetic analyses have shown that the integrity of the first 77 preS1 aa of L including its N-terminal myristic acid is mandatory for HBV infectivity [40, 41]. The precise role of the myristic acid linked to Gly-2 of L is not defined yet, but is suggested to enhance the interaction between preS1 and the HBV receptor, presumably via insertion into the viral or target cell membrane [2]. Aside, the myristic acid has been shown to protect the virion against recognition by neutralizing antibodies and hence may contribute to viral immune evasion [42]. A complementary infectivity determinant was identified in the luminal loop of the S domain of L. Within this loop, distinct amino acids, in particular cysteine residues and the cysteine disulfide bonds network, proved to be essential for HBV uptake [43, 44]. In addition, the TM1 segment of the HBV envelope proteins is required for a productive infection. TM1 shares structural and functional properties with fusion peptides and is thus supposed to participate in the fusion step accompanying HBV uptake [44].

In the late stages of infection, the i-preS form of L is essential for virus assembly, establishing physical interactions of the viral envelope with preformed cytosolic nucleocapsids. Through mutational analyses of L, a short linear sequence that covers the 17 C-terminal residues of preS1, followed by the N-terminal 5 aa of preS2, was identified as a matrix domain for the interaction with the nucleocapsid [45]. A second region predicted as a capsid-binding site is present in the first cytosolic loop of the S domain. Although three independent genetic screens did not show entirely concordant results, they all reveal that small deletions and even point mutations in this loop

impair virus formation [46–48]. In vitro binding assays, performed with envelope-derived peptides and liver-derived capsids, demonstrated that both putative matrix domains can directly interact with the capsid [49]. The involvement of distinct envelope binding sites is further suggested by cryo-electron microscopy of virions, indicating core/envelope contacts in different insertion areas and different dimensions [50, 51]. Apart from its N-terminal 5 aa, the preS2 domain of i-preS does not directly participate in capsid binding, because it can be completely randomized without major effects on HBV assembly [52]. An extensive shortening of preS2, however, is not tolerated, implicating that it may serve as a spacer to ensure appropriate contacts between the envelope matrix domains and the capsid [52, 53].

The preS2 domain of the i-preS form of L has been also implicated in transcriptional transactivation, since it binds and activates the protein kinase C, thereby stimulating the c-Raf/MEK signaling cascade that controls a variety of promoters [54, 55]. Moreover, L has been assigned to regulate viral replication by controlling the amplification of the viral cccDNA genome [56, 57]. Ablation of L expression concomitant with a block in i-preS-mediated nucleocapsid envelopment led to an increase in the level of cccDNA [57], presumably because the nucleocapsids are shunted into the nucleus where the partially double-stranded DNA genome is converted to nuclear cccDNA.

The HBV core protein: structure, function, and host interaction partners

HBV contains a single type of capsid protein, also termed core protein, or HBc antigen, consisting of 183 or 185 aa residues, depending on genotype. Extensive mutational and structural analyses identified its first 140 aa to form the capsid shell [58–64]. This assembly domain is followed by a linker of about 10 aa, and by a basic nucleic acid-binding C-terminal domain (CTD) (Fig. 1c). Assembly of nucleocapsids within the cytosol is thought to initiate by binding of the core protein to a complex of the RT and pgRNA, cosequestering the enzyme and its template into the proper environment for reverse transcription [65–67]. The capsid shell is composed of either 180 or 240 core subunits with triangulation (T) numbers of 3 or 4, respectively. Both capsid species are found in infected cells, but the $T = 4$ capsids seem to be preferentially incorporated into virions [68]. Crystallographic and cryoelectron microscopy studies have elucidated the structure of capsid particles and show that core dimers align to form spikes on the surface of the particle, each composed of four long bundled α-helices connected by a loop [62–64, 68]. While the N-terminal part of the core protein is important for the dimerization and assembly process, its CTD is dispensable. Rather, by virtue of its high content of arginine residues, CTD binds nucleic acids and is hence essential for packaging of the pgRNA/RT complex [65–67].

Apart, the CTD of core harbors distinct nuclear localization signals (NLS) and nuclear export signals (NES) involved in nucleocytoplasmic shuttling of the core/capsid. Nuclear import of nucleocapsids is pivotal for the establishment and persistence of infection, as it allows the generation and amplification of the cccDNA transcriptional template, either from input or progeny HBV DNA genomes. Nuclear capsid entry depends on both capsid phosphorylation and genome maturation that are thought to lead to translocation of the NLS motifs to the exterior of the capsids [69, 70]. Such capsids can be recognized by nuclear transport receptors of the importin-β superfamily that mediate—in conjunction with the nuclear basket protein nucleoporin 153—their nuclear import and subsequent genome release [71]. Nuclear export of capsids in turn involves binding of the cellular factor TAP/NXF1 (Tip-associated protein/nuclear export factor-1) to the NES of core [70].

The CTD also contains three major sites of serine phosphorylation, which, when phosphorylated, partially neutralize the positive charges of the CTD and also induce conformational changes [72, 73]. Core protein phosphorylation correlates with pgRNA packing during capsid assembly, plus strand ssDNA synthesis during reverse transcription, intracellular transport of capsids, and their nuclear import. Dephosphorylation is suggested to accompany capsid maturation and correlates with subsequent envelopment and secretion [72–74]. Many cellular kinases have been reported to be associated with the capsids, including a 46-kDa kinase, a cdc2-like kinase, protein kinase Cα (PKCα), SRPK1, and SRPK2, but their precise actions in HBV replication remain to be clarified [75–78]. In case of PKCα, its inactivation did not affect genome maturation but resulted in capsid accumulation due to defects in capsid envelopment [79]. For SRPK1, a non-canonical chaperone function has been suggested, as the kinase gates capsid assembly [80].

The CTD-specific phosphorylation status is proposed to be part of the maturation signal ensuring the selective envelopment of mature nucleocapsids. Because virus budding does not occur before second-strand DNA (i.e., dsDNA) synthesis [81], a signal must be transmitted from the inside to the outside of the nucleocapsid. In support of this "maturation signal" hypothesis, the comparison of structural maps of RNA- and DNA-containing capsids revealed small but significant differences, distributed over much of the protein shell [68]. One prominent change concerns a hydrophobic pocket (Pro-5, Leu-60, Leu-95, Lys-96, and Ile-97) located at the junction between the

spike and the basis of the core dimer. In the DNA structure, this pocket appears to be more open as compared to RNA-containing capsids, possibly as a consequence of the contact force generated by a ds nucleic acid [68]. This force is thought to make the pocket accessible for contacts with the envelope proteins, as mutations of pocket-forming residues block nucleocapsid envelopment [60]. However, the discrimination between immature and mature capsids may not exclusively occur on the level of capsid–envelope protein interactions. Rather, host factors involved in late steps of HBV assembly (see below) may also contribute to this selection. For instance, the host γ2-adaptin protein associates with the capsid in a reaction depending on the hydrophobic pocket residue Lys-96 [35] whose amenability may be improved in mature, DNA-containing capsids. The cellular Nedd4 ubiquitin ligase is a further partner protein of core and preferentially binds to the PPAY late domain-like motif (aa 129-132) of DNA-containing capsids [35; R. Prange, unpublished observations]. NIRF (Np95/ICBP90-like ring finger protein) is yet another ubiquitin ligase interacting with the core protein [82]. However, unlike Nedd4, NIRF has been shown to control the lifespan of the core protein and to mediate its proteasomal degradation. A similar destabilization function toward the core protein has been assigned to the cytosolic Hsp40 chaperone [83]. Hsp40 is counteracted by the heat shock protein Hsp90 that has been reported to stabilize the capsid [84].

Assembly and release of empty envelope particles

The generation and secretion, and even the massive overproduction of subviral empty envelope particles (SVP), is a hallmark of hepadnaviral infections, which was among the first aspects of the viral life cycle to be discovered, but remains one of the least well-understood issues. Although several works have provided important information about distinct steps in the formation and secretion of SVPs, the basic mechanism underlying the capsid-independent self-assembly reaction is still unknown [4, 7, 15, 18, 85, 86]. Assembly and secretion of SVPs can be mimicked in many mammalian cell lines transfected exclusively with the S gene. Thus, the S protein alone is sufficient for mobilization of cellular lipids, self-assembly of approximately 96 molecules, intracellular transport, and secretion of these defined lipoprotein complexes (Fig. 2a). SVP assembly is initiated by integration of S in the ER membrane, where S monomers rapidly form disulfide-linked dimers, a process facilitated by the PDI chaperone [15]. Interestingly, recent ultrastructural analyses of cells producing only the S protein indicated that transmembrane dimers initially assemble in a filamentous form, resembling chain of beads. These filaments increase progressively in size and are transported via vesicles to the ER-Golgi intermediate compartment (ERGIC) where they are relaxed and converted into spheres [87, 88]. It was proposed that the absence of PDI from the ERGIC facilitates the conversion of filaments into spheres [15]. The budding process involves reorganization of host membrane lipids, as the SVPs do not contain a typical bilayer structure [8, 89]. The SVPs are then transported through the constitutive secretory pathway, thereby traversing the Golgi complex where high mannose carbohydrates, linked to Asn-146 of S, are processed (Fig. 2a) [7]. Since the secretion of SVPs depends on the action of the dynamin-2 GTPase, GTP hydrolysis is seemingly involved in particle budding and/or trafficking [90]. This is in contrast to virions that engage the catalytic activity of a cellular ATPase during budding [36, 91, 92].

Although the S protein is sufficient for SVP formation, authentic empty envelopes also contain the M and L proteins, albeit in different compositions. Extracellular SVPs have a diameter of about 20 nm and are organized as an octahedral sphere or as a filament with variable length [93–95]. The spherical particles are essentially composed of the S protein, but also contain variable amounts of M and trace quantities, if at all, of L molecules. The L protein is enriched in tubular filaments that are estimated to contain L, M, and S proteins in the ratio of 1:1:4 [96]. Cryo-EM studies and image classification of the tubular particles showed that the three proteins assemble as a mixed population of homo- and heterodimers, thereby being closely packed in the membrane with spike-like features projecting from the membrane [96]. Although both particle types are thought to play a role in allowing the virions to escape immune surveillance, their precise—or potentially distinct—biological functions are unclear. Noteworthy, however, the efficiency of virion production does not depend on SVP overproduction [97].

A multitude of mutational analyses led to the identification of S domain-specific amino acids that are critical for SVP assembly and export [47, 85, 86, 98–101]. Overall, the S protein turned out to be sensitive to deletions and even subtle point mutations, while it is more permissive for insertions. In particular, its N-terminal ectodomain tolerates large heterologous insertions derived from, for example, HIV, globin, or green fluorescent protein [85, 102, 103]. While former studies demonstrated an essential role of the TM segments in SVP biogenesis [85, 86], two recent reports showed that TM1 and TM3 of S could be replaced by foreign TM domains derived from human immunodeficiency virus (HIV) and hepatitis C virus envelope proteins [102, 104]. Hence, SVP formation appears to depend on a functional TM domain rather than on S-specific TM sequences. With regard to the flexibility of S to accommodate foreign membrane domains, it has been suggested to be a promising platform for vaccine development against a variety of enveloped viruses.

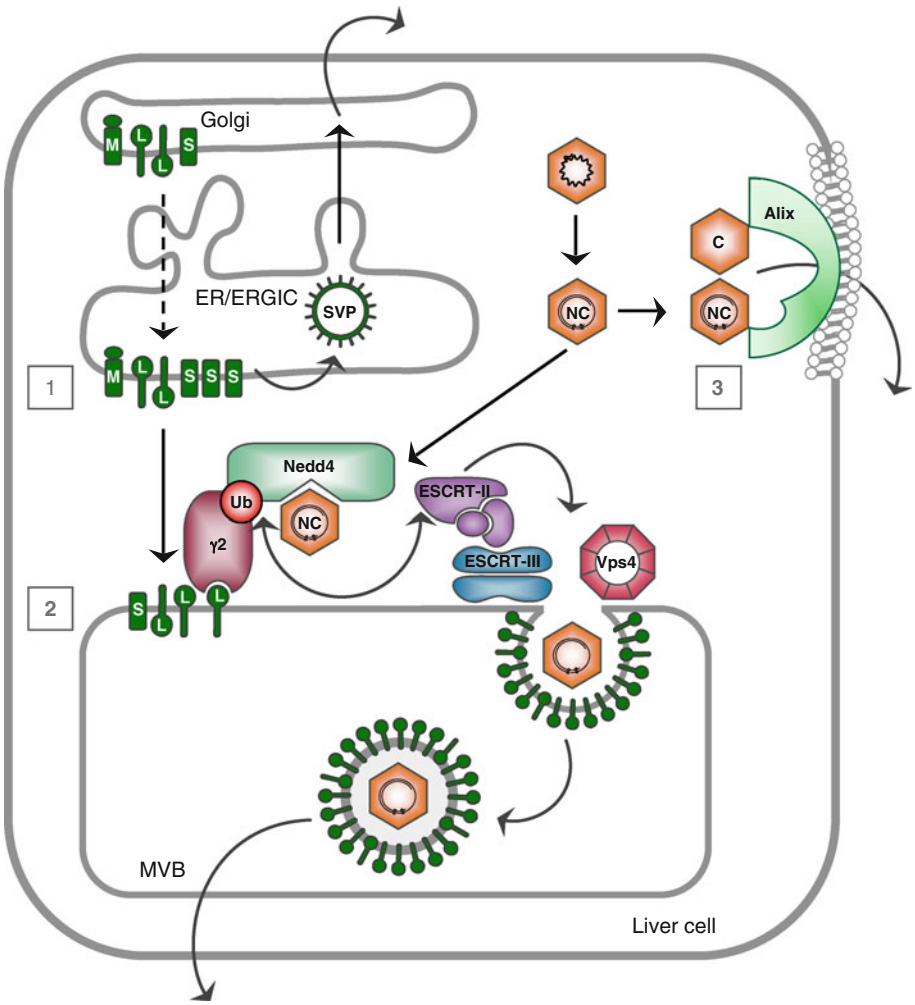

Fig. 2 Model for HBV viral and subviral particle morphogenesis. Pathways in budding and release of SVPs, virions, and naked capsids are indicated by, respectively, A, B, and C. **a** The S, M, and L envelope proteins are synthesized at the ER membrane. Following oligomerization of envelope chains, spherical SVPs bud into the lumen of the ER/ERGIC and are exported through the constitutive secretory pathway, thereby traversing the Golgi complex. **b** For virus assembly, the envelope proteins are transported to the budding site that may involve MVBs. Transport may be facilitated by γ2-adaptin (γ2) that interacts with the i-preS form of L. Viral nucleocapsids (NC) assemble and mature in the cytosol where they are recognized by Nedd4. Ubiquitinated (Ub) Nedd4 in turn is recognized by the ubiquitin-interacting motif of γ2-adaptin thereby bridging the viral substructures. The Ub moiety of the HBV/Nedd4/γ2-pre-assembly complex may then recruit the ubiquitin-binding ESCRT-II complex followed by the recruitment of ESCRT-III and Vps4 that mediate intraluminal budding and membrane fission of viral particles. MVBs and/or MVB-derived exosomes then fuse with the plasma membrane in order to release HBV. **c** Alternatively, pre-assembled capsids (C) and nucleocapsids (NC) can be recognized by Alix that shunts naked capsids into an ESCRT-independent exit route. The mechanism(s) underlying the non-vesicular exocytosis of capsids is unknown but may involve the membrane-deforming ability of Alix

Assembly and release of virions

For decades, it has been assumed that the budding of SVPs reflects the budding of HBV virions. However, in recent years, evidence is increasing that HBV subviral and viral particle assembly and release differ in their budding pathways and requirement of cell functions. Three independent reports have demonstrated that HBV budding and egress depend on functions of the so-called multivesicular body (MVB) pathway [36, 91, 92]. MVBs have the unique ability to generate intraluminal vesicles (ILV) that bud away from the cytosol, a process topologically equivalent to that of enveloped virus budding. Normally, cargo destined for either degradation, lysosomal functions, or exosomal release are sequestered into these inwardly budding vesicles. The MVBs then fuse with the lysosome or the plasma membrane for ILV delivery. The sorting of cargo into MVBs depends on the endosomal sorting complex required for transport (ESCRT) system that consists of the ESCRT-0, -I, -II, and –III complexes together with the Vps4 ATPase, and associated proteins. ESCRT-0, -I, and -II are soluble, contain ubiquitin-binding domains, and are

primarily involved in sorting of ubiquitinated cargo and in the recruitment and activation of ESCRT-III. Activated ESCRT-III appears to be the "core" of the machinery and is responsible for the budding and membrane fission events during ILV formation. Finally, specific ESCRT-III subunits recruit Vps4 that dissembles and releases the complexes upon ATP hydrolysis for recycling [105–107]. The ESCRT complexes and associated proteins have been found to also play roles in enveloped virus budding. Viruses access the MVB pathway through so-called late assembly domains of their structural proteins, which contain three alternative sequences, P(S/T)AP, LYPXL, and PPXY. P(S/T)AP late domains function as recruitment sites for Tsg101, the ubiquitin-binding subunit of ESCRT-I. The LYPXL motifs bind to Alix, an ESCRT-I and -III interaction partner, and the PPXY late domains bind HECT-domain ubiquitin ligases, like Nedd4 [106, 108, 109].

HBV co-opts downstream functions of the ESCRT machinery, as perturbations of ESCRT-III and Vps4 by overexpression of dominant-negative (DN) mutants significantly impair the release of virions [36, 91, 92]. In favor of these observations is a recent proteomic analysis demonstrating that Chmp4B, an ESCRT-III subunit, is significantly up-regulated in HBV-replicating stable cell lines [110]. How HBV gains access to the ESCRT pathway is less clear, but has been implicated to involve cellular proteins, like γ2-adaptin and Nedd4 (Fig. 2b). γ2-Adaptin is classified as a member of the clathrin adaptor protein family that guides vesicular transport processes [111]. However, unlike classic adaptins, γ2-adaptin possesses ubiquitin-binding properties [35]. By means of two-hybrid screening, γ2-adaptin was isolated as an L-specific binding partner that exclusively interacts with the i-preS isoform [34]. Notably, γ2-adaptin was shown to also associate with the HBV capsid in a reaction involving the Lys-96 residue of the core protein, a potential target for ubiquitination [35]. While this observation suggests an ubiquitin-dependent recognition pattern between γ2-adaptin and core, independent studies failed to demonstrate a direct ubiquitin modification of the capsid [35, 112]. The missing link may be provided by Nedd4 that is itself modified by ubiquitin and is able to bind to the late domain-like PPAY motif of core [35, 113]. Since ubiquitinated Nedd4 functionally interacts with γ2-adaptin [113], this reaction cycle may establish a linkage between the viral envelope and the capsid required for assembly. In support of this notion, the functional inactivation of γ2-adaptin and Nedd4 blocks the assembly and release of HBV [35, 36]. Although Nedd4 is not an intrinsic player of the MVB pathway, it is proposed to escort cargos to the ESCRT machinery [106, 108, 109]. Whether Nedd4 may perform a similar role during HBV maturation remains to be clarified. By using an RNA interference approach, my group recently observed that HBV also appropriates the ESCRT-II complex, composed of EAP20, EAP30, and EAP45 [J. Stieler and R. Prange, unpublished observations]. Since the EAP45 subunit contains an ubiquitin-binding module, the HBV/Nedd4/γ2-adaptin/ubiquitin pre-assembly complex may access the scission functions of ESCRT-III/VPS4 via ESCRT-II (Fig. 2b).

An open question that also remains to be answered concerns the HBV budding site. In regard to various immunofluorescence studies, the viral proteins are barely detectable at the plasma membrane, thus favouring intracellular membranes used by HBV for budding. Indeed, EM studies of HBV-infected cells and liver biopsies from HBV-infected patients have shown that the virus assembles in large intracellular compartments that may resemble late endosomes or MVB [114, 115]. Spherical SVPs were also observed inside cytoplasmic vesicles that, however, hardly colocalize with the virus-containing cellular compartment [87], likely as a consequence of different assembly paths. The ESCRT dependency of HBV is another clue for MVBs serving as HBV budding sites. In this context, it should be noted, however, that many ESCRT-dependent viruses, like retroviruses and herpes viruses, do not bud into MVBs, but rather recruit the ESCRT network to their specific budding membranes [106, 108, 109, 116]. If HBV would bud into MVBs and exit the cell by the exosome pathway, the envelope proteins have to traffic from of ER/Golgi complex to the endosomal system via yet uncharacterized transport routes. Consistent with known functions of adaptor protein complex subunits that sort their specific cargo proteins to membrane compartments, γ2-adaptin may contribute to HBV envelope protein trafficking [111]. Recent reports have shown that HBV is able to induce cellular autophagic processes, concomitant with a subversion of the autophagy machinery for virus envelopment [17]. Besides the viral X protein, the S envelope protein was identified as a potent autophagy inducer and found in close association with LC3, an autophagosomal membrane protein [17, 117]. Thus, it seems also conceivable that the HBV envelope proteins may use autophagic pathways to travel to the endosomal system.

Of note, neither of the host factors required for HBV budding (i.e., γ2-adaptin, Nedd4, ESCRT-II, ESCRT-III, Vps4) is needed for the assembly and secretion of spherical SVPs composed exclusively of the S protein [35, 36, 92]. The employment of ESCRT pathway functions in viral but not subviral particle budding may account for the phenomenon why an experimental coinfection of hepatic cell lines with HBV and HIV increases the pool of intracellular hepadnaviral SVPs [118]. Because both HBV and HIV are ESCRT-dependent viruses, they may compete for the MVB system in coinfected cells with the consequence that more HBV envelope proteins may be shunted into the SVP assembly pathway.

Assembly and release of naked capsids

In addition to infectious particles, HBV-replicating cell lines release non-enveloped (nucleo)capsids, but their functional implication and pathways of release are less clear [61, 74, 79, 92, 119]. In a recent study, my group could show that the sole expression of the core protein is sufficient for capsid release [120]. Unlike retroviral Gag-particles, the released HBV capsids are devoid of a detectable membrane bilayer, implicating a non-vesicular exocytosis process. In contrast to HBV viral particles, naked capsid budding does not require ESCRT functions but rather depends on cellular Alix (apotosis-linked gene 2 (ALG-2)-interacting protein), a multifunctional protein with key roles in membrane biology (Fig. 2c) [105, 120, 121]. Conversely, HBV budding does not need Alix. It can be blocked, however, by DN Alix mutants because these variants are able to globally disrupt the ESCRT cascade [92]. The different use of cell functions by HBV viral and capsid particles is puzzling and poses the question how the virus may orchestrate these pathways. One critical determinant assigning the budding pathway may be the stage of capsid maturation, as only mature nucleocapsids containing the dsDNA genome can be enveloped [81]. As outlined above, the degree of capsid maturation is supposed to be signed by the phosphorylation and conformation state of the capsid. Accordingly, mature nucleocapsids may gain access to ESCRT-dependent HBV budding either directly, via capsid–envelope interactions, or indirectly with the help of cellular factors, including γ2-adaptin and Nedd4. Immature capsids may fail to enter this pathway and may rather be recognized by Alix in order to be shunted into an ESCRT-independent exit route. Mapping analyses of Alix domains responsible for the capsid release-stimulating activity identified its boomerang-shaped Bro1 domain to be required and sufficient [120]. By binding to membranes with its convex surface, the Bro1 domain may contribute directly to the generation of negative curvature required for non-lytic capsid budding away from the cytosol (Fig. 2b) [121, 122]. The precise mechanistic action of Bro1/Alix, however, remains to be determined. Whether the release of naked capsids may be beneficial for the infected host or the virus or both also remains to be addressed. Naked capsids are hardly found in the blood of infected patients or chimpanzees [123]. Noteworthy, however, the capsid is extremely immunogenic, and high titers of anti-capsid antibodies are produced in virtually all patients who have been exposed to HBV [124]. The Alix-assisted extracellular export of naked capsids may be one explanation for this hitherto less understood phenomenon. For HBV, the release of naked nucleocapsids may be instrumental in spreading infection, if they approve to be replication-competent upon cellular uptake.

Concluding remarks

The studies reviewed above have significantly enhanced our understanding of some of the cell biological aspects of HBV assembly and release. However, there are a number of significant questions that arise from these findings, as well as additional questions that have proved to be somewhat intractable thus far. For example, we do not unambiguously know which cellular membranes are selected as platforms for virus particle assembly and how this occurs. How does the budded virion then exit the cells? Despite there is compelling evidence for a signal selectively present in mature nucleocapsids, its precise nature is still unclear. If it involves dynamic modification of the nucleocapsid, the identification of the responsible cellular phosphatase would be meaningful. What features of the capsid determine its entry into the ESCRT-dependent virus particle or ESCRT-independent capsid particle assembly pathway? Another poorly understood aspect of HBV biology is how capsids can be extracellularly released in a "naked" state. Finally, although being used as an effective HBV vaccine for more than 30 years, the genesis of SVPs is not thoroughly disclosed, as we do not yet know the underlying mechanism of this budding reaction. Achieving a nearly complete understanding of the assembly, budding, and release of the distinct HBV particle types will be not only fascinating for scientists, but also invaluable for future antiviral strategies.

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