

# **Morphogenesis of hepatitis B virus and its subviral envelope particles**

Romuald Patient , Christophe Hourieux , Philippe Roingeard \*

*Morphogénèse et antigénicité du VIH et des virus des hépatites INSERM : U966 , Université François Rabelais - Tours , FR*

\* Correspondence should be addressed to: Philippe Roingeard <roingeard@med.univ-tours.fr >

## **Abstract**

### **Summary**

After cell hijacking and intracellular amplification, nonlytic enveloped viruses are usually released from the infected cell by budding across internal membranes or through the plasma membrane. The enveloped human hepatitis B virus (HBV) is an example of virus using an intracellular compartment to form new virions. Four decades after its discovery, HBV is still the primary cause of death by cancer due to a viral infection worldwide. Despite numerous studies on HBV genome replication little is known about its morphogenesis process. In addition to viral neogenesis, the HBV envelope proteins have the capability without any other viral component to form empty subviral envelope particles (SVP) which are secreted into the blood of infected patients. A better knowledge of this process may be critical for future antiviral strategies. Previous studies have speculated that the morphogenesis of HBV and its SVP occur through the same mechanisms. However recent data clearly suggest that two different processes, including constitutive Golgi pathway or cellular machinery that generates internal vesicles of multivesicular bodies (MVB), independently form these two viral entities.

**MESH Keywords** Cell Membrane ; virology ; Endoplasmic Reticulum ; virology ; Hepatitis B virus ; physiology ; Host-Pathogen Interactions ; Humans ; Models, Biological ; Virion ; ultrastructure ; Virosomes ; biosynthesis ; Virus Assembly ; Virus Release

**Author Keywords** viral morphogenesis, viral assembly, HBV, subviral particles

## **Introduction**

The human hepatitis B virus (HBV) is a small hepatotropic and highly infectious DNA virus member of the Hepadnaviridae family. HBV represents the prototype virus of the Orthohepadnavirus genus. Discovered almost forty years ago (Dane et al. , 1970 ; Blumberg et al. , 1965 ), HBV still remains a major health problem worldwide, as there is no widely available treatment for the estimated 350 million chronic carriers who have a high risk of liver cirrhosis or cancer. Despite the existence of an effective vaccine designed more than twenty years ago, currently almost 1 million people per year die due to HBV infection (Perz et al. , 2006 ). A thorough understanding of HBV structure and morphogenesis is thus promptly required for the development of innovative antiviral treatment.

The infectious HBV virion (or Dane particle) is a spherical particle, 42 nm in diameter, consisting of an icosahedral capsid about 30 nm in diameter. The virion contains the circular partially double-strand genomic DNA about 3.2 kb in length which is covalently linked to the viral reverse-transcriptase. This nucleocapsid is surrounded by a lipid bilayer in which the three envelope proteins (small [S], medium [M] and large [L]) are anchored as transmembrane proteins playing a major role in HBV morphogenesis and infectivity (Bruss, 2007 ). After being delivered into the nucleus (Rabe et al. , 2003 ), the viral DNA is converted to a covalently closed circular DNA (cccDNA) which is transcribed in: (i) pregenomic RNA (pgRNA) about 3.5 kb in length and (ii) different subgenomic RNAs encoding all the viral proteins necessary for the HBV replication cycle (Beck and Nassal, 2007 ). In the cytosol, a single molecule of pgRNA together with the viral reverse transcriptase is incorporated into an assembling capsid. Once the pgRNA is encapsidated, reverse-transcription into a new genomic DNA occurs (Beck and Nassal, 2007 ). The mature nucleocapsid is then enveloped by the surface proteins and a new generation of infectious virions can be released (Bruss, 2007 ). Interestingly, the production of the HBV surface proteins also leads to the intracellular budding of empty subviral spherical or filamentous envelope particles (SVP). SVP are 20 nm in diameter, lack the nucleocapsid and are secreted in great excess over virions (Ganem, 1991 ; Heermann et al. , 1984 ). It has been clearly shown that this natural phenomenon specific to HBV is linked to the ability of the S envelope protein to spontaneously form empty envelope particles at the rough endoplasmic reticulum (ER) (Dubois et al. , 1980 ). This noninfectious material is currently the basis of most vaccines against hepatitis B (Eddleston, 1990 ). Unfortunately, despite in vitro production of virions using HBV DNA transfection of Huh-7 or HepG2 cells (Yaginuma et al. , 1987 ; Sureau et al. , 1986 ) or HBV infection of the recently developed HepaRG cell line (Gripon et al. , 2002 ), HBV morphogenesis remains highly difficult to observe by electron microscopy (EM). This may be due to the low rate of HBV production approaching 1 to 10 viruses per hepatocyte per day in vivo (Nowak et al. , 1996 ). Thus, the models of HBV and its subviral envelope particles morphogenesis are based on biochemical approaches (Huovila et al. , 1992 ) and lack ultrastructural data that may be helpful for a more complete understanding of these mechanisms, as for other viral models (Roingeard, 2008 ). In this review, we focus on data that have recently been accumulated on both HBV and SVP morphogenesis and illustrate our discussion with new and original EM observations.

## **HBV capsid**

The 22 kD HBV core protein (also called C or Hbc) consists of 183 or 185 amino acid (aa) residues depending on the genotype and is relatively well-conserved among HBV isolates (Chain and Myers, 2005). In the cytosol, the fundamental building unit of the HBV capsid is formed by homodimerization of two core proteins stabilized by a disulfide bridge between their respective Cys-61 residues (Zheng et al., 1992; Zhou and Standring, 1992). Then, dimer oligomerization occurs by unclear cellular processes and leads to the formation of two different types of capsids: (i) a capsid consisting of 90 dimers associated in an icosahedral symmetry ( $T = 3$ ) about 30 nm in diameter and (ii) a larger capsid consisting of 120 dimers associated in an icosahedral symmetry ( $T = 4$ ) about 34 nm in diameter (Wynne et al., 1999). Both capsids are found in infected cells but the  $T = 4$  capsids seem to be preferentially selected for envelopment in infectious particles (Roseman et al., 2005). However  $T = 3$  capsids have also been reported to be incorporated in virions in various percentages (Dryden et al., 2006; Roseman et al., 2005). While the N-terminus of the capsid protein is important for the dimerization process, the basic C-terminal domain contains a nuclear localization sequence (NLS) and an Arg-enriched area. Formed by the last 34 aa, this basic area is probably involved in the pgRNA/reverse-transcriptase complex encapsidation (Nassal, 1992; Gallina et al., 1989). Finally, the HBV core protein structure was solved by crystallization at a resolution of 3.3 Å (Wynne et al., 1999). In a recent review, Bruss described the HBV core dimer as a structure resembling an upside down "T"; with the horizontal bar mediating the inter-dimer association (Bruss, 2007). The perpendicular domain to this bar consists of a spike formed by 4  $\alpha$ -helices, 1  $\alpha$ -helical hairpin for each protein, protruding outwards from the center of the dimer basis (Wynne et al., 1999). The sequence from residue 78 to residue 83, located on the top of the spike, is known as the major epitope of the capsid antigen (HBcAg). Associations of 5 or 6 dimers are arranged around the 12 axes with a five-fold or two-fold (or quasi six-fold) symmetry, respectively. Finally, analysis by cryo-electron microscopy (cryo-EM) showed that the capsid was not continuous but opened by the presence of pores about 12 to 15 Å in diameter (Wynne et al., 1999). It was suggested that these pores are essential to enable the entry of deoxyribonucleotides during the genomic DNA synthesis and/or the expulsion of ribonucleotides after the degradation of the pgRNA by the RNaseH activity of the reverse transcriptase.

As described above, all stages of pgRNA reverse-transcription occur in the nucleocapsid. However, only virions containing a mature circular partially double-strand genomic DNA are secreted into the extracellular environment (Weiser et al., 1983; Mason et al., 1982). Actually, it has been suggested that the presence of the pgRNA could interfere with the incorporation of the nucleocapsid in the virion. The synthesis of genomic DNA would be required and associated with structural changes in the capsid structure leading to a mature nucleocapsid formation capable of being wrapped by the viral envelope (Summers and Mason, 1982). Recently, Roseman et al were able to support this hypothesis by demonstrating a structural difference between a pgRNA-containing capsid and a genomic DNA-containing capsid. The difference is related to the contact force generated by a double-strand nucleic acid on a hydrophobic pocket located at the junction between the spike and the basis of the dimer (Roseman et al., 2005). This pressure would make the pocket accessible to the HBV surface proteins in order to proceed to the nucleocapsid/envelope interaction. Interestingly, the natural mutation I97L found in the "adr" HBV subtype leads to the early envelopment of an immature single-strand DNA-containing nucleocapsid which can be corrected by the P130T point mutation (Yuan and Shih, 2000). In addition, the high infectivity of HBV could be explained by the existence of a quasi-control and/or a strict selection for mature nucleocapsid envelopment and new infectious virions secretion. As a typical example, the duck hepatitis B virus (DHBV, genus Avihepadnavirus) production rate is about 1 infectious virion per physical particle secreted (Jilbert et al., 1996).

## HBV envelope

The three envelope proteins S, M and L are encoded by a single open reading frame referred as ORF-E, 389 or 400 codons in length depending on the "ay" or "ad" viral subtype, respectively. This ORF consists of three 5' in-phase ATG codons for the initiation of translation and one 3' TAA termination codon. Thus, the three HBV envelope proteins only differ by the length of their N-terminal domains. These envelope proteins are present in different proportions in the three types of HBV-related particles: while the S protein represents the major component of the viral envelope, the L protein is only found in the virion and the filamentous SVP (Heermann et al., 1984). The M distribution seems to be the same in all particles.

The 226 aa S protein is a typical membrane protein with a relatively complex topology and is produced at the rough endoplasmic reticulum (ER) (Bruss, 2007). Computer modeling of its secondary structure suggests that three hydrophobic integral domains are separated by two hydrophilic loops. The S protein is translocated at the ER compartment by its N-terminal first transmembrane domain (TM-I) located between residues 4 to 24. The fact that this sequence is not cleaved by any of the cellular signal peptidases allows its co-translational translocation through the ER membrane in order to direct the N-terminus of the protein into the ER lumen. Downstream of this TM-I domain is a cytosolic loop (CYL-1) followed by a second transmembrane domain (TM-II) located between residues 80 to 100 which acts as a type II signal/anchor domain. The TM-II C-terminal end directs a luminal loop known as the antigenic loop (AGL) containing a single N-glycosylation site at Asn-146 (Peterson et al., 1982). The prediction models for the third C-terminal hydrophobic domain show that this region is actually composed of two transmembrane  $\alpha$ -helices TM-III and IV, composed of residues 173 to 193 and 202 to 222 respectively that are separated by a short cytosolic sequence (CYL-2) (Persson and Argos, 1994). The arrangement of these two helices in the ER membrane is probably responsible for the translocation of the C-terminus of S into the ER lumen (Sonveaux et al., 1994). Since S contains fourteen Cys residues some of which are directly involved in disulfide bridge formation and/or particle secretion,

it is clear that the acquisition of a functional structure must be extremely regulated (Mangold and Streeck, 1993 ). Therefore, as with many other transmembrane proteins, the co and post-translational modifications of S are highly monitored by ER chaperones such as the protein disulfide isomerase (PDI) (Helenius and Aebi, 2001 ; Huovila et al. , 1992 ). Several other chaperones are likely involved in this control but still remain undefined. The fact that only half of the small envelope proteins are found N-glycosylated in the particles clearly emphasizes the complexity of its maturation (Peterson et al. , 1982 ). The S protein is an unusual viral surface protein in the sense that it is the necessary and sufficient unit for the formation and the spontaneous budding of HBV subviral envelope particles (SVP), in the absence of any other viral factor. Indeed, *in vitro* production of S alone results in the secretion of empty and non-infectious spherical SVP which are currently industrially produced to generate highly efficient and safe vaccines against HBV (Eddleston, 1990 ; Dubois et al. , 1980 ). Additionally, it is widely accepted that the oligomerization of several S proteins is required to initiate the SVP morphogenesis (Huovila et al. , 1992 ). The exclusion of any other cellular proteins and even envelope proteins from avian hepadnaviruses during this process suggests a specific control of the SVP formation and composition (Gerhardt and Bruss, 1995 ; Stübbe and Gerlich, 1982 ).

The biosynthesis of the 281 aa M protein is quite similar to S. This protein differs from S by the N-terminal addition of a 55 residue sequence named “preS2” which is co-translationally translocated into the ER lumen by the TM-I signal sequence (Eble et al. , 1990 ). In addition to the Asn-201 (Asn-146 on the S protein), the preS2 domain contains an N-glycosylation site at Asn-4 together with an optional O-glycosylation site at Thr-37 depending on the genotype (Tolle et al. , 1998 ; Heermann et al. , 1984 ). Interestingly, a strict correlation between glycan-dependent chaperone binding and secretion of M indicates that ER proteins such as calnexin promote folding and trafficking of M allowing particle secretion (Werr and Prange, 1998 ). However, the exact role of M in the viral cycle remains unclear. Indeed, its *in vivo* absence in infected cells does not disrupt viral morphogenesis or particle functionality (Fernholz et al. , 1991 ). Thereby, this protein seems not to be essential for virus spread and its complete absence from the Avihepadnavirus genomes reinforces this hypothesis.

The L protein is characterized by the addition of a 108 to 119 residue sequence named “preS1” on the N-terminus of M. Thus, this large protein basically incorporates the preS domain (preS1 and preS2) to the N-terminus of S. Although this has not been demonstrated experimentally, the N-terminus of preS1 is probably anchored on the ER surface by a fourteen carbon saturated fatty acid added by myristylation at Gly-2 (Persing et al. , 1987 ). The kinetics of this biochemical modification and its exact role for the virus life cycle are still unclear but cellular enzymes such as the cytosolic N-myristyl transferase are probably involved in this process. Contrary to the M protein, the L protein is only N-glycosylated at Asn located in the S domain (Bruss, 2007 ). Two other potential sites of N-glycosylation are also present at Asn-15 of the preS1 domain and Asn-4 of the preS2 domain but are not glycosylated because of their cytosolic localization (Heermann et al. , 1984 ). Indeed, it seems that initially the N-terminus of L is retained in the cytosol as an internal preS domain in a topology called “i-preS” (Bruss, 2007 ). The translocation of the preS domain into the ER lumen occurs in an obscure but probably post-translational mechanism leading to an external topology of L called “e-preS”. This dual orientation of L is physically linked to the ER membrane, suggesting the involvement of host-cell transmembrane transport machinery. However, an interesting computer-aided analysis suggests that oligomerized S domains could form a channel in the ER membrane for the preS transport (Berting et al. , 1995 ). In parallel, some studies led to the identification of a topogenic element mapped to a preS1 C-terminal sequence located between residues 70 and 94, termed cytosolic anchorage determinant (CAD). CAD interacts with the cognate heat-shock protein Hsc-70 preventing preS co-translational translocation into an “e-preS” topology and glycosylation of the Asn-15 and Asn-4 of the preS1 and preS2 domains, respectively (Lambert and Prange, 2003 ; Löffler-Mary et al. , 1997 ). Luminal chaperones binding to the e-preS domain such as the binding protein BiP could support this process (Cho et al. , 2003 ). Currently, it is clearly recognized that both “i-preS” and “e-preS” topologies of L play a specific role in the viral cycle: recruitment of a mature viral nucleocapsid for virion budding (Bruss, 2007 ) and recognition of the cellular (co-)receptor(s) for virus entry (Glebe and Urban, 2007 ), respectively.

## SVP morphogenesis

As described above, the HBV surface proteins are not only incorporated into the virion envelope but also bud in an empty and non-infectious subviral envelope particle (SVP). The SVP conformation is organized as an octahedral sphere about 20 nm in diameter containing 48 S-dimer subunits or as a filament with the same diameter but with variable length (Gilbert et al. , 2005 ). This variation in the particle topology seems to greatly depend on the L protein quantity and on the S/L ratio incorporated into the particle during the morphogenesis process. Thus, since the spherical SVP are essentially composed by the S protein, the filament organization is greatly dependent on the co-assembly of a large proportion of L protein together with S (Heermann et al. , 1984 ). During the infection, it is currently assumed that these SVP act as decoys, trapping the host's immune system to protect the infectious virus. However, a high proportion of L protein tends to retain the particles inside the infected cell because of specific retention motifs (Persing et al. , 1986 ). Therefore, the inability of infected cells to export long HBV filamentous particles through the cellular secretion pathway seems to be at the origin of a direct cytopathic effect described as an inclusive cellular vesicle called “ground-glass” structure (Roingard and Sureau, 1998 ).

How the SVP formation occurs in the cell is still highly debated, but recent ultrastructural observations of cells producing the small S protein alone have provided a better understanding of this particular question (Patient et al. , 2007 ). The structural unit of the SVP is a block composed by a strongly associated dimer of S protein (Gilbert et al. , 2005 ), and this dimer is probably stabilized by disulfide

bridges which are generated under the control of the ER chaperone PDI (Huovila et al. , 1992 ). The S protein self-assembles initially in a filamentous form within the perinuclear space which is part of the ER of the cell (Fig 1A-a ). These filaments are then clustered and packed into crystal-like structures and transferred by ER-derived vesicles to the rough ER-Golgi intermediate compartment (ERGIC) (Fig 1A-b ). Unfortunately, the mechanisms and the cellular factors favoring all these events are not yet understood. In higher eukaryotes, transport from the ER is initiated by a COP-II-mediated budding of vesicular carriers through a specialized subdomain of the ER named ER-exit sites (ERES) (Appenzeller-Herzog and Hauri, 2006 ). Theoretically the directionality of vesicle transit from one cellular compartment to another is dependent on a motor/cytoskeletal network including microtubule-plus-end-directed kinesin and/or microtubule-minus-end-directed dynein. However, as ERGIC clusters are close to but clearly distinct from ERES, ER to ERGIC transports are known to be microtubule-independent. The ER-derived vesicles containing the S filaments are then transported to the ERGIC where the membrane docking is probably mediated by tethering proteins such as p115, a Rab1 effector, and followed by a SNARE-mediated membrane fusion. Once unleashed into the ERGIC lumen, the filaments are unpacked and relaxed possibly due to a different cellular milieu and/or association with new chaperones (Fig 1A-c ), before being converted into spherical particles for secretion (Fig 1A-d ). This hypothesis is supported by the observation that S secreted particles produced in a transgenic mouse model show a significant heterogeneity in size, a phenomenon that may be linked to the dissociation of filamentous particles (Gilbert et al. , 2005 ). Despite the lack of information indicating a specific release pathway of spherical SVP from the ERGIC, the conversion of the N-linked glycans on the S protein into complex structures before exocytosis provides arguments for the export of the particles using a constitutive cellular secretory pathway through the Golgi apparatus (Patzner et al. , 1986 ). In all cases, COP-I and the Arf family of small GTPases may be involved.

While our understanding of the SVP formation and traffic has progressed, the early mechanisms of their assembly remain unclear. To illustrate this review, we have re-examined cells producing the HBV S protein by EM, paying particular attention to the perinuclear space, to identify the events that precede the budding of vesicles containing packed filaments (Fig 1B ). This observation suggests that the filaments increase progressively in size and number and initially induce a slight dilatation of the perinuclear space (Fig 1B-a to 1B-d ) before being clustered and packaged to initiate the budding of the subsequent transport vesicle. To date, no direct extrusion of the filaments from the membrane of the nuclear envelope has been observed, and intraluminal free ends were clearly identified for some filaments (arrow in Fig 1B-d ). This suggests that these particles self-assemble inside the perinuclear space by a process quite distinct from lipid bilayer vesicle budding. This is supported by biochemical analyses that have shown that lipids which are only 25% by weight in the SVP have a highly restricted composition and are not organized in a typical bilayer configuration, but are instead located on the surface of the particle (Satoh et al. , 2000 ; Gavilanes et al. , 1982 ). The absence of lipid bilayer in the S SVP was also recently confirmed by cryo-EM analysis (Gilbert et al. , 2005 ).

## HBV morphogenesis

Unlike some Lentivirinae able to bud through a lipid bilayer without any envelope protein (Hourieux et al. , 2000 ), the HBV nucleocapsid budding is strictly dependent on the large L protein. The absence of L or its *in vitro* production under an exclusive “e-preS” topology completely abolish the generation of new virions (Bruss and Vieluf, 1995 ). Indeed, the “i-preS” topology of L is obligatory for mature HBV nucleocapsid recruitment. A sequence overlapping the 17 C-terminal residues of the preS1 domain and the 5 N-terminal residues of the preS2 domain seems to be involved in this process (Bruss, 2007 ; Poisson et al. , 1997 ). Moreover, in order to take advantage of all the conditions in the cell, when the ratio between the “i-preS” L protein and the nucleocapsid is not optimal, the genomic viral DNA is preferentially recycled to the nucleus to amplify the cccDNA stock (Summers et al. , 1990 ). When an HBV virion is finally assembled, cryo-EM analyses show that the spike of the core dimer does not actually penetrate the lipid envelope but stays in close apposition by unclear biophysical links (Seitz et al. , 2007 ; Dryden et al. , 2006 ). A second point of interaction between 11 aa residues located at the basis of the core dimer and the preS domain of L also plays a role in particle stability (Ponsel and Bruss, 2003 ).

It has been generally presumed that the budding of SVP reflect the budding of HBV virions. However, although HBV is an enveloped DNA virus, there is growing evidence that HBV maturation and egress depend, as for many enveloped RNA viruses including Retroviridae or Filoviridae , on intraluminal vesicles of maturing endosomes known as multivesicular bodies (MVB) (Fig 2 ) (Lambert et al. , 2007 ; Watanabe et al. , 2007 ; Kian Chua et al. , 2006 ). Usually, MVB are involved in the sorting of misfolded and ubiquitinated proteins for their degradation (Slagsvold et al. , 2006 for review). The proteins driving this machinery are grouped into at least three oligomeric endosomal sorting complexes required for transport ESCRT-I, -II and -III. These complexes are recruited and sequentially assembled to the cytosolic side of a late endosome membrane to generate MVB by multiple invaginations. Once the protein sorting is terminated, the disassembly of these multimers requires an ATPase associated with diverse cellular activities (AAA-type ATPase) named Vps4 (isoforms A or B) in humans. It has been previously shown that the ubiquitin-interacting adaptor  $\gamma$ 2-adaptin and the Nedd4 ubiquitin ligase interact with the HBV core and L envelope proteins, possibly to regulate transport of the viral structures through the budding site ( Rost et al. , 2006 ; Hartmann-Stuhler and Prange, 2001 ). Recently, several studies have demonstrated that the HBV core and envelope proteins co-localize with Vps4A/B and Alix/AIP1 (a functional tie between ESCRT-I and ESCRT-III in mammalian cells) and that dominant negative mutants of these host proteins inhibit virus release (Lambert et al. , 2007 ; Watanabe et al. , 2007 ). Thus, HBV virions would bud into late endosomes or MVB by utilizing ESCRT/Vps4B functions and exit the cell by the exosome pathway, as is the case for

human immunodeficiency virus type-1 (HIV-1) in macrophages and human T-lymphoblastic cell lines (Grigorov et al. , 2006 ). This hypothesis is also supported by the presence of a PPAY sequence (residues 129 to 132) in the HBV core protein related to the tetrapeptide late-budding (L) domain PPxY (also PTAP or YPDL) found in retrovirus core proteins and involved in viral release through the MVB pathway (Slagsvold et al. , 2006 ; Wynne et al. , 1999 ). How ESCRT mediates viral budding is still unclear.

## HBV and SVP assemble in different compartments

Recent studies on HBV morphogenesis have pointed out that the mechanisms of the SVP assembly are clearly distinct from those of the enveloped HBV virions and independent of the MVB functions. The  $\gamma$ 2-adaptin, which strongly interacts with the HBV L protein does not recognize the HBV small (S) protein, the main component of the SVP (Rost et al. , 2006 ). Dominant negative mutants of Alix/AIP1 and/or Vps4A/B have no effect on the release of SVP (Lambert et al. , 2007 ; Watanabe et al. , 2007 ). Thus, the HBV virion and SVP assembly pathways differ in their requirement for cell functions and trafficking routes. Past EM studies of HBV-infected cells have documented that the virus assembles in large intracellular compartments (Roingard, 2003 ; Roingard and Sureau, 1998 ). Regarding the recent advances in understanding HBV morphogenesis, it appears that these large compartments were probably late endosomes or MVB. However, long subviral filaments appeared also to be formed by a tubular budding at the membrane of the same compartments (Roingard, 2003 ; Roingard and Sureau, 1998 ). Actually, it should be noted that these filaments were enriched in L protein. As the L protein interacts with the  $\gamma$ 2-adaptin, it is probable that L-rich subviral filaments may assemble into MVB-related compartments by a conventional membrane-derived budding and by mechanisms quite distinct from those involved in the ER lumen for the HBV S filaments.

## Conclusion

Contrary to the preexisting concept, HBV virion and subviral particle morphogenesis and traffic seem to involve specific cellular partners and specific secretion pathways. EM data obtained on cells producing the HBV S protein alone support the hypothesis that MVB functions required for HBV budding are not involved in the release of the SVP. The fact that these different particles accomplish their assembly in seemingly disparate ways is intriguing. The identity, recruitment, and mechanism of action of host factors involved in the various means of HBV and SVP assembly will be a challenge for future works. Technological advancements in mass spectrometry and large-scale proteome mapping will help to identify new interactions between viral and cellular proteins. Visualization of viral particles budding using real time high-resolution single-particle light microscopy and by using cryoelectron tomography and correlative electron microscopy techniques (Roingard, 2008 ) may also shed light on these processes. As current therapy against HBV involves drugs that induce numerous unwanted side effects and may ultimately be rendered ineffective by drug resistant mutants, a better knowledge of these mechanisms might be a key factor in the development of more effective and better tolerated new anti-viral treatments.

## Acknowledgements:

We apologize to all authors whose work could not be cited due to space restrictions. We are grateful to Dr. Shauna Clark (Liver Diseases Branch, NIDDK, National Institutes of Health, USA) for critical reading of the manuscript. We thank the RIO/IBiSA Electron Microscopy Facility of François Rabelais University for generating EM data.

## References:

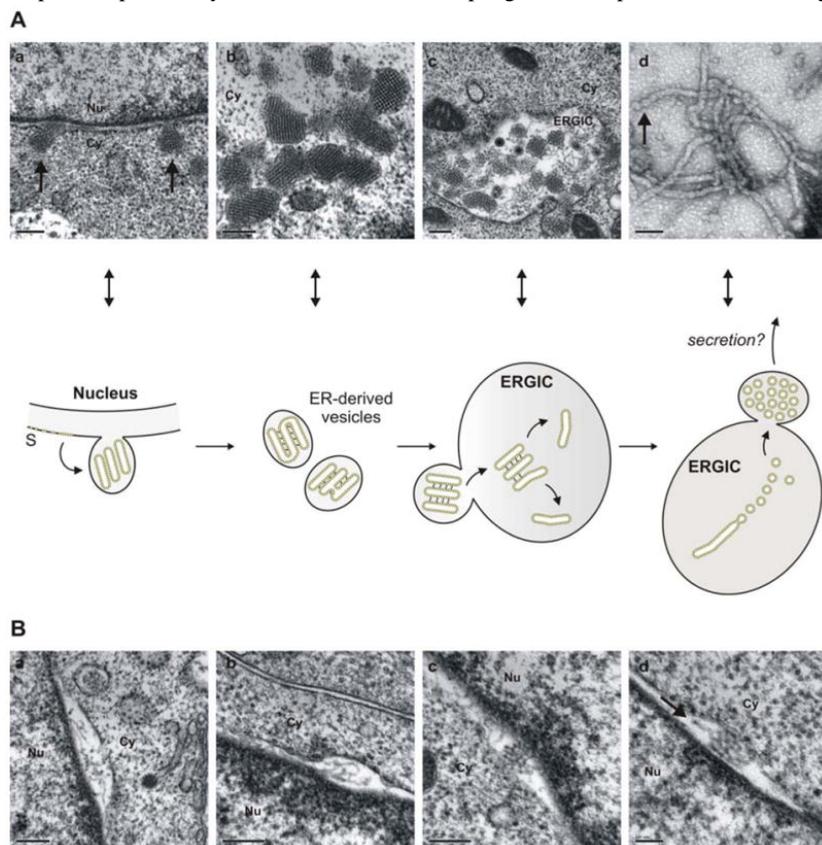
- Appenzeller-Herzog C , Hauri HP . 2006 ; The ER-Golgi intermediate compartment (ERGIC): in search of its identity and function . *J Cell Sci* . 119 : 2173 - 2183
- Beck J , Nassal M . 2007 ; Hepatitis B virus replication . *World J Gastroenterol* . 13 : 48 - 64
- Berting A , Hahnen J , Kroger M , Gerlich WH . 1995 ; Computer-aided studies on the spatial structure of the small hepatitis B surface protein . *Intervirology* . 38 : 8 - 15
- Blumberg BS , Alter HJ , Visnich S . 1965 ; A "New" Antigen in Leukemia Sera . *Jama* . 191 : 541 - 546
- Bruss V . 2007 ; Hepatitis B virus morphogenesis . *World J Gastroenterol* . 13 : 65 - 73
- Bruss V , Vieluf K . 1995 ; Functions of the internal pre-S domain of the large surface protein in hepatitis B virus particle morphogenesis . *J Virol* . 69 : 6652 - 6657
- Chain BM , Myers R . 2005 ; Variability and conservation in hepatitis B virus core protein . *BMC Microbiol* . 5 : 33 -
- Cho DY , Yang GH , Ryu CJ , Hong HJ . 2003 ; Molecular chaperone GRP78/BiP interacts with the large surface protein of hepatitis B virus in vitro and in vivo . *J Virol* . 77 : 2784 - 2788
- Dane DS , Cameron CH , Briggs M . 1970 ; Virus-like particles in serum of patients with Australia-antigen-associated hepatitis . *Lancet* . 1 : 695 - 698
- Dryden KA , Wieland SF , Whitten-Bauer C , Gerin JL , Chisari FV , Yeager M . 2006 ; Native hepatitis B virions and capsids visualized by electron cryomicroscopy . *Mol Cell* . 22 : 843 - 850
- Dubois MF , Pourcel C , Rousset S , Chany C , Tiollais P . 1980 ; Excretion of hepatitis B surface antigen particles from mouse cells transformed with cloned viral DNA . *Proc Natl Acad Sci U S A* . 77 : 4549 - 4553
- Eble BE , Lingappa VR , Ganem D . 1990 ; The N-terminal (pre-S2) domain of a hepatitis B virus surface glycoprotein is translocated across membranes by downstream signal sequences . *J Virol* . 64 : 1414 - 1419
- Eddleston A . 1990 ; Modern vaccines. Hepatitis . *Lancet* . 335 : 1142 - 1145
- Fernholz D , Stemler M , Brunetto M , Bonino F , Will H . 1991 ; Replicating and virion secreting hepatitis B mutant virus unable to produce preS2 protein . *J Hepatol* . 13 : ( Suppl 4 ) S102 - 104
- Gallina A , Bonelli F , Zentilin L , Rindi G , Muttini M , Milanesi G . 1989 ; A recombinant hepatitis B core antigen polypeptide with the protamine-like domain deleted self-assembles into capsid particles but fails to bind nucleic acids . *J Virol* . 63 : 4645 - 4652
- Ganem D . 1991 ; Assembly of hepadnaviral virions and subviral particles . *Curr Top Microbiol Immunol* . 168 : 61 - 83
- Gavilanes F , Gonzalez-Ros JM , Peterson DL . 1982 ; Structure of hepatitis B surface antigen. Characterization of the lipid components and their association with the viral proteins . *J Biol Chem* . 257 : 7770 - 7777
- Gerhardt E , Bruss V . 1995 ; Phenotypic mixing of rodent but not avian hepadnavirus surface proteins into human hepatitis B virus particles . *J Virol* . 69 : 1201 - 1208

- Gilbert RJ, Beales L, Blond D, Simon MN, Lin BY, Chisari FV. 2005; Hepatitis B small surface antigen particles are octahedral. *Proc Natl Acad Sci U S A*. 102 : 14783 - 14788
- Glebe D, Urban S. 2007; Viral and cellular determinants involved in hepadnaviral entry. *World J Gastroenterol*. 13 : 22 - 38
- Grigorov B, Arcanger F, Roingard P, Darlix JL, Muriaux D. 2006; Assembly of infectious HIV-1 in human epithelial and T-lymphoblastic cell lines. *J Mol Biol*. 359 : 848 - 862
- Gripon P, Rumin S, Urban S, Le Seyec J, Glaise D, Cannie I. 2002; Infection of a human hepatoma cell line by hepatitis B virus. *Proc Natl Acad Sci U S A*. 99 : 15655 - 15660
- Hartmann-Stuhler C, Prange R. 2001; Hepatitis B virus large envelope protein interacts with gamma2-adaptin, a clathrin adaptor-related protein. *J Virol*. 75 : 5343 - 5351
- Heermann KH, Goldmann U, Schwartz W, Seyffarth T, Baumgarten H, Gerlich WH. 1984; Large surface proteins of hepatitis B virus containing the pre-s sequence. *J Virol*. 52 : 396 - 402
- Helenius A, Aebi M. 2001; Intracellular functions of N-linked glycans. *Science*. 291 : 2364 - 2369
- Hourieux C, Brand D, Sizaret PY, Lemiale F, Lebigot S, Barin F, Roingard P. 2000; Identification of the glycoprotein 41(TM) cytoplasmic tail domains of human immunodeficiency virus type 1 that interact with Pr55Gag particles. *AIDS Res Hum Retroviruses*. 16 : 1141 - 1147
- Huovila AP, Eder AM, Fuller SD. 1992; Hepatitis B surface antigen assembles in a post-ER, pre-Golgi compartment. *J Cell Biol*. 118 : 1305 - 1320
- Jilbert AR, Miller DS, Scougall CA, Turnbull H, Burrell CJ. 1996; Kinetics of duck hepatitis B virus infection following low dose virus inoculation: one virus DNA genome is infectious in neonatal ducks. *Virology*. 226 : 338 - 345
- Kian Chua P, Lin MH, Shih C. 2006; Potent inhibition of human Hepatitis B virus replication by a host factor Vps4. *Virology*. 354 : 1 - 6
- Lambert C, Prange R. 2003; Chaperone action in the posttranslational topological reorientation of the hepatitis B virus large envelope protein: Implications for translocational regulation. *Proc Natl Acad Sci U S A*. 100 : 5199 - 5204
- Lambert C, Doring T, Prange R. 2007; Hepatitis B virus maturation is sensitive to functional inhibition of ESCRT-III, Vps4, and gamma 2-adaptin. *J Virol*. 81 : 9050 - 9060
- Loffler-Mary H, Werr M, Prange R. 1997; Sequence-specific repression of cotranslational translocation of the hepatitis B virus envelope proteins coincides with binding of heat shock protein Hsc70. *Virology*. 235 : 144 - 152
- Mangold CM, Streeck RE. 1993; Mutational analysis of the cysteine residues in the hepatitis B virus small envelope protein. *J Virol*. 67 : 4588 - 4597
- Mason WS, Aldrich C, Summers J, Taylor JM. 1982; Asymmetric replication of duck hepatitis B virus DNA in liver cells: Free minus-strand DNA. *Proc Natl Acad Sci U S A*. 79 : 3997 - 4001
- Nassal M. 1992; The arginine-rich domain of the hepatitis B virus core protein is required for pregenome encapsidation and productive viral positive-strand DNA synthesis but not for virus assembly. *J Virol*. 66 : 4107 - 4116
- Nowak MA, Bonhoeffer S, Hill AM, Boehme R, Thomas HC, McDade H. 1996; Viral dynamics in hepatitis B virus infection. *Proc Natl Acad Sci U S A*. 93 : 4398 - 4402
- Patient R, Hourieux C, Sizaret PY, Trassard S, Sureau C, Roingard P. 2007; Hepatitis B virus subviral envelope particle morphogenesis and intracellular trafficking. *J Virol*. 81 : 3842 - 3851
- Patzer EJ, Nakamura GR, Simonsen CC, Levinson AD, Brands R. 1986; Intracellular assembly and packaging of hepatitis B surface antigen particles occur in the endoplasmic reticulum. *J Virol*. 58 : 884 - 892
- Persing DH, Varmus HE, Ganem D. 1986; Inhibition of secretion of hepatitis B surface antigen by a related presurface polypeptide. *Science*. 234 : 1388 - 1391
- Persing DH, Varmus HE, Ganem D. 1987; The preS1 protein of hepatitis B virus is acylated at its amino terminus with myristic acid. *J Virol*. 61 : 1672 - 1677
- Persson B, Argos P. 1994; Prediction of transmembrane segments in proteins utilising multiple sequence alignments. *J Mol Biol*. 237 : 182 - 192
- Perz JF, Armstrong GL, Farrington LA, Hutin YJ, Bell BP. 2006; The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. *J Hepatol*. 45 : 529 - 538
- Peterson DL, Nath N, Gavilanes F. 1982; Structure of hepatitis B surface antigen. Correlation of subtype with amino acid sequence and location of the carbohydrate moiety. *J Biol Chem*. 257 : 10414 - 10420
- Poisson F, Severac A, Hourieux C, Goudeau A, Roingard P. 1997; Both pre-S1 and S domains of hepatitis B virus envelope proteins interact with the core particle. *Virology*. 228 : 115 - 120
- Ponsel D, Bruss V. 2003; Mapping of amino acid side chains on the surface of hepatitis B virus capsids required for envelopment and virion formation. *J Virol*. 77 : 416 - 422
- Rabe B, Vlachou A, Pante N, Helenius A, Kann M. 2003; Nuclear import of hepatitis B virus capsids and release of the viral genome. *Proc Natl Acad Sci U S A*. 100 : 9849 - 9854
- Roingard P. 2003; Vacuolization in hepatitis B virus-infected hepatocytes. *Hepatology*. 37 : 1223 - 1224 author reply 1224
- Roingard P. 2008; Viral detection by electron microscopy: past, present and future. *Biol Cell*. 100 : 491 - 501
- Roingard P, Sureau C. 1998; Ultrastructural analysis of hepatitis B virus in HepG2-transfected cells with special emphasis on subviral filament morphogenesis. *Hepatology*. 28 : 1128 - 1133
- Roseman AM, Berriman JA, Wynne SA, Butler PJ, Crowther RA. 2005; A structural model for maturation of the hepatitis B virus core. *Proc Natl Acad Sci U S A*. 102 : 15821 - 15826
- Rost M, Mann S, Lambert C, Doring T, Thome N, Prange R. 2006; Gamma-adaptin, a novel ubiquitin-interacting adaptor, and Nedd4 ubiquitin ligase control hepatitis B virus maturation. *J Biol Chem*. 281 : 29297 - 29308
- Satoh O, Imai H, Yoneyama T, Miyamura T, Utsumi H, Inoue K, Umeda M. 2000; Membrane structure of the hepatitis B virus surface antigen particle. *J Biochem*. 127 : 543 - 550
- Seitz S, Urban S, Antoni C, Bottcher B. 2007; Cryo-electron microscopy of hepatitis B virions reveals variability in envelope capsid interactions. *Embo J*. 26 : 4160 - 4167
- Slagsvold T, Pattni K, Malerod L, Stenmark H. 2006; Endosomal and non-endosomal functions of ESCRT proteins. *Trends Cell Biol*. 16 : 317 - 326
- Sonveaux N, Conrath K, Capiuc C, Brasseur R, Goormaghtigh E, Ruyschaert JM. 1994; The topology of the S protein in the yeast-derived hepatitis B surface antigen particles. *J Biol Chem*. 269 : 25637 - 25645
- Stibbe W, Gerlich WH. 1982; Variable protein composition of hepatitis B surface antigen from different donors. *Virology*. 123 : 436 - 442
- Summers J, Mason WS. 1982; Replication of the genome of a hepatitis B--like virus by reverse transcription of an RNA intermediate. *Cell*. 29 : 403 - 415
- Summers J, Smith PM, Horwich AL. 1990; Hepadnavirus envelope proteins regulate covalently closed circular DNA amplification. *J Virol*. 64 : 2819 - 2824
- Sureau C, Romet-Lemonne JL, Mullins JI, Essex M. 1986; Production of hepatitis B virus by a differentiated human hepatoma cell line after transfection with cloned circular HBV DNA. *Cell*. 47 : 37 - 47
- Tolle TK, Glebe D, Linder M, Linder D, Schmitt S, Geyer R, Gerlich WH. 1998; Structure and glycosylation patterns of surface proteins from woodchuck hepatitis virus. *J Virol*. 72 : 9978 - 9985
- Watanabe T, Sorensen EM, Naito A, Schott M, Kim S, Ahlquist P. 2007; Involvement of host cellular multivesicular body functions in hepatitis B virus budding. *Proc Natl Acad Sci U S A*. 104 : 10205 - 10210
- Weiser B, Ganem D, Seeger C, Varmus HE. 1983; Closed circular viral DNA and asymmetrical heterogeneous forms in livers from animals infected with ground squirrel hepatitis virus. *J Virol*. 48 : 1 - 9
- Werr M, Prange R. 1998; Role for calnexin and N-linked glycosylation in the assembly and secretion of hepatitis B virus middle envelope protein particles. *J Virol*. 72 : 778 - 782
- Wynne SA, Crowther RA, Leslie AG. 1999; The crystal structure of the human hepatitis B virus capsid. *Mol Cell*. 3 : 771 - 780

- Yaginuma K , Shirakata Y , Kobayashi M , Koike K . 1987 ; Hepatitis B virus (HBV) particles are produced in a cell culture system by transient expression of transfected HBV DNA . Proc Natl Acad Sci U S A . 84 : 2678 - 2682
- Yuan TT , Shih C . 2000 ; A frequent, naturally occurring mutation (P130T) of human hepatitis B virus core antigen is compensatory for immature secretion phenotype of another frequent variant (I97L) . J Virol . 74 : 4929 - 4932
- Zheng J , Schodel F , Peterson DL . 1992 ; The structure of hepadnaviral core antigens. Identification of free thiols and determination of the disulfide bonding pattern . J Biol Chem . 267 : 9422 - 9429
- Zhou S , Stranding DN . 1992 ; Hepatitis B virus capsid particles are assembled from core-protein dimer precursors . Proc Natl Acad Sci U S A . 89 : 10046 - 10050

**FIG. 1**

EM observation of BHK-21 cells producing the HBV S protein (yellow dot, materials and methods described in Patient et al , 2007 ) and model for the SVP morphogenesis and traffic. Vesicles about 0.2 to 0.3 μm in diameter (arrows), packed with 20 nm-large HBV S filaments, are observed budding from the nuclear envelope (A-a). After budding, these vesicles are packed with HBV S filaments and appear in lengthwise or crosswise sections with a crystal-like structure (A-b). The fusion of such vesicles with the endoplasmic reticulum-Golgi intermediate compartment (ERGIC) provides the relaxing of these filaments from their crystal-like structure in the ERGIC lumen (A-c). Negative staining of the purified intracellular HBV S filaments shows that these filaments tended to dissociate at their ends (arrow) into subviral spherical particles (A-d). A careful observation of the cell perinuclear space suggests that nascent filaments are self-assembled in the lumen of this compartment rather than by membrane-derived budding (B-a to B-d). For some filaments, intraluminal free ends are identified (arrow in B-d). Bars in A-a, A-b, A-c, B-a, B-b and B-c, 200 nm; Bars in A-d and B-d, 100 nm; Nu, nucleus; Cy, cytosol; the cellular chaperones potentially involved in the SVP morphogenesis are presented as black zig-zag.



**FIG. 2**

Model for HBV morphogenesis. On the left is the representation of a typical endocytosis and multivesicular bodies (MVB) sorting of an activated membrane protein, e.g. the Epithelium Growth Factor Receptor (EGFR). The ubiquitylated (Ub, black square) protein is transported into an early endosome to the ESCRT machinery. In MVB, the ubiquitylated receptor is recognized by ESCRT-I (I, blue) which immediately recruits additional components of the MVB pathway: ESCRT-II (II, red), ESCRT-III (III, green) which is anchored to the MVB membrane by myristylation (zig-zag), Alix/AIP1 (Alix, purple) and Vps4A/B (brown) which is critical for disassembly of the complex following inward budding of intraluminal vesicle (ILV) into the MVB lumen. On the right, the hijacking of MVB sorting machinery for HBV morphogenesis is depicted. See text for details. pgRNA, pre-genomic RNA; gDNA, genomic DNA; the viral reverse-transcriptase located inside the capsid is presented as a black heptagon.

