Intracellular transport and egress of hepatitis B virus

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Summary

Hepatitis B virus (HBV) replicates its genomic information in the nucleus via transcription and therefore has to deliver its partially double stranded DNA genome into the nucleus. Like other viruses with a nuclear replication phase, HBV genomes are transported inside the viral capsids first through the cytoplasm towards the nuclear envelope. Following the arrival at the nuclear pore, the capsids are transported through, using classical cellular nuclear import pathways. The arrest of nuclear import at the nucleoplasmic side of the nuclear pore is unique, however, and is where the capsids efficiently disassemble leading to genome release. In the latter phase of the infection, newly formed nucleocapsids in the cytosol have to move to budding sites at intracellular membranes carrying the three viral envelope proteins. Capsids containing single stranded nucleic acid are not enveloped, in contrast to empty and double stranded DNA containing capsids. A small linear domain in the large envelope protein and two areas on the capsid surface have been mapped, where point mutations strongly block nucleocapsid envelopment. It is possible that these domains are involved in the envelope – with capsid interactions driving the budding process. Like other enveloped viruses, HBV also uses the cellular endosomal sorting complexes required for transport (ESCRT) machinery for catalyzing budding through the membrane and away from the cytosol.

Introduction

Hepatitis B viruses (HBV) exhibit an extraordinary infectivity. This has been shown by Ullrich and colleagues, whereby nearly a single virus was sufficient to cause infection in 80–90% of chimpanzees [1–3]. Such a significant relationship between physical and infectious particles suggests that the numerous steps needed for infection and virion production are efficient and coordinated. This involves i) the arrival at the hepatocytes, ii) the entry into the cell, iii) transport of the viral genome to the compartment where multiplication of the genomic information occurs and also iv) a quality control of virions. The latter is of particular importance as the host RNA polymerase II transcribing the viral RNA pregenome from the viral DNA genome and the viral polymerase, which facilitates reverse transcription of the viral RNA pregenome and subsequent DNA synthesis, both lack a proof reading activity.

Intracellular cytoplasmic transport after entry

Like all retro- and DNA viruses (with the exception of Poxviridae), the genomic information of HBV is multiplied within the nucleus [4]. As a consequence, the HBV genome has to be transported from the place where the capsid is released into the cytosol, to the nuclear envelope and subsequently into the nucleus. The cytoplasm exhibits a high viscosity [5,6] making diffusion inefficient. Microinjection experiments of Luby-Phelps using fluorescent latex beads show that particles up to 50 nm can diffuse, although to a limited extent [6]. Most viruses – or their subviral structure containing the genome – use active directed transport via microtubules. To date, only baculoviruses and vaccinia virus have been described using polymerizing actin filaments as intracellular bacteria do [7–9]. The latter transport is, however, undirected and must be considered to be less efficient. Microtubule-mediated transport is also used for organelle

translocation and the motor protein complex, which determines the direction of the cargos attached [10]. For centripetal transport to the nuclear envelope, cytoplasmic dynein is used, which comprises 13 chains. Cargo attachment is frequently mediated by a dynein-bound multiprotein complex, termed dynactin, which modulates dynein activity.

The absence of an efficient infection system allowing virus propagation is a general obstacle in HBV research. This limits our knowledge about several aspects of the viral life cycle, including HBV shuttling and egress. As a consequence, many data are based on surrogate experimental systems. Lipofection, an experimental technique that has been used to circumvent infection and allows highly efficient infection/transduction of hepatoma and other cell lines, revealed that the genomes were certainly transported within the 32 nm-measuring capsids [11]. This was conclusively shown with fluorescence *in situ* hybridization under native conditions, which only detects capsid-release genomes. The absence of cytosolic signals however does not exclude the possibility that a small minority of capsids may partially disintegrate, which evokes detection by sensors involved in the innate immune response [12]. In fact, a small fraction of cytoplasmic cores fail to protect their DNA to exogenous nucleases and show evidence of proteolysis of the encapsidated polymerase, supporting at least a transient opening of the capsids [13,14].

Treatment of cells with paclitaxel, a microtubule-depolymerizing agent used in breast cancer therapy [15,16], has shown that capsid arrival at the nuclear envelope is microtubule-dependent. These experiments further showed that genome release from the capsids was dependent upon capsid translocation to the nucleus. In agreement with dynein-mediated transport velocity, which varies between 800 and 100 nm/s [17,18] the first released genomes were observed 1 h post lipofection but were exclusively intranuclear. This shows that intracytoplasmic transport of HBV capsids and genome release is rapid, directed and coordinated at least with regard to genome liberation. These observations are also in agreement with unpublished results from our laboratory, showing that capsid arrival at the nuclear envelope after microinjection in much larger *Xenopus laevis* oocytes, was observed after less than 1 h (Osseman, Panté, Kann, unpublished). It is noteworthy that microtubule-mediated transport is highly conserved between species; even plant cells translocate cargos of other eukaryotes [19]. Recent observations from our laboratory showed that HBV capsids bind exclusively to one particular dynein chain, which is conserved between *Xenopus laevis* and man and for which no polymorphisms are known (Osseman, Kann, unpublished). This argues that the intracytoplasmic transport is neither host nor organ-specific. It further leads to the conclusion that this step of the hepadnaviral life cycle is not likely to offer a target for therapy without risking severe side effects through off target interactions with other cellular proteins. However, drugs such as heteroaryldihydropyrimidines (HAPs), which modify the capsid structure, have the potential to interfere with cytosolic transport. Like picornaviruses, HBV capsids have momentary instability also known as capsid breathing [20,21]; an innate immune response that could be promoted. This assumption is supported by observations linking inflammation with cytoplasmic capsids [22–24].

Nuclear transport and genome release

HBV capsids have a diameter of 36 nm [23,24] and are small enough to pass the nuclear pores [25]. Such passage must be concluded from the fact that HBV infects non-dividing cells and that the nuclear pore complexes (NPC) form the only aqueous channels between nucleo- and cytoplasm. There are 400–18500 NPCs per cell (oligodendrocytes, Purkinje cells) [26]; dependent upon a cell's metabolic activity [27]. NPCs allow rapid exchange of ions and macromolecules, the latter reaching a rate of up to 800 to 1000 transport events per second [25]. While ions and other small molecules traverse the nuclear pores by diffusion, macromolecules depend on cytoplasmic transport receptors. Transport receptors can be separated into exportins and importins, the latter comprising of ten members, which act on cargos exposing different nuclear localization signals [26]. Transportin, for example – a member of the importin beta superfamily – binds to so called 'M9 domains', which are glycine-rich, and are present on nuclear housekeeping proteins. Better known is nuclear import via importin alpha and importin beta, which bind to classical nuclear localization signals (NLS) and consist of four to six basic amino acids [27]. Here, importin alpha serves as an adaptor protein binding to the cargo, while importin beta facilitates nuclear translocation after binding to an importin beta binding domain (IBB) on importin alpha. Like NLS, IBB is composed of basic amino acids but is much longer, with 39 amino acids. IBBs, which comprise of 13 basic amino acids in seven clusters [28], not only exist on importin alpha but also on karyophilic proteins, which are then directly imported by importin beta without the need of importin alpha [29,30]. The higher number of interacting amino acids compared to NLSs make the binding more stable than

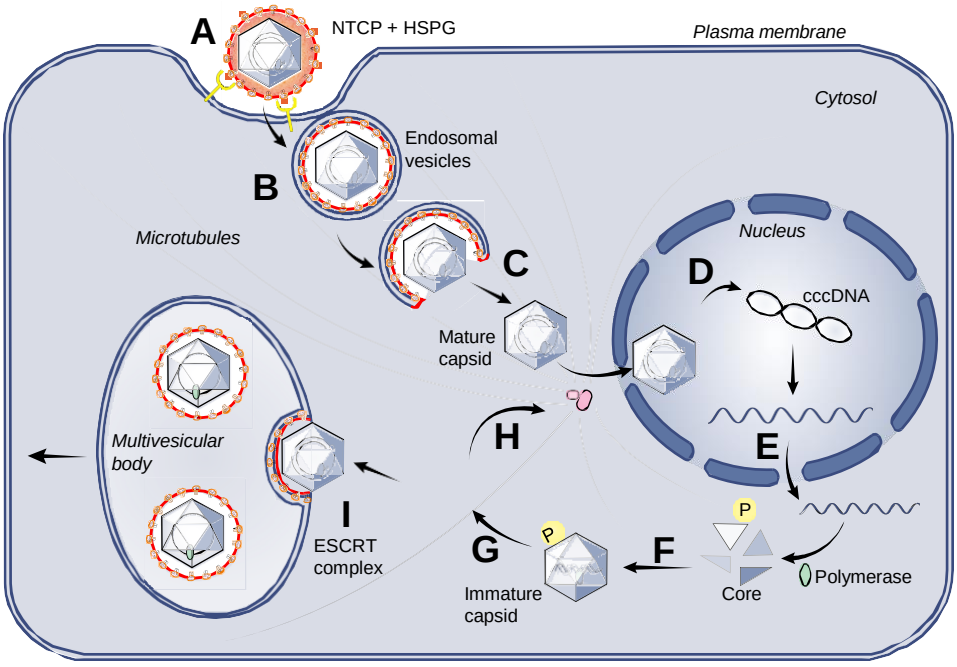

Fig. 1. Overview on HBV transport processes during entry and egress. (A) Hepatitis B virions attach to the hepatocytes via interaction with Na+-taurocholate cotransporting polypeptide (NTCP) and heparan sulfate proteoglycans (HSPG), and become internalized in endosomal vesicles. (B) Deduced from other viruses, transport should occur also in the endosomes along microtubules. (C) Capsid release from the endosomes before significant acidification followed by transport of the capsids to the nuclear periphery using cytoplasmic dynein. (D) Capsid transport through the nuclear pore, genome release and genome repair to cccDNA (for details see Fig. 2). (E) Transcription and mRNA export. (F) Pregenome: after translation of core proteins, polymerase and phosphorylation (P) of at least some core proteins, core, polymerase, heat shock proteins and RNA pregenome form an immature capsid. (G) Genome maturation, dephosphorylation. (H) Transport of the mature capsid to the nucleus when surface proteins not (sufficiently) expressed. (I) Dephosphorylation; interaction with membrane-integrated L protein, budding via the ESCRT machinery. The fate of empty capsids and the release of naked capsids are not depicted. Capsids: blue hexagons, core proteins: blue triangles, polymerase: green circles, (—) strand DNA: red circle, (+) strand DNA/incomplete red circle, viral surface: red line/circle.

the one of importin alpha – (dissociation constant NLS-importin alpha – 1M [31]; IBB-importin alpha – nM [28]).

NPCs are composed of 30 different proteins collectively called nucleoporins (Nups). NPCs have an octagonal symmetry and Nups exist in 8-56 fold copies. Many Nups bearing FXFG or GXFG repeats are essential and are involved in nuclear transport [32,33]. Despite more than two decades of research, the exact mode of translocation is not known. However, the current models show enough similarity to conclude that the cargo import receptor complex attaches to cytoplasmic fibers extruding from the NPCs, and which comprise Nup358 and Nup214, which are important in adenoviral capsid docking to the nuclear pore and to genome liberation [34]. The import complex then passes the central channel of the pore, which is filled with a hydrophobic mesh, limiting diffusion to small molecules of 2 nm. It is of note that there is no clear size or molecular weight cut-off, as other factors including charge and shape are important. After passing the pore, the import complex binds to nuclear filaments, which form the nuclear basket. This interaction leads to the arrest of the import complex unless the small GTPase, Ras-related nuclear protein (Ran) in its GTP-bound form (RanGTP) dissociates importin beta or transportin from the cargo or adaptor molecule [28,35]. The reversibility of cargo import receptor binding is also reflected by the high Michaelis constant (Km). Upon classical nuclear import, the cargo subsequently diffuses deeper in the karyoplasm while RanGTP in complex with the import receptor is exported into the cytoplasm followed by recycling of Ran.

The primary amino acid sequence of the HBV core protein, from which 240 copies form the capsid, comprises an arginine rich C-terminal domain containing NLS [36–38]. This domain also comprises a putative IBB-like sequence, which could allow capsid transport if entirely exposed. In RNA-containing capsids, at least after their expression in *E. coli*, the C termini localize in the lumen of the capsid as shown by Zlotnick *et al.* using nano gold labeling [39]. This is in agreement with data from permeabilized cells showing that RNA-containing capsids do not interact with the nucleus even in the presence

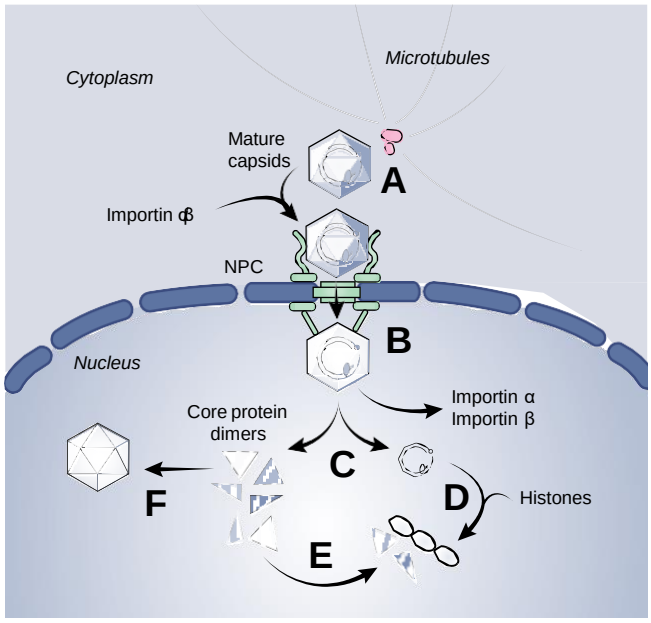

Fig. 2. Pre- and post-nuclear import events. (A) Following transport via microtubules the mature capsids dissociate from the microtubules by an unknown mechanism and attach to importin a/b. (B) The capsids pass the NPC and bind to Nup153 where importin a/b dissociate. (C) Unknown factors dissociate mature capsids to core protein dimers leading to genome release. (D) The genome is repaired to the cccDNA form and associates with histones and (E) core proteins. (F) Once a threshold concentration is reached, the core protein dimers re-associate to capsids, which are either empty or eventually filled with cellular RNA. See Fig. 1 for the detailed legend of the symbols.

of nuclear import factors. Lowering the affinity between RNA and C termini of the core protein by serine phosphorylation resulted in importin alpha/beta mediated interaction with the NPCs and allowed capsid pull-down by these import factors [38] but not by importin beta alone. However, the extent of capsid phosphorylation and the position of phosphorylated serines are not fully understood and more recent data suggests that after initial phosphorylation, further genome maturation coincides with dephosphorylation in HBV and duck hepatitis B virus (DHBV) [40–42]. This allows the conclusion that capsids from infection are likely to be non-phosphorylated and also indicates that parts of the C termini can flip to the capsids surface [43,44]. This hypothesis in turn is in agreement with observations by tryptic cleavage of capsids, showing that in capsids with mature double stranded DNA – to which the C termini bind poorly – the C termini are exposed outside the capsid and do not interact with the NPCs after cleavage [45]. Accordingly, these mature capsids interact with importin alpha/beta and with the NPCs in permeabilized hepatocyte cell lines used to circumvent inefficient entry and also after microinjection into the cytosol of *Xenopus laevis* oocytes. It must therefore be considered that the C termini have a dual topology, which depends upon their affinity to the encapsidated nucleic acids. It is likely that the structural changes lead to presentation of a short NLS but do not comprise the entire C terminus. This can be concluded from experiments with mature and *in vitro* phosphorylated RNA capsids as they interact with importin beta only in the presence of importin alpha. It is notable that these structural changes, which are required for nuclear delivery of the genome, are genetically separable from those allowing envelopment by surface proteins [46].

The data on partial cytoplasmic capsid disassembly raised the question of whether the viral DNA passes the nuclear membrane in the intact capsid or in a liberated complex of with the viral polymerase. In fact, the virion-extracted polymerase DNA-complex becomes actively imported [47] but it has to be considered that the extraction needs chaotropic agents, which severely alter protein conformation including that of the polymerase. However, there is better evidence arguing for a transport of the genome within the capsid. Investigations into the fate of mature capsids upon infection are difficult as the number of capsids entering the cell or at least the cytoplasm is limited [48–51]. As mentioned before, lipofection and permeabilized cells have both been used, allowing the capsids and eventually genomes to be followed by microscopy. For nuclear import studies, permeabilized cells have further advantage, as defined amounts of capsids can be applied. Using this system and adding virus-

derived capsids either from HepG2.2.15 cell culture supernatant or from HBV-infected patients plasma, nuclear import of capsids have been observed, and were combined with the appearance of nuclear released viral DNA [11,45,52]. Quantification revealed that each nucleus released the genomes from hundreds of capsids within minutes, supporting the speed of the reaction [53]. These studies further showed that – within the detection limits – all capsids released their genome. This is in agreement with the very low particle-to-infectious unit-ratio. Interestingly, genome liberation was combined with the dissociation of the capsids into core protein dimers, which is similar to adenoviral disassembly [54,55]. However, the dimers were released into the nucleus where they reassembled to capsids. This observation allows the conclusion that entry of capsids into cells upon infection is not combined with irreversible structural changes by low pH or proteolytic cleavage as it is the case for most other viruses.

Investigation of capsid transport in more detail by electron microscopy has shown that intact capsids pass the nuclear pore intact [56] but that they became arrested within the nuclear basket by interaction with Nup153 [52]. While artificially cross-linked capsids stayed in the basket, the physiological non-linked ones dissociated. Disassembly was further shown to be genome maturation dependent, which supports the importance of this trafficking pathway during infection. Importantly, the underlying mechanisms were observed in different cultured cells indicating that no liver-specific factor is required. While this is the case for attachment of the capsids with the NPC, the current data do not exclude that genome release is more specific.

Of interest for clinicians is the potential to intervene by disturbing the nuclear import pathway and genome release. However, the latter is poorly understood, making it difficult to draw conclusions. Regarding nuclear import, one has to realize that this pathway is well conserved through all eukaryotes indicating the potential for severe side effects. This assumption becomes even more plausible when considering that Nups are not only important for nuclear trafficking but also in cell division, in particular in reformation of the nuclear membranes [57–60]. Uncoating, however, may eventually be addressed, as suggested by Liu *et al.* [61].

Post entry trafficking

As outlined previously, there are probably no irreversible capsid changes upon entry into the cell. As a consequence, progeny capsids – containing mature DNA, as in the virion-derived capsids – should be identical to entering ones. Accordingly, progeny capsids are subject to the same trafficking as capsids upon infection. In fact, progeny capsids can thus deliver their DNA into the nucleus. This is well documented for DHBV capsids exhibiting more than a 19 fold increase in nuclear viral DNA concentration in the absence of the viral surface proteins (420 to 630 copies/cell respectively for 1165A and 1S mutants *vs*. WT 22 copies/cells (10–50 copies/-cell)) [62]. Single cell analysis revealed a significant difference, even in the presence of functional surface proteins: 90% of nuclei contained between 1 and 17 cccDNA molecules and 10% more [63].

For HBV, the situation is less clear but artificial surface protein deficient mutants exhibited a 1.5 to 6 fold increase in cccDNA copy number [13,64–67]. In particular, some reported numbers to be below one copy per cell, showing that these were mostly derived from calculations of DNA molecules per total number of cells and not per number of infected cells.

Core proteins are expressed in excess with regard to the quantity needed for progeny virus synthesis. These core proteins localize as assembled capsids either in the cytoplasm or in the nucleus, depending upon the individual cell and the inflammation state of the patient [68–71]. The nuclear capsids have been found to be empty [72] or at least devoid of the viral genome. The thousands of viral genome copies do not apparently reduce hepatocyte life span, which seems rather to be extended as indicated by clonal expansion of infected cells in chimpanzees [73]. Evidently, these capsids are not derived from infecting capsids but from progeny core proteins. This raises the question on their transport. The current knowledge allows two hypotheses: first, an import as non- or preassembled capsids. This is likely to involve core protein dimers as dimerization occurs rapidly [21,74–76]. In dimers, the entire C termini are exposed meaning that nuclear import could be mediated either by their NLS or directly by their IBB. The latter idea is supported by the higher affinity of importin beta to IBBs (nM range) compared to importin alpha – NLS interaction (1M range) [28]. The second model argues for a transport based on empty capsids formed in the cytosol. This hypothesis is justified by the conclusion that there is no nucleic acid in these capsids, fixing the C termini in the capsid lumen. A structural change to the topology, similar to mature capsids, is further supported by findings showing that empty capsids can be encapsidated by the viral surface proteins leading to empty virions [77,78].

Virus egress

During the productive phase of the HBV infection, at least a fraction of newly formed cytoplasmic nucleocapsids have to escape from the nuclear transport pathway and move towards budding sites for envelopment and egress. These buddings sites are formed at intracellular membranes carrying the three viral envelope proteins S, M, and L (for small, middle, and large) as transmembrane polypeptides. How capsids are transported from their site of formation in the cytosol to budding sites is unknown.

HBV envelope proteins

The viral envelope proteins are encoded by a single open reading frame and translated by the usage of three start codons in this frame [79]. Therefore, the sequence of the 226 amino acid (aa) long S protein is present at the C termini of the M and L proteins. The 119 or 108 aa long sequence (depending on the virus genotype), unique at the N terminus of L is called preS1, and the 55 aa long sequence in the M protein N-terminal to its S domain is referred to as preS2. After translation, the S and M proteins span the membrane probably four times with transmembrane domains (TM) in the S region. N and C termini are oriented to the lumen of the endoplasmic reticulum (ER) [80]. The preS1 domain of L contains a sequence between aa 70 to 94 binding to the cytoplasmic chaperone Hsc70 which blocks the cotranslational translocation of preS1 and preS2 (together referred to as preS) into the ER lumen [81]. Therefore, the preS of L initially stays in the cytoplasmic compartment and TM1 of its S domain does not traverse the membrane, while TM2–4 anchor the L protein in the lipid bilayer [82–84]. All three proteins form disulfide-linked homo- and heterodimers with each other laterally floating in the membrane [85,86].

Independent of the viral capsid, the HBV envelope proteins form higher oligomers which can bud into the lumen of a post-ER/pre-Golgi compartment and in turn, form two morphologically distinct populations of subviral lipoprotein particles of 20 nm diameter: spherical particles containing relatively little L protein (up to 10% of total protein) and filamentous particles of various lengths carrying more L. Overexpression of the L protein blocks subviral particle release in a dose dependent fashion [87,88]. N-terminal truncation of the L protein, which also abolishes myristylation, releases this suppressive effect of L. Subviral particles are released from cells by the constitutive secretion pathway [89,90]. Their formation is not dependent on other viral functions or liver-specific factors since efficient subviral particle secretion can be achieved in a wide variety of eukaryotic cells expressing the envelope proteins.

Budding sites

The viral envelope proteins are required for budding of the HBV capsid [91]. This is different from type C retroviruses, where myristylated Gag proteins bind independently of envelope proteins to the plasma membrane via the N-terminally attached fatty acid moiety, and oligomerization of the Gag proteins drives budding even when no viral envelope proteins are present [92]. Accordingly, the envelopes of type C retroviruses often are not tightly covered with viral surface proteins and in addition, contain membrane proteins from the host which are passively incorporated. Therefore, retroviruses can be pseudotyped just by overexpression of foreign membrane proteins in the plasma membrane of the virus producing cell. In contrast, the HBV L and S protein are both necessary for virus formation while the M protein is not essential [91,93]. This finding is in agreement with data of Ni *et al.*, showing that the preS2 domain is dispensible [94]. The current model for HBV budding proposes that similar to alphaviruses, whereby one or two cytoplasmic domains (matrix domains) of the HBV surface proteins contact the capsid. These contacts then order the envelope protein in the membrane into a tightly packed formation and the envelope protein, associated with capsid interactions, drives the budding process [95]. As a consequence, host membrane proteins are not found in the envelope of HBV particles and pseudotyping with non-related viral envelope proteins is not successful. However, the L protein from the woodchuck hepatitis virus may substitute the L protein from the human virus whereas this has not been possible with the less related L protein of the DHBV [96].

One 22 aa long matrix domain (MD) at the boundary of preS1 and preS2 (aa 103 to 124) in L (MD1) [97,98] and one domain in the C-terminal half of the cytoplasmic loop between TM1 and TM2 in S (MD2) [99–101] has been genetically defined by phenotypic characterization of deletions, substitutions, and point mutants. A spacer with a minimal length of 26 aa between MD1 and the first transmembrane domain of L (TM2 in the S domain) was required for nucleocapsid envelopment [102]. Therefore, MD1 can potentially reach binding sites on the entire capsid surface and is not sterically restricted to bind to, or close to the tip of spikes

protruding from the capsid. Indeed, a genetic screen using a large number of single alanine mutations located at the capsid surface mapped two small areas at the ground of the spikes and between two spikes where point mutations strongly blocked nucleocapsid envelopment [103,104]. The function of these areas is very sensitive to mutations because single exchanges of alanines within these regions invariably block virion formation, and some amino acids may not be exchanged without losing function. These areas might represent binding partners of matrix domains (matrix binding domains, MBD). However, it is also possible that they are involved in other steps of the virion morphogenesis pathway, such as capsid maturation (see below) or transport of the capsid to budding sites. Usage of MBDs as targets for small molecules has been attempted. Indeed, a DNA aptamer selected for binding to one MBD partially blocked virus production in transiently transfected cells [105]. However, peptides that also bind to the tip of the capsid spike inhibit nucleocapsid envelopment [106] and electron microscopic studies have shown an interaction of the spike tip with the viral envelope [107].

It seems possible that MD1 in the L protein is an important factor in redirecting nucleocapsids from nuclear transport to budding sites. For DHBV it has been shown that the absence of L causes a strong enhancement of nuclear cccDNA formation [108,109]. A possible model explaining this observation suggests that without L, no budding sites are generated and nucleocapsids therefore move into the nucleus, delivering the viral genome for cccDNA formation. If a certain threshold of budding sites is reached, nucleocapsids are redirected for egress.

Like many other enveloped viruses [110], HBV also utilizes the ESCRT complexes of the host cell to catalyze the membrane fission that has to occur as the last step of budding [111]. These host factors are involved in the formation of vesicles that bud away from the cytosol into the lumen of multivesicular bodies. Many viruses like HBV also bud away from the cytosol into the lumen of a cellular compartment or into the extracellular space and hijack these factors for this task. So called 'late domains' in viral capsid proteins, interact with factors of the fission machinery. The HBV capsid protein contains the late-domain-like motif, PPAY and binding of the cellular Nedd4 ubiquitin ligase to this motif has been demonstrated [112]. Nedd4 is proposed to escort cargos to the ESCRT machinery. Until now, the requirement for the PPAY motif for envelopment could not be shown by mutational analysis due to the effects that altering it have on capsid assembly and maturation.

Nucleocapsid maturation

The synthesis of the HBV DNA genome occurs within the lumen of the capsid by reverse transcription of an RNA pregenome, generating a single stranded DNA molecule of (—) polarity. Subsequently, this DNA is the template for the synthesis of the (+) DNA strand. Capsids with all intermediates of genome synthesis can be found within the cell. However, secreted virions carry no capsids with single stranded nucleic acid, they contain partially double stranded DNA. This observation has led to the hypothesis that early nucleocapsids are immature and that a maturation signal is generated on the capsid surface during second strand DNA synthesis; that can be sensed by the envelopment machinery and that excludes immature capsids from budding [113]. This model is supported by the observation that a virus with a mutation in the reverse transcriptase blocking DNA synthesis so that the nucleocapsids are frozen in an immature state, is unable to form virions [114]. Recently, however, it has been found that empty capsids containing no nucleic acid are incorporated into virus like particles [115]. This has prompted the proposal of a different paradigm; the single strand blocking model [78]. According to this model, the single stranded RNA pregenome and the single stranded (—) DNA strand, induce a signal in capsids blocking their envelopment and the synthesis of the second DNA strand releases this block. The nature of the accompanying signal in the capsid is not clear. Possibly, differential phosphorylation of serine residues in the C-terminal domain of the core protein play a role or perhaps a conformational change in the core protein [116]. It is also unclear as to which step of the virion formation pathway the envelopment competent and incompetent capsids are separated. For example, this may happen at the level of transport of capsids to budding sites; at the level of interaction with viral envelope proteins; or at the level of interaction with cellular factors necessary for membrane fission. For DHBV, it has been demonstrated that mature capsids associate with intracellular membranes in the absence of viral envelope proteins, in contrast to immature capsids [117]. The basis for this binding is unknown, however.

In virions a conformation of the L protein can be found that is different from the initial conformation: newly synthesized L displays its preS domain at the cytosolic side of the ER membrane. This site is equivalent to the inner side of the viral envelope. However, in virions L proteins expose their preS domain to the outside [82] and this is important for the virus because the preS1 sequence is involved in binding to the host cell during infection [118]. Apparently, the L

protein can switch from an i-preS to an e-preS conformation. How this posttranslational translocation of preS across the membrane is achieved is unclear. Also it is not known when this switch happens. Investigation of the L topology in virions has shown that approximately half of the L chains have the i-preS topology and the other half has the e-preS folding. It is possible that in each virus particle both conformations are present in a roughly 1:1 ratio or that, for example, half of the virions carry L with i-preS and the other half with e-preS. The latter possibility is supported by the observation of two different kind of virions with either a compact or a gapped appearance under the electron microscope [107]. This would imply that the topological switch of L is triggered more or less simultaneously in a virus particle.

Transfected HBV expressing cells release naked capsids (capsids without a surrounding envelope) in addition to subviral lipoprotein particles and virions. It is unclear how the capsids leave the cell without disruption of the plasma membrane. The pathway is independent of the ESCRT machinery but depends on Alix, a multifunctional protein with key roles in membrane biology [119,120]. However, *in vivo* naked capsids have not been found in the serum of virus carriers negative for antibodies against capsids [121].

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Authors' contributions

All authors contributed in writing the review.

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