Background on Hepatitis B Virus

Molecular Virology of Hepatitis B Virus and Targets for Antiviral Intervention

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Introduction

Hepatitis B, caused by infection with hepatitis B virus (HBV), is an infectious disease that is still one of the major global health problems. HBV infection can lead to acute and chronic liver disease. Worldwide, more than 240 million people are currently chronic HBV carriers [1], and more than 620,000 die annually from the long-term sequelae of this disease, despite the fact that the infection is preventable by a protective vaccine that has been available worldwide for over 25 years [2]. Vaccination can especially protect newborns from one of the major natural transmission routes that keep this blood-borne virus within the human community: vertical transmission from chronic HBV-infected mother to the child at birth. Infection during birth or early childhood results in 90% of the cases developing into a chronic carrier state. Chronic HBV infection may proceed after decades of seemingly good health in approximately 20–30% of the virus carriers to liver fibrosis, cirrhosis, and hepatocellular carcinoma. Progression depends on ongoing viral replication [3]. Careful monitoring of the 240 million chronic HBV-infected persons worldwide and successful treatment of the roughly 60 million with active replication is therefore the main goal of antiviral therapy. Understanding the molecular virology of HBV, including its close interconnection during replication with hepatocytes and the immune system, will provide opportunities for devel-
opment of better therapies and vaccines. In this review we describe basic virological aspects of the viral replication cycle with special consideration of current and innovative therapies of chronic hepatitis B.

**Classification of HBV**

HBV belongs to the family of Hepadnaviridae, a family of small enveloped viruses. All Hepadnaviridae are able to cause acute and persistent infections with viremia and antigenemia. Strong hepatotropism combined with relatively strict species specificity are further hallmarks of this viral family. The orthohepadnaviruses of the mammals comprise the species HBV with many genotypes in humans and apes (gorillas, chimpanzees, gibbons, and orangutans) [4]. Another primate orthohepadnavirus species (WMHBV) has been isolated from a New World ‘woolly monkey’ [5]. For long time, the family of Sciuridae (esp. ‘Northern American squirrels’, e.g. woodchuck, ground squirrel, and arctic squirrel) were the only additional animal hosts for mammalian orthohepadnaviruses. In 2013, however, we [6] and another group [7] isolated new hepadnaviruses in bats from Africa, Central America, and Asia. Interestingly, these bat-derived orthohepadnaviruses were phylogenetically very close to HBV genotype F and WMHBV, and have zoonotic potential [6]. For human HBV, at least 9 genotypes (10 genotypes A–J) have been characterized that differ in the nucleotide (nt) level of their genome by more than 8%. The many subgenotypes differ by 4% [4]. For more information on HBV genotypes, see the review by Kramvis [this issue, pp. 141–150].

**Virion Structure**

HBV appears as a spherical particle under native conditions with an outer diameter of approximately 52 nm [8, 9]. Three HBV surface proteins and lipids enclose the icosahedral nucleocapsid core, combining 240 core protein subunits that are around 36 nm in diameter. The nucleocapsid contains the viral DNA genome and several cellular proteins, e.g. protein kinases [10–12]. A hallmark of all Hepadnaviridae is the secretion of HBV surface proteins (for HBV HBsAg particles) as subviral particles in spherical or filamentous form (fig. 1). These particles do not contain viral DNA and are noninfectious. The second major viral antigen, the core protein, may also be secreted as the so-called ‘e-antigen’. Both antigens are produced in large excess and may reach up to hundred or more μg/ml protein per ml serum.

**Genome Organization and Transcriptome**

The small genomes of Hepadnaviridae have a unique structure with a relaxed circular (rc), partially double-stranded DNA of 3.1–3.3 kb [13]. Circularization is achieved by base-pairing of an interconnected overlap between the 5′ ends of the plus and minus strand DNA. The two DNA strands still carry their primers from the replication. The 5′ end of the minus strand is covalently linked to a tyrosine hydroxyl residue in the terminal protein domain of the HBV polymerase. The 5′ end of the plus DNA strand is formed by a 19-nt-long 5′-capped RNA remnant of the pregenomic RNA (pgRNA) [for review, see 14]. The circular HBV genome contains four overlapping open reading frames (ORFs):

1. The longest ORF (Pol) covers around 70% of the viral genome and encodes the viral polymerase, a polyprotein comprising three subdomains: the reverse transcriptase including the DNA-dependent DNA polymerase for plus strand synthesis, the terminal protein domain priming the minus strand DNA synthesis, and the RNase H located at the carboxy-terminus.

2. The surface proteins (S-ORF), which code for three co-carboxy-terminal HBV surface (HBs) proteins: the large (LHBs), middle (MHBs), and small (SHBs) protein. The backbone of the surface proteins is the carboxy-terminal S-domain that is present in all three proteins. The amino-terminal preS1-domain is only present in LHBs [15]; preS2 is available in LHBs and MHBs [for review, see 16, 17].

3. Precore/core encodes the structural core protein and an additional precore sequence. Full translation of precore/core ORF leads to synthesis and processing of HBeAg that can be regarded as a nonstructural form of the core proteins that is efficiently secreted [18, 19].

4. The still enigmatic X protein, encoded by X-ORF, whose function is still not fully understood, is believed to be involved in the epigenetic control of HBV transcription and HBV-associated carcinogenesis [20, 21]. Phenotypically, all viral transcripts are similar to cellular mRNAs, with 5′ cap and 3′ polyadenylation; however, they are usually not spliced. Liver-specific expression of HBV is driven by at least four promoter and two enhancer elements containing binding sites for liver-specific but also ubiquitous transcription factors supporting the efficient transcription of HBV covalently closed circular DNA (cccDNA).
HBV infection of hepatocytes is mediated by low-specific heparan sulfate proteoglycans and high-specific (NTCP) receptors on the plasma membrane of differentiated hepatocytes [1], resulting in uptake in endosomal structures. After the fusion with an intracellular membrane, the released nucleocapsid is actively transported within the cytosol to the nucleus [2]. Disassembly of core particles occurs within the nuclear pore complex, resulting in release of the partially double-stranded HBV DNA genome into the karyoplasma. The repair of HBV rcDNA into cccDNA is subsequently mediated by cellular factors [3]. Cellular histone and nonhistone proteins associate with episomal cccDNA, similar to host chromosomes, allowing epigenetic modification [4]. The cccDNA serves as a template for transcription of pregenomic and subgenomic mRNAs [5] that are actively translocated from the nucleus into the cytosol [6]. Translation of two subgenomic HBV mRNAs (preS/S mRNAs) at the ER leads to synthesis of the three HBV surface proteins, required for envelopment of mature core particles [13] and construction and secretion of subviral particles [7, 8]. pgRNA (orange; with the characteristic 5′ epsilon signal structure indicated) is translated to core proteins and the polymerase [9] that assemble their own template (pgRNA) together with cellular proteins (protein kinases and heat shock proteins) into an immature core particle [10]. Here, reverse transcription and genome maturation starts and the mature core particle [11] is either translocated to the nucleus [12] to replenish the cccDNA pool [3, 4], or enveloped with HBV surface proteins [13] and secreted through multivesicular particles [14]. The constitutive secretory pathway, including the ER-Golgi complex, is the exit pathway of the subviral particles. Synthesis of nonstructural viral proteins HBe and HBx is not shown. Current and potential inhibitors of HBV replication are indicated. The figure is adapted from Glebe and Bremer [16].
of the core particle inside the basket of the nuclear pore complex [26, 27], the rcDNA form is converted into cccDNA [28]. The cccDNA is then complexed with histone and nonhistone proteins, and can thus be regarded as a stable episomal minichromosome, similar to other episomal viral genomes typical for polyomaviruses and papillomaviruses [29]. A reverse transcription step during genome replication is shared by the viral families Hepadnaviridae and Retroviridae (e.g. HIV), but an integration of the HBV genome within the host cell genome will lead to destruction of its circular DNA genome, in contrast to the linear HIV DNA provirus. As mentioned above, all transcribed viral RNAs from HBV cccDNA contain a 5′ cap and a 3′ polyA tail structure. Potential splice sites within the hepadnaviral RNAs are suppressed by a posttranslational regulatory element, which also mediates the nuclear export of the RNAs. pgRNA encodes the complete HBV genome including overlength redundancy with additional sequence elements necessary for viral replication [14]. pgRNA is the template for reverse transcription and genome maturation, and also serves for the translation of core protein and polymerase.

Replication of hepadnaviruses starts with binding of the viral DNA polymerase (Pol) to a stem-loop structure close to the 5′ end of the pgRNA, called ε (epsilon) [14]. This reaction induces encapsidation of the whole complex along with core protein into an immature core particle. Reverse transcription starts with the formation of a phosphodiester bond between the hydroxyl residue of a tyrosine in the terminal protein domain of the Pol protein and the first nucleotide of the minus strand. After a complex shift of the nascent minus DNA strand to the 3′ terminal ε, a complete minus DNA strand is transcribed. The template RNA strand degradation by viral RNase H and the primer shift pave the way to subsequent plus strand DNA synthesis, resulting in a partially double-stranded HBV DNA genome. The HBV polymerase is thereby still attached to a variable 3′ end of the growing plus strand [14].

Budding of HBV is mediated by a multiprotein mechanism, called the ‘endosomal sorting complex required for transport’ (ESCRT) [30, 31]. The preS-domain of LHBs (17 C-terminal AA of preS1 and 5 N-terminal AA), facing towards the cytoplasm [32, 33], interacts with mature nucleocapsids containing viral rcDNA. Some reports indicate that the cytosolic loop of the S-domain might also be involved in HBV assembly [34–37]. Budding of HBV occurs from an intracellular membrane, and involves ESCRT protein complexes that are also necessary for formation of multivesicular bodies, a special intracellular compartment containing intraluminal vesicles [38, 39]. Secreted nonenveloped HBV core particles have not been observed in the plasma of chronically HBV-infected patients without anti-HBc [40]. However, secretion of naked cores is a common phenomenon of HBV-producing hepatoma cell lines, e.g. HepG2.2.15 [11, 31, 41, 42], which are mediated by an ESCRT-independent process at the plasma membrane [43].

HBV subviral particle formation begins with translation and integration of the multitransmembrane-spanning S-domain of the three HBV surface proteins at the membrane of the endoplasmic reticulum (ER). Translational modification involves N- and O-glycosylation [44, 45], but also the formation of intra- and intermolecular disulfide bridges by ER-resident protein disulfide isomerase, connecting the highly conserved free cysteine groups of the S-domain of monomeric HBV surface proteins [46]. This results in assembly of surface protein dimers and, most importantly, formation of the antigenic (a) determinant that is essential for attachment to hep- ran sulfate proteoglycans and recognition by protective antibodies. Those surface protein dimers are reported to be the building blocks of filamentous structures that will be converted into spheres in an ER-Golgi intermediate compartment, followed by secretion through the classical secretory pathway (fig. 1) [47, 48]. Subviral particles are secreted as 20-nm spheres and filaments with variable lengths but similar diameters.

Therapeutic Intervention of the Replicative Cycle of HBV

HBV Entry Inhibitors

Blocking viral infection of host cells at the level of the very early steps of infection, viral binding, and entry of host cells is one of the key goals of antiviral intervention. This can be achieved by either targeting the virus itself or host cell molecules involved in viral binding and entry. Successful vaccination by the HBV vaccine neutralizes HBV infectivity by targeting the antigenic loop of HBV surface proteins, necessary for infection. However, full protection (anti-HBs titer >100 IU/l) with this vaccine can only be achieved by an active immune system, and patients with subtle immune suppression (e.g. dialysis patients) suffer from incomplete vaccine response and are poorly protected against HBV infection. In addition, anti-HBs titers usually show a fast drop during the first year after vaccination [49]. Low anti-HBs titers can protect
against hepatitis B [50], but are often unable to provide sterilizing immunity after exposure to HBV (e.g. of healthcare workers and sexual partners) as described recently [51, 52]. Thus, in those cases with missing or incomplete protection against HBV, postexposure prophylaxis relies on passive administration of anti-HBs antibodies (HBIG) isolated from sera of vaccinees. In clinical practice, this is currently given to newborns to prevent perinatal infection from their HBV-infected mothers in combination with active vaccination (for a current review, see Gerlich [this issue, pp. 131–133]), and during liver transplantation in combination with nucleos(t)ide analogues (for a current review, see Roche and Samuel [this issue, pp. 196–201]). Since the identification of NTCP [25], a bile salt transporter of hepatocytes, as a high-affinity receptor for HBV, many antiviral drug approaches are currently focused on blocking HBV interaction with this receptor [53–55]. One of the straightforward approaches is the use of myristoylated preS1 peptides from the N-terminal preS1-domain of LHBs. This domain is essentially involved in HBV infection due to high-specific receptor-binding to the NTCP [56]. Interestingly, the antiviral potency of these infection-interfering acylated preS1-lipopetides was characterized in vitro and in vivo long before NTCP was known as a high-affinity receptor for HBV [57–61]. For further details, see the review by Lempp and Urban [this issue, pp. 151–157].

Modulation of HBV cccDNA Pool

HBV transcription is maintained from a pool of episomal cccDNA that is associated with histone and nonhistone proteins within the nucleus of the infected hepatocyte (fig. 1) [14, 29]. The cccDNA is very stable and able to endure prolonged times in infected hepatocytes of the liver even after antiviral treatment with immunological control of the infection (for review, see [62]). HBV cccDNA stability itself, but also viral gene expression, is highly dependent on epigenetic factors like posttranslational modification of bound histones (e.g. acetylation and methylation) and alterations of cccDNA itself (e.g. methylation) [29]. Specific control of those factors, responsible for stability or even transcriptional repression of cccDNA, to prevent viral reactivation after stopping antiviral therapy with nucleos(t)ide analogues or during severe immune suppression would be a hallmark for future antiviral therapies [29]. Specific epigenetic modulation of HBV cccDNA versus the host genome will be difficult to achieve, but epigenetic modification of cccDNA has been reported as an essential factor of the antiviral effect of interferon-α in vitro and in vivo [63].

Inhibitors of Reverse Transcription

Based on the success of antiviral intervention of viral polymerases with nucleoside analogues in the case of HIV and herpesviruses, five nucleos(t)ide analogues have finally been approved for antiviral treatment of HBV infection. The first drug, lamivudine (a pyrimidine L-nucleoside) demonstrated antiviral activity against HBV polymerase in vitro [64] and in vivo [65, 66]. Current L-nucleosides also include telbivudine, emtricitabine, clevudine, and torcitabine, but only telbivudine has been licensed so far. The other licensed drugs are adefovir dipivoxil and tenofovir disoproxil fumarate, which are grouped into nucleoside phosphonate prodrugs (nucleotide analogues), and entecavir functioning as a guanosine analogue.

All nucleos(t)ide analogues have the ability to reduce HBV viral load in the serum and improve clinical signs of HBV-associated disease, like serum ALT levels. However, entecavir and tenofovir have proven to be the most potent drugs in this respect, with their high genetic barrier against emerging antiviral resistance and virologic response rates in lowering HBV viral load, often below the limit of detection, in the serum of patients with chronic hepatitis B [67, 68].

All inhibitors of reverse transcription inhibit genome maturation, which is a precondition of envelopment and secretion of infectious viral particles (fig. 1). This block, however, is not curative in the case of HBV and requires ongoing treatment, possibly lifelong if no additional defense mechanisms are active. In addition, those drugs are unable to interfere with transcription of the HBV genome and thus cannot inhibit synthesis and secretion of HBeAg and HBsAg, resulting in persistent antigenemia. Furthermore, reverse transcription of HBV takes place after infection has been already been established with the formation of HBV cccDNA in the nucleus of infected hepatocytes. Therefore, nucleos(t)ide inhibitors are unable to prevent (re)infection of cells. This is in contrast to HIV, where reverse transcription of the viral RNA genome has to take place ultimately after infection in the cytoplasm of the cell before integration of the proviral DNA into the host genome.

Core Assembly Inhibitors

Several nonnucleosidic inhibitors of HBV capsid assembly have been characterized that hamper proper assembly of HBV core particles and thus block reverse transcription of HBV (fig. 1) [69–71]. Although results in vitro and in animal models with these drugs have been very promising, reliable clinical studies have not been reported.
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