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TOPIC HIGHLIGHT

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Viral and cellular determinants involved in hepadnaviral entry

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Abstract

Hepadnaviridae is a family of hepatotropic DNA viruses that is divided into the genera *orthohepadnavirus* of mammals and *avihepadnavirus* of birds. All members of this family can cause acute and chronic hepatic infection, which in the case of human hepatitis B virus (HBV) constitutes a major global health problem. Although our knowledge about the molecular biology of these highly liver-specific viruses has profoundly increased in the last two decades, the mechanisms of attachment and productive entrance into the differentiated host hepatocytes are still enigmatic. The difficulties in studying hepadnaviral entry were primarily caused by the lack of easily accessible *in vitro* infection systems. Thus, for more than twenty years, differentiated primary hepatocytes from the respective species were the only *in vitro* models for both *orthohepadnaviruses* (e.g. HBV) and *avihepadnaviruses* (e.g. duck hepatitis B virus [DHBV]). Two important discoveries have been made recently regarding HBV: (1) primary hepatocytes from tree-shrews; i.e., *Tupaia belangeri*, can be substituted for primary human hepatocytes, and (2) a human hepatoma cell line (HepaRG) was established that gains susceptibility for HBV infection upon induction of differentiation *in vitro*. A number of potential HBV receptor candidates have been described in the past, but none of them have been confirmed to function as a receptor. For DHBV and probably all other avian hepadnaviruses, carboxypeptidase D (CPD) has been shown to be indispensable for infection, although the exact role of this molecule is still under debate. While still restricted to the use of primary duck hepatocytes (PDH), investigations performed with DHBV provided important general concepts on the first steps of hepadnaviral infection. However, with emerging data obtained from the new HBV infection systems, the hope that DHBV utilizes the same mechanism as HBV only partially held true. Nevertheless, both HBV and DHBV *in vitro* infection systems will help to: (1) functionally dissect the hepadnaviral entry pathways, (2) perform reverse genetics (e.g. test the fitness of escape mutants), (3) titrate and map neutralizing antibodies, (4) improve current vaccines to combat acute and chronic infections of hepatitis B, and (5) develop entry inhibitors for future clinical applications.

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Key words: Hepatitis B virus; Duck hepatitis B virus; Infection models; Receptor; Viral attachment; *Tupaia belangeri*; HepaRG; Carboxypeptidase D

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INTRODUCTION

The first step in virus infection is an energy independent attachment of the infectious particle to an accessible structure exposed at the host cell surface. Primary attachment, often characterized by low affinity and reversibility, is usually followed by the passage of the virion to a more specific receptor, which mediates further steps of entry. Both initial attachment and specific receptor recognition often contribute to host specificity and tissue tropism. For enveloped viruses, receptor binding is followed by fusion of the virus with either the plasma or an endosomal membrane. Fusion within intracellular vesicles is regularly triggered by acidification. The universal mechanism of membrane fusion requires conformational changes of virus-encoded fusion proteins leading to a physical approximation and finally merging of viral and cellular membranes[1]. A detailed understanding of receptor binding and membrane fusion is of general interest for molecular virologists and it also provides the basis for therapeutics that interfere with the early steps of infection, as has been successfully accomplished for HIV[2].

HBV and related animal viruses form the family *hepadnaviridae*, which are small, enveloped DNA viruses that cause acute and chronic liver infection. They are

divided into the *orthohepadnaviruses* of mammals and *avihepadnaviruses* of birds[3] (see also the review on HBV taxonomy and genotypes by S. Schaefer in this series). HBV is a serious global infectious diseases and it is assumed that 2 billion people have had contact with that virus[4]. The infection can lead to a chronic carrier state in 5%-10% of immunocompetent adults and up to 90% of infected neonates. Chronic HBV infection is the major cause of liver cirrhosis and hepatocellular carcinoma in numerous regions of the world[5]. While the viral life cycle is still not fully understood, a safe and efficient vaccine has been developed and sensitive tests for HBV surface protein (HBsAg) now allow for reliable diagnosis and screening of blood products. Present therapeutic regimens for HBV address either the host immune system (α-interferon [IFNα]) or inhibit reverse transcription of the viral pregenomic RNA by nucleoside inhibitors (Lamivudine, Adefovir, Entecavir). The latter provoke the selection of resistant or even cross-resistant mutants that will become increasingly problematic to therapeutic control in the future (see also review "Antiviral therapy and resistance of hepatitis B virus infection" by H.L. Tillmann in this series).

To overcome these challenges, antiviral substances that target different replication steps; e.g. inhibitors of viral entry or improved vaccines that counteract the current escape-mutants, are becoming increasingly important. In the past, however, the lack of feasible HBV *in vitro* infection systems hampered investigations aiming in this direction. The only immunocompetent *in vivo* model that could be used for studies related to the infectivity of the virus was based on primary human hepatocytes (PHH) and those of the chimpanzee, which were limited in availability.

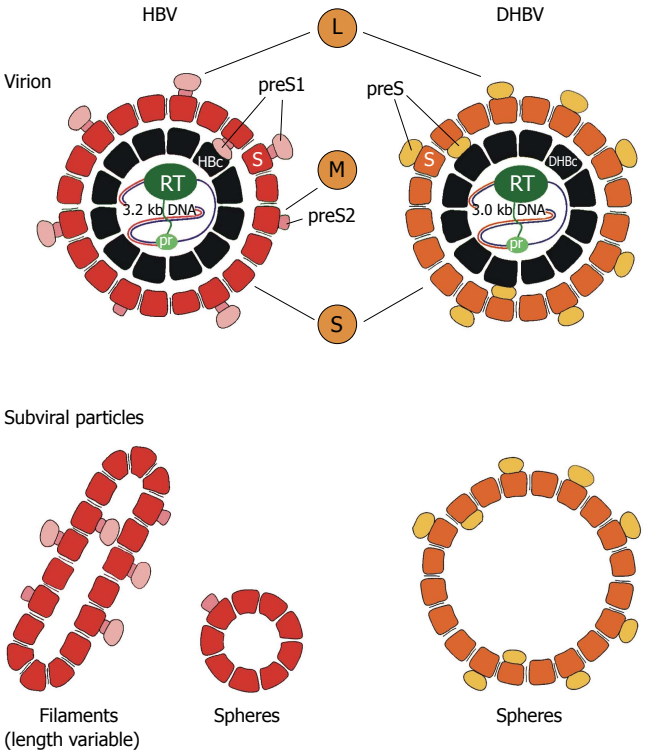


Figure 1 Schematic presentation of human (HBV) and duck (DHBV) hepatitis B virus. The viral DNA is drawn as a single or double line. The viral polymerase is depicted with the primer domain (pr) and the reverse transcription domain (RT). The nucleocapsid (core or HBc/DHBc) is shown in black. Reported encapsidated cellular proteins are omitted. For HBV the surface proteins L, M and S are shown with the S-domain, the preS2-domain and the preS1-domain, whereas for DHBV, L- and S-surface proteins with preS and S-domain are depicted. St, truncated form of DHBV S-surface protein. Non-infectious subviral particles of HBV are shown in filamentous and spherical form and in larger spheroids in the case of DHBV.

ORGANIZATION OF THE HEPADNAVIRAL ENVELOPE

A hallmark of hepadnaviral infection is the constitutive secretion of nucleocapsid-free subviral particles (SVP), mainly composed of the hepadnaviral envelope proteins (large and small in the case of DHBV and large, middle, and small for HBV). HBV-SVPs exist as 22 nm spheres or filaments of the same diameter but variable in length. The HB virions appear in electron microscopy after negative staining as spheres of 45 nm. Virions and SVP contain variable proportions of the three co-carboxyterminal surface (glyco)-proteins; i.e., the large (LHBs), middle (MHBs) and small (SHBs) surface proteins (Figure 1). The SHBs protein is the major component of the virion envelope and the subviral HBsAg particles, while virions and filaments contain more LHB proteins than spheres[6]. In contrast DHBV and DHBV-SVPs are similar in size and shape (55-60 nm)[7] and contain a similar ratio of both envelope proteins (L:S = 1:5). In addition DHBV incorporates a processed version of the DHBs protein consisting of only the two N-terminal transmembrane domains of S and are therefore called S^t[8]. The hepadnaviral surface proteins are products of a single open reading frame and distinguished by three (HBV) or two (DHBV) domains. HBV comprises: preS1 (108 or 119 aa depending on the genotype) only in LHBs, preS2 (55 aa) in LHBs and MHBs, and S (226 aa) common to all three HBs proteins (Figure 2A). All three proteins bear within the S-domain a potential N-glycosylation site (NG) at Asn-146, which is only partially utilised. The second N-glycosylation site at Asn-4 in the preS2-domain is modified in MHBs but not in LHBs[6]. In addition to N-glycans, the preS2 domain of most *orthohepadnaviruses* contains O-glycans[9]. The preS2 domain of both LHBs and MHBs contains a single mucin-type O-glycan (OG) at Thr-37 in genotypes B-H. O-glycans are absent in genotype A, because the O-glycosylation site at Thr-37 is exchanged to Asp in genotype A for an unknown reason[10]. Although potential N- and O-glycosylation sites within the preS1 domain are present, none of them are used due to the cytoplasmic exposition of preS1 during synthesis. In contrast, the two DHBV envelope proteins L (preS+S) and S remain unglycosylated during secretion, but DHBV-L becomes phosphorylated[11] (for more details see review "Avian hepatitis B viruses: molecular and cellular biology, phylogenesis and host tropism" by Funk *et al* in this series). The N-termini of probably all hepadnaviral L-proteins contain recognition sequences that lead to myristoylation at Gly-2[12] while, at least in HBV, more carboxyterminal parts serve as envelopment signals for cytoplasmic core particles[13,14]. Several lines of evidence indicate that hepadnaviral L-proteins adopt dual topologies with half of their preS-domains located inside the particle,

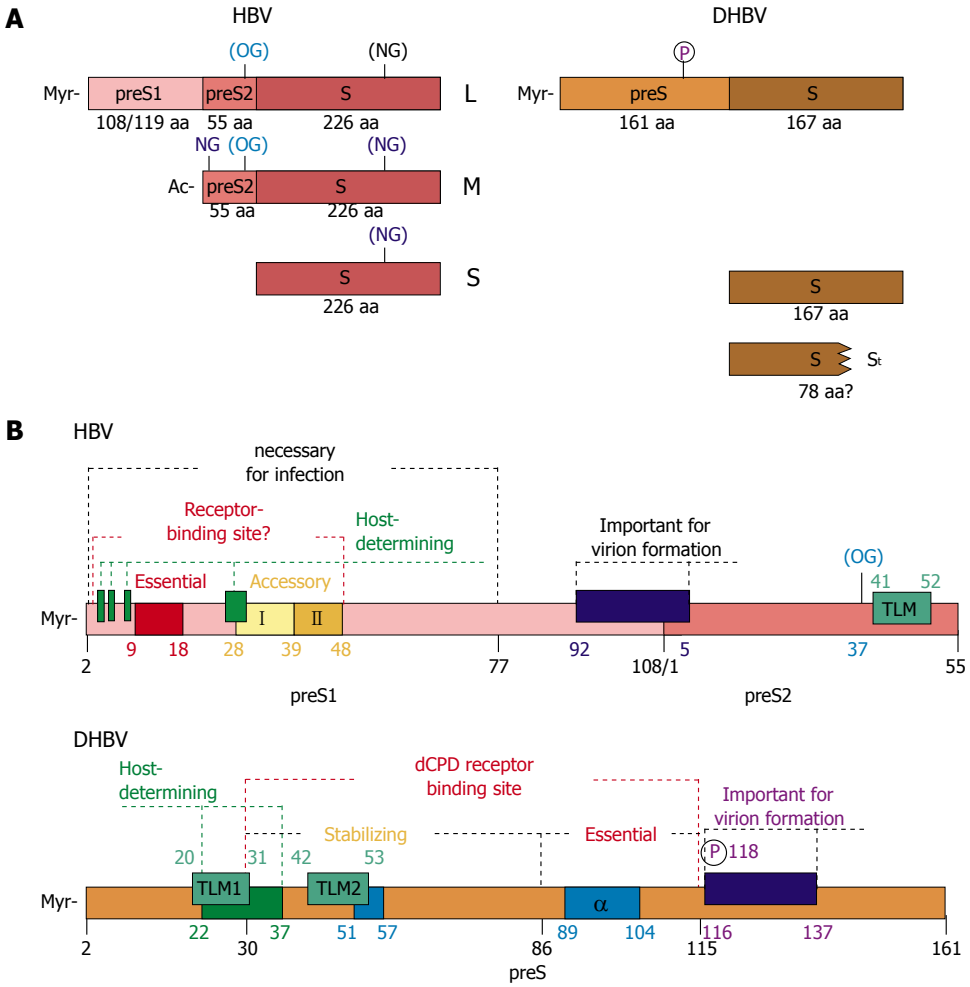


Figure 2 Surface proteins of HBV and DHBV. (**A**) Schematic organisation of the three surface proteins of HBV and the two surface proteins of DHBV. For HBV, used N- and O-glycosylation sites (NG and OG) are shown, (parenthesis indicate partial glycosylation). DHBV surface proteins are non-glycosylated, but the L-protein is phosphorylated at position 118 within its preS-domain. The L-protein of HBV and DHBV is myristoylated (Myr) at Gly-2 of the N-terminus of preS1 or preS, respectively. In case of HBV the preS1-domain encodes for 108 or 119 amino acids, depending on the genotype. The preS2-domain of MHBs is N-terminally acetylated (Ac) and is 55 amino acids long, while a preS2-domain and therefore a special M-protein is missing in DHBV. (**B**) Schematic presentation of preS-domains of HBV and DHBV important for virion formation and viral entry. The host-determining regions are depicted in green. The potential receptor binding site within the preS1-domain of HBV is shown with the essential domain (aa 9-18) and the two accessory domains (aa 28-39 and 39-48, respectively). For DHBV, the carboxypeptidase D (dCPD) binding site is depicted with the essential and stabilizing domains. Alpha helical domains are shown in blue (α). Reported trans-location motives (TLM) are marked in boxes. In DHBV preS, two TLMs are predicted (TLM1 and TLM2). TLM1 overlaps with the host-determining region, while TLM2 is located within the stabilizing sequence of the dCPD receptor binding site. In the case of HBV, the carboxyterminus of the 55 aa long preS2-domain was reported to contain a single TLM. The numbering of HBV preS1 is for genotype D (108 aa). P, phosphorylation site; OG, O-glycosylation; myr, myristoylation.

while the other half is located on the outside[15-18]. Virions and SVP particles bud to a lumen of a post-ER, pre-Golgi intermediate compartment. Therefore the particles contain lipids derived from intracellular compartments, rather than from the plasma membrane. The SHBs subunits of HBV and the subviral particles in the blood are highly cross-linked by disulfide-bonds and do not disassemble in the presence of detergents, unless the disulfide bonds are opened. Interestingly, this tight network of disulfide bridges is not found in DHBV particles. Those can easily be solubilised in mild detergents and both types of surface proteins do not form heterodimeric complexes (unpublished results).

CELL CULTURE SYSTEMS FOR VIRUS PRODUCTION AND ENTRY ANALYSIS

It is known that after artificial delivery of replication competent DNA constructs, later steps of the viral life cycle are not rigidly host-restricted. Transfection of hepatoma cell lines[19-21] or mouse liver cells[22] with replication-competent HBV/DHBV genomes results in the production and secretion of infectious virions. The same holds true for HBV transgenic mice[23]. Furthermore, lipofection of mature core particles, isolated from serum-derived virions into non-permissive hepatoma cells resulted in a full replication cycle of HBV[24]. This suggests that species-specificity of infection is determined at an early step; e.g. viral attachment, entry or fusion. Interestingly, productive infection not only depends on the species origin but also on the state of differentiation of the same cell line. This has been impressively demonstrated in HepaRG cells, which gain susceptibility towards HBV infection only several weeks after induction of differentiation[25]. In case of primary tupaia, human or duck hepatocyte cultures, susceptibility is achieved about one day after attachment

of the perfused and already differentiated hepatocytes, but is lost during prolonged culturing. Since binding and virus accumulation in HepaRG cells is similar in both, differentiated and undifferentiated cells (unpublished data) the restriction may not be caused by the bare presence or absence of a host entry molecule but might be complicated due to the polarisation state of the differentiated cell.

PRIMARY HEPATOCYTE CULTURES AND RELIABLE MARKERS FOR INFECTION

For many years, cultures of primary human hepatocytes (PHH), obtained by immediate perfusion of liver pieces after surgical resection, were the only cells to study viral infectivity[26]. PHH are not easy to handle, cannot be propagated *in vitro* and need particular growth factors for maintenance of their differentiated state[27]. Moreover, the efficiency of HBV infection of PHH *in vitro* was reported to be low leading to only a few percent of cells being infected and a negligible spread within the cell culture. Thus, virus amplification is not achievable. This can be counteracted to some extent by the addition of dimethylsulfoxide (DMSO) during cultivation and the use of 4% polyethylene glycol (PEG) during infection[28-31]. In contrast, infection of PDH with DHBV leads to a spread of infection to virtually every cell in the culture even when very low initial virus titers were supplied. A major drawback of PHH is their limited availability and the heterogeneity in quality with different liver cell preparations resulting in varying susceptibilities towards HBV infection. Furthermore, susceptibility is limited during culture (5-7 d) when DMSO or hydrocortisone is omitted[26,29,30]. Fortunately, primary hepatocyte cultures from *Tupaia belangeri* (PTH) can be infected with HBV as efficiently as PHH cultures of good quality. In contrast to PHH, PTH can also be infected with a primate hepadnavirus from woolly monkey (WMHBV)[32]. Nevertheless, the host range of PTH is restricted to human and primate HBV, since productive infection with rodent hepatitis B virus (woodchuck hepatitis B virus, WHV) is not possible[32].

Hepadnaviruses are non-cytopathic and do not induce conspicuous morphological changes of infected cells. Thus, detection and quantification of infectious virions cannot be achieved by simple virological methods; e.g. plaque test. In general, verification of *in vitro* HBV/DHBV infection should be done by quantification of different markers of an established HBV infection. Conversion of the relaxed circular (rc) DNA genomes of the incoming virus to covalently closed circular (ccc) DNA within the nucleus of an infected hepatocyte is the first marker of a productive HBV/DHBV infection, but is difficult to detect. The amount of cccDNA in just newly infected hepatocytes is very small compared to the large amounts of viral input DNA (as rcDNA), usually needed for efficient *in vitro* infection. Furthermore, DNA-containing particles are also taken up by cells that are not susceptible to infection. This is especially problematic when using embryonic duck hepatocyte preparations because they contain a high percentage of non-parenchymal cells (e.g. antigen-presenting liver sinusoidal endothelial cells). However, quantitative real-time PCR protocols are available that amplify specifically cccDNA, but not rcDNA. With these protocols, specific detection of small amounts of cccDNA is possible, even in the presence of a large excess of rcDNA within infected hepatocytes[24,33]. Verification of specific HBV infection can also be achieved by detection of viral mRNA extracted from cells by either Northern Blot hybridisation or quantitative RT-PCR, usually resulting in higher sensitivity. Less demanding is the detection of secreted viral antigens; i.e., HBeAg or HBsAg, 9-12 d post infection, which could be done by commercially available ELISAs[33,34]. Especially when using enriched or highly purified viral inocula, HBeAg should be the marker of choice, since it is not present in the viral input, in contrast to HBsAg. However, since HBsAg can be detected with higher sensitivity than HBeAg it can be used as a marker, provided that several medium exchanges have been performed prior to the measurement, optimally between day 9-12[33,35]. This is necessary to get rid of input HBsAg, which is released from cells even after removal of the inoculum. There are several reports of alleged infections that determine HBsAg 2 d post infection in the supernatant spuriously assuming that this is progeny viral antigen[36,37]. Using optimized methods, the current detection limit in the PTH-system for purified HBV from human plasma is one HBV particle per hepatocyte in culture plates with 10^5 cells[33,34]. Addition of PEG and DMSO during infection is not necessary to achieve optimal infectivity in PTH[38], as was reported to be beneficial to increase HBV infection of PHH[26,29]. Therefore, *Tupaia belangeri* represent a valuable tool to overcome the restrictions associated with PHH .

HEPATIC CELL LINES

To become independent in the utilization of primary hepatocyte cultures, many groups explored the potentiality of human hepatoma cell lines for infection experiments. HepG2 cells were employed extensively for binding and infection experiments. HepG2-cells exhibit some features of differentiated liver parenchymal cells; e.g. expression of serum albumin[39], and are successfully used for the production of virions after stable or transient transfection of HBV DNA[19,40]. Several studies reported specific binding and uptake of HBV by HepG2 cells[41-46], however, no productive infection was observed by these researchers. In contrast, Bchini *et al*[47] and Paran *et al*[48] reported successful detection of viral antigens upon infection of HepG2 cells that were cultivated with, inter alia, DMSO and 5-aza-2´-deoxycytidine. Unfortunately, these results could not be reproduced by others. In order to search for an explanation for the refractoriness of HepG2 cells towards HBV, Qiao *et al*[43] supposed an inability of the incoming HBV core particles to reach the nucleus, while overexpression of serine protease inhibitor Kazal (SPIK) was suggested by another group[49]. The discovery that the addition of 2% DMSO into the culture medium of primary rat hepatocytes upholds their differentiated state through maintenance of hepatocyte specific detoxification enzymes (e.g. cytochrome P450) was a milestone for hepatic pharmacology[50]. Since hepadnaviral infection depends on the differentiated state of the hepatocyte, this method was

Table 1 Described binding partners for HBV preS1-domain. Numbering of aminoacids (aa) are given for HBV genotype D

Domain	aa	Described interaction partners/binding factors for HBV	Ref.
preS1	10-36	Hepatoma cells bind to preS1 peptide. Inhibition by peptide-antisera	[41]
	19-25	Binding of recombinant subviral particles containing preS1 to human liver. Inhibition by anti-preS1 aa19-25	[142]
	10-36	PreS1-peptide binding not limited to liver cells, also on extrahepatic sites	[143]
	10-36	Partial homology of preS1 aa10-36 with IgA. HBV entry via IgA-binding receptor?	[144]
	18-25	PreS1 aa18-25 crossreact with IgA alpha-1 chain, IgA and HBV use related receptors?	[145]
	10-36	PreS1 aa10-36 binding to 31 kDa protein on HepG2 cells	[146]
	10-36	PreS1-peptide aa10-36 binds to Interleukin 6 (IL6) but not IL3, IL5 and IL7	[147]
	10-36	CHO cells transfected with IL6 cDNA acquire binding sites for preS1 peptide aa10-36	[148]
	10-36	Isolation of 44 kDa protein (HBV-BP) from HepG2 plasma membranes. Homology to SCCA1, human squamous cell carcinoma antigen 1 (human serpin)	[46]
	preS1-GST	Isolation of p80 binding protein from human hepatocytes. Needs preS1 aa12-20/82-90 for binding. p80 binding also to rat hepatocytes	[149]
	preS1	Anti-idiotypic antisera of antibody inhibiting binding of HBV to HepG2. 35kDa protein homology to Glycerinaldehyde-3-phoshate-dehydrgenase (GAPD)	[150,151]
	preS1/ preS2	50 kDa serum glycoprotein interferes with binding of preS1 and preS2 mabs. Soluble form of plasma membrane Protein on human hepatocytes	[152]
	preS1	Yeast two-hybrid assay I: preS1 domain and human liver cDNA library. Isolation of two unknown proteins.	[153]
	preS1	Yeast two-hybrid assay II: preS1 domain and human liver cDNA library. Isolation of cytoplasmic "nascent polypeptide-associated complex alpha polypeptide" (NACA)	[154]
	HBV particles	Asialoglycoproteinreceptor (ASGPR) Ⅰ: preS1-but not preS2-mabs inhibited binding of HBV to ASGPR. SHBs did not bind to ASGPR	[44]
	HBV particles	Asialoglycoproteinreceptor (ASGPR) Ⅱ: HBV uptake by HepG2 and HuH7 (ASGPR+), but not CHO (ASGPR-)	[45]
	HBV particles	Asialoglycoproteinreceptor (ASGPR) Ⅲ: Desialylated HBV only binds to HepG2. Uptake only by susceptible primary human hepatocytes	[155]
	HBV particles	Asialoglycoproteinreceptor (ASGPR) Ⅳ: Increased HBV uptake by HepG2 after desialyation results in HBV infection	[156]

also successfully used for HBV and DHBV infection after preparation of human and duck hepatocytes[26,29,51]. While the method of induced HBV-susceptibility of long-term DMSO-treated hepatoma cell lines was not successful in established hepatoma cell lines (e.g. HepG2 or HuH7), evidence for this principle has been shown for a new hepatoma cell line (HepaRG). This cell line, established from a liver tumour of a female patient suffering from hepatocarcinoma and chronic hepatitis C infection, was shown to become susceptible to HBV and HDV infection upon treatment with DMSO and hydrocortisone[25,52]. The necessity of long term induction of HepaRG differentiation by DMSO and hydrocortisone prior to infection is time consuming, however, it provides the opportunity to decipher cellular determinants of hepatocyte differentiation and their influence on HBV infection for the first time.

In summary, the optimal system for the study of hepadnaviral attachment and entry *in vitro* are primary hepatocyte cultures of Pekin ducks and humans, the latter being very limited in supply and heterogeneous in quality and susceptibility to HBV. Primary hepatocyte cultures from *Tupaia belangeri* and the newly established HepaRG cells can overcome these limitations and provide a nearly unlimited supply of HBV-susceptible hepatic cells for various experimental settings.

CELLULAR AND VIRAL BINDING FACTORS CRUCIAL FOR HBV INFECTIVITY

During the last 25 years, numerous reports on a variety of possible cellular HBV binding partners, involving all three HBV surface proteins, have been published (Tables 1 and 2). Many researchers tried to isolate HBV binding components from plasma membranes of either primary human hepatocytes or established hepatic cell lines (e.g. HepG2) with the help of HBV-peptides or complete (subviral-) particles. In contrast to the DHBV-model discussed later in this review, none of these potential HBV-binding factors has ever been convincingly shown to be essential in HBV entry[53].

FUNCTION OF THE PRES1 DOMAIN

In 1986, Neurath *et al*[41] reported that a synthetic preS1 peptide, comprising amino acids 21-47 and corresponding to amino acids 10-36 in genotype D, E and G binds HepG2 cells and also inhibits HBV binding to this cell line. Furthermore, antibodies directed against this peptide compete with binding of HBV particles to HepG2 cells. They could, however, not show the relevance of their findings for the infection process. These early data were consistent with functional studies of Le Seyec *et al*[54], demonstrating that preS1 amino acids 3-77 of the HBV L-protein are essential for infectivity. In two independent studies, it was further shown that acylation of glycine 2 of preS1 with myristic acid is necessary for efficient hepadnaviral infection[55,56]. Interestingly, a synthetic peptide representing this essential 77 preS1-amino acids, including the N-terminal myristic acid moiety, was able to block HBV infection when added to the medium during infection[25]. Similarly, DHBV infection was sensitive against N-terminal preS-peptides DHBVpreS2-41. Surprisingly the inhibitory activity could be drastically increased by N-terminal myristoylation[57]. The effect of myristoylation

Table 2 Described binding partners for HBV preS2 and S-domains

Domain	aa	Described interaction partners/binding factors for HBV	Ref.
preS2		Binds to polymerised human serum albumin (pHSA). pHSA has affinity to hepatocytes	[157]
		Preincubation of human liver membranes with pHSA induced binding of rec. HBsAg/preS2	[158]
		Natural pHSA only present in minor amount in serum	[159]
	3-16	Natural monomeric HSA binds to preS2 domain aa 3-16	[160]
	3-16	Binding site for pHSA	[161]
	2-24	Polyclonal antisera against preS2 peptide (2-24) neutralised HBV infection of chimpanzees	[71]
	14-32	Immunisation of chimpanzees with preS2 peptide (14-32) protected against infection	[162]
	3-16	PreS2 antisera against aa 3-16 inhibited infection completely, others only partially	[33]
S		Human apolipoprotein H (apo H) binds to small surface protein (SHBs)	[163,164]
		SHBs binds endonexin Ⅱ, now called annexin V	[165]
		Overexpression of annexin V in rat hepatoma cells supported HBV-infection	[166]
		Yeast-derived SHBs alone did not bind to HBV-susceptible hepatocytes	[34]

on the inhibitory potential of preS1 lipopeptides for human HBV[25] was also shown using PTHs[34], and HepaRG cells[58] confirming the fact that myristoylation of the preS-domain of hepadnaviral L-proteins is essential for infectivity, but also indicating that this modification seems to play a role in the context of extracellular inhibition by a peptide. Furthermore, it turned out that the inhibitory activity of the preS1 lipopeptides is dependent on the hydrophobicity of the N-terminal acyl residues. Stepwise increased inhibitory potential of the lipopeptides could be achieved by increasing the chain length from C5 (pentanoyl) to C14 (myristoyl), C16 (palmitoyl) and C18 (stearoyl)[34,58]. The role of the N-terminal myristoylation of preS1 during attachment and entry of HBV is still unclear. One explanation might be that the interaction of preS1 and its receptor might be enhanced by an insertion of myristic acid into the membranes or the receptor. Exposure of a myristoylated peptide or protein ("myristoyl-switch") after attachment is a known element of viral entry mechanisms for some nonenveloped viruses, such as picornaviruses[59] and reoviruses[60].

Using a set of myristoylated HBV preS1 peptides of variable length, the attachment site of HBV was further narrowed down[34]. The data obtained using PTH suggest that: (1) residues containing the first 8 amino acids of preS1 (19 in genotype A) are dispensable, (2) residues in aa 9-18 are essential, (3) residues within aa 19-28 are dispensable, whereas iv) residues of aa 29-48 enhance infection inhibition (Figure 2B). Similar results were obtained in HepaRG cells and PHH in the presence or absence of PEG[58]. Introducing an *E. coli*-based expression system for the production of myristoylated preS-fusion proteins, Engelke *et al*[35] verified that region 9-18 is essential for virion infectivity and identified single amino acids (aa 11, 12, 13 for geno-type D) within this region that are crucial for infection inhibition. Recombinant HBV particles carrying the same point mutations are not infectious. Interestingly, hepatitis delta virus (HDV), an RNA virus that replicates in HBV infected hepatocytes and packages its ribonucleoprotein into the HBV envelope (for a recent review see[61]) can be inhibited by acylated HBV preS-derived peptides with the same specificity[35]. Thus HDV uses at least one common step for entering hepatocytes and is therefore also suitable to study HBV entry events. Surprisingly, HBV-preS1 lipopeptides containing amino acids between aa 49 until aa 78 (the region that has been shown to be important for infectivity of virions[54]) did not further increase but weakened infection inhibition[34,58]. Thus this part of the L-protein may play a role in a different step of infection process. Segment 9-18 is highly conserved with only 3 exchanges in 330 positions of the eleven HBV-genotypes, while in the other segments of 10 aa between aa 1 and 48 there are 4-8 times more exchanges. The unexpected finding that internal deletions of the preS1 sequence 20-27, containing the epitope of the neutralizing monoclonal antibody (mab) MA18/7[6,62]. within lipopeptides 2-48, did not drastically affect infection-inhibition. PreS1-region 20-48 was speculated to contain major B-cell epitopes[63]. Indeed, mab KR359 neutralized HBV infectivity for PHH and binds to aa 19-26[64], whereas another neutralizing mab KR127 binds to aa 37-45. Furthermore, humanised mab KR127 inhibited HBV infection in chimpanzees[65]. Obviously, the binding of mabs to these epitopes hinders the attachment although the essential sequence element needed for infection is elsewhere. While the behaviour of the inhibitory preS1-peptides was very similar in the Tupaia system[34] and PHH/HepaRG cells[58] one point was worth mentioning: the preS1 sequence 20-27 was necessary for full inhibitory potency of the preS1 (2-48) peptide in HepaRG cells. A possible explanation might be that the receptor molecule(s) on Tupaia hepatocytes has binding sites for aa 9-18 and 28-48 comparable to the human receptor(s) but differs with the human binding site at aa 20-27. In a similar approach, Barrera *et al*[66] reported that the preS1-region involved in infection-inhibition of HDV spans residues 5-20 of preS1. However, they needed much higher concentrations of myristoylated preS1 peptides for inhibition in their study (> 5 μmol/L) than in the HBV studies using PTH[34], PHH (< 1 nmol/L)[58] and HepaRG[35]. However this discrepancy might be related to the peptide preparation the authors used, since myristoylated recombinant preS1-proteins obtained from a baculovirus expression system had much higher and comparable specific activities.

The remarkable potency of infection-interference, using acylated preS1-peptide 2-48 at (sub-) nanomolar concentrations, was demonstrated by the kinetics of the peptides using both PTH and HepaRG cells. Even short preincubation periods (30 min) of peptides (100 nmol/L) with the cells are sufficient to block subsequent infection and to induce non-susceptibility of the cells for hours[34,58]. Interestingly, infection could also be blocked by myristoylated peptides (100 nmol/L) after attachment of the viral inoculum at 4℃ had occurred[34]. These experiments supported the assumption that the peptides inhibited infection through binding to the hepatocytes (there possibly addressing a specific receptor), rather than with the virus, although we can presently not exclude the possibility that the peptide might address virions at a specific site on the cell. Using immunohistochemistry, we could demonstrate specific binding of HBV preS1 2-48 peptides to PTH, but not to primary rat hepatocytes or other hepatoma cell lines, such as mouse AML12[34]. The binding was considerably increased when using the respective acylated variants. The binding of the myristoylated HBV-preS-peptides could also prevent binding of highly purified HBV preS1-containing subviral particles, whereas preincubation with myristoylated preS peptides from avian hepadnaviruses did not[34]. However, inhibition of binding required micromolar concentrations of peptides, rather than nanomolar concentrations needed for inhibition of infection[34]. This discrepancy suggested presence of a more abundant low-affinity receptor for HBV on hepatocytes.

This low affinity receptor might be a sulphated glycan, because interaction of HBV and PHH is inhibited by heparin[67]. Furthermore, HBV binds to heparin *in vitro* and could be purified from the plasma of HBV-infected patients by heparin-sepharose columns[68]. Unfortunately, these reports could not clarify the relevance of this interaction for infection. Recently, we could show that HBV infection could be specifically blocked by preincubation of purified virus with heparin, or by treatment of PTH and HepaRG cells with heparinase (unpublished data). Since heparan sulphate proteoglycanes (HSPGs) are enriched in the liver within the space of Dissé, one may speculate that HBV is trapped by liver-specific HSPGs, serving as low-affinity receptors similar to the interaction and entry of apolipoprotein E lipoprotein remnants by liver HSPGs[69]. Specific entry of the virus may subsequently require passage to a yet undefined high affinity receptor(s), which can be blocked by the acylated preS1-derived peptides.

FUNCTION OF THE PRES2 DOMAIN

The M protein of HBV is not essential for infectivity[70], although antibodies against the N-terminal part of preS2 inhibited almost completely HBV infection in PTH cultures[33]. Similar results were reported for polyclonal antisera against HBV preS2 peptides (residue 1-24) *in vivo*[71]. The preS2-domain is present in both M- and L-protein (Figure 2A). However, due to the cytosolic orientation of the preS-domain in L-protein, N-glycosylation at Asn-4 of the preS2-domain occurs only in the M-protein[16,72]. In accordance with this, one of several preS2-antibodies (Q19/10), recognizing aa 1-6 in a glycan dependent manner, strongly bound to the N-terminal part of preS2, but showed the lowest neutralisation potential of all preS2-mabs used in the study[33]. Possibly, the preferential binding of mab Q19/10 to the preS2-domain of MHBs is responsible for the strongly reduced neutralisation potential, which is in agreement with the dispensability of MHBs for infectivity[70]. Furthermore, the HBV M-protein is not essential for infectivity of hepatitis delta virus[73]. A direct role of the N-terminal part of preS2 domain of L-protein for infectivity is still under debate. The carboxyterminal part contains a cell permeable translocation motf (TLM), which was suggested to be involved in HBV entry (Figure 2B)[74]. However, recombinant HBV variants with disturbed or lacking TLM sequence within the preS2 domain of LHBs are still infectious in PHH cultures[75]. The dispensability of the TLM sequence during the entry process of HBV was also detected using the hepatitis delta virus system that uses HBV surface proteins for viral entry. The infectivity of recombinant hepatitis delta virus, containing only the large and small HBV surface proteins, was not affected by the absence or presence of a TLM-sequence within the preS2-domain of the LHBs (Sureau *et al*, Taylor *et al*, personal communication).

FUNCTION OF THE S-DOMAIN

The observation that binding of HBV surface proteins to PTH cultures could be inhibited specifically by myristoylated preS1 peptides[34] argues against a predominant role of the S-domain in initial binding to hepatocytes. PreS1-rich subviral particles from human plasma bound specifically to more than 70% of cultured primary hepatocytes[33,34], while subviral particles containing only S-protein, did not[34]. Addition of the preS1-sequence 2-48 to the S-domain of these particles restored the binding to PTH up to wild-type levels[34]. The main function of the S-domain is morphogenesis, but it contains several elements that participate in entry. The main point is that antibodies against the S-domain (as generated by current S-containing vaccines) could neutralise infection *in vivo* and *in vitro*[33,76-78]. Furthermore, the presence of escape-mutants in HBV-infected patients positive for anti-HBs[79,80] demonstrates the importance of the antigenic domain (residue 100-170) of SHBs for viral spread *in vivo*. Since the S-domain does not contribute directly to binding, the question arises how these antibodies are able to neutralize infection. The S-domain contains 8 Cys residues within the antigenic loop that form inter- and intramolecular disulfide bonds[81,82], resulting in high molecular weight multiprotein complexes[83], while the preS-domain does not contain Cys and forms linear epitopes. We found that a mab recognizing a conformational S-epitope could completely neutralise infection of PTH, while a mab recognising a linear S-epitope failed to inhibit infection completely (90% inhibition)[33]. Therefore, distinct amino acids within the correctly folded antigenic loop of the S-domain might be essential for the uptake process of HBV leading to productive infection. Support for this

assumption comes from infection experiments with HDV, carrying mutant HBV S-proteins in their envelopes. Although short internal deletions, within the antigenic loop of the S-domain (residues 104 to 163), had no effect on HDV morphogenesis, virions with S-deletions between residues 118 and 129 showed reduced infectivity on PHH and HepaRG cells. Single amino acid exchanges within this domain revealed a sequence 119 to 124 (GPCRTC) to be most important for infectivity[52]. This domain contains a CXXC-motive, known to be the active site in protein-disulfide isomerase and related enzymes, involved in catalyzing disulfide-bridge exchanges. In murine leukaemia virus surface proteins, the receptor-binding subunit (SU) domain contains a CXXC motive that is activated after receptor-binding of the envelope transmembrane (TM) subunit. This leads to isomerisation of SU-TM disulfide-bonds and fusion-activation within the TM subunit[84,85]. Whether a CXXC-motive is actively involved in fusion in the case of HDV or HBV, or whether this region binds to a (co)-receptor, is currently unclear.

A clear function in entry can be ascribed to the first transmembrane sequence of the S-domain. It has sequence similarity to type 1 fusion peptides and replacement of the corresponding sequence in influenza virus hemagglutinin with HBV transmembrane sequences confers hemifusion activity of the resulting chimeric influenza virus hemagglutinin[86]. As recently proven for DHBV only the S-domain of the L-protein but not the S-protein itself provides the function of fusion[87]. This observation suggests that the topology of the S-domain in L-protein is different from that of the S-protein. For HBV, this difference is also recognizable in the glycosylation pattern. 50% of SHBs, 30% of MHBs, but 90% of LHBs are N-glycosylated in the S-domain[6].

EARLY EVENTS IN DHBV INFECTION

For more than 20 years, the duck hepatitis B virus model system was successfully used to study hepadnaviral replication. Until recently, one of the exclusive advantages of this system was the possibility to systematically investigate the early events of infection. Even though difficult, a routine preparation of PDH from newly hatched Pekin ducklings, or embryonic hepatocytes from fertilized eggs, has been successfully established in many laboratories to perform *in vitro* infection and infection inhibition studies[51,88]. Guided by the supposition that insights into the DHBV entry process also illuminate the HBV early infection events, a series of studies have been performed, however, only some of them provided satisfactory answers so far. In the following pages we would like to concentrate on the following major issues: (1) What are the roles of the two multifunctional viral envelope proteins, L and S, in entering the hepatocyte and which of their subdomains are involved? (2) Which cellular components have been described to be functionally implicated in these processes? (3) How and where does fusion of the viral and cellular membranes occur and which part of the viral envelope protein acts as a fusion promoter? (4) Which endocytic route is mandatory for the virus in order to productively deliver its nucleocapsid to the nucleus? (5) What determines host specificity of avian hepadnaviruses? (6) Why is the susceptibility of cells towards DHBV infection restricted to differentiated, resting hepatocytes? (7) Finally, taking into account the first functional insights into the HBV entry processes using the recently established *in vitro* systems (HepaRG cells and PTH), we would like to critically scrutinize the question whether there is justified hope that further insights into the DHBV entry processes are relevant for HBV, especially regarding the development of inhibitors for infection.

THE ROLE OF THE DIFFERENT ENVELOPE PROTEINS IN DHBV ENTRY

Soon after the discovery of DHBV and the possibility of replicating the virus *in vitro* using PDH[89,90], several groups characterized DHBV structural proteins biochemically and immunologically, including those that constitute the membranous envelope[91,92]. One approach was the recombinant expression of DHBV-preS fusion proteins to generate antisera. These sera detected two unglycosylated 35 and 37 kDa envelope proteins (the L-protein and its phosphorylated form), co-immunopreciptitated a 17 kDa protein under native conditions (which was identified as the DHBV S-protein by microsequencing) and were able to neutralize DHBV infection *in vitro*[92,93]. Further investigations concentrating on posttranslational modifications of L- and S-proteins and the possible functional implications for DHBV replication revealed, that the N-terminal Glycin-2 of the preS-domain of the DHBV L-protein becomes myristoylated during protein synthesis (Figure 2A)[94]. In addition to myristoylation the DHBV L-protein becomes partially phosphorylated preferentially at Serin 118 in its preS-domain (Figure 2B)[11,95,96]. Both modifications are not required for assembly and secretion of virions. However, while mutations in the phosphorylation sites did not interfere with infectivity of DHBV *in vitro* and *in vivo*[97] the prevention of myristoylation resulted in a loss of infectivity of the mutant in ducklings and a drastically reduced potential to infect PDH *in vitro*[94], (our unpublished results). These findings indicate that at least parts of the DHBV-preS domain, including its N-terminal modification by myristic acid, are involved in virus entry. Functional epitope mapping of mouse mabs obtained after immunization with DHBV particles or recombinant proteins supported this idea[93,98-104]. Three epitopes within the preS domain recognized by neutralizing antibodies were characterized, the first one covering a central part including amino acids 82-109, the second one including a more N-terminal part, amino acids 12-30, and a third part between amino acids 123-137.

The first direct evidence for the participation of the preS-domain of the DHBV L-protein for virus entry came from infection competition experiments using recombinant SVPs composed of only the DHBV L- or DHBV S-protein. These particles were purified from yeast and displayed significant differences in their ability to compete with DHBV infection of PDH with only the L-particles being active[105]. The investigators further showed that only the preS/S-containing particles bind

hepatocytes, supporting the view that the preS-domain as a part of DHBV L plays a pivotal role in attachment and infection of hepatocytes. Following this experimental strategy, Urban *et al* demonstrated that *E. coli*-derived preS-polypeptides, devoid of both the N-terminal myristoyl moiety and the hydrophobic TM-containing S-domain, specifically inhibit DHBV infection *in vitro* with IC_{50}s of about 800 nmol/L. Using a set of terminal and internal deletion mutants it became evident that an uninterrupted innermost preS-domain (amino acids 30-115), including epitopes recognized by neutralizing antibodies, is required for infection inhibition. Since the preS-polypeptide derived from the heron hepatitis B virus (HHBV) also competed with DHBV infection (despite an amino acid variation of 50%), it was evident that the addressed step cannot be responsible for the observed species specificity (see below) between these two avian hepadnaviruses. The striking correlation of the infection competition activity of DHBV-preS polypeptides with their ability to bind duck carboxypeptidase D (including the binding of HHBV preS to dCPD) suggested that it is this molecule which is addressed and inactivated at the surface of hepatocytes[106,107]. Interestingly a second peptide, consisting of the N-terminal 41 amino acids DHBV preS, devoid of CPD-binding and requiring myristoylation of Gly-2 for efficient inhibitory activity (IC_{50} = 200 nmol/L), was identified subsequently[57]. Pre-incubation experiments showed that the peptide addresses a cellular component. Antibodies raised against this peptide and recognizing amino acids 12-23 were able to efficiently block DHBV infection and immunoprecipitate particles indicating that this N-terminal preS-part is exposed on the particle surface and required for infection[108].

Until today, little is known about the role of the DHBV S-protein in DHBV entry. In contrast to the HBV S-protein, DHBV-S is smaller and does not include the antigenic loop, called *a* determinant, which bears epitopes involved in the protective immune response acquired upon vaccination against HBV. Indirect evidence for the involvement of DHBV-S in entry came from the observation that antibodies recognizing the DHBV S-protein, although rarely induced by immunization with DHBV particles[92], can neutralize DHBV-infection of PDH[100]. However, it is not clear if the antibodies directly prevent molecular contacts needed for infection or if they interfere with the formation of preS-dependent interactions by steric hinderance. Both single point mutations within TM-1 of S, resulting in a reduction of hydrophobicity, or the complete replacement of DHBV TM-1 by the HBV TM-1 had no effect on DHBV infectivity (in contrast to the effect the same mutations had when introduced in the TM-1 of L)[100]. It has recently been shown that, besides L- and S-proteins, the DHBV envelope contains a third 10 kDa membrane protein termed S_t[8]. It is a truncated version of the DHBV S-protein, consisting of TM-1, the internal cystein loop and a part of TM-2. S_t, however, seems to play a key role as a chaperone in L-protein translocation. Unfortunately, to date, no further systematic approaches aiming to identify S-specific amino acids that lead to non-infectious virions have been undertaken.

CELLULAR MOLECULES INVOLVED IN DHBV ENTRY

Based on accumulating evidence that the preS-domain plays crucial roles in DHBV infection and also possibly mediates binding to the hepatocyte, Kuroki *et al*[109] performed a biochemical approach for receptor identification and detected a 180 kDa membrane protein in ^{35}S-labeled duck hepatocyte extracts that co-immunoprecipitated with DHBV particles or recombinant envelope proteins. They showed that binding requires only the DHBV-preS part and can be inhibited by neutralizing preS-antibodies. Continuous work by this group and independent efforts by Tong *et al* using GST-preS fusion proteins for affinity purification, identified gp180 or p170 (as named by Tong and co-worker) as the prototype member of a new class of regulatory trans-Golgi network (TGN)-resident carboxypeptidases, soon afterwards termed carboxypeptidase D (CPD)[110,111]. Duck CPD (dCPD) like all other CPDs identified so far, consists of three luminal/extracellular carboxypeptidase E like domains of about 50 kDa each, one transmembrane domain and a highly conserved cytoplasmic tail required for accurate retrieval to the TGN[110,112]. While two of the three luminal/extracellular domains bind Zn^{2+}-ions and exhibit enzymatic carboxypeptidase activity towards yet unidentified cellular proteins that cross the secretory pathway[110,113], the membrane proximal C-domain of dCPD is enzymatically inactive and binds DHBV preS with very high affinity[110,114,115]. However, although the C-domains of human CPD and mouse CPD are homologous to each other and to the dCPD-C domain[116] they do not interact with DHBV preS. Chicken CPD, by comparison, displays only a very weak binding[117]. Interestingly Spangenberg *et al*[117] succeeded in rescuing the binding of DHBV preS to the human CPD C-domain by the introduction of a short dCPD-C domain-derived sequence (amino acids 920-949). Thus, since dCPD is essential for DHBV infection, species specificity could at least partially be explained by the potential of the viral preS-domain to bind CPD.

There is striking experimental evidence that dCPD serves a crucial role in DHBV infection: (1) recombinant DHBV-preS peptides, which are able to bind dCPD *in vitro,* are also active as inhibitors of DHBV infection in PDH[106,107]. (2) soluble dCPD as well as antibodies against dCPD block DHBV infection[115,118]. (3) adenoviral transfer of a dCPD mutant lacking the cytoplasmic TGN-retrieval signal into PDH, abolishes DHBV infection of the transduced cells[119]. (4) dCPD is greatly and selectively down regulated in DHBV infected duck livers and in infected PDH, which is a possible way to exclude superinfection[120], although there is evidence for a second dCPD-independent mechanism[121]. (5) a set of DHBV single point mutants that are deficient in dCPD binding lost their infectivity (unpublished data). However, despite this compelling evidence, it has not been possible to render non-susceptible cell lines that support replication of the viral genome (e.g. LMH cells) susceptible by expression of dCPD[106]. This indicates that either (an) additional factor(s) is/are missing in the dCPD-transduced cell line or that the remarkable and still enigmatic dependency of

hepadnaviral infections on a resting differentiated state of the hepatocyte provide additional constraints that must be overcome as well.

Following the identification of dCPD as a putative DHBV-receptor, extensive work from several groups addressed issues on the sequence requirements of the DHBV preS-domain in order to bind dCPD, as well as details in the mode of dCPD/DHBVpreS interaction[106,111,115,118,122]. Although the results of binding analyses are divergent to some extent, the variations are explainable by the dissimilar techniques that have been applied by the different authors. All findings, however, indicate that a central preS-sequence including amino acids 87-115 (containing major epitopes recognized by neutralizing antibodies) is indispensable for dCPD binding (Figure 2B). The disturbance of the integrity of this sequence abrogated binding entirely. Using quantitative real-time surface plasmon resonance spectroscopy, it became clear that sequences located N-terminal to this essential part (including amino acids 30-86) contribute to the complex stability in a sequence dependent manner, making the interaction of DHBV preS and the C-domain of dCPD to one of the strongest interactions between a viral ligand and a cellular protein[115]. Concerning the mode of interaction, two aspects are noteworthy: First, binding of preS induces conformational changes not only in the viral ligand but also in dCPD. Together with the unusual finding that preS binding to the dCPD C-domain occurs in close proximity to the cellular membrane, the preS-induced dCPD conformational changes indicate that dCPD may play an important role in the fusion of the viral and cellular membrane. If this holds true DHBV entry into hepatocytes would exemplify a novel type of a viral entry mechanism, involving the recruitment of a cellular protein to act as a fusion mediator. However, this hypothesis remains to be supported. Secondly, an extensive 2D NMR structural analysis of the DHBV preS-subdomain that binds dCPD (amino acids 30-115) revealed a mostly unstructured protein with only a short sequence within the essential binding site (amino acids 89-104,) exhibiting the tendency to form an alpha helix (Figure 2B). This is consistent with the observation that a DHBV preS-polypeptide can be treated repeatedly with denaturing agents without losing the ability to bind dCPD with an unaffected K_D of 1.5 nmol/L at 37℃[115]. Thus, the dCPD-binding domain of DHBV represents the first example of a viral protein belonging to the group of intrinsically unstructured/disordered proteins[123] and in that way differs from the well ordered structures found on the surfaces of other enveloped viruses; e.g. influenza virus hemaglutinin or HIV gp120. Structural analyses performed with the whole HBV preS1-polypeptide, as well as with myristoylated N-terminal preS1 peptide fragments, lead to similar results (unpublished data).

Immunization of mice using whole duck hepatocytes and subsequent screening of mabs with respect to their potential to inhibit DHBV binding to and infection of PDH, Guo and Pugh isolated two IgMs exhibiting both activities[124]. They immunoprecipitated a 55 kDa cellular protein that is also detectable in other tissues of ducks and in other birds. Unfortunately, this interesting observation has not been followed up and it therefore remains an open question if the 55 kDa protein represents a primary attachment factor that might be part of the viral entry machinery into hepatocytes.

Following the identification of dCPD as a putative receptor Li *et al*[125] identified a 120 kDa protein preferentially found in liver, pancreas and kidney that displayed binding activity only to some N-terminally and C-terminally truncated variants of GST-preS fusion proteins. Binding depends on the two crucial arginine residues at positions 101 and 102. Purification and mass spectroscopic analysis of p120 identified it as the P-subunit of glycin decarboxylase (GDC), which is an enzyme involved in mitochondrial amino acid metabolism[126]. Recombinant expressed GDC was located to some extent at the cell surface and bound truncated preS-fusion proteins with comparable specificity as the endogenous GDC. Downmodulation of GDC-levels in PDH, either by prolonged cultivation or expression of antisense RNA, resulted in a reduced susceptibility towards infection. Duck GDC has therefore been proposed to act as a co-factor in DHBV infection after proteolytic processing of the DHBV L-protein[127]. Although, the proposed concept of proteolytic activation of the L-protein during entry is attractive, the role of GDC in DHBV infection cannot be exclusive since a DHBV mutant carrying the point mutation R101H is fully infectious, although the respective mutation abolished binding to GDC[108].

MOLECULES INVOLVED IN MEMBRANE FUSION OF DHBV WITH THE HEPATOCYTE MEMBRANE

In contrast to viruses enclosing type 1 fusion proteins on their surface (e.g. HIV, Influenza, Ebola virus), hepadnaviruses do not encode a classical fusion peptide sequence, which becomes proteolytically released from an envelope protein precursor during secretion. It has therefore been hypothesized that instead they use the internally located hydrophobic transmembrane domain 1 (TM-1) as a fusion peptide, similar to the type 2 fusion proteins found in HCV, and alphaviruses[128]. Evidence for this assumption came from experiments with DHBV subviral particles that, upon low pH-treatment, expose hydrophobic domains on their surface, thereby increasing their ability to bind membranes[129]. An elegant subsequent analysis, including reverse genetics, demonstrated that lowering the hydrophobicity of TM-1 in the L- but not the S-protein through alanine substitutions resulted in a loss of DHBV infectivity[87]. Thus, TM-1 serves (a) distinct function(s) in DHBV L- when compared to the DHBV S-protein, with clear involvement in the fusion process on the part of the L-protein.

Subsequent to the observation that a short, possibly amphipatic helix in the C-terminal part of the HBV preS2-domain consisting of amino acids 41-52 is capable of translocating fused proteins, such as GFP or nucleic acids across cellular membranes[74], Stoeckl *et al* predicted two such structural motifs (called trans-location motifs, TLM) also in the N-terminal third of the DHBV preS-domain

(amino acids 20-31 and 42-53, Figure 2B). These two sequence elements are notably conserved among all avian hepadnaviruses. Amino acids 22-41 have previously been shown to be important for a dCPD-independent inhibition of infection mediated by myristoylated DHBV and HHBV preS-peptides[57]. Exchange of 4 highly conserved amino acids in the first motif (D1-mutant) or 3 conserved amino acids in the second motif (D2-mutant) or the concurrent exchange of 7 amino acids (D-1/D2-mutant) resulted in a reduced secretion (D1 mutant) and a loss of infectivity (all mutants) in embryonic hepatocytes, emphasizing the importance of both segments for infection. Interestingly, all mutants bind to and are internalized into cells but cannot be released from an endosomal compartment. This observation supports the preceding idea that the N-terminal preS-part functions at an event downstream of receptor binding and uptake. Based on the loss of the TLM-activity of the mutated sequence, the authors hypothesize that escape from the endosome does not follow a classical fusion mechanism but proceeds via direct translocation of the nucleocapsid and generalize this mechanism for all hepadnaviruses. Although attractive as a model, this hypothesis lacks direct evidence (e.g. that introduction of other TLMs, such as HIV-Tat, can replace the DHBV sequence) and is also not supported by the observation that mutated virions lacking the preS2-containing TLM of the HBV L-protein are infectious *in vitro*[75]. It is also difficult to explain how amino acids 20-53 can contribute to host discrimination between DHBV and HHBV if they provide only a functional TLM in both avian hepadnaviruses (Figure 2B)[130]. Moreover, it has been shown that amino acids 42-51 of TLM-2 as part of DHBV preS does not form an alpha-helix[115]. Thus, the inability of the described DHBV mutants to escape the endosome might be a consequence of the disruption of the interaction with the proposed co-factor and not the disturbance of a TLM-function.

ENDOCYTIC ROUTES USED BY DHBV

Although DHBV infection of PDH is efficient with respect to the percentage of cells that can be synchronously infected, our knowledge of the endocytic routes utilized by the viral particle is still rudimentary. This relates on the one hand to the fact that resting PDH cannot be efficiently transfected by routinely used protocols, making investigations with dominant negative mutants of the endocytic pathway complicated. On the other hand, the low percentage of virus particles that bind to hepatocytes, even when high multiplicities of genome equivalents "MGEs" are offered in the medium[105,131], requires very sensitive methods for a direct visualization of DHBV uptake in hepatocytes by fluorescence microscopy[87]. Consequently, most of our knowledge on the DHBV uptake route comes from results with chemical drugs that are known to interfere with specific intracellular events, which have previously been used to decipher entry routes of other viruses (e.g. binding to charged surface molecules, endosomal acidification, trafficking along microtubules, actin cytoskeleton integrity).

Regarding the question whether productive DHBV infection requires endocytosis and intracellular trafficking events, including acidification as a prerequisite for fusion, early experiments using the lysomotropic reagents ammonium chloride, chloroquine and monensin lead to contradictory results. While Offensperger *et al*[132] showed that infection was abolished with ammonium chloride and chloroquine, Rigg and Schaller reported the contrary[133]. Following DHBV particle uptake using confocal microscopy, Chojnacki *et al* convincingly demonstrated recently that DHBV particles co-localize with fluorescently labeled transferrin in an endosomal compartment 2 h after attachment. The addition of bafilomycin A1, which is a potent inhibitor of vacuolar proton ATPases, at different time points during/after infection clearly showed that transit to the late endosomal compartment is required for infection[87]. Within this compartment, the activation of the DHBV envelope into a fusion competent state is probably not solely triggered by a pH decrease, explaining to some extent the earlier conflicting results, but might include events like CPD-binding and proteolytic cleavage of viral surface proteins. This is consistent with the observation by Breiner and Schaller who, while successfully applying an adenoviral transduction system for PDH, demonstrated that recombinant expression of CPD-mutants lacking the complete TGN retrieval signal abrogated DHBV infection[119]. PH-independent fusion and the dependence of productive infection on endosomal trafficking events have also been confirmed by an independent study[134]. Although there is still some debate on whether the authentic DHBV uptake route into hepatocytes proceeds via dCPD or if dCPD acts at a later stage, these results allow little doubt that accurate vesicular trafficking towards the late endosome, where fusion is expected to occur, is a prerequisite for productive DHBV infection. Using a semiquantitative PCR-based binding assay and chemicals that are known to interfere either with infection of hepadnaviruses or the formation and maintenance of microtubuli and the actin cytoskeleton, Funk *et al* showed that suramin, which is a highly charged urea-derivative and well-known inhibitor of DHBV, RSV and interestingly also HDV infection[135], decreases binding of DHBV to hepatocytes. The authors estimated the number of DHBV binding sites on hepatocytes to be about 10^4/cell, which is remarkably low when compared to other viruses[131]. They further showed that infection at some post-entry step depends on microtubular integrity and that spread in cell culture proceeds via polar egress of new virions from the infected cell[136].

WHAT DETERMINES HOST SPECIFICITY OF AVIAN HEPADNAVIRUSES?

Hepadnaviruses are principally characterized by a narrow host range, restricting *in vivo* infections to only closely related species of their natural hosts. Well-known examples are the restriction of natural HBV infection to humans and chimpanzees. Similar observations have also been made for avian hepadnaviruses. As far as we know, productive DHBV infection exclusively occurs in Pekin

ducks. Related species, such as the Muscovy duck, do not support *in vivo* infection. This *in vivo* species specificity is to some extent reflected by the restricted susceptibility of the respective hepatocyte cultures (e.g., DHBV infects hepatocytes of Pekin ducks but not those from Muscovy ducks or chickens). Interestingly, this is not observed when replication competent viral genomes are artificially transferred into cell lines of different origin. This is best exemplified by the observation that infectious DHBV particles can be produced even in the human hepatoma cell line HuH7[137,138]. It has therefore been assumed that some early step in infection (e.g., attachment, entry, fusion) determines the host range of hepadnaviruses and that the liver specific factors needed for genome replication and virus assembly are not decisive. Comparing the binding of DHBV particles to hepatocytes from Pekin ducks with hepatocytes from Muscovy ducks or chicken hepatocytes and fibroblasts, Pugh *et al* provided evidence for this assumption showing that the difference in susceptibility corresponds to the ability to bind virions and subviral particles. The loss of susceptibility towards infection during prolonged cultivation correlated with a reduction of binding capacity of cells.

The discovery of the heron hepatitis B virus (HHBV) and its property to be not infectious for Pekin ducks and PDH[139], opened the way to investigate host specificity on a molecular level. In that line, Ishikawa and Ganem produced pseudotyped heron hepatitis B viruses (HHBV) with envelopes consisting of HHBV-S and chimeras of the DHBV and HHBV L-protein[130]. They showed that the replacement of the HHBV-preS domain with DHBV-preS rescued the infectivity of HHBV in PDH. This indicated that the preS-domain determines host range without the need for a species-specific "cross-talk" between L- and S-proteins. Further fine mapping revealed that a sequence element containing amino acids 22-37 is sufficient to overcome host restriction *in vitro*[130], (Ishikawa, personal communication). Similar experiments have also been performed with HBV particles that were pseudotyped with chimeric L-proteins carrying WMHBV preS-sequences. Chouteau *et al*[140] found that HBV pseudotyped with a WMHBV envelope lost their infectivity for PHH *in vitro*. However, substitution of only the first 30 amino acids of HBV preS1 could restore infectivity of the chimera, indicating that a short N-proximal region in the L-protein harbors a determinant that contributes to the species specificity of HBV.

Although these data accentuate host restriction of hepadnaviruses as a general theme of this virus family, some unexpected recent observations complicate our understanding. One observation identifies a new hepadnavirus isolated from crown cranes which, despite its close relation to HHBV, infects PDH[12]. Another observation demonstrates that primary hepatocytes from *Tupaia belangeri*, belonging to the order *Scandentia*, are susceptible for HBV infection *in vitro*[32]. Taken together, host specificity of hepadnaviruses is to some extent determined by an early step in infection involving the adaptation of the N-terminal preS-domain of the L-protein to an unknown cellular factor. Moreover, there might be additional viral and host determinants that are to be identified.

WHY IS SUSCEPTIBILITY TOWARDS DHBV INFECTION RESTRICTED TO DIFFERENTIATED, RESTING HEPATOCYTES?

Another hallmark of hepadnaviral infection is its restriction to differentiated resting hepatocytes. Although some attempts have been undertaken there is no proliferating cell line available that supports DHBV infection. The recently described HepaRG cell line, which is the first to support the full replication cycle of HBV, is also not susceptible in a non-differentiated state[25]. HepaRG cells become susceptible for infection only after prolonged treatment with hydrocortisone and DMSO (this process needs at least 2 wk). This induced susceptibility does not correlate with enhanced binding of HBV to differentiated cells. In fact, other hepatoma cell lines, although not susceptible towards infection, bind and accumulate HBV much better than HepaRG cells (unpublished data). This remarkable behavior is supplemented by the observation that initial amplification of cccDNA after *in vitro* infection of embryonic duck hepatocytes increases by the progression of the cell cycle[141]. Thus we have to assume that, in addition to the bare presence or absence of receptor molecules, unknown differentiation-specific and cell cycle-dependent factors of hepatic origin are important key players that are involved in early restriction events.

DO INSIGHTS INTO THE DHBV ENTRY PROCESSES HELP US TO UNDERSTAND HBV INFECTION?

Having now readily available *in vitro* systems to study HBV infection, important questions, such as the nature of the HBV receptor(s) or the characterization of the HBV entry pathway and its inhibition, can now be studied. These investigations are influenced by the results and concepts obtained from the DHBV studies. Just a few examples shall be mentioned: (1) The establishment of transduction systems for PTH, PHH and HepaRG cells will allow us to inspect the relevance of known endocytic pathways for HBV infection. (2) Using highly purified virus preparations, we may further be able to directly follow attachment and entry using sensitive microscopic techniques. (3) The application of microarray-based gene expression profiling will help us identify genes that are becoming up- or down-regulated during differentiation of HepaRG-cells and may therefore also be important regulators of the HBV replication cycle. (4) Having a set of well characterized preS-peptides that interfere with infection, it will be possible to identify the molecule(s) they address. However, it is of utmost importance to be aware of the possible differences in the uptake strategy that might have evolved in the two genera ortho- and avihepadnaviruses with their prototypic members HBV and DHBV, respectively. Two already known examples illustrate this. First, the discovery of dCPD as an important cellular factor for avihepadnavirus infection raised the

question of whether the human homologue plays a similar role in HBV infection. We have performed extensive studies related to that question (e.g., an infection inhibition experiment using soluble human CPD or anti-human CPD antibodies, investigations on whether transfection of human CPD promotes uptake of purified HBV, binding assays using HBV preS and human CPD *etc*.). None of these experiments gave any hint that this molecule is involved in HBV infection (unpublished data). Secondly, Chojnaki *et al*[87] provided unquestionable evidence that DHBV infection depends on the intracellular transport of virions from the early to the late endosome and is thereby blocked by bafilomycin A1. In contrast, HBV infection of HepaRG cells is not influenced by this drug (unpublished results) indicating, along with other evidence, that the two hepadnaviruses enter hepatocytes via different endocytic pathways.

PERSPECTIVES

Since the cloning of the HBV genome, and the discovery of related viruses in the animal kingdom, many aspects of the hepadnaviral life cycle have been unravelled with the help of established hepatoma cell lines and the transfection of replication competent genomes. These cell lines were, however, not suitable for infection experiments, possibly due to the lack of one or more unknown factors required for infection. A huge and still growing list of binding partners for HBV and DHBV have been reported since then, however, none of them have been convincingly shown to be related to HBV infection, and only Carboxypeptidase D has been shown to play a crucial role for the infection of avihepadnaviruses. For over 20 years, primary human hepatocytes were the only possible *in vitro* system for studying HBV infections, which created strong limitations. These limitations have become obsolete with the discovery of the HepaRG cell line and the usability of PTH instead of PHH to study HBV infection in an accurate manner. Although both systems bear their specific difficulties (e.g., Tupaias have to be bred in captivity, and HepaRG cells require a laborious protocol in order to render them susceptible for infection) this should be manageable.

With these models it will be possible to characterise cellular attachment factors and entry receptors for HBV. It will further be possible to decipher the entry pathway(s) of HBV and thereby to relate this important pathogen to other viruses.

In light of the discovery of a crucial domain within the preS1 part of the L-protein, the available HBV vaccines have to be improved. Although the current vaccine has been shown to be safe and effective, it consists only of S-protein containing recombinant particles and relies solely on the generation of protective antibodies recognizing this part of the viral surface protein, which as we now know do not counteract binding of the virus to its target cells. This allows the emergence of escape mutants, frequently arising especially under antiviral therapy, with reverse transcriptase inhibitors (e.g. lamivudine). Inclusion of the preS1 sequences into vaccines should therefore directly protect against infection.

The discovery of HBV preS1-derived lipopeptides as potent inhibitors of HBV entry will not only stimulate further investigations aiming to decipher the early infection events, they also represent a novel antiviral approach for the treatment of acute and chronic hepatitis B and hepatitis delta, similar to the HIV-peptide entry-inhibitor T20 (also called *enfuvirtide* and *fuzeon*). However, compared to T-20, the most active HBV inhibitor (HBVpreS/2-48$^{\text{stearoyl}}$ and also called Myrcludex B) approximately displays a 1000 fold higher specific activity. This substance, which is presently under preclinical development, could be very useful for post-exposure prophylaxis or the inhibiting of re-infection after liver transplantation. Whether efficient entry inhibition will also be beneficial in the treatment of chronic HBV and HDV infections, alone or in combination with current therapies, is an interesting objective to be addressed in a clinical trial in the near future.

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