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TOPIC HIGHLIGHT

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Virus entry mediated by hepatitis B virus envelope proteins

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Abstract

Hepatitis B virus (HBV), a major cause of human liver disease worldwide, encodes three envelope proteins needed for the attachment and entry of the virus into susceptible host cells. A second virus, hepatitis delta virus, which is known to enhance liver disease in HBV infected patients, diverts the same HBV envelope proteins to achieve its own assembly and infection. In the lab, lentiviral vectors based on human immunodeficiency virus type 1 can be assembled using the HBV envelope proteins, and will similarly infect susceptible cells. This article provides a partial review and some personal reflections of how these three viruses infect and of how recipient cells become susceptible, along with some consideration of questions that remain to be answered.

Key words: Hepatitis B virus; Hepatitis delta virus; Receptor; Envelope proteins; Entry

Core tip: The recent identification of a key receptor for hepatitis B virus and hepatitis delta virus provokes a wider discussion of how different cells may become susceptible to infection when the receptor is provided.

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INTRODUCTION

The following is a very brief introduction to the envelope proteins of hepatitis B virus (HBV) and of how they can be used to facilitate the assembly and infection by HBV and two other viruses. More detailed recent reviews are available elsewhere[1,2]. HBV encodes three envelope proteins, commonly referred *via* their size, as large, middle and small, or L, M and S. They have S as a common C-terminal domain. M contains additional N-terminal sequences, referred to as preS2. L, relative to M, has additional N-terminal sequences, referred to as preS1. L, M and S, exist with and without carbohydrate modifications. The L protein undergoes an essential myristoylation of a glycine residue penultimate to the N-terminus.

Hepatitis delta virus (HDV) exists in nature in some patients who are also infected with HBV. While HDV uses a totally different method of genome replication than HBV, its final assembly is entirely dependent upon the envelope proteins of HBV; thus, it only produces infectious progeny in hepatocytes already infected with HBV (or producing envelope proteins from fortuitously integrated HBV DNA). HDV can infect a new hepatocyte in the presence or absence of HBV. In the humanized chimeric uPA mouse model, human hepatocytes infected with HDV alone can persist for at least six weeks in the absence of HBV, a so-called latent infection, with ultimate rescue of virus production dependent on a follow-up infection by HBV[3]. Such studies suggest that in patients conversion of a latent HDV mono-infection may contribute to the persistence of HDV even in patients with low HBV replication.

Many labs have produced retrovirus vectors that have

been engineered to carry novel genes, that can be expressed following integration of the provirus; that is, the DNA copy of the viral RNA genome. Such retrovirus vectors have been assembled using envelope proteins of the wild type retrovirus, as well as those derived from other viruses, such as vesicular stomatitis or Ebola viruses[4]. However, what is relevant here is that such a retrovirus vector was assembled using the envelope proteins of HBV and acquired the host cell specificity of HBV and HDV[5].

ENVELOPE PROTEIN DETERMINANTS ESSENTIAL FOR INFECTIVITY

Several labs have shown that an essential determinant for infectivity using HBV envelope proteins is located near the N-terminus of the preS1 domain of the L protein. The data for this are very good and include evidence that a synthetic peptide containing such sequences, especially if it is myristoylated, will act as a potent inhibitor of virus entry[6]. Interestingly, unlike the L and S proteins, the M protein can be omitted in experimental situations without a loss of assembly or infectivity, at least for HDV[7,8]. Thus, the role of M in HBV infection is unclear. The carbohydrate moieties attached to the three envelope proteins are essential for particle assembly and infectivity[2]. The three envelope proteins share at least four transmembrane domains. One shared loop is presented on the surface of the virus. This loop, containing the so-called "A" determinant, is highly antigenic. Certainly antibodies raised against the S protein will neutralize virus infectivity. A widely used, S protein based vaccine, protects individuals against both HBV and HDV.

WHAT THE HOSTS PROVIDE

Until last year, almost the only cells that could be infected by these viruses were primary human hepatocyte cultures. Such cultures are difficult both to establish, and can rapidly lose susceptibility to infection. Some non-human primary hepatocyte cultures were similarly susceptible; examples include chimpanzee and tupaia hepatocytes. Also, primary woodchuck hepatocytes are susceptible to HDV. Several years ago a cell line, HepaRG, was derived from a human liver tumor, and it is susceptible to infection by HBV and HDV[9]. These cells require specific *in vitro* culture conditions and they are almost as difficult to maintain as primary human hepatocytes.

For many years groups worldwide had struggled to identify, and confirm the functionality of host molecules needed for HBV and HDV entry. Many candidates were identified but none were shown to be sufficient for virus entry and initiation of replication[6]. This situation was changed dramatically in late 2012 by a report from Yan *et al*[10]. They used a synthetic peptide corresponding to the myristoylated N-terminus of the HBV preS1 protein to affinity select a candidate virus receptor from hepatocyte cultures. These hepatocytes were derived the treeshrew (*Tupaia belangeri*). The purification procedure used near-zero-distance photo-cross-linking and tandem-affinity purification and yielded a single protein. Then, with mass spectrometry, they identified the protein as the sodium taurocholate cotransporting polypeptide, NTCP, also known as SLC10A1[10]. NTCP transports bile acids from the blood into the liver. Their subsequent findings included evidence that the cDNA clone of human NTCP, when transfected into human hepatocellular carcinoma cell lines, specifically HepG2 and Huh7, conferred susceptibility to both HBV and HDV. Susceptibility could be inhibited by the synthetic preS1 peptide. Furthermore, in susceptible primary hepatocyte cultures and HepaRG cells, suppression of NTCP with specific small interfering RNAs inhibited susceptibility. It remains to be shown whether the NTCP functions *in vivo* as well as *in vitro*. However, the situation is very promising in that other studies have already shown that the synthetic preS1 peptide to which it binds, is a potent inhibitor of *in vivo* infections of human hepatocytes (as transplanted into mice) by both HBV and HDV[11].

In their initial paper, the authors made use of prior studies by others of the established role of NTCP in the liver. This protein is 335 amino acids in length and is predicted to have 9 trans-membrane domains[12]. They thus compared the sequence of NTCP of the crab-eating monkey (*Macaca fascicularis*) (since hepatocytes from these monkeys are not susceptible to HBV infection) and humans. They noted that the primary sequence of the monkey protein has a limited number of specific differences relative to the human protein. The authors experimentally demonstrated that replacement of just nine contiguous amino acids of monkey NTCP with the corresponding sequence from the human protein produced a cDNA, that when transfected into nonsusceptible liver cell lines, rendered them susceptible to infection by both HBV and HDV.

Several commentaries by others on the NTCP findings have since been published[13-16]. And, several labs, in as yet unpublished studies, have confirmed the ability of recombinant NTCP to facilitate entry of HBV and HDV into otherwise non-susceptible cell lines.

Moreover, two follow-up studies by Wenhui Li and coworkers have been published in 2013. In the first study, the focus was on the woolly monkey hepadnavirus, WMHBV, and its ability to infect tupaia hepatocytes[17]. Their findings include evidence that cDNA of the woolly monkey NTCP when transfected into nonsusceptible liver cell lines renders them susceptible to both WMHBV and HDV pseudotyped with WMHBV envelope proteins. The authors thus suggest that orthologs of NTCP might function as receptors for all known primate hepadnaviruses. In the second study they examined mouse NTCP, and considered many exchanges with the human sequence to determine what might be need to achieve susceptibility[18]. They did find a region, of only two amino acids, that was sufficient to achieve HDV (but, as discussed below, not HBV) susceptibility. These changes

were not at the same location as the changes needed on the crab-eating monkey NTCP[10]. We can infer that the secondary and maybe the tertiary structure of NTCP are needed for susceptibility. It is relevant that a recently published study by Meier *et al*[19] reports that cultured mouse hepatocytes will bind the preS1 peptide even though no infection is detected. This suggests that a *bona fide* binding site may be available on the mouse NTCP, but is insufficient for HBV infection. Scheick *et al*[20] suggest an additional step might be at the level of membrane fusion.

It is important to note that earlier studies showed that HDV can actually infect mouse hepatocytes *in vivo*, although to only a low extent[21]. Therefore, the changes introduced into the mouse NTCP, as described above, may have increased the affinity of preS1 binding without, for HDV, a need to alter a second step (*e.g.*, fusion) in HDV infection. Thus, it is possible that mice made transgenic for the modified NTCP will be readily infected with HDV. (They will not be infected by HBV and as such, may not be sufficient to create a new model of HBV induced hepatocellular carcinoma.) Or, perhaps, even overexpression of the unmodified mouse NTCP would allow more efficient *in vivo* infection by HDV.

ATTACHMENT AND ENTRY

Recent studies have shown that the initial attachment of HBV to susceptible cells depends upon glycosaminoglycans present on the cell surface[22-24]. This is a necessary but not sufficient step. Furthermore, even after virus has attached, entry can be significantly inhibited by a number of agents[25]. Of the latter, the most interesting inhibitor is the previously mentioned synthetic peptide, corresponding to the N-terminus of the preS1 domain[6,11]. It would seem that even after attachment, (additional) interactions with the putative host cell receptor remain to be made[25]. It could also be that multiple such interactions are needed to facilitate entry.

FURTHER DISCUSSION AND OUTLOOK

Clearly, the combination of attachment, entry, and initiation of HBV replication is more complex than that of HDV. A partial explanation of this difference comes from earlier studies: Firstly, transcription of RNA from HBV covalently closed-circular DNA (CCC DNA) depends upon host transcription factors, some of which are specific for the liver[2]. Additionally, CCC DNA formation is inefficient or even absent in transgenic mice[26]. Secondly, HDV replication can be initiated in many mammalian cell lines, not necessarily those that are derived from liver tissue[1]. Nevertheless, it also remains possible that it is the entry of HBV that differs from that of HDV; that is, it requires host contributions in addition to the expression of NTCP.

As exemplified below, there are now multiple questions that can be asked of the entry mechanisms of other members of the hepadnavirus family. Certainly it is time to reinvestigate infection of duck hepatocytes by duck hepatitis B virus (DHBV). It was first found by an affinity strategy, that a host protein identified as carboxy peptidase D, binds the DHBV envelope protein[27]. However, no study has yet been able to confer susceptibility by expression of this protein[28,29]. Some studies have suggested a co-receptor, but again, this has not led to susceptibility[30]. Independently, it has been shown that the myristoylated N-terminus of the DHBV preS region is needed for infectivity[28,31]. Therefore, maybe an analogous application of the powerful peptide affinity strategy used by Wenhui Li and coworkers[10], will identify a functional DHBV receptor. And, an alternative and specific approach would be to directly test whether the duck NTCP can function as receptor.

Also one can examine the newly reported HBV of bats[32]. This virus shows more sequence relationship to the orthohepadnavirus than to the avi hepadnaviruses. Novel questions can now be asked, such as what is the sequence of the bat NTCP, and would the cDNA of this gene when expressed in human cell lines, confer susceptibility to infection.

Another question is why HDV but not HBV will infect primary woodchuck hepatocytes. This may or may not be analogous to the abovementioned situation where human NTCP cDNA transfected into non-liver cell lines will allow HDV but not HBV infection. Presumably the woodchuck hepatocytes provides an acceptable NTCP for HDV infection, but now one can address questions such as whether expression of one or more human cDNAs will make the cells susceptible to HBV.

In all these studies of cells susceptible to HDV or HBV, there is a question of the efficiency with which cells are infected. Even with primary hepatocytes and HepaRG cell line, the efficiency is typically less than 1%. Several possible explanations include low levels of expression of NTCP or low levels of NTCP that is functionally active. This efficiency is increased approximately 10-fold by the presence of 2%-4% polyethylene glycol, PEG[9,33]. Studies have shown that infection in the presence of PEG is still be inhibited by the synthetic preS1 peptide, indicating that the infection is still specific for the NTCP receptor. PEG is most likely causing aggregates of virus particles; certainly, at 6%-10% PEG the virus can even be collected by low speed centrifugation. Thus, it may be that the low levels of PEG produce aggregates that can more efficiently make use of the available NTCP receptor present on the surface of the hepatocytes. Future studies with cDNA transfected cell lines will no doubt test the relevance of the amount of NTCP expressed at the cell surface and infection by un-aggregated *vs* aggregated virus. Perhaps controlled mixtures of functional and non-functional NTCP cDNAs will also help clarify what is involved.

In summary, the important new finding of NTCP as a functional receptor has opened the way for much more basic research concerning the entry of HBV and HDV into susceptible cells. And, in turn, such information will

allow new applied research, possibly providing additional novel ways in which such entry can be interfered with, all new armamentaria for treating chronic HBV and HDV infections.

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