THE ABSENCE OF HEPATITIS B VIRUS M PROTEIN EXPRESSION RESULTS IN IMPAIRED VIRION SECRETION WITHOUT AFFECTING ITS INFECTIVITY

CĂTĂLIN LAZĂR1, CRISTINA DOROBANȚU1, ALINA MACOVEI1, NORICA BRÂNZĂ NICHITA1,

1Institute of Biochemistry of the Romanian Academy, Department of Viral Glycoproteins, Spaiul Independenței 296, Bucharest 17, Romania

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A significant amount of data is accumulating on the function of the envelope proteins in the life cycle of the hepatitis B virus (HBV), an important human pathogen. While the role of the large (L) and small (S) envelope in virion assembly, secretion and infection is well established at molecular level, the role of the middle (M) protein in these processes only begins to be defined. The results are often variable and controversial, depending on the cellular systems or the HBV DNA expression constructs and strategies used.

In this study we analysed the ability of HBV completely lacking the M protein to assemble in different cell lines of both hepatic and non-hepatic origin and the infectivity of the resulted virions, using the HepaRG differentiated cells. Our findings show that HBV was assembly and secretion competent regardless the cell line used for its production. However, only the virions secreted from hepatic cells were infectious. The HBV particles lacking the M protein were secreted less efficiently; however, this effect was not sustained at the level of subviral particles (SVP) secretion. Our results also showed that the M protein was not required in the early steps of HBV infection in HepaRG cells.

Key words: Hepatitis B virus, infection, envelope proteins.

INTRODUCTION

Infection with Hepatitis B virus (HBV) is currently diagnosed in more than 400 million people worldwide and often results in severe liver disease and death (1). The virus is a the Hepadnaviridae family member, with a partially double stranded DNA genome of approximately 3.2 kb protected by a viral envelope consisting of a lipid membrane in which multiple copies of the viral surface proteins are inserted (2). These proteins, designated large (L), middle (M) and small (S) are translated from alternative start codons of the same open reading frame (ORF) and have a common S domain at the C-terminal region (3). The
S protein consists of a membrane spanning domain of 226-amino-acids; the M protein includes the S domain and an additional N-terminal pre-S2 ectodomain that is 55 amino acids in length and the L protein contains the M sequence and an extra pre-S1 domain of 109 amino acids (4). A key characteristic of infection with HBV is its very narrow host range, most likely a consequence of the specific interaction between the viral envelope proteins and the human hepatocytes surface receptor(s).

The development of the HepaRG cell line, permissive for HBV infection in vitro, has enabled the investigation of host cell proteins with a potential role in HBV infection, such as caveolin-1 and dynamin-2, which were shown to be important for viral endocytosis (5). Although the exact mechanism of HBV entry is still poorly understood, and the identity of the receptor remains elusive, infectivity determinants have been recently mapped and assigned to discrete domains within the sequence of the envelope proteins (6). Certain distinct elements, crucial for HBV infectivity have been clearly associated with the pre-S domains of the L protein: the 2-77 amino acid sequence of the pre-S1 domains, the L-myristoylation and the antigenic loop of the S domain (7, 8). While the importance of the L protein in viral infection has been well established and confirmed also using the Hepatitis delta virus (HDV), a surrogate model for studying HBV entry (9), the role of the M protein in this process is still a matter of controversy. Studies published by Le Seyec et al. (10) and very recently by Ni et al. (11) showed that the pre-S2 region and the M protein are dispensable for HBV infection. However, a potential HBV receptor binding region located in the pre-S2 domain was suggested by another report (12). Also, incubation of HepG2 cells with the vesicular stomatitis virus pseudotyped with the M protein resulted in the highest infection rate, compared to all other envelope protein combinations (13). Our own published results showed that treatment of HBV producing cells with protein folding inhibitors (iminosugars), which promote degradation of the M protein, resulted in secretion of virions with reduced infectivity (14).

In this study we analysed the capacity of HBV completely lacking the M protein to assemble in different cell lines of both hepatic and non-hepatic origin and the infectivity of the resulted virions, using the HepaRG differentiated cells.

Unlike previously reported investigations (6, 11), the HBV genome (1.1 fold overlength, genotype ayw) was cloned into the pTriEx 1.1 containing a hybrid cytomegalovirus (CMV) enhancer coupled to a modified chicken β-actin promoter (CAG), which has been shown to drive high expression of proteins in various cell types and tissues (15). This promoter was fused with the nucleotide 1818 of the HBV genome. Our findings show that although HBV was assembly and secretion competent regardless the cell line used for its production, only the virions secreted from hepatic cells were infectious. The HBV particles lacking the M protein were secreted less efficiently, however this protein was dispensable for infection of the HepaRG cells.
MATERIALS AND METHODS

CELLS

HepaRG cells were grown in William’s E medium (GIBCO, UK) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml insulin, and $5 \times 10^{-5}$ M hydrocortisone hemisuccinate, as described (16). HepG2 and HEK 293T were grown in RPMI 1640 medium (Euroclone, UK) containing 10% FBS, supplemented with 100 units/ml penicillin, and 100 µg/ml streptomycin. HepG2 2.2.15 cells stably transfected with two copies of the HBV genome (17) were grown in the same medium as HepG2 cells except that 200 µg/ml of G418 (GIBCO, UK) was added.

PLASMIDS

pTriExHBV1.1 and pTriExHBV1.3 were a kind gift from Dr. David Durantel (INSERM, U871 Lyon, France). The mutation that changes the START codon of the M protein from ATG to ACG (Met→Thr) was introduced by sequential PCR, using two sets of primers and the pTriExHBV1.1 as template. The first set of primers consisted of the following oligonucleotides: 1) 5'- ATAAAAAGCGAGCGGCGGCCGCGCAAC-3' and 5'-GGCAGCACAGCCTAGCAGCCATGGAAACG, while the second set of primers consisted in the sequences 5'-CTCATCCTCAGGCCAGTGGAATTCCAC-3' and 5'GTGGAATTCCACTGCGTGGCCTGAGGATGAG-3', respectively. The resulting plasmid was further called pTriExHBV1.1ΔM.

TRANSFECTION OF MAMMALIAN CELLS

The cells were transfected at 80% confluence, with either lipofectamine LTX (Invitrogen) (Huh7, HepaRG, HepG2) or polyethyleneimine (Sigma) (HEK 293T), according to the manufacturer’s instructions. The plasmid DNA varied between 1–2 µg / per 10 cm² well (~ $10^6$ cells), depending on the transfection reagent used. When 150 cm² flasks were used, the DNA quantity was scaled-up accordingly. Because of the higher cell toxicity observed when lipofectamine was used, the culture medium containing the transfection reagent was replaced with fresh RPMI, at 4h post-transfection.

SDS-PAGE AND WESTERN BLOTTING

Huh7 or HEK293T cells transfected with different HBV DNA-containing vectors were harvested 24 h pi. Cells were lysed in a buffer containing 0.5% Triton X-100, 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, and 2 mM EDTA (Triton TSE buffer) and a mixture of protease inhibitors (Sigma). Cell lysates were clarified by
cenrifugation at 10,000 X g for 15 min and soluble proteins were separated by sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) and transferred to nitrocellulose membranes using a semidyre electroblotter (Millipore). HBV proteins were detected with anti-preS1 monoclonal antibodies (MAb) (Santa Cruz, dilution 1/1,000), anti-preS2 MAb (Abcam, dilution 1/1,000) or with anti-S polyclonal Ab (Fitzgerald, dilution 1/2,000) followed by horseradish peroxidase-conjugated anti-mouse Ab (Amersham, dilution 1/2,000). The proteins were further visualised using an enhanced-chemiluminescence (ECL) detection system (GE Healthcare), according to the manufacturer’s instructions.

HBV PURIFICATION AND INFECTION OF DIFFERENTIATED HepaRG CELLS

HepG2, HepaRG and HEK293T cells and HepG2.2.15 cells transfected with pTriExHBV1.1 or pTriExHBV1.1∆M were grown in 150 cm² flasks. The culture medium was changed with a fresh one every 3 days. After 9 days the medium was harvested and clarified by centrifugation at 10,000 g to eliminate cell debris. The virus particles were concentrated 150 fold by ultracentrifugation through a 20% sucrose cushion, in a SW 41 Ti Beckman rotor at 36,000 rpm, for 4 h. The pellet was resuspended in phosphate-buffered saline (PBS) and quantified using a standard curve. The HepaRG cells were seeded in 6-well plates and differentiated as described (16). Approximately 10⁶ differentiated cells were used for infection with 50 µl of concentrated HBV virus containing 1.2 × 10⁹ genome equivalents (GEq). Next day the viral inoculum was removed, cells were washed 3 times with PBS and 3 ml of fresh medium was added. Infected HepaRG cells were harvested after 9 days and the HBV DNA was analyzed by quantitative (q) reverse-transcription (RT) PCR.

EXTRACTION OF HBV DNA AND SOUTHERN BLOTTING

Encapsidated viral DNA was purified by phenol-chloroform extraction. Briefly, 20 µl of 150 fold concentrated virus were lysed in 500 µl TLB buffer (50 mM Tris-HCl pH 8, 1 mM EDTA, 1% Nonidet P40) for 30 minutes on ice. The supernatant resulted after centrifugation for 5 min at 10,000 g was adjusted to 10 mM Mg-acetate. A mixture of DNase/RNase (Sigma) 10 mg/ml was added and incubated for 2 h at 37°C. Then 12.5 µl EDTA 0.5 M, 10 µl 5 M NaCl, 7.5 µl Proteinase K 20 mg/ml and 11.5 µl SDS 20% were added and incubated over night at 42°C. Viral DNA was extracted in 0.5 ml phenol:chloroform:isoamyl alcohol mix followed by isopropanol precipitation. The DNA pellet was resuspended in DNase/RNase-free water and 15 µl of this sample were separated on a 1.2% agarose gel. The DNA was transferred to a Hybond-N+ membrane (GE Healthcare) using a vacuum transfer blotter (Biorad). The HBV DNA fragments were hybridized with a fluorescein-labelled probe obtained by random priming using the
HBV DNA genome as template. Identification was done with anti-fluorescein AP-conjugated monoclonal antibodies (GE Healthcare).

**QUANTIFICATION OF SECRETED HBV BY qPCR AND ELISA**

A volume of 10 µl of concentrated HBV resulted from transfected HepG2, HepaRG, HEK or HepG2.2.15 cell medium was used to either determine the level of HBsAg expression using the Monolisa HBsAg Ultra kit (Bio-Rad) or for viral DNA extraction, as described earlier. The extracted DNA was quantified using a Corbett Rotor Gene 6000 real-time PCR system and the SensiMix Plus Kit (Quantance). The primers designated to amplify a 279-bp HBV-specific fragment (data not shown).

**QUANTIFICATION OF HBV TRANSCRIPTS FROM HepaRG INFECTED CELLS**

Total RNA from control or HBV-infected cells was isolated using an RNeasy minikit (Qiagen) and used to measure the viral replication. The RNA was quantified using a Corbett Rotor Gene 6000 real-time PCR system, following reverse transcription and amplification using the SensiMix One-Step Kit (Quantance) and the same primers, as for secreted HBV. Amplification of actin-specific RNA was used as loading control in all samples.

**RESULTS AND DISCUSSION**

**HBV ASSEMBLY AND SECRETION DRIVEN BY THE pTriEx PLASMIDS**

To determine the efficiency of HBV replication, assembly and secretion driven by the chicken β-actin promoter, HepaRG cells were transfected with either pTriExHBV1.1 or pTriExHBV1.3 plasmids, containing 1.1 or 1.3 of the HBV genome overlength (Fig. 1). The ability of these constructs to drive HBV pregenomic RNA synthesis and support viral replication was determined by purification of encapsidated virions from both cells and supernatants, followed by Southern blotting analysis. As shown in Fig. 2A, DNA replication intermediates were identified in cells transfected with pTriExHBV1.1 or pTriExHBV1.3 plasmids, as well as their corresponding supernatants, using a HBV-specific probe for hybridization. However, the efficiency of HBV replication and secretion was significantly higher in cells transfected with the pTriExHBV1.1 construct. Similarly, when the level of the HBV envelope protein expression was investigated by Western blotting, a higher amount of the L protein was found in HepaRG cells transfected with pTriExHBV1.1 (Fig. 2B). Thus, in all further studies only the pTriExHBV1.1 construct was used to drive HBV replication, assembly and secretion.
Fig. 1. – Schematic representation of expression vectors used in this study. 1.1 or 1.3 fold of HBV genome length containing the capsid protein (C), viral polymerase (P), envelope proteins (L, M and S) and X protein (X) was cloned in pTriEx vector under the control of the CMV enhancer/chicken β-actin promoter (CAG). An x marks the mutation that changes the START codon of the M protein (ΔM).

Fig. 2. – HBV production in Huh7 transfected cell line. Subconfluent Huh7 cells were transfected with pTriEx vector containing 1.1 and 1.3 over-length HBV genome. A) Cells were washed three times with PBS at 24 h pi and fresh culture medium was added. Three days later, the supernatant was harvested and used for virus purification by ultracentrifugation through a 20% sucrose cushion. Encapsidated HBV DNA was purified from cell lysate (cells) or from concentrated supernatant (medium), separated in 1.2% agarose gel, blotted on nitrocellulose membrane and detected with fluorescein-labeled HBV-specific DNA probe. Non-transfected Huh7 cells (Mock) were included as negative control. B) Cells were harvested and lysed at 24 h post-transfection. The soluble proteins were separated by SDS-PAGE 10% and were blotted on nitrocellulose membrane. The HBV L protein was identified with anti-preS1 MAb. Non-transfected Huh7 as well as HepG2.2.15 cell lysate were included as negative (Mock) and positive control, respectively.
EXPRESSİON PATTERN OF THE HBV ENVELOPE PROTEINS IN THE PRESENCE AND ABSENCE OF THE M PROTEIN

Having confirmed the mutation of the START codon that drives translation of the M envelope protein (Fig. 1) by DNA sequencing, the expression pattern of L, M and S proteins was determined in cells transfected with pTriExHBV1.1 or pTriExHBV1.1ΔM. The HEK 293T cell line was chosen for this experiment as it proved to sustain the highest transfection rate, when compared to cells of hepatic origin, such as HepaRG, HepG2 and Huh7 (data not shown). The cells were lysed and HBV envelope proteins were identified using the anti-S Ab specific for the S domain, which is shared by all three envelope proteins. As shown in Fig. 3, expression of the L, M and S proteins was readily detectable in cells transfected with either pTriExHBV1.1. In contrast, the pTriExHBV1.1ΔM construct was unable to support M protein expression, confirming the mutation of the M protein START codon. It is interesting to note that the expression of the L and S proteins was not affected by the absence of the M protein synthesis.

Fig. 3. – Synthesis of viral envelope proteins in HEK 293T transfected cells. HEK293T cells grown in six wells plate were transfected with pTriExHBV1.1 or pTriExHBV1.1ΔM vectors. At 24 h post-transfection, the cells were lysate, separated by SDS-PAGE and transferred to nitrocellulose membranes. HBV proteins were detected with anti-S polyclonal Ab followed by horseradish peroxidase-conjugated anti-mouse Ab. Mock transfected cells (Mock) were included as negative control.

ASSEMBLY AND SECRETION OF M-DEFICIENT HBV PARTICLES

Both HBV and DHBV were previously shown to be able to replicate in non-hepatic tumour cell lines (18). However, despite good replication rates of the viral genome, not all tested cell lines produced infectious virions, raising the important question of whether or not specific host cell factors are determinants of proper viral assembly.
To test the virion, as well as SVP assembly and secretion efficiency following expression of the pTriExHBV1.1 and pTriExHBV1.1ΔM constructs, three cell lines were chosen for transfection: two of hepatic origin, HepG2 and HepaRG, and the HEK 293T human embryo kidney-derived cell line. As shown in Fig. 4A, encapsidated HBV DNA was efficiently secreted from all cell lines, at levels close to the HepG2.2.2.15 control cells, stably transfected with two copies of the HBV genome. The highest number of HBV genomes was found in the HEK 293T cell supernatants, most probably a consequence of both, the good transfection rates characteristic to this cell line, and the efficient transcription of the pTriex promoter in these cells (19). Interestingly, the HBV particles lacking the M protein were less efficiently secreted in all cell lines, regardless their origin. Analysis of the SVP secretion in the same supernatants, revealed no significant difference for the wild-type particles in the case of HepG2 and HepaRG cells, while a higher amount of SVP devoid of the M protein were secreted from the HEK 293T cells. Together, these results suggest an implication of the M protein in HBV, but not SVP secretion.

Fig. 4. – Secretion of M deficient HBV produced in different cell lines. HepG2, HepaRG and HEK293T cells were grown in 150 cm² flasks and transfected with wild type (pTriExHBV1.1) or mutant (pTriExHBV1.1ΔM) HBV genome. The cell culture medium was replaced every three days. After nine days, the medium was harvested and used to purify virus by ultracentrifugation through a 20% sucrose cushion. Concentrated virus was used (A) to purify encapsidated HBV DNA and quantification by qPCR or (B) to measure the viral envelope proteins using an ELISA HBV-specific kit (Monolisa HBs Ag ULTRA, Biorad). Concentrated HBV produced in HepG2.2.2.15 was used as positive control and non-transfected cells (Mock) were included as negative control.
The role of M protein in HBV life-cycle

The low secretion efficiency of HBV particles lacking the M protein, as compared to the wild-type, made the normalization of the HBV genome number used for infection very difficult to achieve experimentally. Thus, in a first round of infectivity tests, only the virions containing the wild-type envelope, secreted from HepG2, HepaRG or HEK 293T cells, were analyzed. The virus was purified, concentrated and an equal number of GEq/ml was used to infect the differentiated HepaRG cells (Fig. 5A). Although the highest HBV replication rate was obtained in HEK 293T, the virus secreted from these cells was less infectious when compared to HepG2 and HepaRG by 10 and 100 fold, respectively. Interestingly, the wild-type HBV genome number level in HEK293T cells (Fig. 4A) was not accompanied by a similar increase of SVP assembly and secretion, which were similar in all cell lines taken into the study (Fig. 4B). A possible explanation for this effect is that the potent pTriex promoter ensures a high transcription efficiency of the pregenomic (pg) HBV RNA, while transcription from the internal pre-S promoters (4), which occurs similarly in all cell lines, remains the rate limiting factor of proper HBV assembly and envelopment. This is in agreement with a recently published study showing that, when expressed at lower levels, the envelope proteins are preferentially used for virion rather than SVP particles formation (20).

Fig. 5. – Infectivity of HBV or M deficient HBV in differentiated HepaRG cell line. Concentrated HBV produced in HepG2, HepaRG or HEK293T cells was normalized to an equal number of either wild-type (A) or M-deficient (B) HBV GEq/ml before infection of differentiated HepaRG cells. HBV replication was measured at day 9 pi, by RT-qPCR. Mock infected cells (Mock) were included as negative control.
The role of the M protein in HBV infectivity was investigated using the HepaRG cells to produce both the wild-type (as control) and the M protein-lacking virus. The virus particles were purified and adjusted to an equal number of GEq/ml before infection of differentiated HepaRG cells. Evaluation of HBV infection was performed by RT-qPCR, using total RNA isolated form control and infected cells, at day 9 pi. The results obtained show no difference in the ability of HBV to infect the cells, regardless whether or not the M protein is present in the viral envelope (Fig. 5B). This is in agreement with the results obtained in an independent study, using a trans-complementation expression system, which showed that neither the M protein nor the preS2 region of L is important for HBV entry (11). Despite not being involved in early steps of the HBV life cycle, it is important to note that secretion of HBV lacking the M protein was impaired in all cell lines taken into this study. However, unlike the virions, HBV SVP were secreted normally in the absence of the M protein. This result may seem surprising as SVP contain host-derived lipid embedded with the S, but also the M protein. Relying on the observation that M protein influence on virion secretion requires its coexpression with the L protein, a model of M function in HBV assembly was recently proposed (20). In this model, the M protein maintains a simultaneous contact between both the L and the S envelope proteins, thus increasing the efficiency of virion formation and secretion.

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