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Cellular uptake of hepatitis B virus envelope L particles is independent of sodium taurocholate cotransporting polypeptide, but dependent on heparan sulfate proteoglycan

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ABSTRACT

Sodium taurocholate cotransporting polypeptide (NTCP) was recently discovered as a hepatitis B virus (HBV) receptor, however, the detailed mechanism of HBV entry is not yet fully understood. We investigated the cellular entry pathway of HBV using recombinant HBV surface antigen L protein particles (bio-nanocapsules, BNCs). After the modification of L protein in BNCs with myristoyl group, myristoylated BNCs (Myr-BNCs) were found to bind to NTCP *in vitro*, and inhibit *in vitro* HBV infection competitively, suggesting that Myr-BNCs share NTCP-dependent infection machinery with HBV. Nevertheless, the cellular entry rates of Myr-BNCs and plasma-derived HBV surface antigen (HBsAg) particles were the same as those of BNCs in NTCP-overexpressing HepG2 cells. Moreover, the cellular entry of these particles was mainly driven by heparan sulfate proteoglycan-mediated endocytosis regardless of NTCP expression. Taken together, cell-surface NTCP may not be involved in the cellular uptake of HBV, while presumably intracellular NTCP plays a critical role.

1. Introduction

Hepatitis B virus (HBV) infection is one of the major causes of liver-related diseases, including chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (Neuveut et al., 2010). Despite much effort in the last five decades since the discovery of HBV, the life cycle of HBV is not yet fully understood at the molecular level. Generally, viral entry is an important target for antiviral drugs since entry inhibitors can prevent the binding of virions to cellular receptors and subsequent viral membrane fusion with the host cell membrane (Dimitrov, 2004). In regards to the development of entry inhibitors for HBV, sodium taurocholate cotransporting polypeptide (NTCP) was recently identified as a functional cellular receptor for HBV by Yan et al. (2012). Particularly, it was shown that exogenous expression of NTCP conferred susceptibility to HBV infection *in vitro* in otherwise non-susceptible Huh-7 and HepG2 cells (Iwamoto et al., 2014; Yan et al., 2012). However, the detailed mechanism of the NTCP-mediated cellular entry pathway has not been fully elucidated (Li, 2015; Urban et al., 2014; Watashi et al., 2014b; Yan et al., 2015). In addition to NTCP, several other cellular proteins have been proposed as important factors for HBV infectivity. Most importantly, the heparan sulfate proteoglycan (HSPG) was identified as an initial attachment protein of HBV, and especially, glypican-5 may be one of the HSPG that is involved in the cellular entry process (Leistner et al., 2008; Schulze et al., 2007; Verrier et al., 2016). Additionally, several domains in the HBV envelope L protein have been shown to play essential roles in the interactions between HBV and human hepatic cells. For example, Neurath et al. (1986) were the first to find that 10–36 amino acid residues (aa) of the pre-S1 region were essential for the recognition of human hepatic cells. Using HBV deletion mutants, it was shown that N-terminal aa of the pre-S1 region were necessary for HBV infectivity, which was likely due to cellular receptor binding (Blanchet and Sureau, 2007). Additionally, using pre-S1-derived synthetic peptides, 9–15 aa of the pre-S1 region were shown to be crucial for HBV infection as a synthetic peptide corresponding to those residues suppressed infection *in vitro* (Schulze et al., 2010). Recently, it was demonstrated that 9–15 aa of the pre-S1 region participated in the interaction with NTCP (Yan et al.,

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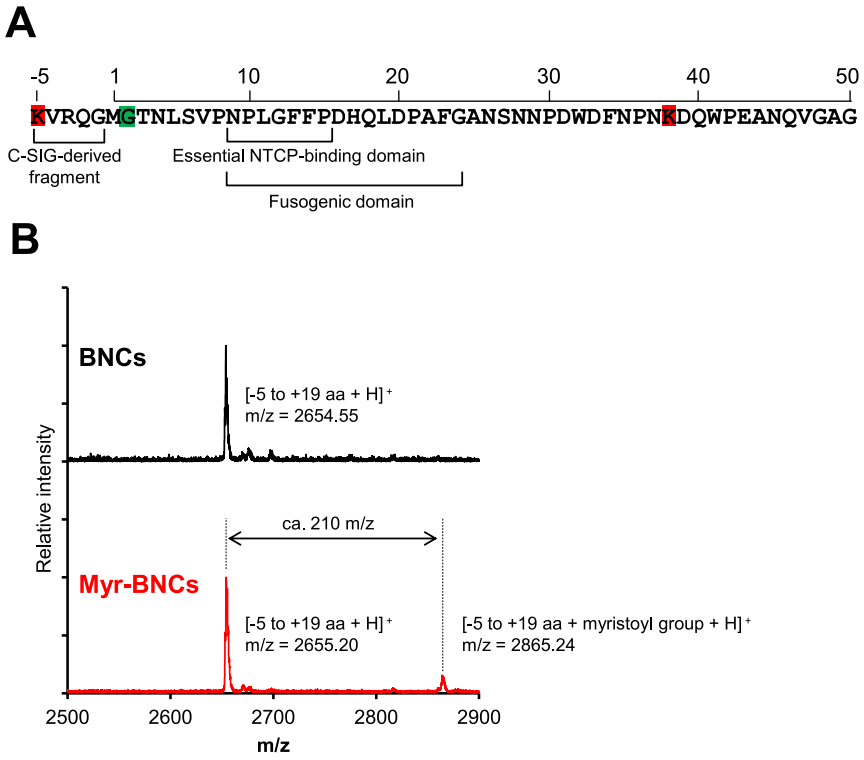

Fig. 1. Preparation of Myr-BNCs. (A) N-terminal amino acid sequence of the L protein of BNCs (Kuroda et al., 1992). Amino acids from −5 to −1 correspond to a residual fragment of the chicken lysozyme signal peptide (C-SIG). Potential lysine residues for myristoylation are highlighted in red. The glycine residue highlighted in green indicates the N-myristoylation site found in the endogenous L protein of HBV genotype D. Essential NTCP-binding domain (Meier et al., 2013) and fusogenic domain (Somiya et al., 2015) are indicated with brackets. (B) MALDI-TOF MS data of protease digests of BNCs and Myr-BNCs.

2012) (see Fig. 1A). Furthermore, the antigenic loop of the S region was shown to be essential for HBV infection (Abou-Jaoudé and Sureau, 2007; Jaoude and Sureau, 2005; Salisse and Sureau, 2009), and was recently demonstrated to interact with HSPG (Sureau and Salisse, 2013). In addition to the aa sequence of the L protein, myristoylation at the glycine-2 residue was tightly linked to HBV infectivity (Gripon et al., 1995), suggesting that myristoylation is necessary for pre-S1 interaction with NTCP (Urban et al., 2014). While the various cellular proteins and viral domains (within the pre-S1 and S regions) described above are indispensable for the cellular attachment and entry of HBV, it remains unclear how these factors contribute to these mechanisms at the molecular level.

Owing to the identification of NTCP as a functional HBV receptor, several hepatic cell lines, in lieu of primary human hepatocytes, have become useful for HBV research because NTCP overexpression confers HBV susceptibility (Iwamoto et al., 2014). To investigate the intracellular trafficking of HBV with biochemical techniques, it is necessary to obtain substantial amounts of HBV. However, it is time-consuming and laborious to purify HBV virions from hepatitis B patient plasma or HBV-containing culture medium. Therefore, we synthesized large amounts of HBV envelope L protein particles in yeast cells (referred to as bio-nanocapsules, BNCs) (Kuroda et al., 1992). BNCs are hollow nanocapsules displaying L proteins as transmembrane proteins in a manner similar to those in HBV. BNCs can bind and enter into a wide variety of human hepatic cells including those not susceptible to HBV or that lack NTCP (Yamada et al., 2012). While BNCs have no myristoyl group at the N-terminus of the L protein, the rate of cellular entry of BNCs was the same as that of hepatitis B patient plasma-derived HBV surface antigen (HBsAg) particles, which are endogenous HBV subviral particles containing myristoylated L proteins. After encapsulation of drugs, BNCs have been utilized as human hepatic cell-specific drug-delivering nanocarriers both *in vitro* and *in vivo* (Somiya and Kuroda, 2015; Yamada et al., 2003). Furthermore, we recently used BNCs to reveal that 9–24 aa of the pre-S1 region exhibit low pH-dependent fusogenic activity (Somiya et al., 2015) (see Fig. 1A), which could induce membrane fusion between the envelope membranes of the BNCs and exogenous membranes, followed by the disruption of both membranes. These observations demonstrated that BNCs could enter cells through a NTCP-independent pathway and escape from endocytic vesicles following membrane fusion.

In the current study, we prepared myristoylated BNCs (Myr-BNCs) by chemical modification of BNCs with myristoyl groups. Unlike BNCs, Myr-BNCs were shown to inhibit HBV infection of NTCP-expressing HepG2 cells through interactions with NTCP in a competitive manner. Meanwhile, Myr-BNCs, BNCs, and HBsAg particles (referred to as HBV-mimicking particles) were shown to enter HepG2 cells regardless of NTCP expression with similar efficiency, which was predominantly mediated by HSPG-dependent endocytosis. Furthermore, the cellular entry of HBV-mimicking particles was inhibited by a myristoylated pre-S1 peptide (2–48 aa) regardless of NTCP expression. Taken together, these results suggest that the pre-S1 domain is important for HSPG-dependent endocytosis, NTCP is not involved in the cellular uptake of HBV-mimicking particles, and intracellular NTCP may exclusively function in the binding of HBV.

2. Materials and methods

2.1. Materials

BNCs were purified from *Saccharomyces cerevisiae* AH22R$^-$ strain harboring an HBsAg L protein expression plasmid as described previously (Jung et al., 2011; Kuroda et al., 1992). *N*-succinimidyl myristate and a mouse monoclonal anti-pre-S1 antibody