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Myristoylation signal transfer from the large to the middle or the small HBV envelope protein leads to a loss of HDV particles infectivity

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Abstract

A myristate linked to the N-terminus of the large hepatitis B virus (HBV) envelope protein was found to be required for infectivity of the hepatitis delta virus (HDV). Myristoylation of the large HBV envelope protein being known as indispensable for HBV infectivity, this result further demonstrates the similarities between the HBV and HDV entry pathways. In addition, the transfer of the *N*-myristoylation signal from the large to the middle or the small HBV envelope protein led in both cases to a loss of HDV infectivity. Hence, it is suggested that viral entry could depend on a physical link, or a spatial association, between the N-terminal receptor-binding polypeptide of the large protein and the myristoyl anchor linked to glycine-2.

Keywords: Myristoylation; HBV; HDV; Envelope protein; Infectivity

Introduction

The *hepatitis B virus* (HBV) displays a very narrow host range mainly determined through a restriction at the initial steps of the virus life cycle, including the interaction of the viral envelope proteins with human hepatocyte-specific receptors (Seeger and Mason, 2000). The HBV envelope proteins designated large (L-HBsAg), middle (M-HBsAg) and small (S-HBsAg) are integral glycoproteins that differ from each other by the size of their N-terminal ectodomain (Heermann and Gerlich, 1992). L-HBsAg contains an N-terminal pre-S1 domain, a central pre-S2 region and a C-terminal S domain. M-HBsAg is shorter than L-HBsAg in lacking the pre-S1 polypeptide (Fig. 1), whereas S-HBsAg consists of the S domain only. L-HBsAg displays a dual topology at the viral membrane: the N-terminal pre-S domain (pre-S1 + pre-S2) is either external (Le-HBsAg) at the surface of the particles, or internal (Li-HBsAg) facing the inner side of the particle (Bruss et al., 1994; Ostapchuk et al., 1994; Prange and Streeck, 1995). The two topologies correspond to two distinct functions: Le-HBsAg is a receptor-binding protein at viral entry, and Li-HBsAg serves as a matrix protein for HBV nucleocapsid envelopment (Bruss and Vieluf, 1995; Le Seyec et al., 1999). The HBV budding mechanism being nucleocapsid-independent, the envelope proteins can self-assemble to form subviral particles (SVPs), and they can also package the *hepatitis delta virus* (HDV) ribonucleoprotein (RNP) in the case of HBV/HDV coinfection (Bonino et al., 1986; Wang et al., 1991). This interaction leads to the formation of mature HDV particles, the infectivity of which depends upon the presence of L-HBsAg in the envelope (Sureau et al., 1993).

Several lines of evidence, obtained with both the HBV and HDV models, indicate that pre-S1 contains a primary receptor-binding site responsible for tissue and species specificity (Barrera et al., 2005; Glebe et al., 2005; Gripon et al., 2005) (Le Seyec et al., 1999). Furthermore, the pre-S1 domain is myristoylated at glycine-2, a modification required for infectivity but dispensable for HBV assembly (Bruss et al., 1996; Gripon et al., 1995). The lipid anchor is also partially

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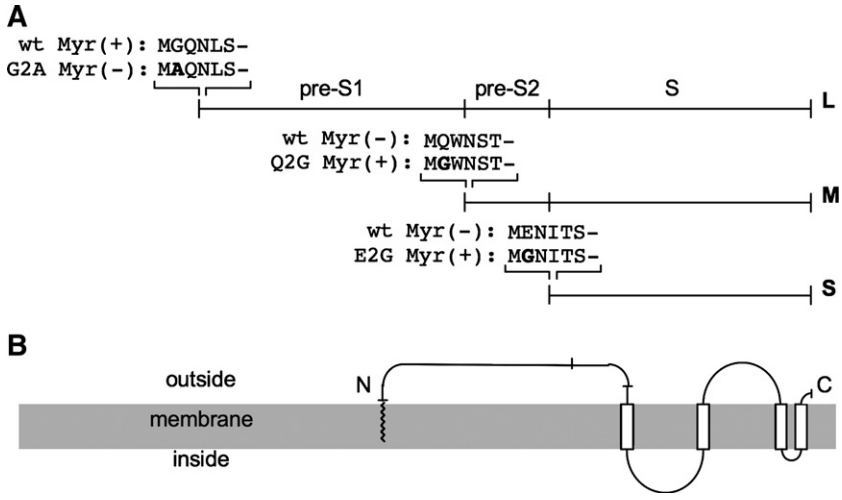

Fig. 1. Schematic representations of HBV envelope proteins. (A) The L-, M- and S-HBsAg proteins are depicted by horizontal lines (L, M and S, respectively). The N-terminal amino acid sequences of the wt and mutant proteins are indicated. The presence (Myr(+)) or absence (Myr(−)) of a myristoyl attachment signal is indicated. The mutations are designated by the one-letter code of the wt amino acid followed by its position in the envelope protein, and the one-letter code of the substituted amino acid. (B) The topology of L-HBsAg at the viral membrane is represented. Broken line represents the myristate group linked to the N-terminus of wt L-HBsAg. Open boxes represent transmembrane regions in the S domain.

responsible for the retention of L-HBsAg at the ER membrane (Prange et al., 1991). Myristoylation is catalyzed by the *N*-myristoyl transferase that functions in the cytosol on nascent proteins by linking a myristic acid molecule to glycine-2 (Wilcox et al., 1987). In general, a myristoylation consensus signal includes, in addition to glycine-2, a serine or a threonine at position 6 (Utsumi et al., 2004).

The role of L-HBsAg myristoylation in infectivity has been clearly demonstrated in the HBV and the *duck hepatitis B virus* (DHBV) models, either directly by reverse genetic analysis (Gripon et al., 1995; Macrae et al., 1991) or indirectly using myristoylated pre-S1-specific peptide competitors in *in vitro* infection assays (Gripon et al., 2005). However, the mechanism by which the lipid anchor participates in viral entry remains unclear. One possibility is that the receptor-binding site located at the N-terminus of pre-S1, together with the myristic acid linked to glycine-2, forms the structure that is competent for interaction with a receptor. The myristic acid could also act independently at a fusion step by inserting reversibly with the viral or target cell membrane, following a process analogous to the "myristoyl switch mechanism" that has been described elsewhere (Resh, 1999). Several questions remain unanswered regarding the exact function(s) of the lipid anchor and the stoichiometry of Le-HBsAg or Li-HBsAg acylation. Although the inhibitory activity of pre-S1-specific peptides has been demonstrated to be drastically improved upon their N-terminal acylation, it is not proven that, at the virion surface, the pre-S1 receptor-binding polypeptide and the myristoyl anchor need to be physically linked to each other for function at viral entry.

Results and discussion

In this study, we used a site-directed mutagenesis approach to eliminate the N-terminal myristoylation signal of L-HBsAg in order to directly address the function of the lipid anchor in infectivity of HDV particles and to transfer the myristoyl attachment signal to the N-terminus of M- or S-HBsAg to determine whether these modifications could substitute for the L-HBsAg-borne signal at viral entry.

For production of HDV virions, Huh-7 cells were cotransfected with plasmid pSVLD3 for expression of the HDV RNPs (Kuo et al., 1989) and either (i) pT7HB2.7 for the expression of the wt S-, M- and L-HBsAg proteins, (ii) pHB2730 for expression of wt S- and M-HBsAg and G2A L-HBsAg mutant (lacking the myristoylation signal) or (iii) p123 for expression of wt S-HBsAg only (Sureau et al., 2003). As shown in Fig. 2, secretion of non-myristoylated L-HBsAg was not significantly increased compared to that of the wt protein (Fig. 2A), and particles bearing wt or G2A L-HBsAg contained equivalent amounts of HDV RNA.

For infectivity assays, primary cultures of human hepatocytes were inoculated with wt or mutant HDV particles and cells were harvested at day 9 postinoculation for measurement of antigenomic HDV RNA. As expected, viral RNA was present in hepatocytes that had been exposed to wt HDV particles and absent in cells inoculated with particles lacking L-HBsAg (Fig. 2B). As observed in experiments previously conducted in the DHBV and HBV models (Gripon et al., 1995; Macrae et al., 1991), HDV particles lacking a myristoylation site at the N-terminus of L-HBsAg were not infectious. This result represents a direct proof that L-HBsAg-linked myristic acid is required for HDV entry, and it is in agreement with recent studies that reported the potent inhibition activity of myristoylated pre-S1-specific peptides in an *in vitro* HDV infection assay (Engelke et al., 2006).

For a better interpretation of our data, we sought to verify that the pre-S domain of non-myristoylated L-HBsAg was indeed exposed at the outside of HDV virions, a topology that is required for receptor recognition. To this end, we produced HDV particles covered with wt S-HBsAg+G2A L-HBsAg