論文内容要旨

Study on particle formation mechanism of hepatitis B virus

(B型肝炎ウイルスの粒子形成機構に関する研究)

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Hepatitis B virus (HBV), which belongs to the family *Hepadnaviridae*, causes hepatitis in humans with complications of liver cirrhosis and carcinoma, and HBV infection is a serious health problem. Seven proteins are encoded by HBV: preCore (preC-core), core, polymerase (pol), X (HBx), and three envelope proteins, L (PreS1-PreS2-S), M (PreS2-S) and S (HBs). HBV or HBV-related proteins are released from cells in multiple forms. Dane particles, which are mature infectious virions, are composed of an icosahedral nucleocapsid containing an incomplete double-stranded DNA genome and core proteins wrapped by a viral envelope containing S, M and L. Subviral particles with circular or long shapes that are free from the nucleocapsid are found in patients' sera. HBV proteins are also released in other forms such as HBe antigen, which is generated from preCore, and a Dane-like particle containing a variant core protein p22cr that is free from genomic DNA. A naked nucleocapsid without an envelope can also be released from cultured cells.

In the case of enveloped viruses, expression of one or a few viral proteins causes shedding of viral protein-driven virus-like particles (VLPs) from cells. In the case of HBV, at least S and core proteins are known to be released from cells as VLPs when expressed alone. The use of VLPs could be an experimental measure to investigate the mechanism of virion formation.

For the VLPs formed by the S protein (S-VLPs), we compared them with released HBV proteins by introducing HBV genomic DNA into cultured cells and with HBV-containing sera of a chronic HBV patient in terms of density and protein composition and we found that S-VLPs have properties similar to those of HBV subviral particles of HBV.

Tetherin, also named CD317, BST2 or HM1.24, is an interferon (IFN)-inducible protein that inhibits the release of human immunodeficiency virus (HIV), Ebola virus, Lassa virus, herpesvirus and other enveloped viruses from infected cells by tethering progeny virions to the membranes of host cells. We demonstrated that the release of VLPs by the M proteins of Nipah virus and Sendai virus is inhibited by tetherin. Furthermore, tetherin can induce NF-kB-mediated signal transduction that leads to the production of proinflammatory cytokines, thereby acting as an innate sensor of viral release. In the case of HBV, tetherin has also been shown to interact with the S protein and to moderately reduce HBV release from cultured cells. On the other hand, many viral proteins can inactivate tetherin in multiple ways. For example, HIV-1 Vpu can interact with tetherin via its transmembrane domain and downregulate tetherin from the plasma membrane. Ebola virus glycoprotein can directly bind to tetherin for antagonizing its function.

In the present study, we showed that tetherin efficiently inhibits the release of S-VLPs. This inhibition was cancelled by co-expression of Vpu. On the other hand, tetherin did not significantly inhibit either the release of HBV or core-mediated virus-like particles (Core-VLPs). It was confirmed by

fluorescent staining that the S protein and tetherin were colocalized in the cell. In the cultured cells carrying HBV genomic DNA and supporting continuous HBV replication (T23 cells), release of genomic DNA, which is an indicator of HBV mature particles, did not decrease even when tetherin was overexpressed. Furthermore, when tetherin was knocked down by siRNAs, there was no change in release of HBV DNA. In addition, in mice carrying human livers, expression of endogenous tetherin in the liver was moderately increased in HBV infection, while HBV continuously propagated in the liver. The growth of HBV did not appear to be significantly hindered even in the presence of tetherin. Therefore, the inhibitory effect of tetherin on virus *in vivo* was thought to be limited. Considering reports that the number of released subviral particle reaches 100,000 times more than that of infectious HBV particles, it is speculated that abundant subviral particles act as a decoy to protect HBV infectious particles from the action of tetherin.

The core protein is composed of the N-terminal assembly domain and the C-terminal arginine-rich domain (ARD). Core-VLPs are released as a naked capsid-like structure when the core protein is expressed in cultured cells like HuH-7 cells (data not shown). The mechanism of core-VLP release is unknown. The core protein is known to interact with Alix, a component of ESCRT (endosomal sorting complex required for transport). Core-VLP release is inhibited by co-expression of a dominant negative form of CHMP4B or Vps4A, which are components of the ESCRT system, and we also confirmed the results (data not shown).

We then analyzed the interaction between the core mutant proteins and Alix. Core deletion mutants from the N-terminus and those from the C-terminus indicated that the α-3 helix in the assembly domain is involved in the interaction. We further generated a series of core mutants in which three consecutive amino acid residues within the α-3 helix region were replaced with alanine residues (alanine scanning). We found that the mutant 56AAA58, in which amino acid residues 56-58 were replaced with alanine residues, did not interact with Alix. Furthermore, only this mutant did not cause core-VLP release, indicating that interaction with Alix is important for core-VLP release.

We analyzed C-terminal ARD deletion mutants and found that complete deletion of ARD inhibited core-VLP release, indicating a role of this region for core-VLP release. We next analyzed ARD charge mutants in which abundant positively charged arginine residues were converted to negatively charged glutamic acids (core-RtoE) or the ARD domain was replaced with neutral charged HA tags (core-HA) or a glycine-rich linker

(core-Gs) of a similar length. Core-RtoE completely lacked release and core-HA and core-Gs partially lacked release, showing charge-dependent ability to aid core-VLP release. The Blue-Native PAGE method was used to investigate whether the core protein formed a multimer or not. Large amounts of monomers and oligomers were observed in Core-GtoE, indicating a failure of nucleocapsid assembly. Smaller amounts of monomers and oligomers were found in core-HA and core-Gs mutants. Although the ARD domain positive charge is thought to be important for holding viral genomic DNA, the charge may also be involved in core assembly and subsequent core-VLP budding. Similar failure of assembly was also observed in the complete ARD deletion mutant and the 56AAA58 mutant. These results suggest that proper capsid assembly is a prerequisite for core-VLP release. The results further suggest that there is link between capsid assembly and binding of the core protein with Alix, which remains to be elucidated.

The use of VLP formation by the S protein revealed that release of S-VLPs, which are similar to subviral particles, was inhibited by tetherin. We showed the possibility that S-VLPs protect mature infectious virus particles from an interferon-inducible anti-viral protein, tetherin. The core-VLP release itself is an enigma. For the release of core-VLPs, prior assembly to a capsid was shown to be necessary. Involvement of Alix in the core-VLP release was shown, but the detailed mechanism is still unknown.