論　文　の　内　容　の　要　旨

獣医学　　専攻

平成 18 年度博士課程　　入学

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論文題目　Studies on the molecular biological interactions of bovine viral diarrhea virus nonstructural protein 5A with the host cell

（牛ウイルス性下痢ウイルス非構造蛋白質 5A と宿主細胞の分子生物学的相互作用に関する研究）

Bovine viral diarrhea virus (BVDV) is an enveloped, positive-stranded RNA virus which belongs to the genus *Pestivirus* within the family *Flaviviridae*. As one of the best characterized prototype pestivirus, BVDV represents an attractive model system to study the replication of the closely related hepacivirus, hepatitis C virus (HCV). The viral genomes of BVDV and HCV encode single polyproteins, which are processed co- and posttranslationally by the viral and cellular proteases, yielding several functionally conserved gene products. The order and nomenclature of the cleavage products in the BVDV polyprotein are as follows: NH2-Npro-C-Erns-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH. Nonstructural protein 5A (NS5A) is a 56-58 kDa phosphorylated zinc metalloprotein and an essential component of HCV and BVDV replicase complexes Extensive studies on HCV NS5A and its molecular interactions with the host cell are

available; however the exact function(s) of NS5A in the BVDV pathogenesis remains unknown. Since, no intrinsic enzymatic activities are performed by NS5A during BVDV pathogenesis it is assumed that it plays its functions through interactions with the host cell proteins. Unraveling these functions may be a clue to better understand the mechanisms involved in the replication and pathogenesis of BVDV.

In order to work with NS5A protein, it was essential to have a specific antiserum available. Therefore, with this aim, in CHAPTER 1, the author described the generation and characterization of monoclonal antibodies against the BVDV NS5A, by fusion of P3U1 myeloma cells with spleen cells from mice immunized with recombinant *E. coli*-expressed GST-NS5A protein. Two MAbs (1H12 and 2F9) were established on the basis of Immunofluorescence and Western blot analysis. Both the MAbs were of IgG1 subclass and recognized an epitope clustered within the N-terminal region of NS5A. The MAb 1H12 was used successfully to detect NS5A protein in BVDV field isolates belonging to genotypes 1 and 2. Temporal expression pattern studies during an infectious cycle revealed that BVDV NS5A could be detected 12–60 h post-infection. Confocal microscopy studies showed a cytoplasmic staining pattern and revealed that NS5A is localized along with NS3 on the endoplasmic reticulum membrane in BVDV infected cells.

In the second CHAPTER of thesis, the author identified a protein kinase which phosphorylates and binds BVDV NS5A. It is well known that same or similar serine/threonine protein kinase phosphorylates NS5As from HCV and BVDV. Several cellular protein kinases, including protein kinase A (PKA), casein kinase I (CKI), CKII, MAPK and MEK family members were demonstrated as candidates for HCV NS5A

phosphorylation; however which particular protein kinase is responsible for BVDV NS5A phosphorylation is not known. In an attempt, *E. coli* expressed GST-NS5A fusion protein was used as a substrate for PKA, CKII and cdc2 cyclin-dependant kinase (CDK1). *In vitro* kinase assays were performed and a proline-directed cdc2/cyclin B1 complex was found to be responsible for phosphorylation of BVDV NS5A, consistent with the previous kinase inhibitor studies whereas PKA and CKII were unable to phosphorylate BVDV NS5A. Using KinasePhos, a putative cdc2 site was found in NS5A protein at Thr292 which was mutated to alanine as GST-NS5AmutT292A to block phosphorylation. Assessment of this NS5A mutant in kinase assay suggested that Thr292 in NS5A was the target site of cdc2 protein kinase. Moreover, in consistent with a previous report on HCV NS5A, cellular cdc2 was coimmunoprecipitated with NS5A in BVDV infected cells indicating a physical association of cdc2 with NS5A.

Although there is a growing list of proteins that interact with HCV NS5A, knowledge about the binding partners of BVDV NS5A is scarce. Therefore, one of the major goals of this study was the identification of novel binding partner(s) of NS5A through yeast two-hybrid technique. In CHAPTER 3, the author described a novel protein-protein interaction between BVDV NS5A and the host which is involved during BVDV RNA replication. To identify the cellular proteins, a yeast two-hybrid screen using MDBK cDNA library was performed using NS5A N-terminus as bait. As a result, a celluar protein termed *NIK (nuclear factor kappa B inducing kinase) and IKK-β binding protein (NIBP)* was identified which is implicated in NF-κB signaling and membrane trafficking in the cells. The interaction of NS5A with NIBP was confirmed by both *in vitro* and *in-vivo*. Complementing the GST-pull down and immunoprecipitation data are the confocal

immunofluorescence results indicating that NS5A co-localized with NIBP in the cytoplasm of BVDV infected cells. Moreover, the minimal residues of NIBP which interact with NS5A were mapped from amino acids 597 to 623. In addition, over-expression of NS5A inhibits tumor necrosis factor (TNF)-α induced NF-κB activation in HEK293 and LB9.K cells as determined by luciferase gene reporter assay. Further it was shown that inhibition of endogenous NIBP by small interfering RNA molecules enhanced viral replication indicating the importance of NIBP implications in BVDV pathogenesis.

As stated above, the author described the first attempt towards the generation and characterization of MAbs against BVDV NS5A and by utilizing these MAbs, he was able to characterize the NS5A in BVDV infected cells. Since NS5A is a phosphor-protein, possibly it might have a regulatory role in viral replication as is the case with HCV NS5A. Using NS5A as a substrate, the author identified a cdc2 cyclin-dependent kinase which binds and phosphorylates NS5A at Threonine 292. The finding may suggest that BVDV perhaps affects the cellular growth activities. Since BVDV cause persistent infection in the host and cdc2 has been shown to regulate the cell cycle, the role of NS5A phosphorylation in BVDV persistent infections will be helpful in devising the future studies. To further explore the possible implicated signaling pathways during BVDV pathogenesis, he was able to identify a novel protein named NIBP using NS5A as bait in yeast two-hybrid technique. Since NIBP is involved in the cytokine-mediated NF-κB activation, these findings will contribute to better understand the mechanism of BVDV pathogenesis. Moreover, being the first reported interaction between NIBP and a viral protein, this finding suggest a new mechanism whereby viruses may subvert host cell machinery for mediating trafficking as well as NF-κB signaling.