Physical and functional interaction between hepatitis C virus NS5A protein and ovarian tumor protein deubiquitinase 7B

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学位論文の内容要旨

Physical and functional interaction between hepatitis C virus NS5A protein and ovarian tumor protein deubiquitinase 7B

C型肝炎ウイルス NS5A 蛋白と卵巣腫瘍蛋白脱ユビキチン化酵素７B との蛋白質間及び機能的相互作用

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INTRODUCTION

Hepatitis C virus (HCV) infection often causes chronic liver diseases, including chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC). HCV infects approx. 130–170 million people worldwide (2.2%–3% of the global population), based on the World Health Organization estimation. Therefore, HCV infection poses extensive global public health problems. Many viruses modulate various cellular pathways by manipulating ubiquitylation and deubiquitylation machineries to enhance viral replication and pathogenesis. It was reported that several other HCV proteins, including NS5A protein, are also degraded through the ubiquitin-proteasome pathway. However, the physiological roles of ubiquitylation and deubiquitylation in HCV infection are largely unknown. To elucidate the roles of ubiquitylation and deubiquitylation in HCV infection, we sought to identify a deubiquitinase (DUB) that can interact with HCV proteins.

In the present study, we aimed to identify DUB proteins involved in HCV replication or viral pathogenesis. Here we identified OTUD7B as an NS5A-binding protein. OTUD7B is a 93-kDa member of the ovarian tumor protein (OTU) domain-A20-like DUB family and widely expressed in many tissues, including the liver. We investigated a role of OTUD7B in HCV replication and viral pathogenesis.

METHODS

1. The human hepatoma cell lines Huh-7, Huh-7.5 cells and Huh-7 cells stably harboring an HCV-1b RNA replicon (FGR) derived from Con1 were used. The expression plasmids for NS5A, a series of deletion NS5A mutants as HA-tagged and myc-tagged proteins, the substitution mutants of HA-NS5A(1-126) were used. To express the OTUD7B protein as a FLAG-tagged fusion protein in mammalian cells,

pCAG-FLAG-OTUD7B was constructed.

2. Cells were lysed in RIPA buffer and sonicated at 4°C. Lysates were subjected to immunoprecipitation with either anti-FLAG-M2 agarose or appropriate antibodies followed by incubation with Protein A Sepharose 4 Fast Flow. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. The membranes were incubated with appropriate primary and secondary antibodies and then visualized using ECL Western blotting detection reagents.

3. Cells were transfected with pCAG-FLAG-OTUD7B and then infected with HCV J6/JFH1. At 24 h postinfection, cells were washed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. The coverslips were incubated with the appropriate primary and secondary antibody, then counterstained and examined under a BZ-9000 microscope and an LSM 700 confocal microscope. The coverslips were also incubated with appropriate primary antibody and PLA probes, and then processed for probe ligation, signal amplification, and mounting following the manufacturer's instructions for in situ PLA Assay.

4. Huh-7.5 cells were transfected with 10 or 20 pmol of either OTUD7B-specific small interfering RNA using Lipofectamine RNAiMAX.

5. The total RNA was extracted from the cells and the cDNA was synthesized from one μg of total RNA and subjected to a quantitative real-time PCR analysis. As an internal control, the human GAPDH gene expression levels were measured.

RESULTS

HCV NS5A protein interacts with OTUD7B.

To determine whether HCV NS5A protein physically interacts with OTUD7B protein in cultured cells, we cotransfected Huh-7 cells with the myc-His_6-tagged NS5A plasmid together with the FLAG-OTUD7B plasmid. The immunoprecipitation analysis revealed that NS5A protein was co-immunoprecipitated with FLAG-OTUD7B protein. In contrast, HCV core protein was not co-immunoprecipitated with FLAG-OTUD7B protein. These results suggest that NS5A protein specifically interacts with OTUD7B.

To determine whether HCV NS5A protein expressed from replicating HCV can interact with endogenous OTUD7B, we used HCV 1b full-length replicon (RCYM1). Immunoprecipitation analysis revealed that NS5A but not core protein was co-immunoprecipitated with endogenous OTUD7B using anti-OTUD7B pAb. These results suggest that HCV NS5A protein specifically interacts with endogenous OTUD7B in HCV-replicating cells.

HCV NS5A protein colocalizes with OTUD7B protein

To determine whether NS5A protein colocalizes with OTUD7B in cells, Huh-7.5 cells were cotransfected with FLAG-OTUD7B expression plasmid and then infected with HCV J6/JFH1. Immunofluorescence staining demonstrated that OTUD7B localizes mainly in the cytoplasm and also in the nucleus to some extent without HCV infection. In the HCV-infected cells, NS5A and OTUD7B were colocalized in the cytoplasm. Interestingly, we observed that the HCV-uninfected cells exhibited approx. 40% nuclear+cytoplasmic localization. In contrast, HCV-infected cells showed more than 90% nuclear+cytoplasmic localization. These results suggest that

HCV infection enhances the nuclear localization of OTUD7B.

To further investigate the presence and localization of NS5A-OTUD7B interactions in the context of the HCV infection, we performed an *in situ* PLA. The result of PLA demonstrated a bright red signal in the cytoplasm where NS5A protein and OTUD7B protein showed a physical interaction. Taken together, these results indicate that HCV NS5A protein colocalizes with OTUD7B in HCV-infected cells.

The NS5A domain I interacts with the OTUD7B protein

To determine the OTUD7B-binding domain on NS5A protein, we performed co-immunoprecipitation analyses using a series of NS5A deletion mutants. When the N-terminal deletion mutants of NS5A were used, all of the HA-NS5A proteins except HA-NS5A(357–447), HA-NS5A(250–447), or HA-NS5A(214–447) were co-immunoprecipitated with OTUD7B. These results suggest that the domain I of NS5A consisting of aa 1 to 213 is important for the OTUD7B binding. In addition, FLAG-OTUD7B was co-immunoprecipitated with NS5A(1–126)-myc-His_6 and NS5A(1–147)-myc-His_6, but not with HA-NS5A(1–83), HA-NS5A(1–100), or HA-NS5A(1–120). These data suggest that the region from aa 121 to 126 of NS5A is essential for the specific interaction with OTUD7B.

To identify the specific aa residues of NS5A required for interaction with OTUD7B, we used alanine substitution mutants of HA-NS5A(1-126). The immunoprecipitation analysis revealed that both mutant HA-NS5A V121A(1-126) and HA-NS5A V124A(1-126) failed to interact with FLAG-OTUD7B. These results suggest that NS5A Val^{121} and Val^{124} are crucial for the interaction between NS5A and OTUD7B.

Knockdown of endogenous OTUD7B by RNAi increases HCV replication.

To investigate the role of OTUD7B in HCV replication, we used an RNA interference (RNAi) technique. The expression of endogenous OTUD7B was efficiently knocked down by siRNA. The intracellular HCV RNA levels in the HCV J6/JFH1-infected cells were slightly increased after siRNA transfection. The extracellular HCV infectious titer was increased 2-fold compared to the control cells. These data suggest that OTUD7B has some negative effects on HCV replication and the production of HCV infectious particles.

HCV NS5A enhances OTUD7B deubiquitinase activity.

To determine whether HCV NS5A affects OTUD7B DUB activity, we cotransfected Huh-7 cells with myc-His_6-tagged NS5A plasmid together with the FLAG-OTUD7B plasmid and HA-Ubiquitin plasmid. Transfection of the HCV NS5A plasmid resulted in a remarkable decrease in ubiquitylation signals. These data suggest that HCV NS5A enhances the OTUD7B DUB activity.

DISCUSSION

Here we identified OTUD7B as a novel NS5A-binding protein. Our immunoprecipitation analyses revealed that HCV NS5A protein specifically interacts with OTUD7B in a transient expression system and in HCV RNA replicon cells. The immunofluorescence staining analysis and the *in situ* PLA assay revealed that HCV NS5A protein colocalizes with OTUD7B in the cytoplasm. We also obtained evidence suggesting that the nuclear localization of OTUD7B is enhanced in HCV-infected cells. Together these findings suggest that the NS5A-OTUD7B interaction

affects the subcellular localization of OTUD7B and/or the stability of OTUD7B in the nucleus.

Using a series of NS5A deletion mutants, we demonstrated that domain I of NS5A is important for the interaction with OTUD7B. Domain I of NS5A is highly conserved among all the HCV genotypes, and has a conserved tetracysteine zinc-binding motif that is essential for HCV RNA replication, with an N-terminal amphipathic α-helix that is responsible for membrane association. The NS5A domain I has been shown to interact with many host proteins. Our mapping study of the OTUD7B-binding domain on NS5A protein also demonstrated that the region from aa 121 to 126 of NS5A is essential for the interaction with OTUD7B. NS5A Val^{121} and Val^{124} in particular are crucial for the interaction with OTUD7B. Okamoto et al. reported that NS5A Val^{121} is important for the interaction with FK506-binding protein 8 (FKBP8) and is crucial for HCV replication. Interestingly, the NS5A V124A mutation disrupted the interaction with OTUD7B, but not with HNF-1α or FKBP8. These findings may give us a clue to understand the roles of NS5A in physical and functional interactions with these three different host factors, i.e., FKBP8, HNF-1α, and OTUD7B.

We used an RNAi technique to investigate the role of OTUD7B in HCV replication and the production of infectious HCV particles. The efficient knockdown of endogenous OTUD7B resulted in slight increases of HCV replication and the production of infectious HCV particles. Further studies are required to validate the role of OTUD7B in HCV replication and the production of infectious HCV particles.

We also obtained evidence suggesting that HCV NS5A enhances OTUD7B DUB activity. There are at least two possibilities to explain this result. First, the interaction between OTUD7B and NS5A protein may enhance the DUB activity of OTUD7B. Second, NS5A may affect the subcellular localization of OTUD7B, thereby reducing the ubiquitylation.

CONCLUSION

HCV NS5A protein interacts with OTUD7B, thereby modulating its DUB activity. The identification of OTUD7B as the NS5A-binding protein may contribute to a better understanding of the ubiquitylation and deubiquitylation in the HCV life cycle as well as pathogenesis. To our knowledge, this is the first report of a specific interaction between HCV protein and DUB.

神戸大学大学院医学(系)研究科（博士課程）

論文審査の結果の要旨			
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論文題目 Title of Dissertation	Physical and functional interaction between hepatitis C virus NS5A protein and ovarian tumor protein deubiquitinase 7B Ｃ型肝炎ウイルスNS5A 蛋白と卵巣腫瘍蛋白脱ユビキチン化酵素７Ｂとの蛋白質間及び機能的相互作用		
審査委員 Examiner	主　査 Chief Examiner 林 祥剛 副　査 Vice-examiner 東 健 副　査 Vice-examiner 匂坂 敏朗		

（要旨は１，０００字～２，０００字程度）

要旨

Hepatitis C virus (HCV) infection often causes chronic liver diseases, including chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC). HCV infects approx. 130–170 million people worldwide (2.2%–3% of the global population), based on the World Health Organization estimation. Therefore, HCV infection poses extensive global public health problems. Many viruses modulate various cellular pathways by manipulating ubiquitylation and deubiquitylation machineries to enhance viral replication and pathogenesis. It was reported that several other HCV proteins, including NS5A protein, are also degraded through the ubiquitin-proteasome pathway. However, the physiological roles of ubiquitylation and deubiquitylation in HCV infection are largely unknown. To elucidate the roles of ubiquitylation and deubiquitylation in HCV infection, the candidate sought to identify a deubiquitinase (DUB) that can interact with HCV proteins. Here the candidate identified ovarian tumor protein 7B (OTUD7B) as an NS5A-binding protein. OTUD7B is a 93-kDa member of the OTU domain-A20-like DUB family and widely expressed in many tissues, including the liver. To determine whether HCV NS5A protein physically interacts with OTUD7B protein in cultured cells, the candidate cotransfected Huh-7 cells with the myc-His_6-tagged NS5A plasmid together with the FLAG-OTUD7B plasmid. The immunoprecipitation analysis revealed that NS5A protein was co-immunoprecipitated with FLAG-OTUD7B protein. In contrast, HCV core protein was not co-immunoprecipitated with FLAG-OTUD7B protein. These results suggest that NS5A protein specifically interacts with OTUD7B. To determine whether HCV NS5A protein expressed from replicating HCV can interact with endogenous OTUD7B, the candidate used HCV 1b full-length replicon (RCYM1). Immunoprecipitation analysis revealed that NS5A but not core protein was co-immunoprecipitated with endogenous OTUD7B using anti-OTUD7B pAb. These results suggest that HCV NS5A protein specifically interacts with endogenous OTUD7B in HCV-replicating cells. To determine whether NS5A protein colocalizes with OTUD7B in cells, Huh-7.5 cells were cotransfected with FLAG-OTUD7B expression plasmid and then infected with HCV J6/JFH1. Immunofluorescence staining demonstrated that OTUD7B localizes mainly in the cytoplasm and also in the nucleus to some extent without HCV infection. In the HCV-infected cells, NS5A and OTUD7B were colocalized in the cytoplasm. Interestingly, the candidate observed that the HCV-uninfected cells exhibited approx. 40% nuclear+cytoplasmic localization. In contrast, HCV-infected cells showed more than 90% nuclear+cytoplasmic localization. These results suggest that HCV infection enhances the nuclear localization of OTUD7B. To further investigate the presence and localization of NS5A-OTUD7B interactions in the context of the HCV infection, the

candidate performed an *in situ* proximity ligation assay (PLA). The result of PLA demonstrated a bright red signal in the cytoplasm where NS5A protein and OTUD7B protein showed a physical interaction. Taken together, these results indicate that HCV NS5A protein colocalizes with OTUD7B in HCV-infected cells. To determine the OTUD7B-binding domain on NS5A protein, the candidate performed co-immunoprecipitation analyses using a series of NS5A deletion mutants. When the N-terminal deletion mutants of NS5A were used, all of the HA-NS5A proteins except HA-NS5A(357–447), HA-NS5A(250–447), or HA-NS5A(214–447) were co-immunoprecipitated with OTUD7B. These results suggest that the domain I of NS5A consisting of aa 1 to 213 is important for the OTUD7B binding. In addition, FLAG-OTUD7B was co-immunoprecipitated with NS5A(1–126)-myc-His_6 and NS5A(1–147)-myc-His_6, but not with HA-NS5A(1–83), HA-NS5A(1–100), or HA-NS5A(1–120). These data suggest that the region from aa 121 to 126 of NS5A is essential for the specific interaction with OTUD7B. To identify the specific aa residues of NS5A required for interaction with OTUD7B, the candidate used alanine substitution mutants of HA-NS5A(1-126). The immunoprecipitation analysis revealed that both mutant HA-NS5A V121A(1-126) and HA-NS5A V124A(1-126) failed to interact with FLAG-OTUD7B. These results suggest that NS5A Val^{121} and Val^{124} are crucial for the interaction between NS5A and OTUD7B. To investigate the role of OTUD7B in HCV replication, the candidate used an RNA interference (RNAi) technique. The expression of endogenous OTUD7B was efficiently knocked down by siRNA. The intracellular HCV RNA levels in the HCV J6/JFH1-infected cells were slightly increased after siRNA transfection. The extracellular HCV infectious titer was increased 2-fold compared to the control cells. These data suggest that OTUD7B has some negative effects on HCV replication and the production of HCV infectious particles. To determine whether HCV NS5A affects OTUD7B DUB activity, we cotransfected Huh-7 cells with myc-His_6-tagged NS5A plasmid together with the FLAG-OTUD7B plasmid and HA-Ubiquitin plasmid. Transfection of the HCV NS5A plasmid resulted in a remarkable decrease in ubiquitylation signals. These data suggest that HCV NS5A enhances the OTUD7B DUB activity.

Here the candidate identified OTUD7B as a novel NS5A-binding protein. The candidate's immunoprecipitation analyses revealed that HCV NS5A protein specifically interacts with OTUD7B in a transient expression system and in HCV RNA replicon cells. The immunofluorescence staining analysis and the *in situ* PLA assay revealed that HCV NS5A protein colocalizes with OTUD7B in the cytoplasm. The candidate also obtained evidence suggesting that the nuclear localization of OTUD7B is enhanced in HCV-infected cells. Together these findings suggest that the NS5A-OTUD7B interaction affects the subcellular localization of OTUD7B and/or the stability of OTUD7B in the nucleus. Using a series of NS5A deletion mutants, we demonstrated that domain I of NS5A is important for the interaction with OTUD7B. Domain I of NS5A is highly conserved among all the HCV genotypes, and has a conserved tetracysteine zinc-binding motif that is essential for HCV RNA replication, with an N-terminal amphipathic α-helix that is responsible for membrane association. The NS5A domain I has been shown to interact with many host proteins. The candidate's mapping study of the OTUD7B-binding domain on NS5A protein also demonstrated that the region from aa 121 to 126 of NS5A is essential for the interaction with OTUD7B. NS5A Val^{121} and Val^{124} in particular are crucial for the interaction with OTUD7B. Okamoto et al. reported that NS5A Val^{121} is important for the interaction with FK506-binding protein 8 (FKBP8) and is crucial for HCV replication. Interestingly, the NS5A V124A mutation disrupted the interaction with OTUD7B, but not with HNF-1α or FKBP8. These findings may give the candidate a clue to understand the roles of NS5A in physical and functional interactions with these three different host factors, i.e., FKBP8, HNF-1α, and OTUD7B. The candidate used an RNAi technique to investigate the role of OTUD7B in HCV replication and the production of infectious HCV particles. The efficient knockdown of endogenous OTUD7B resulted in slight increases of HCV replication and the production of infectious HCV particles. Further studies are required to validate the role of OTUD7B in HCV replication and the production of infectious HCV particles. The candidate also obtained evidence suggesting that HCV NS5A enhances OTUD7B DUB activity. There are at least two possibilities to explain this result. First, the interaction between OTUD7B and NS5A protein may enhance the DUB activity of OTUD7B. Second, NS5A may affect the subcellular localization of OTUD7B, thereby reducing the ubiquitylation. HCV NS5A protein interacts with OTUD7B, thereby modulating its DUB activity. The identification of OTUD7B as the NS5A-binding protein may contribute to a better understanding of the ubiquitylation and deubiquitylation in the HCV life cycle as well as pathogenesis. This may be the first report of a specific interaction between HCV protein and DUB. The candidate, having completed studies on molecular basis, with a specialty in interaction between HCV protein and DUB, and having advanced the field of knowledge in the area of physiological roles of ubiquitylation and deubiquitylation in HCV infection, is hereby recognized as having qualified for the degree of Ph.D.(Medicine).