cells. In PH/Ctr cells, IRF3 was distributed in a perinuclear and/or cytoplasmic context. However, in PH/NS5B cells as well as PH5CH8 cells treated with poly (IC), IRF3 was distributed to the nucleus, a finding consistent with its activated state (Fig. 10D). Taken together, our findings indicate that the RdRp activity of HCV NS5B anchoring on ER membrane is necessary and sufficient to activate the TLR3 signaling pathway.

PH/NS5B cells are more susceptible than PH/Ctr cells to DNA-damaging reagents

To better understand the effect of IFN-β induction in PH/NS5B cells, we next examined the susceptibilities of PH/NS5B and PH/Ctr cells against various types of DNA-damaging reagents. A clonogenic assay using PH/NS5B and PH/Ctr cells was performed by treatment with MMS (a DNA alkylating reagent) and H_2O_2 (a DNA oxidative reagent) and by UV-B irradiation, which induces DNA single-strand breaks and/or thymidine dimer formation in DNA. ADR and NCS, which induce DNA double-strand breaks, were also used for the clonogenic assay. As shown in Fig. 11A, PH/NS5B and PH/Ctr cells were susceptible to the MMS treatment, the H_2O_2 treatment, and the UV-B irradiation, and no differences were observed between their susceptibilities. Interestingly, however, PH/NS5B cells were more susceptible than PH/Ctr cells against ADR or NCS treatment (Fig. 11A). These results suggest that PH/NS5B cells are more sensitive than PH/Ctr cells to damage in the form of DNA double-strand breaks. To clarify whether or not IFN-β induction increases the susceptibility against ADR or NCS treatment, we examined the effect of ADR or NCS in PH/Ctr cells treated with IFN-β. In this treatment, the cells changed to susceptible phenotype against ADR or NCS treatment, as observed in PH/NS5B cells (Fig. 11B). These results suggest that IFN-β induced by NS5B in PH5CH8 cells changes the cells into the hypersensitive phenotype, making them susceptible to DNA damage in the form of double-strand breaks.

Discussion

In the present study, we found that HCV NS5B induced IFN-β in two kinds of immortalized human hepatocyte cell lines, PH5CH8 and NKNT-3. We showed that NS5B's induction of IFN-β was mediated through the TLR3 but not the RIG-I signaling pathway. The induction of IFN-β caused the delay of cell cycle progression through the S phase in these cells. Since it has been generally known that the activation of the TLR3 signaling pathway is caused by dsRNA, a molecular pattern associated with replicating viral genomes, we first obtained data suggesting that dsRNA is generated by NS5B even without replication of the viral genome.

TLRs belong to a family of evolutionarily conserved innate immune recognition molecules, and ten members of the TLR family have been identified in human (Medzhitov, 2001; Takeda et al., 2003). TLR3 recognizes dsRNA and induces the antiviral immune responses (Alexopoulou et al., 2001; Matsumoto et al., 2002). TLR3 activates transcription factor IRF3 through TRIF, leading to IFN-β production (Oshiumi et al., 2003; Yamamoto et al., 2002, 2003). We speculated on two possible mechanisms underlying the activation of the TLR3 signaling pathway by NS5B. The first possibility is that protein–protein interaction between NS5B and TRIF is involved in the activation of the TLR3 signaling pathway. However, we failed to obtain evidence of direct interaction between NS5B and TRIF. The second possibility is that the RdRp activity of NS5B contributes to the activation of TLR3. To evaluate this hypothesis, we examined whether or not several NS5B mutants, including carboxyl-truncated mutants or an RdRp activity-defective mutant (G2736V), could induce IFN-β. The experimental data clearly showed that neither the G2736V mutant nor the carboxyl-truncated mutants could induce IFN-β. Therefore, we suggested that NS5B RdRp activity anchoring the ER membrane is critical for the activation of the TLR3 signaling pathway.

The finding that NS5B RdRp activity on the ER membrane was a critical factor for the induction of IFN-β surprised us because we expected that dsRNA, a natural ligand for TLR3, was produced in NS5B-expressing hepatocyte cells without replication of the viral RNA genome. Therefore, we now presume a daring hypothesis: that NS5B can produce dsRNA using cellular RNA as a template on the ER membrane. Since no direct evidence has been found to support this hypothesis at this stage, further experiments are necessary to evaluate this hypothesis. For instance, if possible, the detection of newly synthesized dsRNA in NS5B-expressing cells or the detection of newly synthesized dsRNA by recombinant NS5B using cellular RNA in vitro may become positive evidence. Furthermore, since the formation of a membrane-associated replication complex is a characteristic of positive-stranded RNA viruses, including HCV (Shi et al., 2003), it will also be interesting to examine whether or not the RdRps of the other RNA viruses possess novel activity similar to that observed in this study. At least we recently detected that NS5B derived from an HCV-2a genome designated JFH-1, which produces virus particles infectious for HuH-7 cells (Wakita et al., 2005), also strongly induced IFN-β in PH5CH8 cells (Ikeda et al., unpublished data). In addition, we are not able to completely exclude the possibility that NS5B-encoding RNA, but not NS5B, specifically activates the TLR3 signaling pathway. However, this possibility is unlikely because the G2736V mutant with only one nucleotide substitution could not activate the TLR3 signaling pathway.

Since the activation of the TLR3 signaling pathway in NS5B-expressing PH5CH8 or NKNT-3 cells is considered to be due to a novel function of NS5B, it is important to clarify whether or not this function occurs in the HCV life cycle. Although HCV replicon systems carrying autonomously replicating HCV RNA genomes developed using HuH-7 (Blight et al., 2000; Ikeda et al., 2002; Lohmann et al., 1999) and HeLa (Zhu et al., 2003) cells have become powerful tools for basic studies of HCV, these systems would not be suitable to prove our hypothesis because the induction of IFN-β by NS5B was not observed in HuH-7 or HeLa cells, in which the TLR3 signaling pathway was suggested to be defective. In fact, it has been recently reported that HuH-7 cells lack a TLR3

response to external dsRNA (Lanford et al., 2003). Therefore, a new HCV replicon system needs to be developed using other human cell lines possessing intact TLR3 signaling pathways. We are currently making a trial to establish an HCV replicon system using PH5CH8 or NKNT-3 cells.

On the other hand, it has been recently found that an HCV serine protease, NS3-4A, can block the phosphorylation and effector action of IRF3 (Foy et al., 2003). This finding using HuH-7 cells suggests that NS3-4A mediates the proteolysis of cellular proteins within an antiviral signaling pathway upstream of IRF3, leading to persistent viral infection. The recently identified TRIF (Li et al., 2005a, 2005b) and RIG-I (Foy et al., 2005) are possible candidates for these cellular proteins. Therefore, it was thought that IFN-β induction by NS5B through the activation of TLR3 might be suppressed by NS3-4A in PH5CH8 cells. In fact, our recent study showed that NS3-4A, in a serine protease activity-dependent manner, suppressed NS5B's activation of the IFN system (Dansako et al., 2005). However, the synergistic induction of IFN-β in PH5CH8 cells co-expressing Core and NS5B was only partially suppressed by NS3-4A, whereas the induction of IFN-β by NS5B only was drastically suppressed by NS3-4A (Dansako et al., 2005). We speculate that a biological implication of this phenomenon is that HCV proteins contribute to the maintenance of a low steady state of the virus by controlling the expression level of IFN-β in the infected cells.

In addition to the delay of cell cycle progression through the S phase, enhanced susceptibility to DNA-damaging reagents was found in NS5B-expressing PH5CH8 cells. This phenomenon was attributed to IFN-β induced by NS5B. Further characterization of this phenomenon may contribute to the understanding of IFN-β's biological effects on hepatocytes and effects on the pathogenesis of hepatocellular carcinoma caused by HCV. Furthermore, the findings of the present study may contribute to an understanding of the mechanisms underlying the TLR3 activation involved in innate immunity against viral infection. In addition, our findings suggest that an antiviral state in uninfected cells may be induced by the expression of a viral protein, NS5B.

Materials and methods

Cell culture and cell cycle analysis

The non-neoplastic immortalized human hepatocyte cell lines, PH5CH8 and NKNT-3 cells, were maintained as described previously (Ikeda et al., 1998; Kobayashi et al., 2000). Human hepatoma cell line HuH-7 cells, human cervical carcinoma HeLa cells, and HEK 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

To synchronize the cells at the G1/S transition, growing cells were treated with thymidine (Sigma, St. Louis, MO) (2.5 mM) for 19 h, washed in PBS, and released into fresh medium for 11 h. The cells were then treated with aphidicolin (Sigma) (5 μM) for 13 h, washed in PBS, and released into fresh medium. The cells were pulse-labeled with 10 μM bromodeoxyuridine (BrdUrd; Sigma) for 1 h, fixed with 70% ethanol at indicated time points, stained with fluorescein-isothiocyanate (FITC)-conjugated mouse monoclonal antibody to BrdUrd (BD Pharmingen, San Diego, CA), and counterstained with propidium iodide (PI) (Sigma). The cellular content of DNA was determined by flow cytometry with FACScalibur instrument, and data were analyzed with CELL Quest software (BD Biosciences, San Jose, CA) (Naka et al., 2004). To determine the population of G2-M phase reached cells, the cells were treated with Nocodazole (Noc; Sigma) (200 ng/ml) at 5 h after release into the S phase. Then, after 7 h (post release from 12 h), the cell population that had accumulated in the G2-M phase was analyzed by flow cytometry. To examine the effects of IFN-β, PH5CH8 and NKNT-3 cells were treated with or without IFN-β (500 IU/ml) at 12 h prior to release, and cell cycle progression was analyzed. To assess the effect of anti-IFN-β neutralizing antibody, NS5B-expressing cells were treated with anti-IFN-β antibody (70 U/ml, OBT0377, Oxford Biotechnology, Oxfordshire, UK) during cell cycle synchronization and after release from the G1/S boundary.

Vector construction and retrovirus infection

Retroviral vectors pCXbsr (Akagi et al., 2000) and pCX4bsr (Akagi et al., 2003), which contain the resistance gene for blasticidin, were used in this study. The DNA fragments encoding the influenza hemagglutinin tagged (HA)-core, NS3, HA-NS4B, HA-NS5A, HA-NS5B, and NS5B were amplified from pMILE (HCV 1B-1 strain belonging to genotype 1b; accession no. AB080299) by PCR using KOD-plus DNA polymerase (Toyobo, Osaka, Japan). The obtained DNA fragments were subcloned into the *Eco*RI (*Bam*HI for NS5B) and *Not*I sites of pCXbsr or pCX4bsr. The DNA fragment encoding myc-tagged NS5A was also amplified from pMILE by PCR. The obtained DNA fragment was subcloned into the *Eco*RI and *Not*I sites of pCXpur (Akagi et al., 2000), which contains the resistance gene for puromycin. The DNA fragments encoding TLR3 (accession no. NM_003265), Toll-IL-1 receptor (TIR) domain-containing adaptor-inducing IFN-β (TRIF or TICAM-1, accession no. NM_182919), and RIG-IC, a dominant negative inhibitor of retinoic acid-inducible gene-I (RIG-I) (Yoneyama et al., 2004), were amplified from cDNAs obtained from PH5CH8 cells. The primer sequences containing the *Sph*I (for forward) or *Not*I (for reverse) recognition sites for TLR3, TRIF, and RIG-IC were designed to enable expression of the TLR3, TRIF, and RIG-IC open-reading frames, respectively. The obtained DNA fragments were subcloned into the *Sph*I and *Not*I sites of pCXpur/myc, which can express myc-tagged protein. The *IFN-β* gene promoter region (−125 to +19) described previously (Fujita et al., 1988) was amplified using genomic DNA derived from PH5CH8 cells and a primer set of 5′-ACGGGGTACC-GAGTTTTAGAAACTACTAAAATG-3′ containing the *Kpn*I recognition site (underlined) and 5′-AGGAAGATCTTC-GAAAGGTTGCAGTTAGAATG-3′ containing the *Bgl*II recognition site (underlined). The obtained DNA fragment was

subcloned into the *Kpn*I and *Bgl*II sites of pGL3-Basic (Promega) and was termed pIFN-β(-125)-Luc. Retrovirus infections were performed as described previously (Naganuma et al., 2004).

RT-PCR and RNA interference

RT-PCR was carried out as described previously (Dansako et al., 2003). The sequences of sense and antisense primers for IFN-β (accession no. V00547) were 5′-CCCTGAGGAGATTAAGCAGCTGC-3′ and 5′-AGTTCCTTAGGATTTCCACTCTGAC-3′. The sequences of primer set for ISG56 (accession no. X03557) were 5′-AGAAGCAGGCAATCACAGAAAAGCTG-3′ and 5′-CCAGGGCTTCATTCATATTTCCTTCC-3′. Small-interference RNA (siRNA) duplexes targeting the coding regions of human TLR3, TLR4, and luciferase GL2 (Elbashir et al., 2001) as a control were chemically synthesized (Greiner, Tokyo, Japan). The sequences of the human TLR3 oligonucleotides were: 5′-CCUCCAGCACAAUGAGCUATT-3′ and 5′-UAGCUCAUUGUGCUGGAGGTT-3′. The sequences of the human TLR4 oligonucleotides were: 5′-CCUCCCCUUCUCAACCAAGTT-3′ and 5′-CUUGGUUGAGAAGGGGAGGTT-3′. The cells were transfected with the indicated siRNA duplex using OligofectAMINE (Invitrogen, Carlsbad, CA). Total RNAs were extracted after 3 days, and RT-PCR was performed using primer sets for TLR3 (Kadowaki et al., 2001), TLR4 (Kadowaki et al., 2001), and GAPDH (Dansako et al., 2003).

Western blot analysis

The preparation of cell lysates, SDS-PAGE, and immunoblotting analysis were performed according to standard procedures using primary antibodies, rat monoclonal anti-HA (3F10; Roche Molecular Biochemicals, Mannheim, Germany), mouse monoclonal anti-NS3 (clone MMM33; Novacastra Laboratories, Newcastle upon Tyne, UK), anti-NS5B (a gift from Dr. M. Kohara), anti-myc (PL14; Medical and biological laboratories, Nagoya, Japan), anti-β-actin (AC-15, Sigma), anti-STAT1 (clone 42; BD Transduction Laboratories, San Diego, CA), rabbit polyclonal anti-phospho-STAT1(Y701) (Cell Signaling Technology, Beverly, MA), and anti-IRF7 (H-246, Santa Cruz Biotechnology, Santa Cruz, CA), and horseradish-peroxidase-conjugated secondary antibodies. The immune complex was visualized using the ECL Western blot detection system (Amersham Bioscience, Piscataway, NJ).

Reporter assay

The luciferase activity was measured by dual-luciferase assay system (Promega, Madison, WI) as previously described (Dansako et al., 2003). Briefly, cells were transfected with pISRE-Luc (Stratagene, LaJolla, CA) or pIFN-β(-125)-Luc reporter plasmid together with phRL-CMV (Promega) as an internal control reporter plasmid by FuGENE6 (Roche). After 48 h of transfection, cell lysates were then prepared and assayed for luciferase activities; transfection efficiency was normalized by renilla luciferase activity (internal control) derived from phRL-CMV. Three independent triplicate transfection experiments were conducted in order to verify the reproducibly of the results.

Immunoprecipitation

Cells were lysed in a buffer containing 50 mM Tris (pH 7.4), 125 mM NaCl, 0.1%(v/v) Nonidet P-40 (NP-40; Sigma), 5 mM EDTA, 0.1 M NaF, and a mixture of protease inhibitors (Complete; Roche). Pre-cleared cell lysates were subjected to immunoprecipitation using agarose-conjugated anti-myc antibody (PL14, MBL). Bound proteins were eluted from beads by boiling in SDS sample buffer, and immunoblotting analysis was performed using anti-myc or anti-NS5B antibody, and HRP-conjugated anti-mouse IgG TrueBlot (eBioscience, San Diego, CA).

Immunofluorescence analysis

To examine the intracellular protein localization, 2×10^4 cells were cultured and treated on chamber slides then fixed and probed with polyclonal rabbit anti-IRF3 antibody (FL-425, Santa Cruz Biotechnology) and FITC-conjugated donkey anti-rabbit secondary antibody according to a method described previously (Foy et al., 2003). PH5CH8 cells treated with poly (IC) (2.5 μg/ml for 6 h; Amersham Biosciences) were used as a positive control for the activation of IRF3.

Evaluation of sensitivity to DNA damage

Cells in an exponential growth phase were plated onto 10-cm plates (5×10^3 cells/plate) and cultured for 4 days. The cells were treated with hydrogen peroxide (H_2O_2; Wako Pure Chemical, Osaka, Japan), methylmethane sulfonate (MMS; Sigma), Adriamycin (ADR; doxorubicin; Sigma), and neocarzinostatin chromophore (NCS; generously provided by Kayaku, Tokyo, Japan) for 2 h at 37 °C. For UV-B treatment (UV-B radiation at 302 nm), the medium was aspirated prior to exposure, the cells were washed twice with PBS, and then a fresh culture medium was added. Ten days later, the cells were fixed and stained with Coomassie brilliant blue as described previously (Naganuma et al., 2004). Only colonies containing >50 cells were scored as being derived from viable clonogenic cells.

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Hepatitis C virus core protein exerts an inhibitory effect on suppressor of cytokine signaling *(SOCS)-1* gene expression

Hideyuki Miyoshi[1], Hajime Fujie[1], Yoshizumi Shintani[1], Takeya Tsutsumi[1], Seiko Shinzawa[1], Masatoshi Makuuchi[2], Norihiro Kokudo[2], Yoshiharu Matsuura[3], Tetsuro Suzuki[4], Tatsuo Miyamura[4], Kyoji Moriya[1], Kazuhiko Koike[1,*]

[1]*Department of Internal Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan*
[2]*Department of Hepatobiliary, Pancreatic and Transplantation Surgery, Graduate School of Medicine, University of Tokyo, Tokyo, Japan*
[3]*Research Center for Emerging Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan*
[4]*Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan*

***Background/Aims*: Suppressor of cytokine signaling (SOCS)-1, a negative feedback regulator of cytokine signaling pathway, also has a tumor suppressor activity, the silencing of its gene by hypermethylation is suggested to contribute to hepatocarcinogenesis. We studied the effect of the core protein of hepatitis C virus (HCV) on the expression of *SOCS-1* gene.**

***Methods*: HCV core gene transgenic mice, which develop hepatocellular carcinoma late in life, HepG2 cells expressing the core protein, and human liver tissues were analyzed.**

***Results*: The expression of *SOCS-1* gene was significantly suppressed in the liver of core gene transgenic mice and HepG2 cells expressing the core protein, while that of *SOCS-3* gene was conserved. *SOCS-1* expression levels also decreased in HCV-positive human liver tissues. The core protein differentially down-regulated the expression of signal transducer and activator of transcription (STAT) target genes, but rather enhanced STAT1 and STAT3 activation after interleukin-6 stimulation in mouse liver tissues and cells.**

***Conclusions*: HCV core protein down-regulates the expression of *SOCS-1* gene. This is a mechanism leading to *SOCS-1* silencing, an alternative to the hypermethylation of the gene; this effect of the core protein may modulate the intracellular signaling pathway, contributing to the pathogenesis in HCV infection including hepatocarcinogenesis.**

***Keywords*: Tumor suppressor gene; Hepatocellular carcinoma; Transgenic mouse; STAT3**

1. Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis. A substantial proportion of patients with chronic hepatitis C eventually develop hepatocellular carcinoma (HCC), which is one of the leading causes of death worldwide [1,2]. Despite the absence of appropriate in vitro replication systems or practical infectious animal model systems, the mechanism underlying hepatocarcinogenesis in human HCV infection is gradually clarified. Both the direct and indirect effects of HCV on HCC development are demonstrated [3–6]. The accumulation of gene aberrations, such as the inactivation of tumor suppressor genes or the activation of oncogenes, which are induced through the inflammation-mediated continuous death of hepatocytes followed by regeneration, may be one of the mechanisms underlying hepatocarcinogenesis [3]. On the other hand, the viral gene products are suggested to contribute to the development of HCC by their direct effects on hepatocytes [4]. Such direct effects have been demonstrated by the use of model systems including mice

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* Corresponding author. Address: Department of Infectious Diseases, Internal Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Tel.: +81 3 5800 8801; fax: +81 3 5800 8807.
E-mail address: kkoike-tky@umin.ac.jp (K. Koike).

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[7–9]. HCV-infected hepatocytes produce viral structural and nonstructural proteins. Some of these confer certain phenotypes to hepatocytes and may be associated with the pathogenesis of HCV infection including the development of HCC. Among such viral proteins, the core protein of HCV has a variety of biological activities, including oncogenic activity, which substantially affects host cellular functions [7–11].

Suppressor of cytokine signaling (SOCS)-1, also called signal transducer and activator of transcription (STAT)-induced STAT inhibitor-1 or Jak binding protein-1, is a negative feedback regulator of cytokine signaling through the Jak/STAT pathway. SOCS-1 contains the SH2 domain and directly interacts with the kinase domain of Jak to suppress Jak activity. *SOCS-1* gene expression is augmented by various cytokines, such as interferon (IFN)-γ, interleukin (IL)-6 or leukemia inhibitory factor (LIF), resulting in the suppression of the signal transduction downstream pathways of these cytokines [12–14]. Moreover, SOCS-1 has been recently shown to exhibit a tumor suppressor activity. SOCS-1 suppresses the expression of several oncogenes or growth-related genes acting as a negative regulator of cell proliferation: the loss of function of SOCS-1 facilitates tumor progression [15–17]. As a mechanism underlying the loss of function of SOCS-1, a recent study has revealed a frequent silencing of the *SOCS-1* gene by CpG methylation in HCC tissues [18–20]. Alternatively, however, it may be possible that HCV infection, particularly, the proteins that the HCV genome encodes per se, may render the *SOCS-1* gene unable to exhibit its function by gene silencing.

We examined such a possibility using a mouse model for HCV infection that is destined to develop HCC [7,9], as well as cultured cells expressing the HCV core protein [21]. The core protein markedly suppressed the expression of the *SOCS-1* gene in both liver tissues and cultured cells. This silencing of the *SOCS-1* gene may be one of explanations for the pathogenicity of HCV in humans.

2. Materials and methods

2.1. Transgenic mouse and cell lines

HCV core gene transgenic mice have been described previously [7]. These mice develop HCC late in life [7,9]. The mice were cared for according to the institutional guidelines and maintained in a specific pathogen-free state. All the animals received humane care and the study protocol complied with the institution's guidelines for the care and use of experimental animals. HepG2 cell lines expressing the HCV core protein under the control of CAG promoter (Hep39J, Hep396 and Hep397) or a control HepG2 line (Hepswx) carrying the empty vector were described previously [21,22].

2.2. IL-6 Stimulation

For the in vivo experiments, 0.05–0.5 μg/g BW murine IL-6 (Diaclone, Besançon, France) was administered into 8 w.o. male mice i.p., and liver tissues were obtained 60 min later. Cultured cells were treated with human IL-6 (Diaclone) at 10–100 ng/ml or IFN-α at 1.0–10.0 ng/ml and then were harvested 60 min later.

2.3. Reverse transcription (RT)-PCR analysis

Total RNA was extracted from liver tissues or cultured cells before and after the treatment with IL-6 using TRIzol (Invitrogen). RNA was reverse-transcribed using oligo(dT) primers and Superscript II (Invitrogen). Equal amounts of cDNA were then subjected to PCR. The primer pairs used were:

5′-CACTCACTTCCGCACCTTCC-3′ (forward) and
5′-TCCAGCAGCTCGAAAAGGCA-3′ (reverse) for murine *SOCS-1*,
5′-CACGCACTTCCGCACATTCC-3′ (forward) and
5′-TCCAGCAGCTCGAAGAGGCA-3′ (reverse) for human *SOCS-1*,
5′-TCACCCACAGCAAGTTTCCCGC-3′ (forward) and
5′-GTTGACAGTCTTCCGACAAAGATGC-3′ (reverse) for murine *SOCS-3*,
5′-CACGCACTTCCGCACATTCC-3′ (forward), and
5′-GTTGACGGTCTTCCGACAGAGATGC-3′ (reverse) for human *SOCS-3*.

For the RT-PCR analysis, the quantity of cDNA template and the number of amplification cycles were optimized to ensure that the reaction was terminated during the linear phase of product amplification, so that the semiquantitative comparison of mRNA abundance between different samples was possible [23]. The intensities of the bands were determined using a densitometer. RT-PCR was also done using GAPDH primers to adjust the amounts of RNA in each experiment.

2.4. Human liver tissue samples and real-time PCR

Nine patients with HCC who had underlying chronic hepatitis C were studied for *SOCS-1* expression in noncancerous tissues. Additional nine patients, who were found to be negative for both HBsAg and anti-HCV at the time of operation, were also studied. The latter patients underwent liver resection for metastatic liver tumors from colon cancer. The experimental protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Ethics Review Committee for Human Experimentation. Informed consent was obtained from each patient. The noncancerous liver tissues obtained from these patients were immediately frozen and stored at −80 °C until further use.

Taqman real-time RT-PCR was performed as described previously [24], using an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Primers and the TaqMan probe for *SOCS-1* were as follows:

Forward primer:5′-CTGGCCCCGGAGCAT-3′
Reverse primer:5′-GTTGTGTGCTACCATCCTACAGA-3′
Probe:5′-FAM-CCGGACGCTATGGCCCA-MGB-3′

Primers and probes for *SOCS-3*, β-*actin*, interferon regulatory factor (*IRF*)-1, c-*myc* and *bcl*-X_L genes were purchased from ABI by Assays-on-Demand system.

2.5. Methylation status

The methylation status of the *SOCS-1* gene was analyzed by methylation-specific PCR as described previously [20].

2.6. Western blotting and immunoprecipitation

Nuclear and cytoplasmic fractions were prepared from HepG2 cells, and Western blotting was performed as described previously [25]. Anti-STAT1 and anti-STAT3 polyclonal antibodies (Cell Signaling Technology, Inc., Beverly, MA), anti-phosphorylated STAT3 (Tyr705) polyclonal antibody (Cell Signaling Technology), anti-phosphorylated STAT3 monoclonal antibody (Upstate Biotechnology Inc., Lake Placid, NY), and anti-protein inhibitor of activated *STAT* (*PIAS*)*1*, *anti-PIAS3* and *anti-SOCS-1* antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were

used. Immunoprecipitation was done as described previously using antibodies followed by protein A-Sepharose [26].

2.7. Immunocytofluorescence

HepG2 cell lines with or without the core gene were grown overnight on chamber slides and treated with 10 ng/ml human IL-6 for 60 min. Cells were fixed with 4% paraformaldehyde plus methanol, and reacted with the anti-STAT3 antibody followed by incubation with a FITC-labeled secondary antibody.

2.8. Statistical analysis

The results are expressed as means ± SD. The significance of the difference in means was determined by Mann–Whitney's *U*-test. $P<0.05$ was considered significant.

3. Results

3.1. HCV core protein suppresses SOCS-1 gene expression

To examine the impact of the core protein on *SOCS-1* gene expression, we analyzed mRNA expression levels by semi-quantitative RT-PCR in liver tissues from the HCV core gene transgenic and nontransgenic control mice. *SOCS-1* mRNA expression levels in mouse liver tissues of nontransgenic mice were increased in a dose-dependent manner of IL-6, but were only marginal in the liver tissues from the core gene transgenic mice even in those treated with the maximal dose of IL-6 (0.5 μg/g BW, $n=5$ each) (Fig. 1(A) and (C)). In contrast, the expression levels of *SOCS-3* mRNA in the core gene transgenic mice were comparable to or rather higher than those in nontransgenic mice, before and after stimulation with IL-6 (Fig. 1(A) and (E)) [27,28].

We then examined whether or not this observation in mice is reproducible in HepG2 cell lines that constitutively express the core protein. *SOCS-1* gene expression was suppressed in the core-expressing HepG2 cell lines Hep396, Hep397 and Hep39J, even after stimulation with IL-6, while control bulk HepG2 cells or a control Hepswx cell line expressed *SOCS-1* mRNA at high levels (Fig. 1(B) and (D)). In contrast, the levels of *SOCS-3* gene expression were similar among the core-expressing HepG2 cell lines and control HepG2 cells, and were augmented by stimulation with IL-6 (Fig. 1(B) and (F)). These observations indicate that the core protein selectively suppresses *SOCS-1* gene expression before the translational level. The SOCS-1 protein was not detectable by Western blotting either in the mouse liver or HepG2 cells using currently available anti-SOCS-1 antibodies.

These results, obtained in HepG2 cell lines constitutively expressing the core protein, were then evaluated using a transient expression system. In this system, HepG2 cells were infected with baculovirus, expressing the core protein as described previously [29], and *SOCS-1* expression was determined by semiquantitative RT-PCR. The introduction

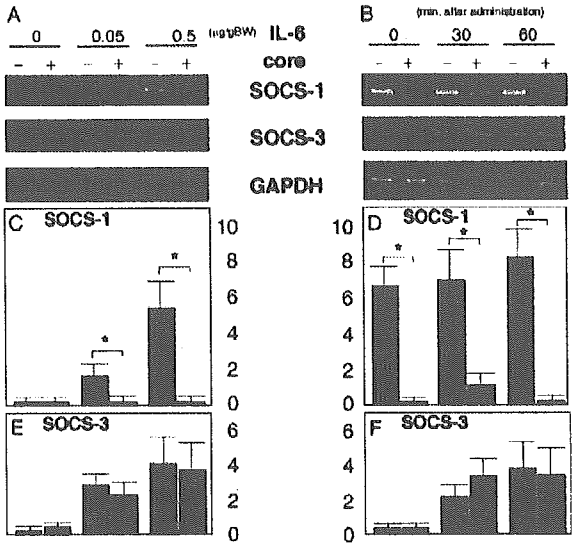

Fig. 1. Suppression of *SOCS-1* gene expression by hepatitis C virus core protein. (A) and (B) RNA from mouse liver tissues (A) or HepG2 cells (B) with or without the core protein was subjected to RT-PCR for the determination of *SOCS-1* and *SOCS-3* gene expression. Liver tissues were taken from mice one hour after inoculation of 0, 0.05 or 0.5 μg/g BW of IL-6. HepG2 cells (Hep396 and Hepswx) were treated with 10 ng/ml of IL-6 for 0, 30 or 60 min before RNA extraction. Bottom panels in (A) and (B) show the expression level of the housekeeping gene GAPDH as an internal control. (C) and (D) Represent means ± SD. of five independent experiments on *SOCS-1* gene expression corresponding to the lanes in (A) and (B), respectively. *, $P<0.05$. (E) and (F) Represent means ± SD. of five independent experiments on *SOCS-3* gene expression corresponding to the lanes in (A) and (B), respectively.

of the core protein selectively suppressed the expression level of *SOCS-1* mRNA even after stimulation with IL-6 (data not shown).

Modulation of expression by the core protein of STAT-target genes other than *SOCS*s was then examined by determining the mRNA levels in mouse liver tissues. Expression of *IRF-1* gene was suppressed in the presence of the core protein under the stimulation with IL-6, while that of c-*myc* was not affected (Fig. 2(A) and (B)). The expression of *bcl*-X_L gene was rather augmented by the core protein although the difference was not statistically significant (Fig. 2(C)).

The methylation status of *SOCS-1* gene was then explored in liver tissues from the core gene transgenic mice by a method described previously [20], to determine whether or not the *SOCS-1* gene expression may be suppressed by hypermethylation. No hypermethylation was observed in the *SOCS-1* gene of the core gene transgenic mice either at the 5′-noncoding region or the CpG island in the coding region (Fig. 3).

In the analysis of *SOCS-1* expression in noncancerous liver tissues from patients with HCV infection, the *SOCS-1* mRNA expression levels were 0.494 ± 0.352 in HCV-positive patients ($n=9$) and 0.862 ± 0.465 in the control subjects without HCV infection ($n=9$) (in arbitrary units,

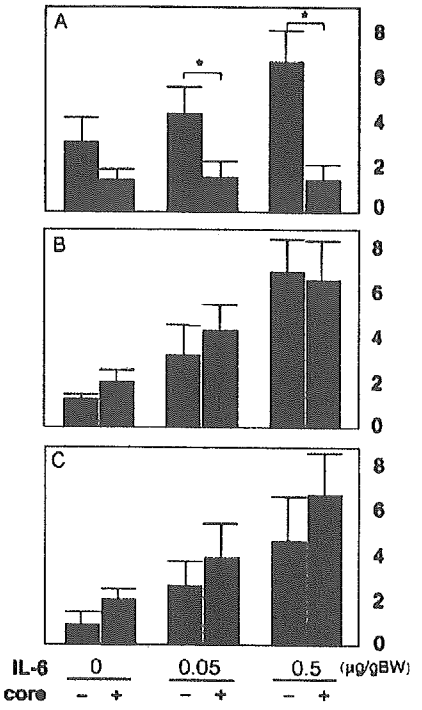

Fig. 2. Effect of hepatitis C virus core protein on the expression of STAT-target genes. RNA from mouse liver tissues with or without the core protein was subjected to RT-PCR for the determination of *IRF1* (A), c-*myc* (B) and *bcl*-X_L (C) gene expressions. Liver tissues were taken from mice one hour after inoculation of 0, 0.05 or 0.5 μg/g BW of IL-6. *, $P<0.05$.

$P=0.0345$). Thus, the SOCS-1 levels in the liver tissues of chronic hepatitis C patients were significantly lower than those of subjects without HCV infection.

3.2. The core protein did not suppress phosphorylation of STAT3 or STAT1

The activation of STAT3 enhances *SOCS-1* expression, thereby forming a negative feedback loop to the STAT3 status [17]. To determine whether or not STAT3 activation is involved in the *SOCS-1* gene suppression in this system, the tyrosine phosphorylation of STAT3 in the mouse liver was

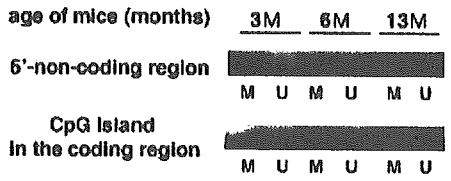

Fig. 3. Methylation status of *SOCS-1* gene in liver from hepatitis C virus core gene transgenic mice. DNA from the liver tissues of core gene transgenic mice at the age of 3 months (3M), 6 months (6M) or 13 months (13M) was subjected to methylation-specific PCR. Only PCR with unmethylation-specific primers yielded bands indicating that the *SOCS-1* gene was unmethylated in the liver tissues of core gene transgenic mice. M, methylation-specific primers; U, unmethylation-specific primers.

examined by Western blotting using an anti-phospho-STAT3 (tyrosine (Tyr)^{705}) antibody. At baseline, Tyr^{705} phosphorylation of STAT3 was low in both the core gene transgenic and nontransgenic mice. However, in response to stimulation with IL-6, the levels of Tyr^{705} phosphorylation of STAT3 was higher in the liver tissues from the core gene transgenic mice than that from nontransgenic mice. A representative result is shown in Fig. 4(A). Similarly, the levels of Tyr^{705} phosphorylation of STAT3 were higher in HepG2 cell lines expressing the core protein than those in control cells (Fig. 4(B)). These results observed in HepG2 cells constitutively expressing the core protein was also evaluated in a transient expression system using a recombinant baculovirus, as described above. The Tyr^{705} phosphorylation of STAT3 was enhanced in HepG2 cells infected with baculovirus expressing the core protein compared with mock-infected HepG2 cells (data not shown). The activation of STAT1 was also analyzed using HepG2 cell lines. As shown in Fig. 4(C), the levels of STAT1 phosphorylation was higher in HepG2 cells expressing the core protein than in control cells similar to the result on STAT3.

3.3. Subcellular localizations of STAT3 and STAT1

STAT activation by tyrosine phosphorylation results in the migration of STAT from the cytoplasm to the nucleus to bind to genomic DNA, modulating of cellular gene expression. We thereby evaluated the subcellular localization of STAT3 and STAT1 by preparing cytoplasmic and nuclear fractions from HepG2 cells followed by Western blotting. The amounts of STAT3 in the nuclei of core-expressing HepG2 cells were similar to or slightly larger

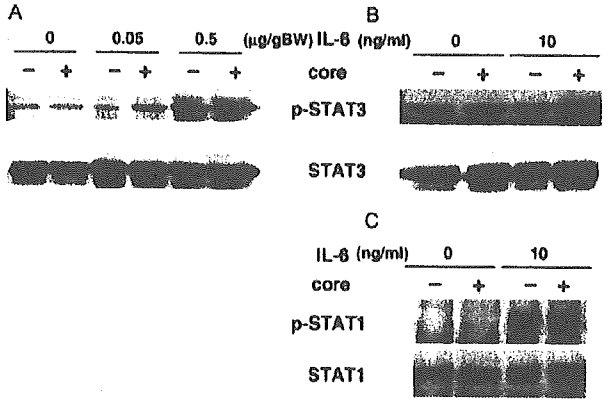

Fig. 4. Increase in the level of tyrosine phosphorylation of STAT3 and STAT1 by hepatitis C virus core protein. Whole cell lysates from mouse liver tissues (A) and HepG2 cells (B) and (C) were subjected to SDS-PAGE followed by Western blotting with anti-STAT3 and anti-*P*-STAT3 (A) and (B) or with anti-STAT1 and anti-*P*-STAT1 (C). Liver tissues were obtained from the mice treated as described in the Fig. 1 legends. HepG2 cells were treated with 10 ng/ml IL-6 or vehicle for 1 h. *P*-STAT3, phosphorylated STAT3; STAT3, total STAT3; p-STAT1, phosphorylated STAT1; STAT1, total STAT1. The experiments were repeated three times.

than those in control HepG2 cells in the presence or absence of IL-6 (Fig. 5(A)). This result was confirmed by an immunofluorescence study (Fig. 5(B)). A similar result was obtained in the analysis of STAT1 subcellular localization (Fig. 5(C)). These observations indicate that the HCV core protein does not inhibit the translocation of STAT3 or STAT1 to the nucleus, and the feedback mechanism is not the cause of *SOCS-1* gene suppression.

Because PIAS3 blocks the nuclear translocation of STAT3 or binding of STAT3 to genomic DNA [30], the expression of PIAS3 was examined by Western blotting. However, there was no significant difference in the levels of PIAS3 between core-expressing HepG2 cells and control HepG2 cells (data not shown). Co-immunoprecipitation analysis was also performed using HepG2 cell lines to know whether or not the core protein affects the association of PIAS1 with STAT1 or PIAS3 with STAT3. However, neither co-immunoprecipitation of STAT1 with anti-PIAS1 antibody nor that of STAT3 with anti-PIAS3 antibody was affected by the presence of the core protein (Fig. 6). We also examined the possibility of the interaction of the core protein with STAT3, which blocks the binding of STAT3 to the promoter of *SOCS-1* gene. For this purpose, a co-immunoprecipitation technique was utilized with whole-cell extracts of core-expressing HepG2 cells. However, no association was observed between these two proteins.

4. Discussion

In the current study, we demonstrated that the core protein of HCV suppresses the expression of *SOCS-1* mRNA in the liver tissues of mice that develop HCC late in their life [4,7]. This observation was reproduced in cultured cells that expressed the core protein. This phenomenon may contribute to the modification of the IFN signaling systems

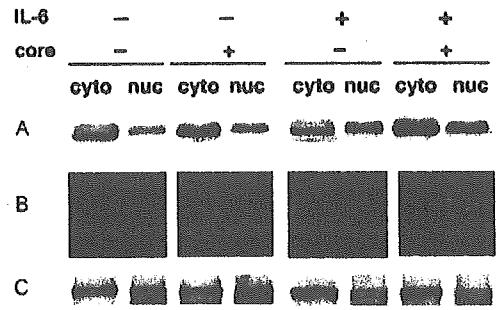

Fig. 5. Hepatitis C virus core protein did not affect subcellular localization of STAT3 or STAT1. Cytoplasmic and nuclear fractions from HepG2 cells with or without the core protein were subjected to Western blotting with the anti-STAT3 antibody (A) or anti-STAT1 antibody (C). HepG2 cells were fixed and an immunocytofluorescence study was performed using the anti-STAT3 antibody (B). Cells were processed before or 60 min after the treatment with 10 ng/ml of IL-6. cyto, cytoplasmic fraction; nuc, nuclear fraction [This figure appears in colour on the web].

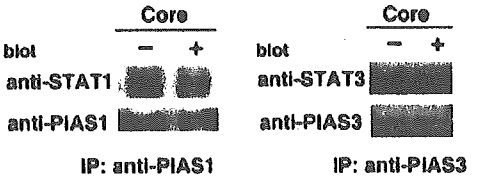

Fig. 6. Effect of the core protein on the interaction of STATs and PIASs. Cell lysates were immunoprecipitated with anti-PIAS1 or anti-PIAS3 antibody, and immunoblotted with anti-STAT1 or STAT3 antibody, respectively. There was no difference in the amounts of STAT1 or STAT3 that were co-immunoprecipitated with anti-PIAS antibodies.

in HCV infection, because SOCS-1 and SOCS-3 play central roles in the Jak/STAT pathway as negative feedback regulators [12–14]. In addition, since SOCS-1 also possesses a tumor suppressor activity [15–17], the down-regulation of *SOCS-1* may contribute to hepatocarcinogenesis in HCV infection. It has been reported that the silencing of the *SOCS-1* gene by hypermethylation is associated with the development of HCC [18–20]. Among patients with HCV infection, a major cause of chronic hepatitis worldwide, HCC develops at a very high incidence [1,2]. Hence, there may be an alternative mechanism of *SOCS-1* silencing to gene methylation in HCV infection. Our current data suggest a possibility of such a mechanism in that HCV per se acts as a negative regulator of SOCS-1, a tumor suppressor. The expression levels of *SOCS-1* mRNA in noncancerous liver tissues in chronic hepatitis C patients were also significantly lower than those in HCV-negative subjects, although the 'shut-off' of the *SOCS-1* gene observed in the experimental systems was not the case. This may be due to the presence of other factors influencing *SOCS-1* gene expression in vivo, including inflammation.

In the exploration of the mechanism underlying the down-regulation of *SOCS-1* expression, we first examined the methylation status of the *SOCS-1* gene in liver tissues from core gene transgenic mice by methylation-specific PCR. Neither the 5′-non-coding region nor the CpG island in the coding region of the *SOCS-1* gene [18–20] was hypermethylated, refuting methylation as a mechanism of SOCS-1 suppression.

We next determined whether or not STAT3, a transcription factor for the *SOCS-1* gene, is involved in the suppression of *SOCS-1* by the core protein: a decreased level or a disturbed phosphorylation of STAT3 may account for the suppression of *SOCS-1*. It was found, however, that STAT3 was rather activated by the core protein, consistent with a previous report [28]. The effect on STAT3 activation by the core protein is yet controversial [31]. Similarly, the activation and nuclear translocation of STAT1 was not disturbed by the presence of the core protein. The core protein differentially affected the expression of STAT-target genes such as *IRF1*, c-*myc* or *bcl*-X_L. The core protein suppressed *IRF1* expression in the mouse liver but did not those of c-*myc* and *bcl*-X_L genes. Regulation of *IRF1*

expression is STAT1-dependent in general, although STAT3 is also involved when stimulated by IL-6 [32]. c-*myc* and *bcl*-X_L inductions by IL-6 are chiefly mediated by STAT3 [33]. Thus, the modulation of expression by the core protein may occur in some other STAT-target genes, suggesting somewhere in Jak/STAT signaling pathway including STAT1 activation is impaired by the core protein. However, no defect was identified in the activation and nuclear translocation of STAT1 and STAT3 in the current study. Thus, although we could not define the precise role of the core protein in *SOCS-1* gene suppression, the direct effect of the core protein on the transcription of the gene is the most likely.

In summary, we found that the HCV core protein selectively suppresses *SOCS-1* gene expression in the liver tissues of animals and cultured cells. These findings may provide a basis for an alternative mechanism of the switch-off of SOCS-1 in the pathogenesis of HCV infection by modulating a tumor suppressor activity or responses to IFNs.

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Hepatitis C as a metabolic disease: Implication for the pathogenesis of NASH

Kazuhiko Koike *

Department of Infectious Diseases, Internal Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113 8655, Japan

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Abstract

In addition to the link with development of hepatocellular carcinoma (HCC), hepatitis C virus (HCV) infection is associated with several extrahepatic manifestations such as essential mixed cryoglobulinemia, porphyria cutanea tarda or Sjögren's syndrome. A role of hepatic steatosis in the pathogenesis of chronic hepatitis C has also been known, implying hepatitis C as a metabolic disease. In addition, recent epidemiological studies have suggested a linkage between type 2 diabetes and chronic HCV infection. However, the presence of additional factors in patients, such as obesity, aging or cirrhosis, prevents the establishment of a definite relationship between HCV infection and these two conditions, lipid metabolism disturbance and diabetes. In addition to the data indicating the presence of dyslipidemia and diabetes or insulin resistance in our cohort of chronic hepatitis C patients, we found a series of evidence showing the association between the conditions and HCV infection in mouse models that are transgenic for the HCV genes.

In patients with chronic hepatitis C, a significant decrease in the serum levels of total cholesterol and apolipoproteins C2 and C3 was observed compared to those with chronic hepatitis B that were comparable in liver function. In an animal model, C18:1 mono-unsaturated fatty acids were significantly increased in the liver from HCV core gene transgenic mice, which was similarly observed in the liver from human hepatitis C patients. Thus, a disturbance in lipid metabolism was observed in both humans and an HCV mouse model, supporting that it is a specific event in HCV infection.

A significant increase in the value of an indicator for homeostasis model assessment of insulin resistance (HOMA-IR), was observed in patients with chronic hepatitis C, even at the very early stage of chronic hepatitis. In the animal model, a marked insulin resistance was exhibited from a very young age in HCV core gene transgenic mice. Insulin resistance observed in the core gene transgenic mice was chiefly due to the shortage of insulin action on the suppression of glucose production in the liver. Thus, the ability of insulin to lower the plasma glucose level in the HCV transgenic mice was impaired, as observed in chronic hepatitis C patients. These results provide a direct experimental evidence for the contribution of HCV in the development of insulin resistance in human HCV infection, which finally leads to the development of type 2 diabetes.

Insulin resistance may be a critical factor in the pathogenesis of chronic hepatitis C as recently suggested in non-alcoholic steatohepatitis (NASH), along with impairment in lipid metabolism. Our results would provide a clue for further understanding of pathobiology of HCV infection, and may provide an implication for the pathogenesis of NASH.

Keywords: Diabetes; Hepatitis C virus; Insulin resistance; NASH; Hepatocarcinogenesis; Transgenic mouse

1. Introduction

Approximately 1.8 million people in Japan and 200 million people in the world are chronically infected with hepatitis C virus (HCV). Chronic HCV infection may lead to cirrhosis and hepatocellular carcinoma (HCC), thereby being a worldwide problem both in medical and socio-economical aspects [1]. In addition, chronic HCV infection is a multifaceted disease, which is associated with numerous clinical manifestations, such as type II mixed cryoglobulinemia, porphyria cutanea tarda and membranoproliferative glomerulonephritis [2]. Furthermore, a strong association of HCV infection with Sjögren's syndrome and lichen

* Tel.: +81 3 5800 8800; fax: +81 3 5800 8799.
E-mail address: kkoike-tky@umin.ac.jp.

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planus have been noted, which is validated in the animal model [3].

In addition, recently, there have been increasing lines of evidence to indicate metabolic disturbances in HCV infection, which would influence the pathogenesis of chronic hepatitis C. The discovery of HCV in 1989 enabled the comparison between chronic hepatitis C and the other chronic hepatitis, resulting in repeated reports that steatosis is significantly associated with chronic hepatitis C [4,5]. Steatosis in HCV infection is reproduced in animal models [6] or cultured cells [7], strengthening a pathologic role of HCV in it. Furthermore, patients infected with HCV have abnormalities in serum lipids, such as hypocholesterolemia or abnormal levels of apolipoproteins in serum [8,9]; they are corrected in sustained virological responders to antiviral treatment [9]. Thus, the association between HCV infection and disturbance in lipid metabolism has become increasingly strong both in patients and experimental systems including animals. Finally, patients with chronic hepatitis C accompanied by severe steatosis develop hepatic fibrosis more rapidly [10]. Thus, abnormal lipid metabolism in HCV infection would be deeply involved in the pathogenesis of hepatitis C.

2. Diabetes may also be a manifestation of HCV infection

The next character appearing as a metabolic aspect of HCV infection is type 2 diabetes. In 1994, Allison et al. [11] reported an epidemiological link between diabetes and HCV infection, but in a cirrhotic cohort. There was little impact, however, in view of well-known impaired glucose tolerance in advanced chronic liver disease. Several reports followed along this line from the same group and others. The trend to accept a positive association between diabetes and HCV infection seems to have been triggered by the population-based study in the United States [12], in which a solid association was found between them. The association between diabetes and HCV infection, however, is blemished by factors such as the development of cirrhosis, obesity and ageing common in patients with hepatitis C; they would make it difficult to prove this association as real. Hence, there is a need to evaluate the association using experimental systems.

3. HCV induces insulin resistance in vivo

We used mice transgenic for the HCV core gene [6,13] to assess the association between HCV infection and diabetes. These mice carry the core gene of genotype 1b HCV, and express HCV core protein of an expected size in the liver, in levels comparable to those in patients with chronic hepatitis C (Fig. 1).

They develop HCC late in life [13]. These transgenic mice were maintained and fed together with their normal littermates, and the glucose metabolism was studied [14]. Although the core gene transgenic mice did not develop overt diabetes, they had markedly elevated serum levels of insulin. Plasma glucose levels were somewhat higher in transgenic mice than their normal littermates control mice with no significant difference between them. In contrast, serum insulin levels were significantly higher in transgenic than normal control mice in both fasting and fed conditions (Fig. 2).

Since such a combination of normal glucose levels and hyperinsulinemia points to the insulin resistance, then, we conducted glucose and insulin resistance. The core gene transgenic mice exhibited glucose levels a little higher than normal littermates without any significant differences between them. In insulin resistance tests, glucose levels were significantly higher in transgenic than normal control mice both 40 and 60 min after injection with insulin (Fig. 3). These

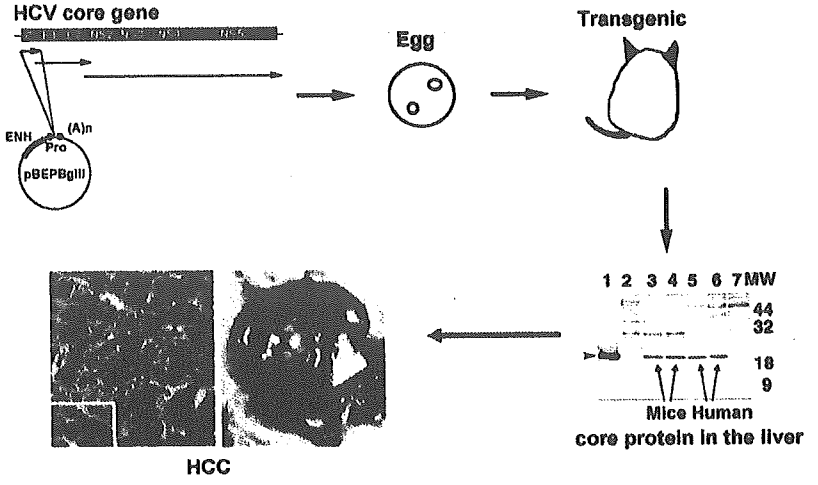

Fig. 1. Hepatitis C virus core gene transgenic mouse. These mice carry the core gene alone of genotype 1b HCV and express the core protein of an expected size in the liver, at levels comparable to those in human chronic hepatitis C patients. The mice eventually develop HCC late in life.

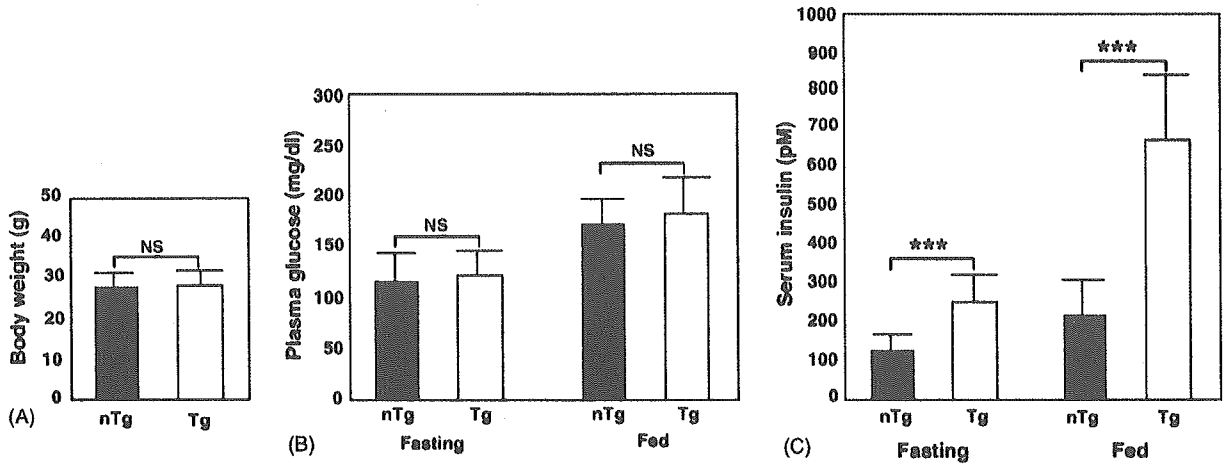

Fig. 2. Altered glucose homeostasis in hepatitis C virus core gene transgenic mice: (A) body weight of 2-month-old mice; (B) plasma glucose levels in fasting or fed mice; (C) serum insulin levels in fasting or fed mice. The insulin level was significantly higher in the core gene transgenic mice than in control mice. Values are mean ± S.E.; ***$p < 0.001$; NS, statistically not significant; nTg, nontransgenic mice; Tg, transgenic mice.

results indicate the presence of insulin resistance in the core gene transgenic mice. Since only the HCV core gene had been incorporated into these transgenic mice, the core protein of HCV would be able to induce insulin resistance in vivo.

By what mechanism, then, insulin resistance observed in the animal model would arise? The insulin resistance is considered to involve two factors. They are the central and peripheral insulin resistances (Table 1) [15]. The hyperinsulinemic–euglycemic clamp method was employed for differentiating between them. In this method, hepatic glucose production (HGP) is calculated on the basis of amounts of glucose required for keeping plasma glucose levels within a certain range at serum insulin levels higher than physiological ones. In normal control mice, HPG was suppressed by 60% by the administration of insulin, in contrast to core gene transgenic mice, in which there was only marginal suppression of HGP by insulin. These results indicate a hepatic (central) origin of insulin resistance in the transgenic mice. In further confirmation of this, uptake of glucose into the muscle was determined. There was no difference in the uptake in response to administration of insulin between transgenic

Table 1
Insulin resistance

1. Peripheral insulin resistance: a shortage of insulin action in the muscle (deficit in the insulin-induced glucose uptake into the muscles)
2. Central insulin resistance: a shortage of insulin action in the liver (deficit in the insulin-induced suppression of glucose production in the liver)

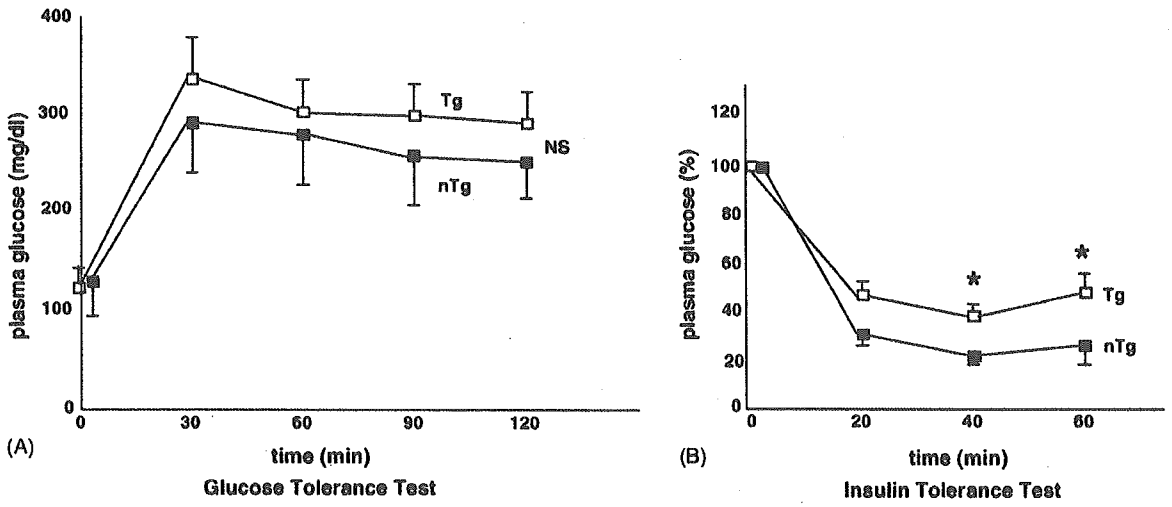

Fig. 3. Insulin resistance in the core gene transgenic mice: (A) Glucose tolerance test. Animals were fasted overnight. D-Glucose (1 g/kg body weight) was administered by i.p. injection to conscious mice, and plasma glucose levels were determined at the time points indicated; (B) Insulin tolerance test. Human insulin (1 U/kg body weight) was administered by i.p. injection to fasted conscious mice and glucose concentrations were determined. Values were normalized to the baseline glucose concentration at the time of insulin administration. Values are mean ± S.E.; *$p < 0.05$; NS, statistically not significant; nTg, nontransgenic mice; Tg, transgenic mice.

and normal control mice. The insulin resistance in mice transgenic for the HCV core gene, therefore, is central and hepatic.

4. The mechanism underlying insulin resistance in HCV infection

Next, we evaluated how insulin resistance emerges in the mouse model. For this purpose, liver homogenate was immunoblotted with anti-phosphotyrosine and anti-phosphserine antibodies after insulin receptor substrate (IRS)-1 and IRS-2 had been immunoprecipitated. Tyrosines in IRS-1 were weakly phosphorylated both in normal and transgenic mice before they received insulin, with no differences between them. After the administration of insulin, however, the phosphorylation of tyrosines in IRS-1 increased in normal but not transgenic mice. Obtained results suggested disturbance in tyrosine phosphorylation as one of the factors for insulin resistance in the liver. There were no differences in phosphorylation of serines in IRS-1 or tyrosines in IRS-2 between transgenic and normal control mice. Overall, they provided experimental evidence for development of insulin resistance by the presence of HCV in the liver that would disturb the transduction of insulin signaling in hepatocytes (Fig. 4). There remains a possibility for the HCV core protein that would directly prohibit phosphorylation of tyrosines. Or else, it might inhibit tyrosine phosphorylation via certain cytokines.

In our extensive searches for the expression of cytokines in the liver of core gene transgenic mice, only TNF-α and IL-1β levels have been found increased [16]. For the purpose of evaluating the role of TNF-α in insulin resistance in transgenic mice, therefore, serum insulin was determined and insulin resistance test performed in them after they had received anti-TNF-α intraperitoneally. Pretreatment with anti-TNF-α partially restored insulin sensitivity in the core gene transgenic mice. Albeit a direct anti-insulin activity of the core protein cannot be excluded, high levels of TNF-α in the liver would be one of the factors for induction of insulin resistance in this mouse model.

5. Insulin resistance as a risk factor for progression of hepatic fibrosis

Insulin resistance in HCV infection may have an additional significant clinical implication. In 260 patients with chronic hepatitis C, Hui et al. [17] have tried to establish the relationship between liver histology and indicators of glucose metabolism as well as insulin resistance represented by homeostasis model assessment of insulin resistance (HOMA-IR). They have found that insulin resistance already exists in hepatitis C patients with stage 0 or 1 fibrosis in the liver. This indicates that insulin resistance in HCV infection is not attributable to advanced liver disease. HOMA-IR was a significant and independent predictor for the stage and velocity of hepatic fibrosis. The results of their study are important, because they implicate a role of hyperinsulinemia, and insulin resistance by inference, in promoting the progression of hepatic fibrosis. Insulin has been proven for an aggravating factor not only in atherosclerosis but also systemic inflammation and fibrosis. The liver would not be an exception in this respect.

6. Similarities and differences between hepatitis C and NASH: implication for the pathogenesis of NASH

We have demonstrated that HCV per se induces insulin resistance in the animal model. High-fat diet and obesity superimposed on it lead to overt diabetes [14]. In view of the progression of chronic hepatitis C accelerated by insulin resistance, insulin resistance would naturally influence the development of HCC. Although the association has not been

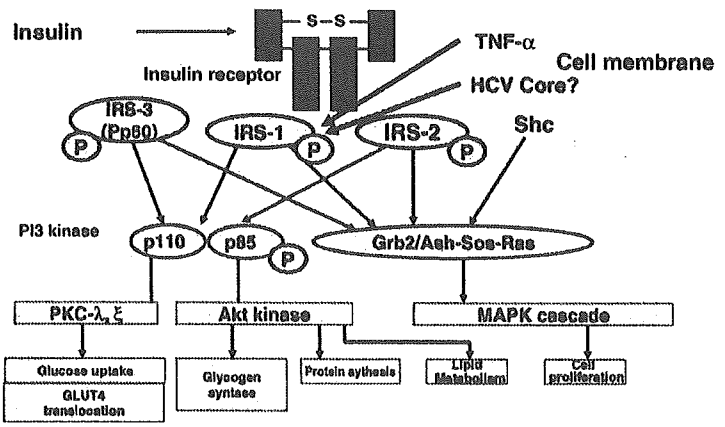

Fig. 4. Insulin resistance and HCV infection, HCV core protein or elevated intrahepatic TNF-α inhibits tyrosine phosphorylation of IRS-1 in the liver, suppresses insulin intracellular signal transduction and leads to insulin resistance. PKC, protein kinase C; PI3-kinase, phosphatidyl inositol 3 kinase; MAPK, mitogen-activated protein kinase; IRS, insulin receptor substrate.

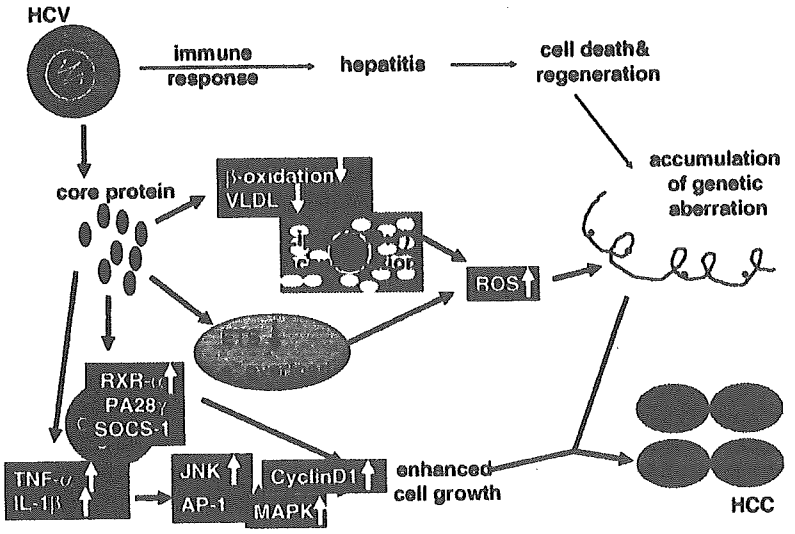

Fig. 5. Molecular hepatocarcinogenesis in HCV infection. Oxidative stress together with hepatic steatosis induced by the HCV core protein would play a pivotal role in the development of HCC. Alterations in cellular gene expressions, such as TNF-α or SOCS-1, and those in the intracellular signaling pathways including JNK would be co-accelerators to hepatocarcinogenesis in HCV infection. The latter pathway has not been found in NASH while the former may be common in the pathogenesis of hepatitis C and NASH. HCC, hepatocellular carcinoma; TNF-α, tumor necrosis factor-α; SOCS-1, suppressor of cytokine signaling-1; NASH, non-alcoholic steatohepatitis.

established between non-alcoholic steatohepatitis (NASH) and HCC, it needs to be pursued energetically in view of histological resemblance of NASH to chronic hepatitis C.

When hepatitis C and NASH are compared, there are a number of similarities between these two medical conditions (Table 2). Steatosis, which is one of the definitions in NASH, is a characteristic trait of chronic hepatitis C [4–6,13]. Disturbances in the lipid metabolism are present in both conditions, although the phenotypes may show a distinction: hypo-β-lipoproteinemia in hepatitis C but hyperlipidemia in NASH. As described above, insulin resistance often arises in chronic hepatitis C, and is also a feature frequently observed in NASH [18]. Some cytokines, such as TNF-α, are considered to be critical in the pathogenesis of these conditions. TNF-α levels are increased in patients with hepatitis C and are implicated in insulin resistance. Single nucleotide polymorphism in TNF-α gene is significantly found in NASH patients [18]. Overproduction of oxidative stress or reactive oxygen species (ROS) plays a pivotal role in the progression of hepatitis and development of HCC in both the conditions: ROS is overproduced in the liver of the core gene transgenic mice in the absence of inflammation, contributing, at least in part, to the development of HCC [13,19,20]. Functional abnormalities in the mitochondria are implicated, in both hepatitis C and NASH, in the pathogenesis of liver diseases including HCC. In HCV core gene transgenic mice, the malfunction of the electron transfer system of mitochondria has been suggested and is assumed to be an origin of ROS overproduction (Table 2).

Table 2
Comparison of hepatitis C and NASH

Hepatitis C	NASH
Steatosis	Steatosis
Hypo-β-lipoproteinemia	Hyperlipidemia
Insulin resistance	Insulin resistance
Cytokines (TNF-α, etc.)	Cytokines (TNF-α, etc.)
Oxidative stress	Oxidative stress
Mitochondrial abnormality	Mitochondrial abnormality
Obesity?	Obesity
HCC	HCC?

Finally, HCC develops both in chronic hepatitis C and NASH. However, the association between NASH and HCC is not strong yet while there is a definite connection in the case of hepatitis C. Nevertheless, HCC develops in patients with NASH, regardless of the frequency. Hence the underlying mechanism of HCC development in NASH awaits further investigation. The analogy between chronic hepatitis C and NASH, as described above, may be a clue to solve a puzzle in the pathogenesis of NASH including hepatocarcinogenesis. In the pathogenesis of HCC in HCV infection, one of intracellular signaling MAPK systems, JNK, is activated in the liver. In the downstream of JNK, transcription factor AP1 and cell cycle machineries, CDK4 and cyclin D1, are subsequently activated, conferring advantage to cell proliferation [16,20]. However, such activations in cellular genes or signaling systems have not been identified yet for NASH. Overproduction of oxidative stress together with the presence of steatosis may be a common pathway to liver hepatocarcinogenesis in both hepatitis C and NASH (Fig. 5, upper half).

However, the alterations in cellular gene expressions and/or intracellular signaling systems are solely with hepatitis C in the presence of the viral protein(s), putting chronic hepatitis C onto the fast track for the development of HCC (Fig. 5, upper half). This aspect of HASH should be investigated. The

analogy between hepatitis C and NASH would give a solution to problems in the pathogenesis of NASH.

7. Conclusion

Although HCV targets at the liver, it has become increasingly evident that HCV can induce diseases of many organs. Recently, much attention is drawn to metabolic disorders in HCV infection. First, hepatic steatosis and derangement in lipid metabolism have been found characteristic of HCV infection, and later on correlation was noted between HCV infection and diabetes as well as insulin resistance. We have demonstrated that HCV by itself can induce insulin resistance by means of disturbing the insulin signaling pathway by an HCV protein. The fact that HCV infection induces insulin resistance by the virus itself may influence the progression of chronic liver disease and open up novel therapeutic approaches. HCV infection would need to be viewed not only as liver disease but also a metabolic disease, which would be a clue to open up a novel way to the molecular understanding of pathogenesis of NASH.

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Human VAP-B Is Involved in Hepatitis C Virus Replication through Interaction with NS5A and NS5B

Itsuki Hamamoto,[1] Yorihiro Nishimura,[1,2] Toru Okamoto,[1] Hideki Aizaki,[2,3] Minyi Liu,[3] Yoshio Mori,[1] Takayuki Abe,[1] Tetsuro Suzuki,[2] Michael M. C. Lai,[3] Tatsuo Miyamura,[2] Kohji Moriishi,[1] and Yoshiharu Matsuura[1]*

Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka,[1] and Department of Virology II, National Institute of Infectious Diseases, Tokyo,[2] Japan, and Department of Molecular Microbiology and Immunology, Keck School of Medicine, University of Southern California, Los Angeles, California[3]

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The hepatitis C virus (HCV) nonstructural protein (NS) 5A is a phosphoprotein that associates with various cellular proteins and participates in the replication of the HCV genome. Human vesicle-associated membrane protein-associated protein (VAP) subtype A (VAP-A) is known to be a host factor essential for HCV replication by binding to both NS5A and NS5B. To obtain more information on the NS5A protein in HCV replication, we screened human brain and liver libraries by a yeast two-hybrid system using NS5A as bait and identified VAP-B as an NS5A-binding protein. Immunoprecipitation and mutation analyses revealed that VAP-B binds to both NS5A and NS5B in mammalian cells and forms homo- and heterodimers with VAP-A. VAP-A interacts with VAP-B through the transmembrane domain. NS5A interacts with the coiled-coil domain of VAP-B via 70 residues in the N-terminal and 341 to 344 amino acids in the C-terminal polyproline cluster region. NS5A was colocalized with VAP-B in the endoplasmic reticulum and Golgi apparatus. The specific antibody to VAP-B suppressed HCV RNA replication in a cell-free assay. Overexpression of VAP-B, but not of a mutant lacking its transmembrane domain, enhanced the expression of NS5A and NS5B and the replication of HCV RNA in Huh-7 cells harboring a subgenomic replicon. In the HCV replicon cells, the knockdown of endogenous VAP-B by small interfering RNA decreased expression of NS5B, but not of NS5A. These results suggest that VAP-B, in addition to VAP-A, plays an important role in the replication of the HCV genome.

Hepatitis C virus (HCV) infects 170 million people worldwide and frequently leads to cirrhosis or hepatocellular carcinoma (6, 29). HCV is classified in the family *Flaviviridae* and possesses a single-stranded positive-sense RNA with a length of 9.6 kb. The HCV genome encodes a single large precursor polyprotein composed of about 3,000 amino acids (aa) that is processed by cellular and viral proteases, resulting in at least 10 structural and nonstructural (NS) proteins (29). Details of HCV's replication cycle are unknown because of the low viral load in the sera of HCV-infected individuals and the lack of a reliable and robust cell culture system to support HCV infection and replication. The development of HCV RNA replicons in which a synthetic HCV genomic or subgenomic RNA replicates efficiently in the human hepatocarcinoma cell line Huh-7 has enabled the study of viral RNA replication in cell culture (4, 20, 24). The HCV RNA replication complex, composed of the viral NS proteins and host cellular proteins, replicates the viral RNA genome at the intracellular membrane. Thus far, the HCV replicon system has greatly contributed to the understanding of HCV replication and pathogenesis associated with the expression of viral NS proteins. Replication of positive-strand RNA viruses generally involves certain intracellular membrane structures, including the endoplasmic reticulum (ER), Golgi apparatus, endosome, and lysosome (39). Recently, several groups have succeeded in demonstrating cell-free replication activities of replication complexes in crude membrane fractions of HCV subgenomic replicon cells (2, 3, 14, 53). These cell-free systems provide semi-intact polymerase assays for biochemical dissection of HCV RNA replication and are a useful source for the isolation of HCV replication complexes. Replication complexes were detected in detergent-resistant membrane structures, most likely lipid raft structures (2, 14). Although HCV NS proteins presumably form a membrane-associated RNA replication complex with host proteins, the precise components and mechanisms for replication are poorly understood.

HCV NS5A is a phosphoprotein that appears to possess multiple and diverse functions in viral replication, interferon resistance, and pathogenesis (26, 35). Cell culture-adaptive mutations have been shown to cluster in the central portion of NS5A in subgenomic HCV replicons, indicating that NS5A is involved in the viral replication process either directly or by interacting with host cellular proteins (4, 55). This observation, together with the modulation of NS5A hyperphosphorylation by NS3, NS4A, and NS4B and physical interaction with other viral NS proteins, strongly supports the notion that NS5A is an essential component of the HCV replication complex (21, 30, 36). NS5A has been shown to be associated with a range of cellular proteins involved in cellular signaling pathways, such as interferon-induced kinase PKR (11), growth factor receptor-binding protein 2 (Grb2) (45), p53 (27, 37), phosphoinositide-3-kinase p85 subunit (15), and proteins in protein trafficking and membrane morphology, such as karyopherin β3 (8),

* Corresponding author. Mailing address: Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, 3-1, Yamadaoka, Suita, Osaka 565-0871, Japan. Phone: 81-6-6879-8340. Fax: 81-6-6879-8269. E-mail: matsuura@biken.osaka-u.ac.jp.

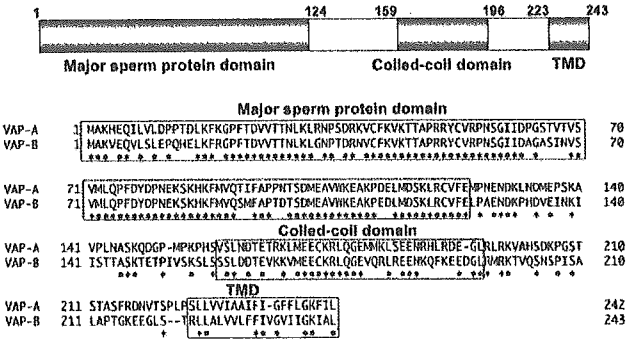

FIG. 1. Schematic representation of VAP-B and alignment of amino acid sequences of VAP-A and VAP-B. The major sperm protein domain, coiled-coil domain, and TMD are indicated. The asterisks indicate identical amino acid residues between VAP-A and VAP-B.

apolipoprotein A1 (40), amphiphysin II (56), and vesicle-associated membrane protein (VAMP)-associated protein (VAP) subtype A (VAP-A), also called VAP-33 (48). Host fatty acids and geranylgeranylation appear to modulate the host and viral proteins involved in HCV RNA replication (19, 49, 54). Gao et al. showed that small interfering RNA (siRNA) or the dominant-negative mutant of VAP-A resulted in relocation of NS5B from detergent-resistant to detergent-sensitive membranes and reduced HCV RNA replication (12). In addition, Evans et al. suggested that NS5A hyperphosphorylation disrupts interaction with VAP-A and negatively regulates HCV RNA replication (9). Like many of the fusion proteins, VAP is a tail-anchored protein with a globular amino-terminal domain followed by a stalk region containing a coiled coil (Fig. 1), and it is ubiquitously expressed in human tissues (7). In humans, there are two isoforms of VAP, VAP-A and VAP-B, encoded by separate genes, and VAP-C is a splicing variant of VAP-B missing the C-terminal two-thirds (23, 32). VAP-B shows 63% amino acid identity to VAP-A (32, 51). The first proposed function for VAP arose from its initial identification as an interactor with the membrane fusion protein synaptobrevin/VAMP in *Aplysia* (43). Since then, it has been shown to be involved in vesicle transport, including the regulation of COP-I vesicle transport in the ER/Golgi pathway (13, 44), VAMP/synaptobrevin-mediated neurotransmitter release (38), and VAMP-2-mediated Glut-4 trafficking at the plasma membrane (10); it is also involved in the interaction between the microtubule network and tight junctions (22). Recently, VAP has been linked to the function of mammalian neurons, where VAP is enriched on microtubules (42), because a mutation in human VAP-B causes familial amyotrophic lateral sclerosis type 8 (32).

To gain a better understanding of the interactions between NS5A and host proteins involved in HCV replication, we screened human libraries by a yeast two-hybrid system using NS5A as bait and identified VAP-B as an NS5A-binding protein. In this study, we examined the biological significance of the interaction between VAP-B and NS proteins in HCV replication and found that VAP-B binds to both NS5A and NS5B in mammalian cells and forms homo- and heterodimers with VAP-A. Immunodepletion of VAP-B suppressed the replication of HCV RNA in a cell-free replication assay, and the knockdown of endogenous VAP-B by siRNA decreased the expression of NS5B but not that of NS5A. These results suggest that VAP-B plays an important role in HCV replication through interaction with NS5A and NS5B.

MATERIALS AND METHODS

Cells. Human embryo kidney 293T, human cervical carcinoma HeLa, and human hepatoma Huh-7 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, Mo) containing 10% fetal calf serum (FCS), while the Huh-9-13 cell line, which possesses an HCV subgenomic replicon (4, 20, 23), was cultured in DMEM supplemented with 10% FCS and 1 mg/ml G418. All cells were cultured at 37°C in a humidified atmosphere with 5% CO_2.

Antibodies. Chicken anti-human VAP-B antibody was prepared by immunization using the synthetic peptides of residues from 188 to 203, KQFKEEDGL RMRKTVQ, of human VAP-B. A mouse monoclonal antibody to human VAP-A was purchased from BD Pharmingen (San Diego, CA). Mouse monoclonal antibodies to giantin, influenza virus hemagglutinin (HA), and GluGlu (EE) tag were from Covance (Richmond, CA). Mouse anti-FLAG antibody M2, horseradish peroxidase-conjugated antibody, and mouse monoclonal anti-beta-actin antibody were from Sigma. A mouse monoclonal antibody to protein disulfide isomerase (PDI) was from Affinity Bioreagents (Golden, CO). Rabbit polyclonal antibody to NS5A was prepared by immunization using peptides of residues from 409 to 422, DVESYSSMPPLEGE. Mouse monoclonal antibody to NS5B was described previously (41).

Plasmids. For expression in mammalian cells, a DNA fragment encoding NS5A was generated from HCV genotype 1b strain J1 (1) (GenBank database accession number D89815), and another was generated from genotype 1a strain H77 (52) (GenBank database accession number AF009606) by PCR using *Pfu* turbo DNA polymerase (Stratagene, La Jolla, CA). The fragments were then cloned into the appropriate sites in pEF-FLAG pGBK puro (18) and pEGFP-C3 (Clontech, Palo Alto, CA). The mutations of the NS5A gene were generated by a method known as "splicing by overlapping extension" (16, 17) and cloned into pEF-FLAG pGBK puro. The DNA fragment encoding NS5B of the J1 strain was generated by PCR and cloned into pCAGGs-PUR (33). The DNA fragment encoding human VAP-A was amplified by PCR from a human fetal-brain library (Clontech) and was introduced into pEF-FLAG pGBK puro, pEF-EE hygro (34), pCHA3 (34), and pcDNA3.1-N-HA, in which an HA tag is inserted in the N terminus of the cloning site of pcDNA3.1(+) (Invitrogen, Carlsbad, CA). The cDNAs of human VAP-A and -B were amplified by PCR and cloned into pEF-FLAG pGBK puro, pEF-EE hygro, pcDNA3.1-N-HA, and pEGFP-C3. The genes encoding VAP lacking the transmembrane domain were amplified and cloned into pEF-FLAG pGBK puro. The DNA fragment encoding the human VAP-B protein lacking a coiled-coil region was introduced into pEF-EE hygro. All PCR products were confirmed by sequencing them with an ABI PRSM 310 genetic analyzer (Applied Biosystems, Tokyo, Japan).

Yeast two-hybrid assay and library screening. The NS5A-binding protein was identified by a yeast two-hybrid assay according to the user manual of MATCHMAKER GAL4 Two-Hybrid System 3 (Clontech). The DNA fragment encoding amino acids 1973 to 2419 was amplified from HCV strain J1 by PCR and then was cloned into pGBKT7 (Clontech). The resulting plasmid was designated pGBK T7 HCV NS5A. A human brain library based on pACT2 was purchased from Clontech. The yeast *Saccharomyces cerevisiae* strain AH109, which secretes alpha-galactosidase under the control of MEL1 upstream activation sequence, was grown in yeast extract-peptone-dextrose medium and transformed with the bait and library plasmids. The transformed yeast cells were grown on 2.0% agar plates of dropout medium lacking tryptophan, leucine, histidine, and adenine. The resulting colonies were inoculated on the new dropout plate containing 20 μg/ml X-alpha-Gal (5-bromo-4-chloro-3-indolyl-alpha-*O*-galactopyranoside) and lacking leucine and tryptophan. The total DNA was prepared from all positive clones and then introduced into *Escherichia coli* strain JM109. The prey plasmids of isolated yeast cells were recovered from the clones grown on LB agar plates containing 10 μg/ml ampicillin and then purified. The insert DNA fragments of isolated clones were determined by sequencing. Finally, 48 alpha-galactosidase-positive clones were identified from 2 million clones screened in the fetal-brain library. One of the positive clones contained the complete cDNA of human VAP-B in frame.

Transfection, immunoblotting, and immunoprecipitation. Cells were seeded onto a six-well tissue culture plate 24 h before transfection. The plasmids were transfected into cells by liposome-mediated transfection using Lipofectamine 2000 (Invitrogen). Cells were harvested 36 h posttransfection, washed five times