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Human Butyrate-Induced Transcript 1 Interacts with Hepatitis C Virus NS5A and Regulates Viral Replication[∇]

Shuhei Taguwa,¹ Toru Okamoto,¹ Takayuki Abe,¹ Yoshio Mori,¹ Tetsuro Suzuki,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

Received 2 October 2007/Accepted 18 December 2007

Hepatitis C virus (HCV) nonstructural protein 5A (NS5A) is required for the replication of the viral genome and is involved in several host signaling pathways. To gain further insight into the functional role of NS5A in HCV replication, we screened human cDNA libraries by a yeast two-hybrid system using NS5A as the bait and identified human butyrate-induced transcript 1 (hB-ind1) as a novel NS5A-binding protein. Endogenously and exogenously expressed hB-ind1 was coimmunoprecipitated with NS5A of various genotypes through the coiled-coil domain of hB-ind1. The small interfering RNA (siRNA)-mediated knockdown of hB-ind1 in human hepatoma cell lines suppressed the replication of HCV RNA replicons and the production of infectious particles of HCV genotype 2a strain JFH1. Furthermore, these reductions were canceled by the expression of an siRNA-resistant hB-ind1 mutant. Among the NS5A-binding host proteins involved in HCV replication, hB-ind1 exhibited binding with FKBP8, and hB-ind1 interacted with Hsp90 through the FxxW motif in its N-terminal p23 homology domain. The impairment of the replication of HCV RNA replicons and of the production of infectious particles of JFH1 virus in the hB-ind1 knockdown cell lines was not reversed by the expression of an siRNA-resistant hB-ind1 mutant in which the FxxW motif was replaced by AxxA. These results suggest that hB-ind1 plays a crucial role in HCV RNA replication and the propagation of JFH1 virus through interaction with viral and host proteins.

Hepatitis C virus (HCV) infects approximately 170 million people worldwide and induces serious chronic hepatitis that results in steatosis, cirrhosis, and ultimately hepatocellular carcinoma (7, 64). More than two-thirds of the HCV-positive population in Western countries and Japan face chronic infection by genotypes 1a and 1b. The current combination therapy using pegylated alpha interferon (IFN) plus ribavirin has achieved a sustained virological response in 50% of individuals infected with HCV genotypes 1a and 1b (37, 53).

HCV belongs to the genus *Hepacivirus* of the family *Flaviviridae* and has a single-stranded, positive-sense RNA genome of approximately 9.6 kb, encoding a large polyprotein composed of approximately 3,000 amino acid residues. The polyprotein is cleaved by host and viral proteases, resulting in viral structural proteins (core, E1, and E2), a putative ion channel-forming protein (p7), and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) (40, 55). Highly structured untranslated regions are flanked at both the 5' and 3' ends of the open reading frame. The initiation of translation of the viral RNA is dependent on an internal ribosome entry site (IRES) localized in the 5' untranslated region (28, 58).

The HCV RNA is suggested to replicate in a replication complex composed of the viral nonstructural proteins and several host proteins. An HCV replicon system established as a representative functional system was composed of an antibiotic gene for selection and HCV genomic RNA for autonomous

replication in the intracellular compartments of human hepatoma cell line Huh7 without production of infectious particles (34). Recently, cell culture systems for production of an infectious HCV have been established based on HCV genotype 2a (32, 62, 74). Furthermore, a mouse model consisting of an immunodeficient mouse xenotransplanted with human liver fragments has been established for the study of *in vivo* replication of HCV (38). These *in vitro* and *in vivo* systems have enabled us to investigate the life cycle of HCV and to develop antiviral drugs for chronic hepatitis C.

NS5A is a phosphoprotein that possesses multiple functions in viral replication, IFN resistance, and pathogenesis (35). Adaptive mutations to increase RNA replication are frequently mapped to the coding region of NS5A, indicating that NS5A is critical for HCV replication (1, 71). NS5A has been shown to be associated with a range of cellular proteins involved in cellular signaling pathways, such as IFN-induced kinase PKR (14), growth factor receptor-binding protein 2 (Grb2) (56), p53 (36, 48), and the phosphoinositide-3-kinase p85 subunit (18), and with proteins involved in protein trafficking and membrane morphology, such as karyopherin b3 (8), apolipoprotein A1 (52), amphiphysin II (73), F-box and leucine-rich repeat protein 2 (FBL2) (26, 63, 70), and vesicle-associated membrane protein-associated protein subtype A (VAP-A) (59). We have previously reported that the host proteins VAP-B and FKBP8, a member of the FK506-binding protein (FKBP) family, interact with NS5A and that these interactions are required for efficient replication of HCV (16, 45), further supporting the hypothesis that NS5A is a pivotal component of the HCV replication complex.

To gain a better understanding of the functional role of

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

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NS5A in HCV replication, we screened human libraries by employing a yeast two-hybrid system and using NS5A as the bait. We thus identified human butyrate-induced transcript 1 (hB-ind1) as an NS5A-binding protein. Murine B-ind1 has been identified as a transcript induced by treatment with sodium butyrate in BALB/c BP-A31 mouse fibroblasts (10). hB-ind1 is a multiple-membrane-spanning protein, consisting of 362 amino acids, that possesses significant homology with protein tyrosine phosphatase-like, member A (PTPLA), and co-chaperone p23 and is suggested to be involved in the Rac1 signaling pathway (10). In this study we examine the biological effects of the interaction of hB-ind1 with NS5A and other host proteins on the replication of HCV.

MATERIALS AND METHODS

Plasmids. The plasmids encoding NS5A, FKBPs, VAP-A, VAP-B, and heat shock protein 90 (Hsp90) have been described previously (45). The human FBL2 gene was amplified from the total cDNA of Huh7 by PCR. A cDNA clone containing hB-ind1 cDNA was isolated from a human fetal brain library (Clontech, Palo Alto, CA) by the advanced yeast two-hybrid system Matchmaker Two-Hybrid System 3 (Clontech) using an HCV NS5A protein as bait. Each cDNA of N-terminally FLAG-tagged hB-ind1 and its mutants was generated by cloning into pEF-FlagGS-pGKpuro (23). pSilencer-hB-ind1, carrying a short hairpin RNA (shRNA) targeted to hB-ind1 under the control of the U6 promoter, was constructed by cloning of the oligonucleotide pair 5'-GATCCGGAAAGGCGACCACTGTTTCTCAAGGAAAGCAAGTGGCTTTTCTTTTCTTTGAGAAA-3'-AGCTTTTCCAAAAGGAAAAGCGACCACTGTTTCTTTGAGAAAAGCTGGTGGCTTTTTCGCG-3' between the BamHI and HindIII sites of pSilencer 2.1-U6 hygro (Ambion, Austin, TX). A plasmid encoding a mutant hB-ind1 resistant to shRNA was prepared by introduction of five silent mutations (nucleotides were changed from A to G, G to A, A to C, A to T, and C to T at positions 291, 294, 297, 300, and 301, respectively) into hB-ind1 cDNA by the method of splicing by overlap extension (19). The pSilencer negative-control plasmid (Ambion) has no homology to any human gene. The pFK-I₅₈₉ neo/NS3-3'/NK5.1 plasmid (46) was kindly provided by R. Bartsch-Schlager, and the neomycin-resistant gene was replaced with a firefly luciferase gene. The resulting plasmid was designated pFK-I₅₈₉ FL/NS3-3'/NK5.1. The plasmids used in this study were confirmed by sequencing with ABI Prism genetic analyzer 3130 (Applied Biosystems, Tokyo, Japan).

Cells and virus infection. All cell lines were cultured at 37°C under a humidified atmosphere with 5% CO₂. Human embryo kidney 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal calf serum (FCS). The human hepatoma cell line Huh7.5.1 was kindly provided by F. Chisari (74). The Huh7 and Huh7.5.1 cell lines were maintained in DMEM containing nonessential amino acids (NEAA), 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% FCS. The Huh9-13 cell line, a Huh7-derived cell line harboring a subgenomic HCV replicon (34), was maintained in DMEM containing 10% FCS, NEAA, and 1 mg/ml G418 (Nacal Tesque, Kyoto, Japan). Huh7.5.1 cells were transfected with pSilencer-hB-ind1 or an empty plasmid, and drug-resistant clones were selected by treatment with hygromycin (Wako, Tokyo, Japan) at a final concentration of 10 µg/ml. Plasmids encoding a full-length or truncated (amino acid residues 101 to 277) version of hB-ind1 were transfected into Huh7.5.1 cells, and the cells surviving after selection with 0.1 µg/ml of puromycin for 1 week were used for virus infection. The viral RNA of JFH1 was introduced into Huh7.5.1 cells according to the method of Wakita et al. (62). The supernatant was collected at 7 days posttransfection and used as HCV particles that are infectious in cell culture (HCVcc).

Antibodies. A rabbit anti-hB-ind1 antibody was prepared by immunization with synthetic peptides corresponding to amino acid residues 106 to 117 of hB-ind1. A mouse monoclonal antibody to influenza virus hemagglutinin (HA) was purchased from Covance (Richmond, CA). The mouse anti-FLAG M2 antibody that was conjugated with a horseradish peroxidase and a mouse anti-β-actin monoclonal antibody were purchased from Sigma. The mouse monoclonal antibody to HCV NS5A was obtained from Austral Biologicals (San Ramon, CA).

Yeast two-hybrid assay and library screening. A human fetal brain library prepared with pAct2 was purchased from Clontech and was screened by the yeast two-hybrid system Matchmaker GAL4 Two-Hybrid System 3 (Clontech) accord-

ing to the manufacturer's protocol. The NS5A cDNA fragment encoding amino acid residues 1973 to 2419 of HCV strain Con1 was amplified by PCR and cloned into pGBKT7 (Clontech); the resulting plasmid was designated pGBKT7 HCV NS5A. The yeast *Saccharomyces cerevisiae* strain AH109, which secretes α-galactosidase under the control of the MEL1 region, was transformed with pGBKT7 HCV NS5A and grown on a medium lacking tryptophan. The clone including the bait plasmid was transformed with the library plasmids. The transformed yeast cells were grown on 2% agar plates of a dropout medium lacking tryptophan, leucine, histidine, and adenine. The resulting colonies grown on the dropout plate were inoculated again on a new dropout plate containing 20 µg/ml X-α-Gal (5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside) and incubated at 30°C for 7 days. The total DNA was prepared from all blue colonies and then introduced into *Escherichia coli* strain JM109. The prey plasmids were recovered from the clones grown on LB agar plates containing 10 µg/ml ampicillin. One positive clone was isolated from among 2 million colonies of the human fetal brain library, and the nucleotide sequence of this clone includes the complete cDNA of hB-ind1 in its frame.

Transfection, immunoblotting, and immunoprecipitation. Transfection and immunoprecipitation analyses were carried out as described previously (16, 45). Immunoprecipitates boiled in loading buffer were subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and were reacted with the appropriate antibodies. The immune complexes were visualized with SuperSignal West Femto substrate (Pierce, Rockford, IL) and detected by an LAS-3000 image analyzer system (Fujifilm, Tokyo, Japan).

Gene silencing by siRNA. The short interfering RNAs (siRNAs) Target-4 (5'-GCUGAGUACGUACAGAAC-3') and Target-6 (5'-GGAAAAGCGAC CACUGUUU-3') were obtained for knockdown of endogenous hB-ind1 (Ambion, Austin, TX). The negative control, siCONTROL Non-Targeting siRNA 2, which exhibits no downregulation of any human genes, was purchased from Dharmacon (Buckinghamshire, United Kingdom). Huh9-13 cells harboring a subgenomic HCV replicon grown on 6-well plates were transfected with 20 nM siRNA by using siFECTOR (B-Bridge International, Sunnyvale, CA) according to the manufacturer's protocol. The transfected cells were incubated in DMEM supplemented with 10% FCS and were then harvested at 96 h posttransfection.

Real-time PCR. The HCV RNA level was estimated by the method described previously (16, 45). Total RNA was prepared from cells by using the RNeasy minikit (Qiagen, Tokyo, Japan). First-strand cDNA was synthesized using an RNA LA PCR kit (Takara Bio Inc., Shiga, Japan) and random primers. Each cDNA was estimated by Platinum Sybr green qPCR SuperMix UDG (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Fluorescent signals were analyzed by an ABI Prism 7000 system (Applied Biosystems). The HCV IRES, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and hB-ind1 genes were amplified using primer pairs 5'-GAGTGTGGTGGAGCCCTCA-3'-5'-CACTCGCAAGCACCTATCA-3', 5'-GAAGGTGAAGTCCGGAGTCC-3'-5'-GAAGGTGAAGTCCGGAGTCC-3', and 5'-CACCTGGAGTTCITAGACCTGTGTG-3'-5'-CAGTCCGGAGTTATTTAGCCGCTC-3', respectively. The values for HCV genomic RNA and hB-ind1 mRNA were normalized to that for GAPDH mRNA. Each PCR product was detected as a single band of the correct size by agarose gel electrophoresis (data not shown).

In vitro transcription and RNA transfection. Plasmids pFK-I₅₈₉ neo/NS3-3'/NK5.1 and pFK-I₅₈₉ FL/NS3-3'/NK5.1 were linearized at the ScaI site and then transcribed in vitro using the MEGAscript T7 kit (Ambion) according to the manufacturer's protocol. To generate capped mRNA encoding *Renilla* luciferase, pRL-CMV was cleaved with BamHI and then transcribed using the mMESSAGE mMACHINE kit (Ambion) according to the manufacturer's protocol. These in vitro-transcribed RNAs were introduced into Huh7.5.1 cells at 4 million cells/0.4 ml by electroporation at 270 V and 960 µF using Gene Pulser (Bio-Rad, Hercules, CA).

Colony formation assay. The colony formation assay has been described previously (45). Briefly, in vitro-transcribed RNA was electroporated into Huh7 cells and plated in DMEM containing 10% FCS and NEAA. The medium was replaced with fresh DMEM containing 10% FCS, NEAA, and 1 mg/ml G418 at 24 h posttransfection. The remaining colonies were fixed with 4% paraformaldehyde and stained with crystal violet at 4 weeks after electroporation.

Luciferase assay. Transfected cells were seeded in a 12-well plate and then lysed in 200 µl of passive lysis buffer (Promega, Madison, WI) at 24 h posttransfection. Luciferase activity was measured in 20-µl aliquots of cell lysates using the Dual-Luciferase reporter assay system (Promega). Firefly luciferase activity was standardized to that of *Renilla* luciferase, and the results are expressed as the increases in relative luciferase units (RLU).

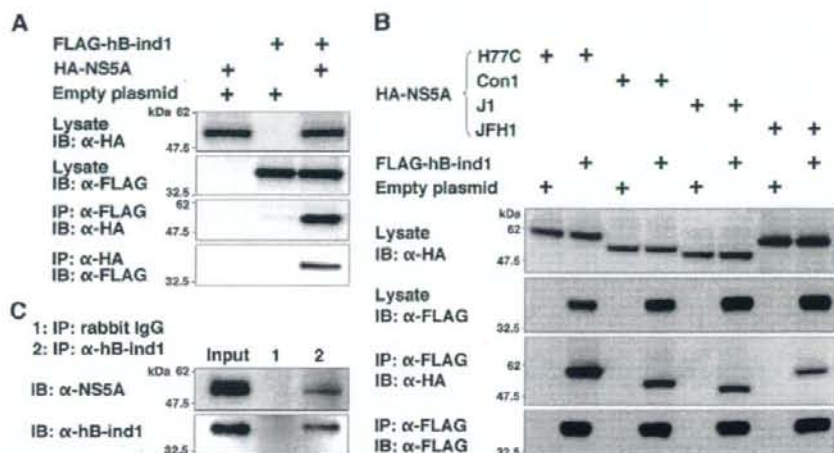


FIG. 1. Interaction of NS5A with hB-ind1 in mammalian cells. (A) HA-NS5A of strain Con1 and FLAG-tagged hB-ind1 were expressed in 293T cells and immunoprecipitated (IP) with an anti-HA or anti-FLAG antibody. Immunoprecipitates were subjected to Western blotting (IB) to detect coprecipitated counterparts. As a negative control, an empty plasmid was used instead of the plasmid encoding FLAG-hB-ind1 or HA-NS5A. Anti-FLAG and anti-HA antibodies did not recognize HA-tagged NS5A and FLAG-tagged hB-ind1, respectively. (B) HA-NS5A protein derived from genotype 1b strain Con1 or J1, genotype 1a strain H77C, or genotype 2a strain JFH1 was coexpressed with FLAG-hB-ind1 in 293T cells, immunoprecipitated with an isotype control or anti-FLAG antibody, and analyzed by Western blotting with an antibody to the FLAG or HA tag. An empty plasmid was used instead of the plasmid encoding FLAG-hB-ind1 as a negative control. (C) Endogenous hB-ind1 in Huh9-13 cells harboring subgenomic HCV replicon RNA was immunoprecipitated with normal rabbit immunoglobulin G (IgG) (lane 1) or anti-hB-ind1 rabbit IgG (lane 2), and immunoprecipitates were analyzed by Western blotting with specific antibodies.

Statistical analysis. Results are expressed as means \pm standard deviations. The significance of differences between the means was determined by Student's *t* test.

RESULTS

hB-ind1 interacts with HCV NS5A of various genotypes. NS5A derived from the genotype 1b strain Con1 was used as bait to screen the human fetal brain cDNA library by a yeast two-hybrid system, and one clone including a gene encoding the open reading frame of the hB-ind1 gene was isolated. To examine whether hB-ind1 could interact with NS5A in mammalian cells, HA-tagged NS5A (HA-NS5A) was coexpressed with FLAG-tagged hB-ind1 (FLAG-hB-ind1) in 293T cells and immunoprecipitated with an antibody to the HA or the FLAG tag. FLAG-hB-ind1 and HA-NS5A were coimmunoprecipitated by either antibody (Fig. 1A). To determine the interaction of various genotypes of NS5A with hB-ind1, HA-NS5A of the genotype 1a strain H77C, the genotype 1b strain J1, or the genotype 2a strain JFH1 was coexpressed with FLAG-hB-ind1 and immunoprecipitated with the anti-FLAG antibody. An empty plasmid was used as a negative control. FLAG-hB-ind1 was immunoprecipitated with the anti-FLAG antibody at similar levels in cells coexpressing FLAG-hB-ind1 and HA-NS5A of all genotypes. HA-NS5A of various genotypes was coprecipitated with FLAG-hB-ind1 by the anti-FLAG antibody, whereas the anti-HA antibody did not precipitate any HA-NS5A of the various genotypes used in this study (Fig. 1B). To further confirm the interaction between hB-ind1 and HCV NS5A in the functional setting, lysates of Huh9-13 cells harboring subgenomic HCV replicon RNA were subjected to im-

muno-precipitation analysis with a rabbit polyclonal antibody raised against hB-ind1. NS5A was coimmunoprecipitated with endogenous hB-ind1 in the lysates of replicon cells (Fig. 1C). These results indicate that hB-ind1 interacts with NS5A of various HCV genotypes in mammalian cells.

hB-ind1 interacts with NS5A through the amino acid residues from 114 to 134 including the coiled-coil domain. hB-ind1 is composed of 362 amino acid residues and has domains homologous with p23 and PTPLA in the regions from Pro⁶ to Asp¹¹² and from Gln¹⁹⁶ to Leu³⁴⁶, respectively (Fig. 2A). To determine the region responsible for the interaction with NS5A, various deletion mutants of FLAG-hB-ind1 were constructed (Fig. 2B). Each of the mutants was coexpressed with Con1 HA-NS5A in 293T cells and immunoprecipitated with an anti-HA antibody. An empty plasmid was used as a negative control in the immunoprecipitation analyses. HA-NS5A was coimmunoprecipitated with full-length hB-ind1 and with mutants possessing amino acid residues 114 to 134, corresponding to the coiled-coil domain, which generally participates in protein-protein interactions (Fig. 2B and C), whereas HA-NS5A was not coimmunoprecipitated with hB-ind1 mutants lacking the coiled-coil domain. The anti-HA antibody did not coprecipitate FLAG-hB-ind1 or its mutants. These results indicate that hB-ind1 interacts with HCV NS5A through the coiled-coil domain.

hB-ind1 participates in the replication of HCV RNA and the propagation of infectious HCV particles. To investigate the role(s) of endogenous hB-ind1 in the replication of HCV RNA, an siRNA targeted to hB-ind1 or a control siRNA was transfected into Huh9-13 cells harboring subgenomic HCV

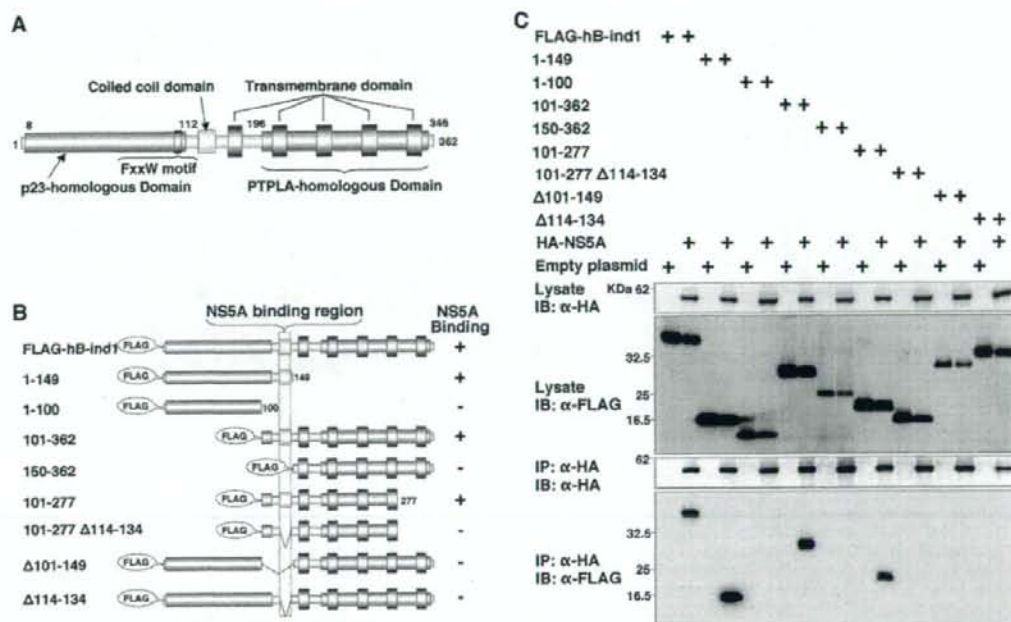


FIG. 2. Determination of the NSSA-binding region in hB-ind1. (A) Structure and functional domains of hB-ind1. (B) Deletion mutants of hB-ind1 used in this study and the results of binding to NSSA. N-terminally FLAG-tagged hB-ind1 mutants encoding the region from residue 1 to 149, 1 to 100, 101 to 362, 150 to 362, or 101 to 277 were designated 1-149, 1-100, 101-362, 150-362, or 101-277, respectively. An N-terminally FLAG-tagged hB-ind1 mutant spanning the region from residue 101 to residue 277 but lacking residues 114 to 134 was designated 101-277 Δ 114-134. In addition, N-terminally FLAG-tagged hB-ind1 mutants lacking the region from 101 to 149 or from 114 to 134 were designated Δ 101-149 or Δ 114-134, respectively. The coiled-coil domain was located at residues 114 to 134. Each mutant gene was inserted into pEF FLAGGs pGKpuro. A summary of immunoprecipitation results is given on the right. (C) Each hB-ind1 mutant was cocexpressed with Con1 HA-NSSA in 293T cells, immunoprecipitated with an anti-HA antibody, and analyzed by Western blotting with an anti-FLAG antibody. As a negative control, an empty plasmid was used instead of the plasmid encoding HA-NSSA. The anti-HA antibody did not recognize FLAG-tagged hB-ind1 or its mutants.

replicon RNA. Total RNA was extracted from the transfected cells, and levels of hB-ind1 mRNA and HCV RNA were determined by real-time PCR. At 72 h posttransfection, hB-ind1 mRNA and HCV subgenomic RNA levels in cells transfected with each of the hB-ind1 siRNAs were reduced more than 60% from the levels in cells treated with the control siRNA (Fig. 3A). The levels of expression of hB-ind1 and the HCV NSSA protein were decreased in HCV replicon cells transfected with the hB-ind1 siRNA but not in those transfected with the control siRNA (Fig. 3B).

To examine the effects of the knockdown of hB-ind1 on the replication of HCV RNA and the propagation of HCVcc, we established Huh7.5.1 cell lines stably expressing an shRNA targeted to hB-ind1. Dozens of colonies were obtained from cells transfected with a plasmid encoding the cDNA of the shRNA to hB-ind1 after selection with hygromycin. Although the levels of mRNA and expression of endogenous hB-ind1 were not changed in cells bearing a nonspecific shRNA, they were reduced in the clones bearing shRNAs targeted to hB-ind1, except for clone 1 (Fig. 3C and D). There was no significant difference in growth among the cell lines (Fig. 3E).

The replicon RNA transcribed from pFK-I₃₈₉ neo/NS3-3'/

NK5.1 was transfected into the hB-ind1 knockdown cell lines Huh-si2 and Huh-si5, which were cultured for 4 weeks in the presence of G418. The numbers of colonies in the knockdown cell lines were less than one-fourth of those in the control cell line (Huh-c) (Fig. 4A). A FLAG-tagged hB-ind1 wobble mutant (FLAG-rB-ind1), which is resistant to the shRNA targeted to hB-ind1 due to the introduction of silent mutations, was capable of expressing an siRNA-resistant hB-ind1 upon introduction into cells at a level similar to that of the endogenous hB-ind1 (eB-ind1) detected in the control cell line (Fig. 4B). The reduction of colony formation by the knockdown of eB-ind1 in the hB-ind1 knockdown cell lines Huh-si2 and Huh-si5 was canceled by the expression of FLAG-rB-ind1 (Fig. 4A). To further examine the involvement of hB-ind1 in the replication of HCV, a chimeric HCV RNA encoding a firefly luciferase gene under the control of HCV IRES (Fig. 4C) was transfected into the knockdown cell lines. Knockdown of hB-ind1 reduced the RLU in Huh-si2 and Huh-si5 cells by 40% and 70%, respectively, and this reduction was also canceled by the expression of FLAG-rB-ind1. To further examine the effect of hB-ind1 knockdown on the production of HCV infectious particles, HCVcc were inoculated into the hB-ind1 knockdown

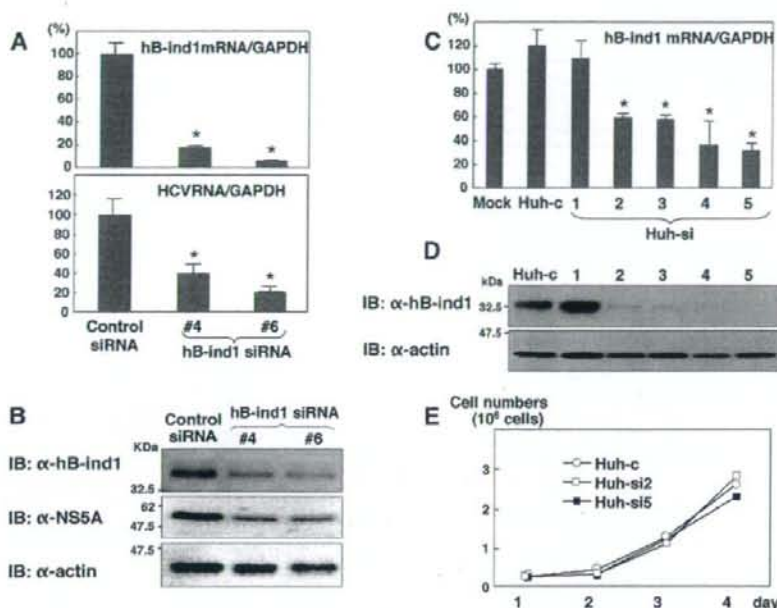


FIG. 3. Effects of hB-ind1 knockdown on HCV replication. (A) Huh9-13 cells were transfected with siRNA 4 or siRNA 6 (#4 or #6, respectively), targeted to the hB-ind1 gene, or with a nonspecific siRNA, at a final concentration of 20 nM, and were harvested at 72 h posttransfection. hB-ind1 mRNA and HCV RNA levels were determined by real-time PCR. The levels of hB-ind1 mRNA and HCV RNA were normalized to the amount of GAPDH mRNA and expressed as percentages of the control value. (B) Huh9-13 cells transfected with siRNAs were lysed at 72 h posttransfection and subjected to Western blotting (IB) with an antibody to hB-ind1, NS5A, or β -actin. (C) Establishment of hB-ind1 knockdown Huh7.5.1 cell lines. Plasmids encoding shRNAs targeted to hB-ind1 (siRNA 6) or nonspecific targets were transfected into Huh7.5.1 cells and cultivated in the presence of hygromycin. Independent clones were established by limiting dilution. The value for hB-ind1 mRNA was normalized to the amount of GAPDH mRNA and expressed as a percentage of the control value. Huh7.5.1 cell lines expressing siRNAs targeted to hB-ind1 (Huh-si1 to Huh-si5) and to a nonspecific target (Huh-c) were established. (D) Expression of hB-ind1 in knockdown cells. The knockdown cell lines were lysed and subjected to Western blotting with an antibody to hB-ind1 or β -actin. (E) Growth curves of the knockdown cell lines were determined by the method of trypan blue dye exclusion. Data in this figure are representative of three independent experiments. Error bars, standard deviations. Asterisks indicate significant differences ($P < 0.01$) from the control value.

cell lines. Both virus titers, determined by focus-forming units at 72 h postinfection in culture supernatants, and HCV RNA levels in Huh-si2 and Huh-si5 cells were significantly reduced, and these reductions were canceled by the expression of FLAG-rB-ind1 (Fig. 4D). These results suggest that hB-ind1 is involved in the replication of HCV RNA and the propagation of HCVcc.

An hB-ind1 mutant retaining the binding region to NS5A has a dominant-negative effect on the replication of HCV. To examine the involvement of hB-ind1 in the replication of HCV in greater detail, deletion mutants of hB-ind1 retaining or lacking the binding region to NS5A were expressed in Huh9-13 cells harboring subgenomic HCV replicon RNA (Fig. 5A). Although the hB-ind1 mutant possessing the NS5A binding region (101-277) and full-length hB-ind1 were detected at similar levels in replicon cells transfected with the expression plasmids (Fig. 5B), HCV RNA replication was reduced only in cells expressing the mutant retaining the binding region to NS5A, not in those expressing full-length hB-ind1 or the mutant lacking the binding region to NS5A (101-277 Δ 114-134) (Fig. 5C). However, no significant difference in NS5A expres-

sion was observed in Huh9-13 cells transfected with the expression plasmids (Fig. 5B). Production of the infectious HCV particles was also reduced in the culture supernatants of Huh7.5.1 cells expressing the hB-ind1 mutant retaining the binding region to NS5A (101-277) but not in those expressing full-length hB-ind1 or the hB-ind1 101-277 Δ 114-134 mutant (Fig. 5D). These dominant-negative effects of the hB-ind1 mutant retaining the binding region to NS5A on the replication of HCV RNA in Huh9-13 cells and on the production of infectious particles in Huh7.5.1 cells further support the notion that hB-ind1 regulates the replication of HCV RNA and the propagation of HCVcc.

hB-ind1 interacts with FKBP8 and Hsp90. Previous reports have suggested that HCV NS5A interacts with several host proteins such as FBL2 (63), VAP-A (59), VAP-B (16), and FKBP8 (45) and that these interactions participate in the replication of HCV. To determine the interplay of the NS5A-binding proteins, FLAG-tagged hB-ind1 was coexpressed with HA-tagged FBL2, VAP-A, VAP-B, or FKBP8 in 293T cells and immunoprecipitated with an anti-FLAG antibody, and FKBP8 was shown to specifically interact with hB-ind1 (Fig. 6A). We have

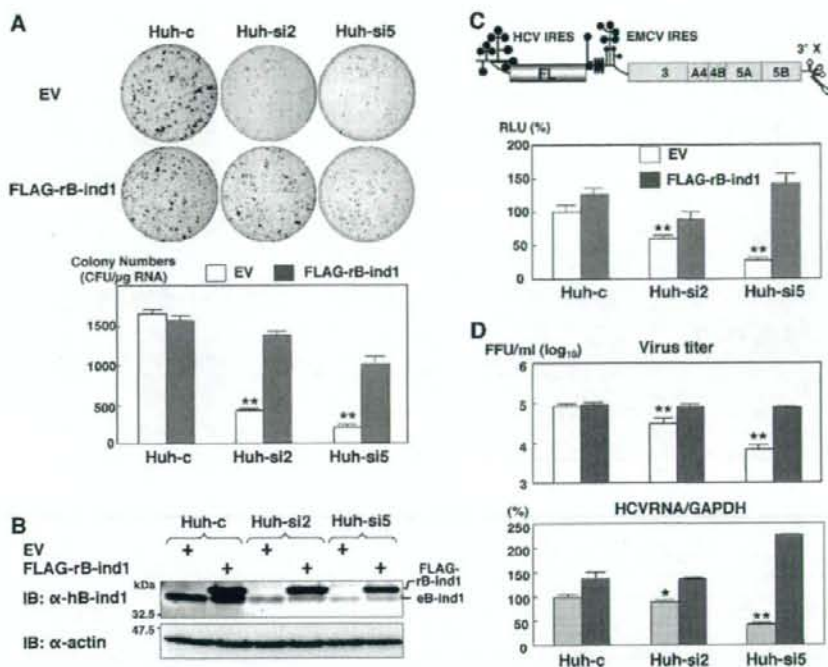


FIG. 4. Effects of hB-ind1 knockdown on the replication of HCV RNA and the production of infectious particles. (A) The hB-ind1 knockdown (Huh-si2 and Huh-si5) and control (Huh-c) cell lines were first transfected with either a plasmid encoding hB-ind1 resistant to siRNA by virtue of the introduction of silent mutations (FLAG-rB-ind1) or an empty vector (EV) and then further transfected with replicon RNA transcribed from pFK-1₃₈₉ neo/NS3-3'/NK5.1. (Upper panel) The cell colonies remaining after cultivation for 4 weeks in the presence of G418 were fixed with 4% paraformaldehyde and stained with crystal violet. (Lower panel) The number of colonies was standardized to the amount of transfected RNA. (B) The expression of the siRNA-resistant hB-ind1 (FLAG-rB-ind1) and the endogenous hB-ind1 (eB-ind1) in Huh-c, Huh-si2, and Huh-si5 cells transfected with either a plasmid encoding FLAG-rB-ind1 or an empty vector was analyzed by Western blotting (IB) with an antibody to hB-ind1 or β -actin. (C) HCV subgenomic replicon RNA transcribed from pFK-1₃₈₉ FL/NS3-3'/NK5.1 and capped *Renilla* luciferase RNA transcribed from pRL-CMV were cotransfected into Huh-c, Huh-si2, and Huh-si5 cells pretransfected with either a plasmid encoding FLAG-rB-ind1 or an empty vector. The firefly luciferase activity was normalized to that of *Renilla* luciferase. HCV IRES-dependent translational activity was expressed as a percentage of the RLU of Huh-c cells transfected with an empty plasmid. EMCV, encephalomyocarditis virus. (D) HCVcc were inoculated into Huh-c, Huh-si2, and Huh-si5 cells pretransfected with either a plasmid encoding FLAG-rB-ind1 or an empty vector. (Upper panel) The culture supernatants at 72 h postinoculation were subjected to a focus-forming assay, and virus titers are expressed as focus-forming units (FFU) per milliliter. (Lower panel) The amount of intracellular HCV RNA was measured by real-time PCR and normalized to the amount of GAPDH mRNA. The HCV RNA level is expressed as a percentage of that of Huh-c cells transfected with an empty plasmid. Data in this figure are representative of three independent experiments. Error bars, standard deviations. Asterisks indicate significant differences (**, $P < 0.01$; *, $P < 0.05$) from the control value.

previously shown that FKBP8 is capable of binding to both NS5A and Hsp90 through the tetratricopeptide repeat (TPR) domain and that the recruitment of Hsp90 to the replication complex plays a crucial role in the replication of HCV (45). Hsp90 is a molecular chaperone and requires various cochaperone proteins such as p23 for efficient chaperone activity. hB-ind1 shows homology to p23 (Fig. 2A), and the FxxW motif, essential for the binding to Hsp90, is conserved in residues Phe¹⁰⁷xTrp¹¹⁰ of hB-ind1 (11, 27, 68). To determine whether hB-ind1 interacts with Hsp90 through the FxxW motif as reported for p23, FLAG-tagged hB-ind1 or an hB-ind1 mutant in which Phe¹⁰⁷ and Trp¹¹⁰ had been replaced with Ala (FLAG-hB-ind1AxxA) was coexpressed with HA-tagged Hsp90 in 293T cells and immunoprecipitated with an anti-FLAG antibody. Hsp90 was coimmunoprecipitated with wild-type hB-ind1 but not with the

mutant hB-ind1, indicating that hB-ind1 interacts with Hsp90 through the FxxW motif (Fig. 6B).

Previously, we showed that the amino acid residues of the carboxylate clump position in the TPR domain of FKBP8 attach to the C-terminal MEEVD motif of Hsp90 (45). To examine the interaction of hB-ind1 with Hsp90 in the absence of association with FKBP8, FLAG-tagged hB-ind1 was first coexpressed with HA-tagged Hsp90 or mutant Hsp90 lacking the MEEVD motif in 293T cells and then immunoprecipitated with an anti-FLAG antibody. Similar levels of hB-ind1 were coprecipitated with Hsp90 irrespective of the deletion of the MEEVD motif of Hsp90 (Fig. 6C), suggesting that hB-ind1 alone is capable of binding to Hsp90 through the FxxW motif irrespective of the association of FKBP8. To further clarify the interplay among hB-ind1, FKBP8, and Hsp90, FLAG-tagged

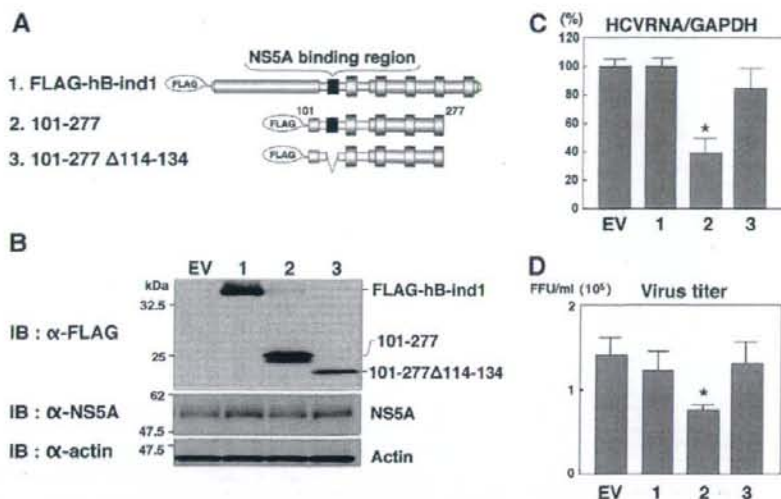


FIG. 5. Dominant-negative effect of an hB-ind1 mutant on the replication of HCV. (A) Plasmids encoding full-length hB-ind1 (construct 1) or deletion mutants of hB-ind1 retaining (construct 2) or lacking (construct 3) the NS5A binding region. (B) One of the three plasmids or an empty vector (EV) was transfected into Huh9-13 cells harboring a subgenomic HCV replicon RNA and was subjected to Western blotting (IB) with specific antibodies at 72 h posttransfection. (C) The amount of intracellular HCV RNA in the Huh9-13 cells was measured at 72 h posttransfection by real-time PCR, normalized to the amount of GAPDH mRNA, and expressed as the percentage of the value for control cells transfected with an empty plasmid. (D) One of the three plasmids or an empty vector was transfected into Huh7.5.1 cells, and then HCVcc were inoculated. Virus production in the culture supernatants at 72 h postinoculation was determined by a focus-forming assay. FFU, focus-forming units. Data in this figure are representative of three independent experiments. Error bars, standard deviations. Asterisks indicate significant differences ($P < 0.01$) from the control value.

hB-ind1 was coexpressed with HA-tagged Hsp90 and/or FKBP8 and then immunoprecipitated with an anti-FLAG antibody. Coprecipitation of Hsp90 with hB-ind1 was increased by additional expression of FKBP8 (Fig. 6D). These results suggest that hB-ind1 interacts with Hsp90 through the FxxW motif and that FKBP8 also participates in the complex formation to enhance the interaction.

hB-ind1 participates in HCV propagation through the interaction with Hsp90. Next, to examine the role of the interaction of hB-ind1 with Hsp90 in the replication of HCV RNA, the replicon RNA transcribed from pFK-I₃₈₉ neo/NS3-3'/NK5.1 was transfected into hB-ind1 knockdown Huh-si5 cells expressing siRNA-resistant FLAG-rB-ind1 or FLAG-rB-ind1AxxA, in which the Hsp90 binding motif FxxW was changed to AxxA. The colony formation in Huh-si5 cells transfected with an empty plasmid was 10% of that in Huh-c cells. The expression of FLAG-rB-ind in Huh-si5 cells recovered the colony formation in Huh-si5 cells to 98% of that in Huh-c cells, although that of FLAG-rB-ind1 AxxA in Huh-si5 cells exhibited only 40% recovery (Fig. 7A). To further examine the role of the interaction between hB-ind1 and Hsp90 in the production of HCVcc, Huh-si5 cells expressing either FLAG-rB-ind1 or FLAG-rB-ind1AxxA were infected with HCVcc, and the virus titer in the culture supernatants and the intracellular HCV RNA level at 72 h postinfection were determined. Virus production was reduced in the culture supernatants, and viral RNA replication in the hB-ind1 knockdown cells was restored by the expression of FLAG-rB-ind1 but not by that of FLAG-rB-ind1AxxA, as seen in colony formation by the replicon

RNA (Fig. 7B). Collectively, these results suggest that the interaction of hB-ind1 with Hsp90 through the FxxW motif is required for genomic RNA replication and particle production of HCV.

DISCUSSION

In this study we have shown that hB-ind1 participates in HCV RNA replication and particle production through interaction with NS5A, FKBP8, and Hsp90. hB-ind1 was initially identified as a downstream transducer of Rac1, a member of the small GTP-binding proteins, in mouse fibroblasts treated with sodium butyrate, a multifunctional agent known to inhibit cell proliferation and to induce differentiation by modulating transcription (6, 10). Rac1 possesses diverse biological functions, including cytoskeletal dynamics, membrane ruffling, cell cycle progression, gene transcription, and cell survival (4, 31, 49). Previous studies have suggested that hB-ind1 mediates Rac1 and Jun N-terminal protein kinase-NF- κ B signaling and is involved in the regulation of gene expression (6, 10). Inhibition of Rac1 function leads to disruption of cytoskeleton dynamics, resulting in impairment of cell growth (17, 69).

Inhibition of cell growth downregulates HCV RNA replication in the replicon cell line (41, 51), and cell cycle regulation affects HCV IRES-mediated translation (20, 61). Furthermore, cytoskeletal regulation is required for HCV RNA synthesis (3). However, knockdown of hB-ind1 and expression of the deletion mutants exhibited neither morphological change nor suppression of cell growth, suggesting that the suppression

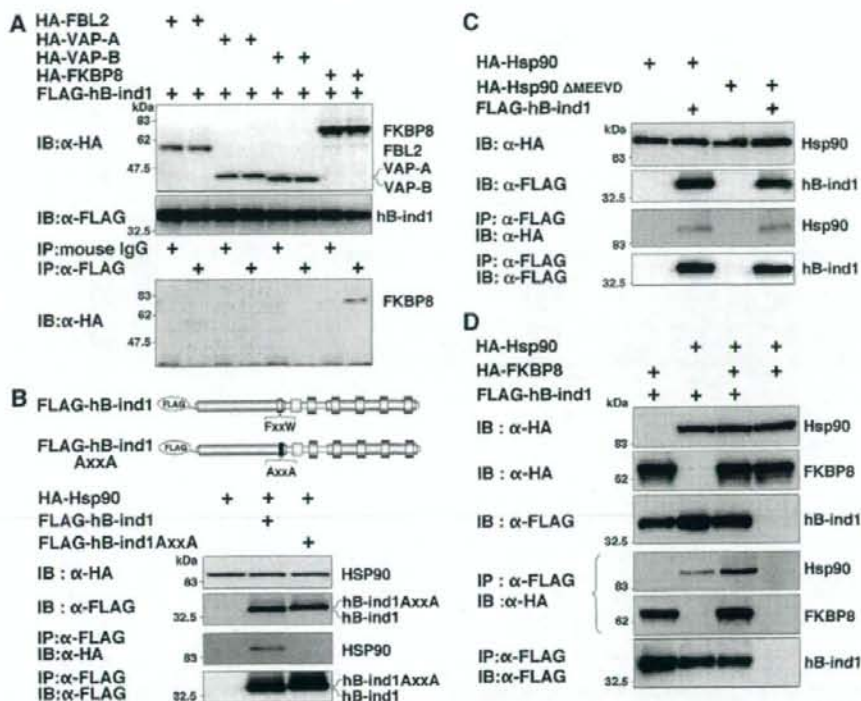


FIG. 6. Interaction of hB-ind1 with other NS5A-binding host proteins. (A) FLAG-hB-ind1 was first coexpressed with HA-tagged FBL2, VAP-A, VAP-B, or FKBP8 in 293T cells and then immunoprecipitated with an anti-FLAG or control antibody. The immunoprecipitates were detected by Western blotting (IB) with an anti-HA antibody. (B) FLAG-hB-ind1 or FLAG-hB-ind1AxxA, in which Phe¹⁰⁷ and Trp¹¹⁰ had been replaced with Ala, was coexpressed with HA-Hsp90 in 293T cells and immunoprecipitated with an anti-FLAG antibody. The immunoprecipitates were detected by Western blotting with an anti-HA or anti-FLAG antibody. (C) FLAG-hB-ind1 was coexpressed with HA-Hsp90 or mutant Hsp90 lacking the MEEVD motif (HA-Hsp90 Δ MEEVD) in 293T cells and was immunoprecipitated with an anti-FLAG antibody. The immunoprecipitates were detected by Western blotting with an anti-HA or anti-FLAG antibody. (D) HA-Hsp90, HA-FKBP8, and FLAG-hB-ind1 were coexpressed in various combinations in 293T cells and immunoprecipitated with an anti-FLAG antibody. The immunoprecipitates were detected by Western blotting with an anti-HA or anti-FLAG antibody. Data in this figure are representative of three independent experiments.

of HCV replication by dysfunction of hB-ind1 is not due to cell growth arrest or cytoskeletal disruption. Murine B-ind1 has been reported to be expressed in all mouse tissues examined, with abundant expression detected in the testis, kidney, brain, and liver (10). Significant levels of endogenous hB-ind1 expression have been detected in the human hepatic cell lines Huh7, HepG2, Hep3B, and FLC4 and in the nonhepatic human cell lines HeLa, 293T, and THP-1 (data not shown); therefore, the tissue specificity of HCV replication could not be explained by the expression of hB-ind1.

Combination therapy with IFN and cyclosporine A has been shown to be effective for patients infected with a high viral load of HCV genotype 1b (24), and cyclosporine A has been shown to suppress HCV RNA replication in vitro through deactivation of the interaction between NS5B and cyclophilin B (66). Cyclophilin and FKBP are classified as immunophilins capable of binding to immunosuppressants cyclosporine A and FK506, respectively (33). The immunophilins do not share a homologous domain with each other, based on their amino acid sequences, substrate specificities, and inhibitor sensitivities. We

have recently reported that NS5A binds specifically to FKBP8 but not to other homologous immunophilins such as FKBP52 and cyclophilin D. FKBP8 forms both a homomultimer and a heteromultimer with the chaperone protein Hsp90. Mutation analyses of FKBP8 and Hsp90 suggest that FKBP8 acts as an intermediate between NS5A and Hsp90 via the different position of the TPR domain in FKBP8 and regulates HCV genome replication (45).

The molecular chaperone Hsp90 is one of the most abundant proteins in unstressed cells and generally requires various cochaperone proteins in multiple steps to promote the folding, functional maturation, and stability of its client proteins. Newly synthesized unfolded client proteins are delivered to the Hsp70 complex via Hsp40. In most cases, Hsp70 is able to process the client proteins on its own. Certain substrates require Hsp90 for proper folding or activation. In this case, the scaffold protein Hop connects elements of the Hsp70 and Hsp90 machineries to form an intermediate complex (2, 12, 13, 47). In the late stage, the Hsp70 component dissociates, and at the same time, p23 and immunophilins enter the complex (44, 54) and the

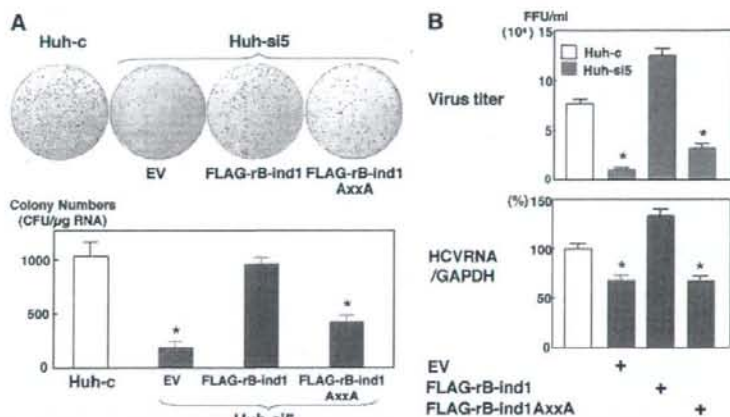


FIG. 7. Role of the interaction of hB-ind1 with Hsp90 in the replication of HCV. (A) hB-ind1 knockdown (Huh-si5) and control (Huh-c) cell lines were transfected either with a plasmid encoding the FLAG-tagged siRNA-resistant hB-ind1 (FLAG-rB-ind1) or FLAG-rB-ind1AxxA (with substitutions in the motif required for binding to Hsp90) or with an empty vector (EV) and were then further transfected with replicon RNA transcribed from pFK-1₃₈₉ neo/NS3-3'/NK5.1. (Upper panel) The cell colonies remaining after cultivation for 4 weeks in the presence of G418 were fixed with 4% paraformaldehyde and stained with crystal violet. (Lower panel) The number of colonies was standardized to the amount of transfected RNA. (B) (Upper panel) Huh-si5 cells expressing either FLAG-rB-ind1 or FLAG-rB-ind1AxxA were infected with HCVcc, and virus production in the culture supernatants at 72 h postinoculation was determined by a focus-forming assay. (Lower panel) The amount of intracellular HCV RNA was measured at 72 h posttransfection by real-time PCR, normalized to the amount of GAPDH mRNA, and expressed as a percentage of the value for control cells transfected with an empty plasmid. Data in this figure are representative of three independent experiments. Error bars, standard deviations. Asterisks indicate significant differences ($P < 0.01$) from the control value.

client proteins are refolded by Hsp90 chaperone activity to achieve the mature form. After that, p23 enhances the dissociation of the mature client protein from the final complex, and the released Hsp90 enters in the next chaperone cycle (72). It has been reported that Hsp90 cochaperone frequencies differ among client proteins (50). FKBP8 interacts with the C-terminal MEEVD motif of Hsp90 through the carboxylate clump position in the TPR domain of FKBP8 (45).

The C-terminal region of hB-ind1 shares homology with PTPBLA (60). Protein tyrosine phosphatases are generally involved in the signaling pathways regulating metabolism, cell growth, differentiation, and cytoskeletal dynamics through the conserved HC(x)₂R motif (57). NS5A also interacts with signal transducer and activator of transcription 1 (STAT1) and impairs IFN signaling through the suppression of STAT1 phosphorylation (30). In addition, intracellular uptake of apoptotic cells expressing NS5A by dendritic cells leads to an increase in the secretion of CXCL-8 and impairment of IFN-induced tyrosine phosphorylation of STAT1 and STAT2 (67). Although hB-ind1 lacks the conserved active motif, the interaction of NS5A with the coiled-coil domain in the central region of hB-ind1 may have an effect on the phosphorylation of host proteins involved in the replication of HCV.

Hsp90 has been shown to be involved in the enzymatic activity and intracellular localization of several viral polymerases, including those of influenza virus (39, 42), herpes simplex virus type 1 (5), and Flock house virus (25). Knockdown and treatment with an Hsp90 inhibitor have revealed that Hsp90 activity is important for the rapid growth of negative-strand RNA viruses (9). Furthermore, Hsp90 has been shown to be required for the activity of hepatitis B virus reverse

transcriptase (21, 22). Although the precise mechanisms by which Hsp90 and FKBP8 cooperate with NS5A to improve the *in vivo* replication of HCV have not been clarified yet, treatment with Hsp90 inhibitors in combination with IFN reduced HCV replication in mice xenotransplanted with human liver fragments (43).

In this study, hB-ind1 was shown to interact with Hsp90 through the FxxW motif in the N-terminal p23 homology domain, and the interaction of hB-ind1 with Hsp90 was shown to be further intensified by the expression of FKBP8, suggesting that FKBP8 and hB-ind1 cooperatively recruit Hsp90 to the HCV replication complex. Furthermore, hB-ind1 was shown to be involved in HCV genomic RNA replication and particle production through the interaction with NS5A and Hsp90. These results suggest that hB-ind1 may be involved in the Hsp90 chaperone pathway in a function similar to that of p23 in cooperation with immunophilins such as FKBP8 and that it plays a crucial role in HCV replication in terms of the correct folding of the replication complex required for efficient enzymatic activity. In addition, cyclophilin B may also participate in the translocation of NS5B, as seen in the polymerase subunits of influenza virus, to facilitate binding to the viral RNA. In contrast to cyclosporine A, FK506 per se exhibits no inhibition of RNA replication in HCV replicon cells (65). FKBP8 is a member of the FKBP family but lacks several amino acid residues required for peptidyl-prolyl *cis-trans* isomerase and FK506 binding activities (29). Therefore, nonimmunosuppressive FK506 derivatives that are capable of binding to FKBP8 may exhibit anti-HCV activity. Recently, geldanamycin, an inhibitor of Hsp90, was shown to drastically impair the replication of poliovirus without any escape mutant emerging (15).

Therefore, elucidation of host proteins, including immunophilins, cochaperones, and chaperones, participating in the HCV replication complex may lead to the development of new therapeutics for chronic hepatitis C with a broad spectrum and a low possibility of emergence of breakthrough viruses against antiviral drugs.

In conclusion, in this study we demonstrated that hB-ind1 is involved in HCV replication through interactions with NS5A, FKBP8, and Hsp90. Further clarification of the relationship between viral and host proteins is needed in order to understand the precise mechanism of HCV replication.

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PPAR α activation is essential for HCV core protein-induced hepatic steatosis and hepatocellular carcinoma in mice

Naoki Tanaka,^{1,2} Kyoji Moriya,³ Kendo Kiyosawa,² Kazuhiko Koike,³ Frank J. Gonzalez,⁴ and Toshifumi Aoyama¹

¹Department of Metabolic Regulation, Institute on Aging and Adaptation, Shinshu University Graduate School of Medicine, Matsumoto, Nagano, Japan.

²Division of Gastroenterology, Department of Internal Medicine, Shinshu University School of Medicine, Matsumoto, Nagano, Japan.

³Department of Infectious Diseases, Internal Medicine, Graduate School of Medicine, University of Tokyo, Tokyo, Japan.

⁴Laboratory of Metabolism, National Cancer Institute, NIH, Bethesda, Maryland, USA.

Transgenic mice expressing HCV core protein develop hepatic steatosis and hepatocellular carcinoma (HCC), but the mechanism underlying this process remains unclear. Because PPAR α is a central regulator of triglyceride homeostasis and mediates hepatocarcinogenesis in rodents, we determined whether PPAR α contributes to HCV core protein-induced diseases. We generated PPAR α -homozygous, -heterozygous, and -null mice with liver-specific transgenic expression of the core protein gene (*Ppara*^{+/+}:HCVcpTg, *Ppara*^{+/-}:HCVcpTg, and *Ppara*^{-/-}:HCVcpTg mice. Severe steatosis was unexpectedly observed only in *Ppara*^{+/+}:HCVcpTg mice, which resulted from enhanced fatty acid uptake and decreased mitochondrial β -oxidation due to breakdown of mitochondrial outer membranes. Interestingly, HCC developed in approximately 35% of 24-month-old *Ppara*^{+/+}:HCVcpTg mice, but tumors were not observed in the other genotypes. These phenomena were found to be closely associated with sustained PPAR α activation. In *Ppara*^{+/+}:HCVcpTg mice, PPAR α activation and the related changes did not occur despite the presence of a functional *Ppara* allele. However, long-term treatment of these mice with clofibrate, a PPAR α activator, induced HCC with mitochondrial abnormalities and hepatic steatosis. Thus, our results indicate that persistent activation of PPAR α is essential for the pathogenesis of hepatic steatosis and HCC induced by HCV infection.

Introduction

HCV is one of the major causes of chronic hepatitis, whereas patients with persistent HCV infection have a high incidence of hepatocellular carcinoma (HCC) (1, 2). Occurrence of HCC associated with chronic HCV infection has increased over the past 2 decades (3–5), and chronic HCV infection is recognized as a serious debilitating disease. However, the mechanism in which chronic HCV infection mediates hepatocarcinogenesis remains unclear.

HCV core protein was shown to have oncogenic potential (6). To examine how HCV core protein participates in HCV-related hepatocarcinogenesis, transgenic mouse lines were established in which HCV core protein is expressed constitutively in liver at cellular levels similar to those found in chronic HCV-infected patients (7). These mice exhibited hepatic steatosis (7) and insulin resistance (8) as early as 3 months of age; on further aging, these symptoms worsened and hepatic adenomas developed in approximately 30% of mice between 16 and 18 months of age (9). Finally, HCC was found within hepatic adenomas in a classic "nodule-in-nodule" pathology (9). Interestingly, no hepatic inflammation or fibrosis was found in these mice throughout

the course of HCC development (9), which suggested that the HCV core protein itself induces hepatic steatosis and HCC independently of hepatitis.

Several studies support the contention that hepatic steatosis promotes the development of HCC (10). Epidemiologic data have identified hepatic steatosis as a major accelerating factor of hepatocarcinogenesis in chronic HCV-infected patients (11). Moreover, increases in ROS production that can cause oxidative DNA damage, mitochondrial abnormalities, and accelerated hepatocyte proliferation were observed in the steatotic livers (12–14). Thus, an intriguing possibility has emerged that alteration of fatty acid metabolism in hepatocytes may be central to the pathogenesis of HCC induced by HCV core protein.

PPARs are ligand-activated nuclear receptors belonging to the steroid/thyroid hormone receptor superfamily; 3 isoforms designated as α , β/δ , and γ exist, all of which are involved in lipid homeostasis (15). PPAR α regulates constitutive transcription of genes encoding fatty acid-metabolizing enzymes (16) and is associated with the maintenance of fatty acid transport and metabolism, primarily in liver, kidney, and heart. Administration of PPAR α agonists, such as the widely prescribed fibrate drugs clofibrate, gemfibrozil, and fenofibrate, ameliorate hyperlipidemia in humans (17) and hepatic steatosis in mice (18).

On the other hand, long-term administration of PPAR α ligands to rodents causes accelerated hepatocyte proliferation, increased ROS generation, and development of HCC (19, 20). Disruption of the PPAR α gene was shown to prevent the development of HCC caused by long-term exposure to PPAR α activators (21). Interestingly, accumulation of fatty acids/triglycerides in hepatocytes

Nonstandard abbreviations used: ACC, acetyl-CoA carboxylase; AOX, acyl-CoA oxidase; CDK, cyclin-dependent kinase; CYP4A1, cytochrome P450 4A1; FAS, fatty acid synthase; FAT, fatty acid translocase; FATP, fatty acid transport protein; HCC, hepatocellular carcinoma; HCVcpTg, HCV core protein-expressing transgenic; L-FABP, liver fatty acid-binding protein; MCAD, medium-chain acyl-CoA dehydrogenase; MTP, microsomal transfer protein; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; PCNA, proliferating cell nuclear antigen; RXR α , retinoid X receptor α .

Conflict of interest: The authors have declared that no conflict of interest exists.

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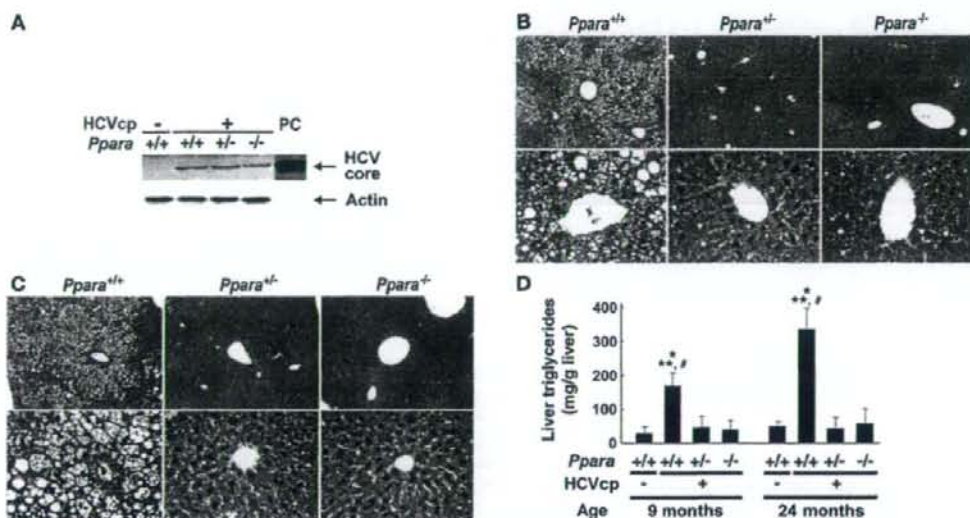


Figure 1

Phenotype changes in transgenic mouse liver. (A) Immunoblot analysis of HCV core protein expression in livers of 9-month-old mice. Because no significant individual differences in the same mouse group were found in the preliminary experiments, 10 mg of liver prepared from each mouse ($n = 6$ /group) was mixed and homogenized. Whole-liver lysate (50 μ g protein) was loaded in each well. The band of actin was used as the loading control. Results are representative of 4 independent experiments. PC, lysate prepared from COS-1 cells overexpressing HCV core protein as a positive control. (B) Histological appearance of hematoxylin- and eosin-stained liver sections from 9-month-old HCVcpTg mice. Upper and lower rows show a lower ($\times 40$) and higher ($\times 400$) magnification, respectively. Microvesicular and macrovesicular steatosis was found only in $Ppara^{+/+}$:HCVcpTg mice. No inflammation or hepatocyte degeneration was evident in any of the genotypes. (C) Histological appearance of hematoxylin- and eosin-stained liver sections from 24-month-old HCVcpTg mice. Upper and lower rows show a lower ($\times 40$) and higher ($\times 400$) magnification, respectively. Hepatic steatosis was marked in $Ppara^{+/+}$:HCVcpTg mice, but not in other mice. Hepatic inflammation, fibrosis, and hepatocyte degeneration were not observed. In $Ppara^{+/-}$:HCVcpTg and $Ppara^{-/-}$:HCVcpTg mice, dysplastic hepatocytes and precancerous lesions were not detected throughout the entire liver. (D) Content of liver triglycerides. Results are expressed as the mean \pm SD ($n = 6$ /group) and compared between genotypes at the same age. * $P < 0.05$ compared with $Ppara^{+/+}$ nontransgenic mice; ** $P < 0.05$ compared with $Ppara^{+/-}$:HCVcpTg mice; # $P < 0.05$ compared with $Ppara^{-/-}$:HCVcpTg mice.

could lead to continuous PPAR α activation because of the presence of fatty acid metabolites that serve as natural PPAR α ligands. For example, mice lacking expression of the peroxisomal acyl-CoA oxidase (AOX) gene showed massive accumulation of very-long-chain fatty acids in hepatocytes, severe microvesicular steatosis, chronic PPAR α activation, and development of hepatic adenoma and HCC by 15 months of age (22). These results suggest a strong contribution of activated PPAR α to liver tumorigenesis.

On the basis of several lines of evidence, we hypothesized that PPAR α might contribute to hepatocarcinogenesis in HCV core protein-expressing transgenic (HCVcpTg) mice. To explore this possibility, PPAR α -homozygous ($Ppara^{+/+}$), PPAR α -heterozygous ($Ppara^{+/-}$), and PPAR α -null ($Ppara^{-/-}$) mice bearing the HCV core protein gene, designated $Ppara^{+/+}$:HCVcpTg, $Ppara^{+/-}$:HCVcpTg, and $Ppara^{-/-}$:HCVcpTg mice, were generated, and phenotypic changes were examined. Surprisingly, we found that severe hepatic steatosis and HCC induced by HCV core protein developed only in $Ppara^{+/+}$ mice, which were related to persistent PPAR α activation.

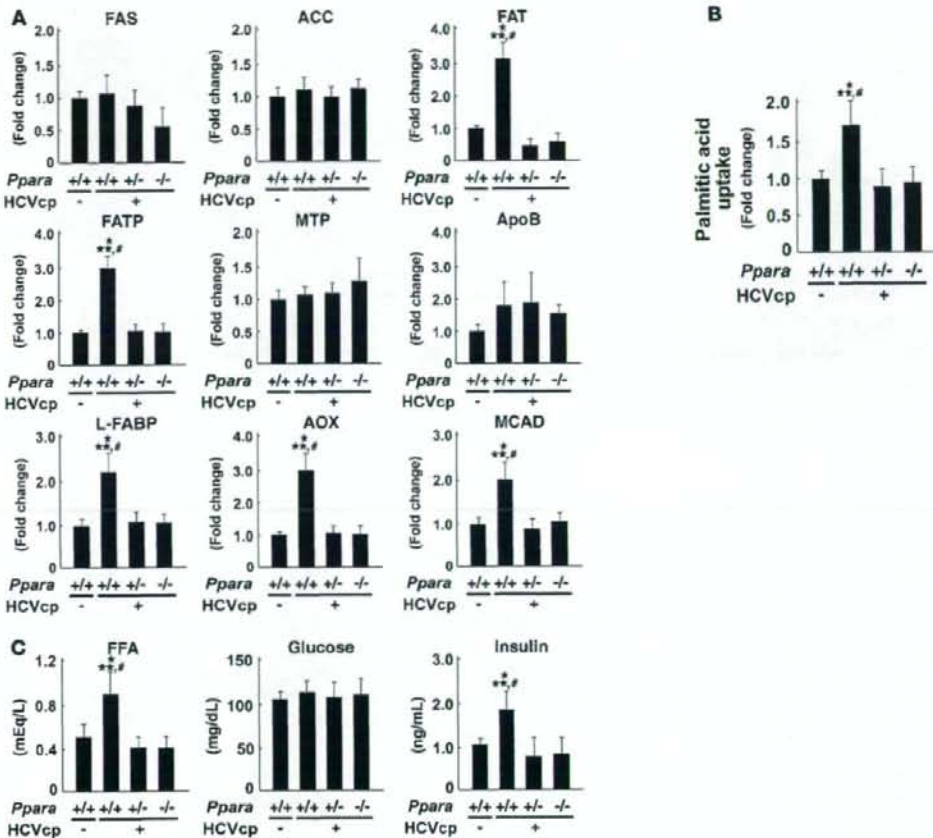
Results

Expression of HCV core protein in transgenic mice. $Ppara^{+/-}$:HCVcpTg and $Ppara^{-/-}$:HCVcpTg mice appeared healthy, and body weight in both genotypes was similar to that of $Ppara^{+/+}$:HCVcpTg and $Ppara^{+/+}$ mice

without the transgene. When hepatic expression of HCV core protein in 9-month-old transgenic mice was examined by immunoblot analysis, it was similar among $Ppara^{+/+}$:HCVcpTg, $Ppara^{+/-}$:HCVcpTg, and $Ppara^{-/-}$:HCVcpTg mice (Figure 1A) and was also similar to expression in HCVcpTg mice reported previously (7, 9). Age and sex had only a minor influence on the hepatic expression of HCV core protein.

Requirement of homozygous PPAR α for the development of hepatic steatosis in transgenic mice. Livers of 9-month-old male HCVcpTg mice with or without the $Ppara$ allele were examined. Those of $Ppara^{+/+}$:HCVcpTg mice were soft, slightly enlarged, and light in color and histologically showed macrovesicular and microvesicular steatosis with no apparent inflammation or hepatocyte necrosis (Figure 1B), in agreement with previous reports (7, 9). Biochemical analysis of liver extracts showed marked hepatic accumulation of triglycerides (Figure 1D). In contrast, livers of 9-month-old $Ppara^{+/-}$:HCVcpTg and $Ppara^{-/-}$:HCVcpTg mice showed neither histological abnormalities nor accumulation of triglycerides (Figure 1, B and D). Hepatic levels of free fatty acids in $Ppara^{+/-}$:HCVcpTg mice were approximately 3 times those in $Ppara^{+/+}$:HCVcpTg and $Ppara^{-/-}$:HCVcpTg mice or $Ppara^{+/+}$ mice not expressing the HCV core protein.

In 24-month-old $Ppara^{+/+}$:HCVcpTg mice, hepatic steatosis was found (Figure 1C), and the hepatic levels of triglycerides were further increased (Figure 1D). Apparent inflammation, hepatocyte

**Figure 2**

Analyses of factors associated with hepatic fatty acid and triglyceride metabolism. (A) Expression of genes associated with fatty acid and triglyceride metabolism in 9-month-old mouse livers. Total RNA was extracted from each mouse liver, and mRNA levels were determined by RT-PCR. mRNA levels were normalized by those of GAPDH and subsequently normalized by those in *Ppara*^{+/+} nontransgenic mice. Results are expressed as the mean \pm SD ($n = 6$ /group). * $P < 0.05$ compared with *Ppara*^{+/+} nontransgenic mice; ** $P < 0.05$ compared with *Ppara*^{+/+}:HCVcpTg mice; # $P < 0.05$ compared with *Ppara*^{+/-}:HCVcpTg mice. (B) Uptake of fatty acids in 9-month-old mouse livers. Liver slices obtained from 3 mice in each group were incubated in medium containing 0.8 mM [¹⁴C]palmitic acid for 7 h. Fatty acid uptake ability was estimated by the sum of palmitic acid converted to CO₂ and ketone bodies with that incorporated into total cellular lipids after incubation. The experiment was repeated 3 times. Results were normalized by those of *Ppara*^{+/+} nontransgenic mice and expressed as the mean \pm SD. (C) Plasma concentrations of free fatty acids, glucose, and insulin. After an overnight fast, blood was obtained from each mouse and the above variables were determined. Results are expressed as the mean \pm SD ($n = 6$ /group).

degeneration and necrosis, and fibrosis were not detected. On the other hand, *Ppara*^{+/-}:HCVcpTg and *Ppara*^{-/-}:HCVcpTg mice showed no steatosis (Figure 1, C and D). These results indicate that hepatic steatosis develops in *Ppara*^{+/+}:HCVcpTg mice, but not in *Ppara*^{+/-}:HCVcpTg and *Ppara*^{-/-}:HCVcpTg mice.

Hepatic fatty acid and triglyceride metabolism in transgenic mice. To investigate the mechanism responsible for the development of severe steatosis in *Ppara*^{+/+}:HCVcpTg mice, the expression of genes associated with fatty acid and triglyceride metabolism in the livers of 9-month-old mice was analyzed using the quantitative RT-PCR method. As shown in Figure 2A, the mRNA levels of genes related

to de novo lipogenesis (fatty acid synthase [FAS] and acetyl-CoA carboxylase [ACC]) and secretion of VLDL (microsomal transfer protein [MTP] and apoB) were constant in all groups. The mRNA levels of fatty acid translocase (FAT) and fatty acid transport protein (FATP), which are associated with the uptake of fatty acids into hepatocytes, were significantly increased only in *Ppara*^{+/+}:HCVcpTg mice, but the mRNA levels of hepatic triglyceride lipase, another contributor to fatty acid uptake, remained unchanged (data not shown). The mRNA levels of liver fatty acid binding protein (L-FABP) were also elevated only in *Ppara*^{+/+}:HCVcpTg mice. Surprisingly, the mRNA levels of AOX and medium-chain acyl-CoA

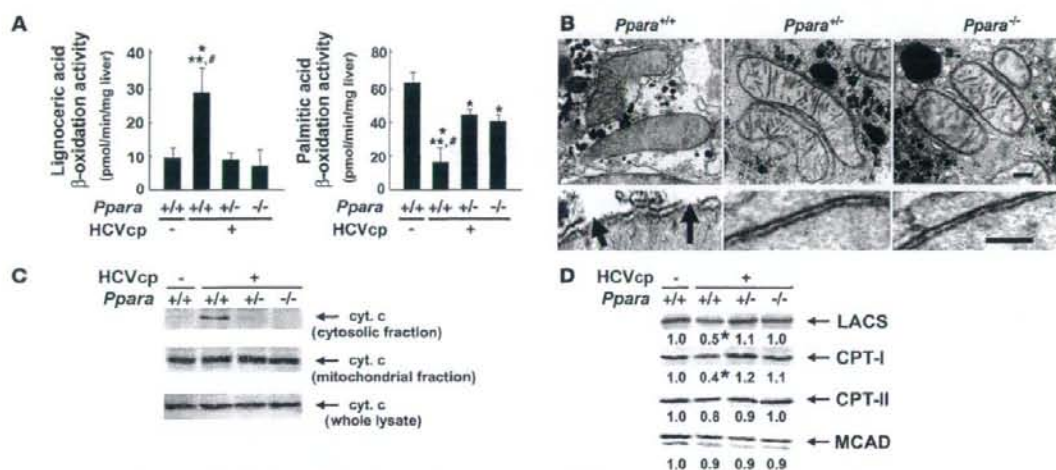


Figure 3

Analyses of mitochondrial abnormalities. (A) Lignoceric and palmitic acid β -oxidation activities in 9-month-old mice. Results are expressed as the mean \pm SD ($n = 6$ /group). * $P < 0.05$ compared with *Ppara*^{+/+} nontransgenic mice; ** $P < 0.05$ compared with *Ppara*^{+/+}:HCVcpTg mice; # $P < 0.05$ compared with *Ppara*^{+/-}:HCVcpTg mice. (B) Electron microscopic features of hepatic mitochondria of 9-month-old HCVcpTg mice. Upper and lower rows show a lower and higher magnification, respectively. In *Ppara*^{+/+}:HCVcpTg mice, some mitochondria showing discontinuance of outer membranes (arrows) and amorphous inner structures were observed. In *Ppara*^{+/-}:HCVcpTg and *Ppara*^{-/-}:HCVcpTg mice, mitochondria appeared normal; the scale bars represent 200 nm (top) and 30 nm (bottom), respectively. (C) Immunoblot analysis of cytochrome *c* in 9-month-old mice. Whole-liver lysate, mitochondrial fraction, or cytosolic fraction (50 μ g protein) was loaded in each well. Results are representative of 4 independent experiments. (D) Immunoblot analysis of representative mitochondrial β -oxidation enzymes using a mitochondrial fraction prepared from 9-month-old mouse livers. The mitochondrial fraction (20 μ g protein) was loaded in each well. Results are representative of 4 independent experiments. The band intensity was quantified densitometrically and normalized by that in *Ppara*^{+/+} nontransgenic mouse. The mean value of the fold changes is shown under the representative band. LACS, long-chain acyl-CoA synthase; CPT, carnitine palmitoyl-CoA transferase.

dehydrogenase (MCAD), a rate-limiting enzymes in the peroxisomal and mitochondrial β -oxidation pathways, respectively, were significantly increased in *Ppara*^{+/-}:HCVcpTg mice. When fatty acid uptake ability was measured in fresh liver slices, it was significantly enhanced only in *Ppara*^{+/-}:HCVcpTg mice (Figure 2B). Additionally, plasma free fatty acid levels were higher in these mice than in mice in the other groups. Although there were no differences in fasting plasma glucose levels between the groups, hyperinsulinemia was observed only in *Ppara*^{+/-}:HCVcpTg mice (Figure 2C), in agreement with the previous observation that significant insulin resistance developed in these mice (8). Similar results were obtained from 24-month-old mice (data not shown). These results combined show that the increased plasma fatty acid levels, which were likely due to enhanced peripheral fatty acid release caused by insulin resistance, and the increase in fatty acid uptake ability are consistent with steatogenesis in *Ppara*^{+/-}:HCVcpTg mice.

Decreased mitochondrial β -oxidation in transgenic mice. Although the transcriptional activities of major β -oxidation enzymes were markedly increased, *Ppara*^{+/-}:HCVcpTg mice had severe steatosis. To explore this discrepant result, peroxisomal and mitochondrial β -oxidation activities were measured using lignoceric and palmitic acids as substrates, respectively. The lignoceric acid-degrading capacity was increased only in *Ppara*^{+/-}:HCVcpTg mice, where it corresponded to an increase in AOX expression. However, the capacity for palmitic acid degradation, which occurs particularly in mitochondria, was significantly lower in *Ppara*^{+/-}:HCVcpTg mice than in *Ppara*^{+/+}:HCVcpTg and *Ppara*^{-/-}:HCVcpTg mice (Figure 3A).

Thus, decreased mitochondrial β -oxidation ability was considered to be another important mechanism for the development of steatosis induced by the core protein.

We further evaluated mitochondrial abnormalities. In electron microscopic examination, discontinuous outer membranes (Figure 3B, arrows) and lack of an internal structure were observed in some mitochondria of *Ppara*^{+/-}:HCVcpTg mouse livers, in agreement with the previous report (9). However, these abnormalities were not seen in *Ppara*^{+/+}:HCVcpTg and *Ppara*^{-/-}:HCVcpTg mice (Figure 3B). Immunoblot analysis showed that cytochrome *c*, which is usually localized in the mitochondrial intermembrane space, was present in the cytosolic fractions of *Ppara*^{+/-}:HCVcpTg mice (Figure 3C). Moreover, immunoblot analysis using mitochondrial fractions showed that the expression levels of long-chain acyl-CoA synthase and carnitine palmitoyl-CoA transferase-I, which are enzymes indispensable to the initial step of mitochondrial β -oxidation and are localized mainly in mitochondrial outer membranes, were significantly decreased only in *Ppara*^{+/-}:HCVcpTg mice (Figure 3D).

Overall, these results suggest that homozygous PPAR α is essential to the pathogenesis of hepatic steatosis induced by the HCV core protein, which results from a decrease in mitochondrial fatty acid degradation capacity caused by the breakdown of mitochondrial outer membranes and a disproportionate increase in the uptake of fatty acids. Interestingly, steatosis and the related changes did not occur in *Ppara*^{+/-} and *Ppara*^{-/-} mice expressing the HCV core protein, which suggested that these changes were not caused by the core protein itself.

Table 1
Incidence of HCC in 24-month-old mice

HCV core protein	<i>Ppara</i>	Mice (n)	Mice with HCC (n)	Incidence (%)
-	+/+	20	0	0
-	+/-	18	0	0
-	-/-	20	0	0
+	+/+	17	6	35.3 ^A
+	+/-	16	0	0
+	-/-	14	0	0

Mice were killed at 24 months of age for analysis. HCC was diagnosed according to histological findings. ^A*P* < 0.05 compared with *Ppara*^{+/+} nontransgenic mice, *P* < 0.05 compared with *Ppara*^{+/+}:HCVcpTg mice, *P* < 0.05 compared with *Ppara*^{-/-}:HCVcpTg mice.

Requirement of homozygous PPAR α for hepatic tumor development in transgenic mice. At 9 months of age, hepatic nodules were not observed at all in transgenic mice, whereas, at 24 months, approximately 35% of *Ppara*^{+/+}:HCVcpTg mice had macroscopically evident hepatic nodules (Table 1). Microscopically, these nodules had the appearance of well-differentiated HCC with trabecular features, which was consistent with the previous report (9). Surprisingly, *Ppara*^{-/-}:HCVcpTg and *Ppara*^{+/-}:HCVcpTg mice of the same ages developed no evidence of hepatic tumors, despite the expression of HCV core protein at similar levels to those found in *Ppara*^{+/+}:HCVcpTg mice (Table 1). Microscopic examination showed that there were no dysplastic cells

or precancerous lesions throughout the livers in *Ppara*^{-/-}:HCVcpTg and *Ppara*^{+/-}:HCVcpTg mice (Figure 1C). These results provide strong evidence that homozygous PPAR α is essential for hepatic tumorigenesis induced by HCV core protein.

Increased hepatocyte proliferation only in *Ppara*^{+/+}:HCVcpTg mice. Because sustained acceleration of hepatocyte proliferation relative to apoptosis may promote the development of HCC, these opposing processes were quantified in the livers of 24-month-old mice. Both the liver-to-body weight ratio and the number of hepatocytes expressing proliferating cell nuclear antigen (PCNA) were increased only in *Ppara*^{+/+}:HCVcpTg mice (Figure 4, A and B). In contrast, the number of TUNEL-positive hepatocytes and the hepatic caspase 3 activity, indicators of hepatocyte apoptosis, remained similar among the 3 mouse strains (Figure 4, C and D). Interestingly, despite the presence of HCV core protein, the amounts of these proliferative and apoptotic markers in *Ppara*^{-/-}:HCVcpTg and *Ppara*^{+/-}:HCVcpTg mice were similar to those in *Ppara*^{+/+} nontransgenic mice. Expression levels of several proteins, such as protooncogenes (c-Fos and c-Myc), cell-cycle regulators (cyclin D1, cyclin-dependent kinase [CDK] 4, and PCNA), and phosphorylated ERK 1 and 2, all of which are associated with hepatocyte proliferation, were elevated in *Ppara*^{+/+}:HCVcpTg mice but not in other genotypes (Figure 4, E and F).

Increased oxidative stress and DNA damage only in *Ppara*^{+/+}:HCVcpTg mice. HCV core protein is associated with increased production of ROS (23). Enhanced ROS production induces nuclear DNA damage, which results in the initiation of hepatocarcinogenesis, and can also injure organelles, which can result in disorders in their

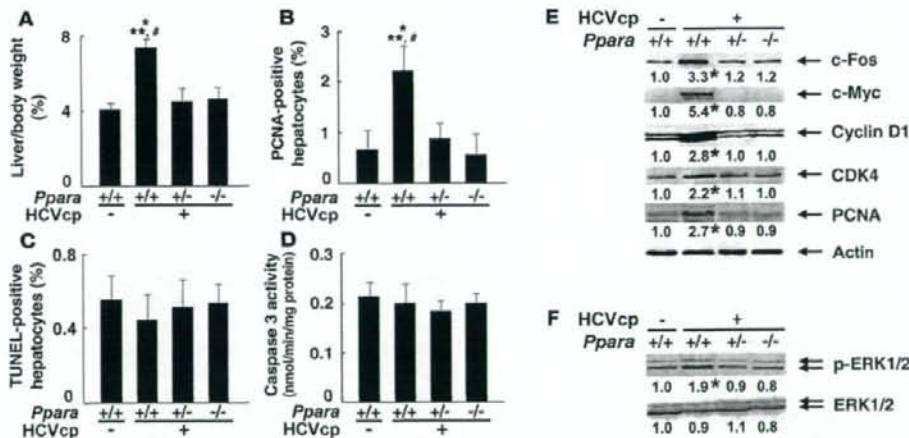


Figure 4

Increased hepatocyte proliferation in *Ppara*^{+/+}:HCVcpTg mice at 24 months of age. (A) Liver-to-body-weight ratio. Results are expressed as the mean \pm SD (*n* = 6/group). (B) Numbers of proliferating hepatocytes. Two thousand hepatocytes were examined in each mouse, and hepatocyte nuclei positive for anti-PCNA antibody were counted. Results are expressed as the mean \pm SD (*n* = 6/group). For A and B, comparisons are designated as follows: ^{*}*P* < 0.05 compared with *Ppara*^{+/+} nontransgenic mice; ^{**}*P* < 0.05 compared with *Ppara*^{-/-}:HCVcpTg mice; [#]*P* < 0.05 compared with *Ppara*^{+/-}:HCVcpTg mice. (C) Numbers of apoptotic hepatocytes. Liver sections were subjected to TUNEL staining, and TUNEL-positive hepatocyte nuclei were counted in 2,000 hepatocytes from each mouse. Results are expressed as the mean \pm SD (*n* = 6/group). (D) Caspase 3 activity. Results are expressed as the mean \pm SD (*n* = 6/group). (E) Immunoblot analysis of oncogene products and cell cycle regulators. The same sample used in Figure 1A (whole-liver lysate, 50 μ g protein) was loaded in each well. The band of actin was used as the loading control. Results are representative of 4 independent experiments. The band intensity was quantified densitometrically, normalized by that of actin, and subsequently normalized by that in *Ppara*^{+/+} nontransgenic mice. The mean value of the fold changes is expressed under each band. (F) Immunoblot analysis of phosphorylated ERK1/2 and total ERK1/2. The same samples in Figure 4E (50 μ g protein) were used.

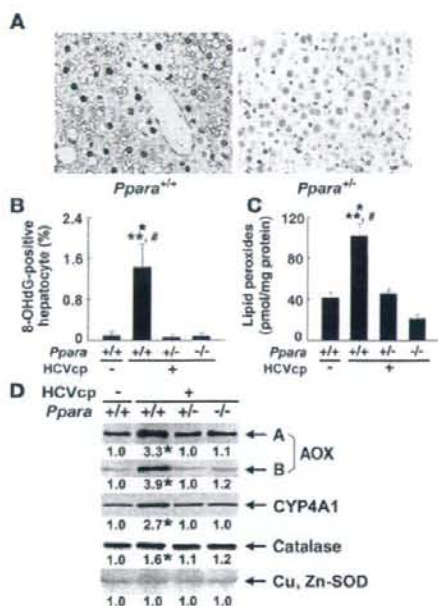


Figure 5

Increased oxidative stress and DNA damage in *Ppara*^{-/-}:HCVcpTg mice at 24 months of age. (A) Immunohistochemical staining using antibody against 8-OHdG. In *Ppara*^{-/-}:HCVcpTg mice, some steatotic hepatocytes were positive for 8-OHdG. Original magnification, ×400. (B) Numbers of 8-OHdG-positive hepatocytes. Hepatocyte nuclei stained with anti-8-OHdG antibody were counted in 2,000 hepatocytes of each mouse. Results are expressed as the mean ± SD (*n* = 6/group). (C) Hepatic content of lipid peroxides. Results are expressed as the mean ± SD (*n* = 6 /group). **P* < 0.05 compared with *Ppara*^{+/+} nontransgenic mice; ***P* < 0.05 compared with *Ppara*^{-/-}:HCVcpTg mice; #*P* < 0.05 compared with *Ppara*^{-/-}:HCVcpTg mice. (D) Immunoblot analysis of AOX, CYP4A1, catalase, and Cu, Zn-SOD. The whole-liver lysate used in the experiment in Figure 4E (20 μg protein for AOX and CYP4A1 and 50 μg for others) was loaded in each lane. The band of actin was used as the loading control. Results are representative of 4 independent experiments. A and B indicate full-length and truncated AOX, respectively. The band intensity was quantified densitometrically, normalized by that of actin, and subsequently normalized by that in *Ppara*^{+/+} nontransgenic mice. The mean value of the fold changes is expressed under each band. **P* < 0.05 compared with *Ppara*^{+/+} nontransgenic mice.

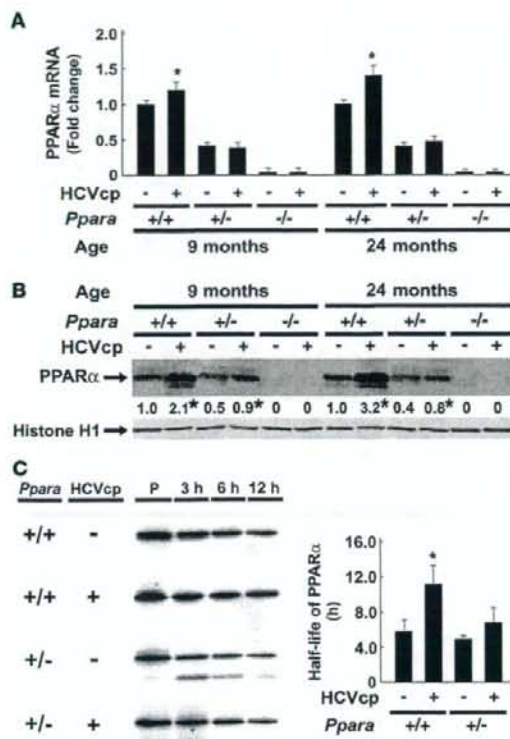
function. The number of hepatocytes positive for 8-hydroxy-2'-deoxyguanosine (8-OHdG), an indicator of oxidative damage to nuclear DNA, was increased only in 24-month-old *Ppara*^{-/-}:HCVcpTg mice (Figure 5, A and B). Lipid peroxides were slightly increased in the livers of 9-month-old *Ppara*^{-/-}:HCVcpTg mice (data not shown) and were more abundant in the livers of 24-month-old *Ppara*^{-/-}:HCVcpTg mice than in those of *Ppara*^{-/-}:HCVcpTg and *Ppara*^{-/-}:HCVcpTg mice or *Ppara*^{+/+} nontransgenic mice (Figure 5C). Expression of typical ROS-generating enzymes (AOX and cytochrome P450 4A1 [CYP4A1]) and ROS-eliminating enzymes (catalase and Cu, Zn-SOD) was examined. Immunoblot analysis showed marked increases in the expression of AOX and CYP4A1 and mild increases in that of catalase only in *Ppara*^{-/-}:HCVcpTg mice. No changes in Cu, Zn-SOD were found in the subgroups of transgenic mice (Figure 5D). These results suggest that enhanced oxidative stress causes damage in nuclear DNA and probably in mitochondria in the *Ppara*^{-/-}:HCVcpTg mice.

Persistent and spontaneous PPARα activation in *Ppara*^{-/-}:HCVcpTg mice. Liver tumorigenesis induced by long-term exposure to peroxisome proliferators and the related changes, such as sustained hepatocyte proliferation and increased oxidative stress, are associated with persistent PPARα activation (19–21). To examine the activation of PPARα, we quantified the level of PPARα mRNA, which is induced by PPARα activation (24, 25). PPARα mRNA levels were higher in 9-month-old *Ppara*^{-/-}:HCVcpTg mice than in *Ppara*^{-/-} nontransgenic mice (Figure 6A). These increases were more pronounced at 24 months of age. However, there were no differences in PPARα mRNA levels between *Ppara*^{-/-}:HCVcpTg and *Ppara*^{-/-} nontransgenic mice at either 9 or 24 months of age. The expression levels of typical PPARα target genes (16, 25, 26) — such as FAT, FATP, L-FABP, AOX, and MCAD (Figure 2); c-Myc, cyclin D1, CDK4, and PCNA (Figure 4); and CYP4A1 (Figure 5)

— were simultaneously and synchronously increased in *Ppara*^{-/-}:HCVcpTg mice, but not in *Ppara*^{-/-}:HCVcpTg or *Ppara*^{-/-}:HCVcpTg mice. These results confirm that persistent activation of PPARα occurs only in *Ppara*^{-/-}:HCVcpTg mice. Various changes observed in *Ppara*^{-/-}:HCVcpTg mice, i.e., increased fatty acid uptake, mitochondrial abnormalities, steatosis, ROS overproduction, accelerated hepatocyte proliferation, and hepatocarcinogenesis, were considered to be closely linked with sustained PPARα activation.

Nuclear PPARα content. The results described above suggest that persistent PPARα activation is critical to the steatogenesis and hepatocarcinogenesis induced by the HCV core protein. A question arises as to why *Ppara*^{-/-}:HCVcpTg mice with an active *Ppara* allele do not exhibit the hallmarks of PPARα activation and do not develop HCC. To address this issue, the nuclear PPARα content was analyzed. Immunoblot analysis for PPARα showed that the amount of nuclear PPARα protein in *Ppara*^{-/-}:HCVcpTg mice was approximately 2- to 3-fold that of *Ppara*^{-/-} nontransgenic mice, which was disproportionate to the higher PPARα mRNA levels (approximately 1.2- to 1.6-fold) (Figure 6, A and B). The level of nuclear PPARα in *Ppara*^{-/-}:HCVcpTg mice was significantly lower than that in *Ppara*^{-/-}:HCVcpTg mice and was similar to that in *Ppara*^{-/-} nontransgenic mice (Figure 6B). Thus, the lower amount of nuclear PPARα in *Ppara*^{-/-}:HCVcpTg mice than in *Ppara*^{-/-}:HCVcpTg mice might have heightened the threshold of expression required for long-term spontaneous PPARα activation.

The degree of an increase in nuclear PPARα levels was evidently higher than the degree of an increase in PPARα mRNA levels in HCVcpTg mice (Figure 6, A and B). To investigate this disparity, the stability of nuclear PPARα was evaluated by pulse-chase experiments using isolated hepatocytes obtained from these mice. The half-life of nuclear PPARα was significantly longer (*P* < 0.05) in *Ppara*^{-/-}:HCVcpTg mice (11.5 ± 2.3 h) than in *Ppara*^{-/-} nontransgenic mice (5.8 ± 1.4 h) (Figure 6C). The half-life of nuclear PPARα in *Ppara*^{-/-}:HCVcpTg mice tended to be prolonged compared with that in *Ppara*^{-/-} nontransgenic mice (Figure 6C). These results suggest that the stability of nuclear PPARα was increased as a result of HCV core protein expression. Because it is known that the core protein interacts with retinoid X receptor α (RXRα) (27) and that

**Figure 6**

Persistent PPAR α activation in *Ppara*^{+/+}:HCVcpTg mice. (A) PPAR α mRNA levels. Total RNA was prepared from each mouse, and PPAR α mRNA levels were determined by RT-PCR, normalized by those of GAPDH, and subsequently normalized by those of 9-month-old *Ppara*^{+/+} nontransgenic mice. Results are expressed as the mean \pm SD ($n = 6$ /group). (B) Immunoblot analysis of nuclear PPAR α . The nuclear fraction obtained from each mouse (100 μ g protein) was loaded in each well. The band of histone H1 was used as the loading control. Results are representative of 6 independent experiments. The band intensity was quantified densitometrically, normalized by that of histone H1, and subsequently normalized by that in 9-month-old *Ppara*^{+/+} nontransgenic mice. The mean value is expressed under each band. * $P < 0.05$ compared with nontransgenic mice of the same age and *Ppara* genotype. (C) Pulse-chase experiments for 3, 6, and 12 h and pulse-label (P) experiments for nuclear PPAR α using isolated mouse hepatocytes. Left: labeled PPAR α bands on x-ray film. Pulse-label and pulse-chase experiments were performed as described in Methods. Results are representative of 4 independent experiments. Right: half-life of PPAR α . The band intensity was measured densitometrically and subsequently normalized by that of the pulse-label experiments. The percentage of the band intensity was plotted, and the half-life of PPAR α was calculated. Results obtained from 4 independent experiments are expressed as the mean \pm SD. * $P < 0.05$ compared with nontransgenic mice in the same *Ppara* genotype.

PPAR α influences the stability of RXR α (28), it is plausible that the core protein would affect its action in nuclei through an interaction with the PPAR α -RXR α heterodimer and stabilization of PPAR α .

Development of hepatic steatosis and HCC with long-term clofibrate treatment in *Ppara*^{+/+}:HCVcpTg mice. To further confirm the significance of persistent PPAR α activation on core protein-induced pathological changes, *Ppara*^{+/+} and *Ppara*^{+/+}:HCVcpTg mice were fed a standard diet containing 0.05% clofibrate for 24 months. Interestingly, hepatic steatosis appeared in the clofibrate-treated *Ppara*^{+/+}:HCVcpTg mice, but not in the *Ppara*^{+/+} mice under the same treatment conditions (Figure 7, A and B). Similar to our observations in *Ppara*^{+/+}:HCVcpTg mice not treated with clofibrate, aberrant mitochondria with discontinuous outer membranes and decreased palmitic acid β -oxidation activity were found only in the clofibrate-treated *Ppara*^{+/+}:HCVcpTg mice (Figure 7, A and C). In addition, levels of MCAD mRNA; AOX, and CYP4A1 proteins; PPAR α mRNA; and nuclear PPAR α protein were higher in the clofibrate-treated *Ppara*^{+/+}:HCVcpTg mice than in the clofibrate-treated *Ppara*^{+/+} mice (Figure 7, D-F), which suggests that the degree of PPAR α activation in the former group was greater than that in the latter group and similar to that in *Ppara*^{+/+}:HCVcpTg mice not treated with clofibrate. Finally, the incidence of HCC after clofibrate treatment was higher in *Ppara*^{+/+}:HCVcpTg mice (25%; 5 in 20 mice) than in *Ppara*^{+/+} mice (5%; 1 in 20 mice). Therefore, these results corroborate the importance of constant PPAR α activation to the pathogenesis of hepatic steatosis and HCC in the transgenic mice.

Discussion

A novel and striking finding in this study is the absolute requirement of persistent PPAR α activation for the development of HCV core protein-induced steatosis and HCC. Our data also show that the HCV core protein alone cannot induce steatosis and HCC in transgenic mice.

Mechanisms of development of steatosis in HCVcpTg mice were previously explained as an enhancement of de novo synthesis of fatty acids (29) and a decrease in MTP expression, the latter of which results in insufficient VLDL secretion from hepatocytes (30). In the present study, we revealed 2 novel mechanisms of steatogenesis in the transgenic mice, i.e., an impairment of mitochondrial β -oxidation due to the breakdown of mitochondrial outer membranes and an increase in fatty acid uptake into hepatocytes, associated with PPAR α activation. PPAR α activation, mitochondrial dysfunction, and hepatic steatosis appeared in 9-month-old *Ppara*^{+/+}:HCVcpTg mice and continued until 24 months of age, clearly preceding development of HCC. These findings thereby indicate a correlation between PPAR α activation, hepatic steatosis, and HCC.

We obtained the novel and rather paradoxical finding that significant PPAR α activation, which generally is expected to reduce hepatic triglyceride levels, is essential for the development of severe steatosis induced by HCV core protein. According to the results of this study, the following hypothesis concerning the development of steatosis in *Ppara*^{+/+}:HCVcpTg mice is proposed. First, the HCV core protein localizes partly in mitochondria (9). A recent study

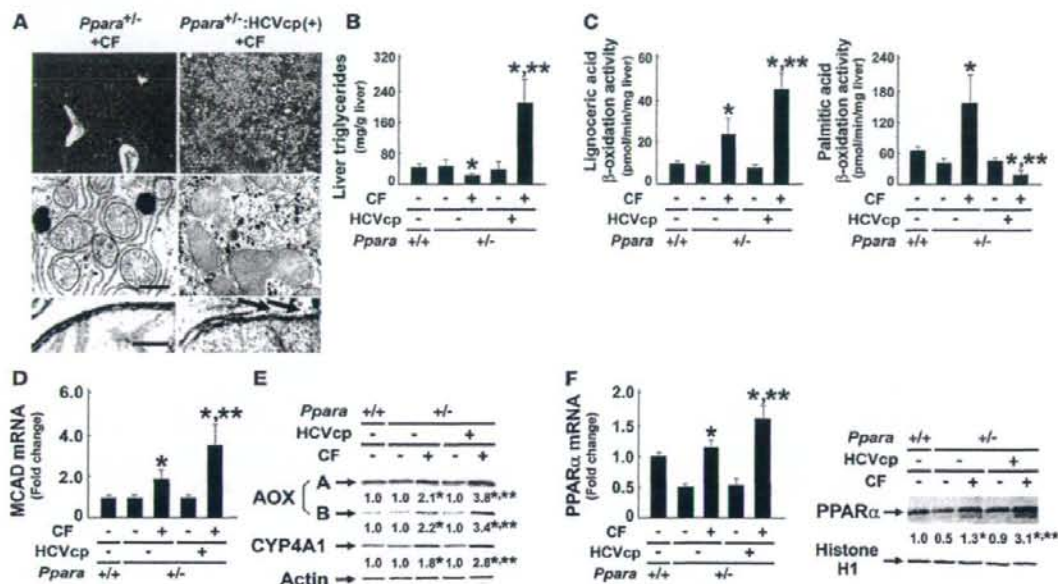


Figure 7

Development of hepatic steatosis by long-term treatment of clofibrate in *Ppara*^{+/-}:HCVcpTg mice. (A) Histological examination of *Ppara*^{+/-} and *Ppara*^{+/-}:HCVcpTg mice treated with diet containing 0.05% (w/w) clofibrate for 24 months (CF). Top: Histological appearance of H&E-stained liver sections. Magnification, ×40. Microvesicular and macrovesicular steatosis were detected only in clofibrate-treated *Ppara*^{+/-}:HCVcpTg mice. Middle and bottom: Electron microscopic features of hepatic mitochondria. Some C-shaped mitochondria showing discontinuance of outer membranes (arrows) were found in clofibrate-treated *Ppara*^{+/-}:HCVcpTg mice. Scale bars: 400 nm (middle), 30 nm (bottom). (B and C) Content of liver triglycerides and lignoceric and palmitic acid β-oxidation activities. (D) MCAD mRNA levels. mRNA levels were normalized to those of GAPDH and subsequently normalized to those in *Ppara*^{+/-} nontransgenic mice. (E) Immunoblot analysis of AOX and CYP4A1. Whole-liver lysate (20 μg protein) was loaded in each lane. Actin was used as a loading control. Results are representative of 6 independent experiments. (F) PPARα mRNA levels and nuclear PPARα contents. Left: PPARα mRNA levels. The same samples used in D were adopted. Right: Immunoblot analysis of nuclear PPARα. Nuclear fraction obtained from each mouse (100 μg protein) was loaded in each well. Histone H1 was used as a loading control. In E and F, the mean value of the fold changes is shown under each band. Results are representative of 6 independent experiments. Band intensity was quantified densitometrically, normalized to that of the loading control, and subsequently normalized to that in *Ppara*^{+/-} nontransgenic mice. **P* < 0.05 compared with untreated mice of the same genotype; ***P* < 0.05 compared with clofibrate-treated *Ppara*^{+/-} mice without core protein gene. Results are expressed as mean ± SD (*n* = 6/group).

showed that, in isolated mitochondria, the core protein directly increased Ca²⁺ influx, inhibited electron transport complex I activity, and induced ROS production (31), all of which can increase the fragility of mitochondria and depress mitochondrial function. In addition, the HCV core protein also localizes in nuclei (9) and can coexist in PPARα-RXRα heterodimer through a direct interaction with the DNA-binding domain of RXRα, which enhances the transcriptional activity of PPARα target genes, such as AOX, despite the absence of PPARα ligands in cultured cells (27). The HCV core protein can also be involved in the PPARα-RXRα complex through a direct interaction with cyclic-AMP responsive element binding protein-binding protein (32), which is able to bind to PPARα (33). Thus, the core protein probably serves as a coactivator and stabilizer of PPARα in vivo, which was further confirmed in this study. Moreover, because it is also known that the core protein itself activates ERK1/2 and p38 mitogen-activated protein kinase (34), these activations might phosphorylate PPARα and thereby transactivate it (35). The core protein-induced PPARα activation enhances the basal expression of AOX and CYP4A1, which leads to increased

production of ROS and dicarboxylic acids. These toxic compounds can damage mitochondrial outer membranes, which impairs the mitochondrial β-oxidation system. These damages directly induce the accumulation of long-chain fatty acids in hepatocytes. Furthermore, PPARα activation increases the expression of FAT and FATP, which promotes the influx of fatty acids from blood. Long-chain fatty acids and their CoA esters accumulated in hepatocytes are likely to act as potent detergents, which further damages the outer membranes of mitochondria. Fatty acids and their derivatives function as natural ligands of PPARα, which results in the activation of PPARα and the induction of FAT, FATP, AOX, and CYP4A1, which further accelerates mitochondrial damage, the reduction of mitochondrial β-oxidation activity, and the accumulation of fatty acids in a vicious cycle.

Persistent PPARα activation increases oxidative DNA damage because of a disproportionate increase in ROS-generating enzymes relative to the levels of degrading enzymes such as catalase and SOD, which can predispose hepatocytes to malignant transformation. In addition, persistent PPARα activation leads to increased