subjected to thin-layer chromatography with a high-performance TLC plate (Merck) by the two-step method[5]. The plate was charred by a copper acetate phosphoric acid solution at 180°C.

In vitro transcription

RNA for transfection was synthesized using MEGAscript T7 (Ambion, TX, USA). Plasmids carrying the JFH1 RNA sequence were linearized with *Xba*I and used as templates for transcription. Probes for *in situ* hybridization were synthesized using MAXIscript Sp6 or T7 (Ambion) in the presence of the DIG RNA labeling mix (Roche). Probes for Northern blots were synthesized with MAXIscript Sp6 or T7 in the presence of 1.85 MBq of [α-^{32}P] UTP (Amersham Biosciences). For detection of plus-strand HCV RNA, minus-strand RNA probes were generated using pcDNA3-TME2 (*Hin*dIII for linearization), pcDNA3-NS3 (*Hin*dIII), and pcDNA3-NS5B (*Bam*HI) as templates for *in vitro* transcription. For detection of minus-strand HCV RNA, plus-strand RNA probes were generated using pcDNA3-TME2 (*Eco*RI), pcDNA3-NS3 (*Xba*I), and pcDNA3-NS5B (*Bam*HI) as templates. The RNA probes used for *in situ* hybridization were subjected to alkaline hydrolysis to generate fragments of ~170 nucleotides in length. Synthesized RNA probes were treated with DNase I (Ambion) and size fractionated using MicroSpin G-50 columns (Amersham Biosciences).

Sucrose density gradient centrifugation of culture medium

The 100-time concentrated medium from JFH1-bearing cells was loaded onto 20-50% [w/v] sucrose gradient containing 50 mM Hepes-KOH (pH 7.4), 100 mM NaCl and 1mM EDTA followed by centrifugation at 100,000 x g for 16 hrs using RPS40T rotor of HITACHI ultracentrifuge. The gradient was fractionated into 31 fractions. Buoyant density of each fraction was analyzed by Abe refractometer (ATAGO Inc., Japan). Each fraction was dialyzed against serum free DMEM and was used for the infection experiment as well as quantification of Core and HCV RNA titer as described above.

Infection experiments

Cells were cultured in DMEM containing 5% FBS. The medium was collected and mixed with a 0.01 volume of 1 M HEPES (pH 7.4). After filtering the sample through a 0.22-μm filter (Millipore), the filtrate was concentrated by reducing the volume to

© 2007 Nature Publishing Group

between 1/50 and 1/100 of the original volume with an Amicon Ultra-15 centrifugal filter with Ultracel-100 membrane (Millipore). Huh-7.5 cells seeded on a collagen-coated Labtech II 8-well chamber were incubated with 100 µl of the concentrated medium for 120 min. Then, the cells were washed three times with DMEM. Twenty-four hours after the inoculation, the cells were labeled with serum from HCV-infected patients to determine the infectivity level.

***In situ* hybridization analysis**

Huh-7 cells transfected with JFH1 RNA were seeded on a collagen-coated Labtech II 8-well chamber (Nunc). Three days after seeding, the cells were washed twice with PBS and fixed with fixation solution for 15 min at room temperature. Then, the cells were permeabilized with 0.05% Triton X-100 in fixation solution for 15 min at room temperature. After washing the cells twice with cold DEPC-treated PBS, the cells were incubated in 95% formamide and 0.1x SSC (1x SSC: 150 mM NaCl and 15 mM sodium citrate) for 15 min at 65°C. After chilling the chamber on ice, the cells were incubated in 100 µl of pre-hybridization solution for 60 min at room temperature. Pre-hybridization solution was composed of 50% formamide, 2x SSC, 1 µg/ml of salmon sperm DNA (sonicated to 1-2 kb pieces, Roche), 1 µg/ml of yeast tRNA (Roche), and 2 mM vanadyl ribonucleoside complex (NEB). Then, the cells were incubated in 100 µl of hybridization solution (pre-hybridization solution containing 10% dextran sulfate and 100 to 500 ng/ml of the RNA probes) for 40 hrs at 42°C. After the hybridization, the slide glass in the chamber was transferred to a bucket filled with wash solution 1 (50% formamide and 2x SSC at pH 7.4) and washed three times for 20 min at 50°C with gentle agitation. Then, the slide was washed three more times in wash solution 2 (0.1x SSC at pH 7.4) for 20 min at 50°C with gentle agitation. The slide was incubated in blocking solution for 30 min at room temperature. To detect DIG-labeled probes, sheep anti-DIG antibodies (Roche) and Alexa 488 or Alexa 568 anti-sheep IgG antibodies (Invitrogen) were used as primary and secondary antibodies, respectively. When HCV RNA, Core, and NS5A were simultaneously labeled in the same sample, anti-DIG antibodies and the Alexa-conjugated antibodies were incubated with the samples separately to avoid cross-reaction of the Alexa 488 or Alexa 568 anti-sheep IgG antibodies with mouse and rabbit IgG. Briefly, the incubation with the anti-DIG antibodies and the Alexa 488 anti-sheep IgG antibodies was performed first. After

© 2007 Nature Publishing Group

washing with PBS followed by a second fixation procedure, the cells were incubated with anti-Core and anti-NS5A antibodies followed by Alexa 568 anti-rabbit IgG and Alexa 647 anti-mouse IgG antibodies. For treatment with nuclease, digitonin-permeabilized cells were treated with 1 μg/ml of RNase A in the presence or absence of 0.05% Triton X-100 for 15 min at 37°C. After the treatment, RNase A was inactivated by incubation with 4% formaldehyde. Then, the cells were completely permeabilized with 0.05% Triton X-100 for 5 min at room temperature.

Statistical analysis of the recruitment of viral components to the LD

Only the cells that have any LDs surrounded by HCV proteins, PDI, or HCV RNA were counted as positive under immunofluorescence microscopy, and those adjacent to HCV signal were not included. The obtained cell number was divided by the total number of HCV replicating cells and is shown as "% cells with HCV protein-LD colocalization". In case of the chimeras Con1/C3 and H77/C3 LD colocalization with HCV proteins was additionally analyzed by using the ImageJ RG2B software package (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2006.). Approximately 200 cells were examined for each antigen.

RNA-protein binding precipitation assay

In vitro translated [^{35}S]-labeled products (Core^{Wt} and Core$^{PP/AA}$) were incubated with poly-U or protein G Sepharose resin in 50 mM HEPES (pH7.4), 100 mM NaCl, 0.1 % NP-40, and RNase inhibitor at 4°C for 2 hrs. After five washes, resin-bound radiolabeled proteins were analyzed by gel electrophoresis followed by autoradiography.

© 2007 Nature Publishing Group

Supplementary References

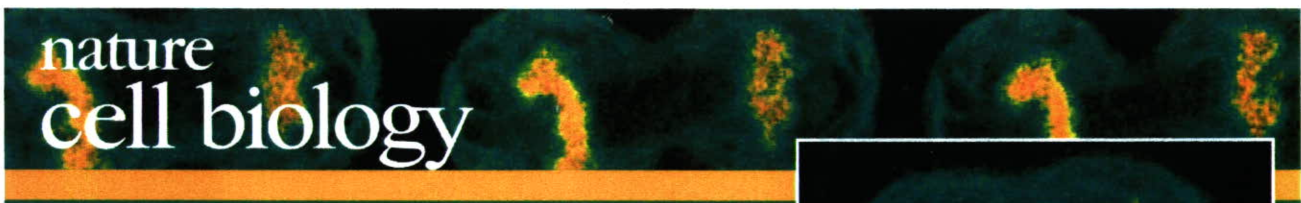
1. Blight, K. J., McKeating, J. A. & Rice, C. M. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *J Virol* **76**, 13001-14 (2002).
2. Lohmann, V. et al. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **285**, 110-3 (1999).
3. Miyanari, Y. et al. Hepatitis C virus non-structural proteins in the probable membranous compartment function in viral genome replication. *J Biol Chem* **278**, 50301-8 (2003).
4. Takeuchi, T. et al. Real-time detection system for quantification of hepatitis C virus genome. *Gastroenterology* **116**, 636-42 (1999).
5. Tauchi-Sato, K., Ozeki, S., Houjou, T., Taguchi, R. & Fujimoto, T. The surface of lipid droplets is a phospholipid monolayer with a unique Fatty Acid composition. *J Biol Chem* **277**, 44507-12 (2002).
6. Pietschmann, T. et al. Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. *Proc Natl Acad Sci U S A* **103**, 7408-13 (2006).

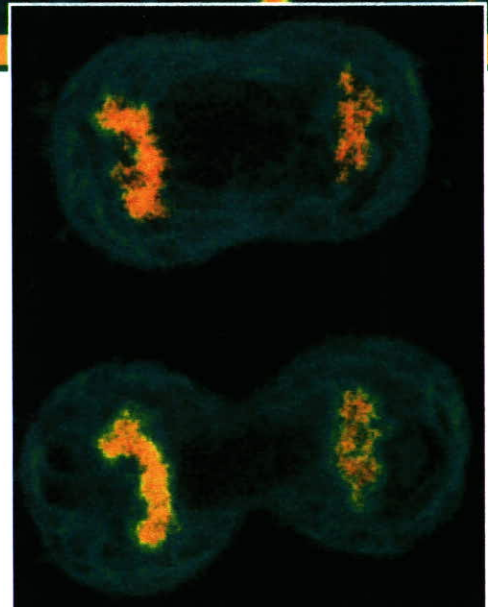
© 2007 Nature Publishing Group

ERRATUM

In the letter by Miyanari *et al.* (*Nature Cell Biol.* **9**, 1089–1097), Kunitada Shimotohno's affiliations were incorrectly listed. His affiliations should have been listed as 1, 2 and 6.

[1]Department of Viral Oncology, Institute for Virus Research, Kyoto University, Kyoto 606-8507, Japan.
[2]Graduate School of Biostudies, Kyoto University, Kyoto 606-8507, Japan.
[6]Correspondence should be addressed to K.S. (e-mail:shimkuni@z8.keio.jp).





NEWS AND VIEWS

Our News and Views section covers recent advances in cell biology and aims to be accessible to a wide audience. Many News and Views pieces highlight papers that appear in *Nature Cell Biology*, but some focus on papers of significance that are published in other journals. As well as placing new studies in the broader context of the field, we encourage News and Views authors to include personal 'views', criticisms and predictions.

We encourage researchers to bring their papers 'in press' elsewhere to our attention, to allow timely coverage of their work (publication embargo dates are fully respected). Unsolicited contributions will not normally be considered, although prospective authors of News and Views articles are welcome to make proposals to the Editor.

© 2007 Nature Publishing Group

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 45, pp. 32765–32772, November 9, 2007
© 2007 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.

Anti-hepatitis C Virus Activity of Tamoxifen Reveals the Functional Association of Estrogen Receptor with Viral RNA Polymerase NS5B*

Received for publication, May 30, 2007, and in revised form, August 15, 2007 Published, JBC Papers in Press, August 17, 2007, DOI 10.1074/jbc.M704418200

Koichi Watashi, Daisuke Inoue, Makoto Hijikata, Kaku Goto, Hussein H. Aly, and Kunitada Shimotohno[1]

From the Department of Viral Oncology, Institute for Virus Research, Kyoto University, 53 Kawaharacho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan

Hepatitis C virus (HCV) is a major causative agent of hepatocellular carcinoma. HCV genome replication occurs in the replication complex (RC) around the endoplasmic reticulum membrane. However, the mechanisms regulating the HCV RC remain widely unknown. Here, we used a chemical biology approach to show that estrogen receptor (ESR) is functionally associated with HCV replication. We found that tamoxifen suppressed HCV genome replication. Part of ESRα resided on the endoplasmic reticulum membranes and interacted with HCV RNA polymerase NS5B. RNA interference-mediated knockdown of endogenous ESRα reduced HCV replication. Mechanistic analysis suggested that ESRα promoted NS5B association with the RC and that tamoxifen abrogated NS5B-RC association. Thus, ESRα regulated the presence of NS5B in the RC and stimulated HCV replication. Moreover, the ability of ESRα to regulate NS5B was suggested to serve as a potential novel target for anti-HCV therapeutics.

Estrogen receptor (ESR)[2] belongs to the steroid hormone receptor family of the nuclear receptor superfamily (1). ESR consists of two subtypes, ESRα and ESRβ. As a primary physiological function, ESR is involved in the transcription for downstream genes in response to stimulation by the ligand, estradiol. In the normal state, ESR is mainly located in the cytoplasm and nucleus. Upon binding of the ligand, ESR dimerizes and translocates into the nucleus, where it binds to the ESR-responsive elements (ERE) in the DNA promoter of downstream genes and drives transcription. In addition to this classical genomic action, a portion of ESR is located on the membrane, such as the plasma membrane, and involved in the nongenomic function of triggering signal transduction pathways, such as mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and protein kinase C (2–4). Although the molecular basis of ESR membrane retention is not fully understood, one mechanism involves a membrane protein, caveolin (CAV); ESRα interacted with CAV, and this interaction facilitated ESRα localization to the membrane (5, 6). It was also reported that ESRα localizes to the lipid rafts on the plasma membrane (7). The lipid rafts are microdomains of the membrane that form platforms enriched in cholesterol and glycosphingolipids. However, the characteristics and relevance of membrane-associated ESR have not been fully disclosed. Here, we report the novel role of ESRα in the regulation of viral replication.

Hepatitis C virus (HCV), a causative agent of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma, constitutes a serious health problem worldwide (8). HCV has a positive strand RNA genome that produces at least 10 functional viral proteins: core, envelope 1, envelope 2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (9, 10). NS5B is an RNA-dependent RNA polymerase, which plays a central role in viral genome replication (11, 12). HCV genome replication can be evaluated using a HCV subgenomic replicon system, which Lohmann *et al.* (13) first established. In this system, cells carry an HCV subgenome RNA encoding NS3 to NS5B. Using this system, it has been proposed that HCV genome replication occurs in the replication complex (RC), which contains the viral genome RNA and HCV NS proteins. The RC forms on the surface of the intracellular membranes, including the endoplasmic reticulum (ER) membrane, and is surrounded by a membrane structure (14–17). It also has been reported that HCV genome replication associates with the lipid rafts on these intracellular membranes, such as the ER membrane (14, 18). These lipid rafts accumulate CAV2, and HCV proteins involved in viral genome replication cofractionate with CAV2 (18). However, it is largely unknown how the RC is formed and under what mechanism the HCV proteins participate in the RC.

A chemical biology approach is a useful method to analyze the molecular mechanism of viral life cycles as well as cellular physiological processes (19). We employed forward chemical genetics in which we analyzed HCV replication activity as a phenotypic indicator of a cell-based assay to screen chemical

* This work was supported by grants-in-aid for cancer research and for the second term comprehensive 10-year strategies for cancer control from the Ministry of Health, Labor, and Welfare; by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science, and Technology; by grants-in-aid for the Research for the Future Program from the Japanese Society for the Promotion of Science; and by grants-in-aid for the Program for Promotion of Fundamental Studies in Health Science from the Organization for Pharmaceutical Safety. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence should be addressed: Dept. of Viral Oncology, Institute for Virus Research, Kyoto University, 53 Kawaharacho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan. Tel.: 81-75-751-4000; Fax: 81-75-751-3998; E-mail: kshimoto@virus.kyoto-u.ac.jp.

[2] The abbreviations used are: ESR, estrogen receptor; HCV, hepatitis C virus; RC, replication complex; ER, endoplasmic reticulum; TAM, tamoxifen; ERE, ESR-responsive element(s); CAV, caveolin; NS, nonstructural protein; MM, microsomal membrane; siRNA, small interfering RNA; si-ESR, small interfering ESR; GST, glutathione S-transferase; aa, amino acid(s); RT, reverse transcription; NS3, NS4A, NS4B, NS5A, and NS5B, nonstructural protein 3, 4A, 4B, 5A, and 5B, respectively.

Downloaded from www.jbc.org at Kyoto University on March 25, 2008

The Journal of Biological Chemistry

compounds that inhibited HCV replication. Using this system, we previously identified an immunosuppressant, cyclosporin A, as an anti-HCV compound (20). We also reported that cyclophilin B regulated the RNA binding activity of NS5B (21). In the current study, this chemical screening approach linked ESRα to HCV replication. We showed that tamoxifen (TAM) suppressed HCV genome replication. Using TAM as a bioprobe, we found that ESRα interacted with NS5B and regulated the participation of NS5B in the RC.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Huh-7 and cured MH-14 cells (21) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, minimal essential medium nonessential amino acid (Invitrogen), and kanamycin (Meiji). MH-14 cells, carrying HCV subgenomic replicon (16), and LucNeo#2 cells, carrying luciferase-containing subgenomic replicon (22), were cultured in the same medium supplemented with 300 μg/ml G418 (Invitrogen). Hus-E7/DN24 cells, a human hepatocyte cell line established by immortalization with HPV E6E7 and hTERT from human primary hepatocytes and introduction with a dominant negative form of interferon regulatory factor-7 (23), were cultured with Dulbecco's modified Eagle's medium with 20 mM Hepes (Invitrogen), 15 g/ml L-proline, 0.25 g/ml insulin (Sigma), 50 nM dexamethasone (Sigma), 44 mM $NaHCO_3$, 10 mM nicotinamide, 5 ng/ml epidermal growth factor, 0.1 mM Asc-2P, 100 IU/ml penicillin G (Invitrogen), 100 μg/ml streptomycin (Invitrogen), 5% fetal bovine serum, 1% Dulbecco's modified Eagle's medium, and 2 UG/ml Fungizone (Invitrogen) (24). Plasmid transfection was performed with FuGENE 6 transfection reagent (Roche Applied Science), as described previously (25). RNA transfection was achieved using DMrie-C transfection reagent (Invitrogen), as described previously (21). siRNA was transfected by using siLentFect (Bio-Rad) (21).

Plasmid Construction—pCMV-FL-ESRα, encoding the whole open reading frame of ESRα fused with a FLAG tag, was generated by inserting the PCR product using 5′-GTTGAATTCATGACCATGACCCTCCAC-3′ and 5′-GTTGATCTCGAGTCAGACTGTGGCAGGGAAAC-3′ as primer set and human lymphocyte cDNA library (Clontech) as a template into the EcoRI-XhoI site of pCMV-FLAG vector (21). pCAG-HA-NS5B, encoding the NS5B protein fused with a hemagglutinin tag, was made by subcloning the PCR product with 5′-GTTGCGGCCGCTATGTCAATGTCCTACTCA-3′ and 5′-GTTCTCGAGTCACCGGTTGGGGAGCAGGTA-3′ as primers and pMH14 as a template into NotI-XhoI digestion of PCAG-HA vector (21). Expression plasmids for HCV NS3, NS4B, NS5A, and NS5B (pcDNA-NS3, pcDNA-NS4B, pcDNA-NS5A, and pcDNA-NS5B, respectively) were described in Ref. 21. pGEX-ESRα A/B, C, D, and E/F, expressing the fusion protein of the domain A/B, C, D, and E/F of ESRα with GST, were prepared by the insertion of the PCR product with pCMV-FL-ESRα as a template and appropriate primers into the EcoRI-XhoI site of pGEX-6P1 vector (Clontech). The expression plasmids for the point mutants of ESRα, ESRα(L540Q), ESRα(255M), and ESRα(258M), of which Leu at aa 540, IRK at aa 255–257, and DRR at aa 258–260 were replaced by Gln, TGT, and ANT, respectively, was generated by oligonucleotide-directed mutagenesis. pCMV-FL-CAV2, encoding FLAG-tagged CAV2, was prepared by inserting the PCR product amplified with 5′-GTTGTCGACTATGGGGCTGGAGAC-3′ and 5′-GTTAAGCTTTCAATCCTGGCTC-3′ as primers and human liver cDNA library (Clontech) as a template into the SalI-HindIII site of pCMV-FLAG vector (21). The mammalian expression vector for the C domain of ESRα was generated by replacing the EcoRI-XhoI digestion of pCMV-FLAG vector (21) by that of pGEX-ESRα C. pLMH14 was described previously (26). pGL3-EREX3-TATA-Luc, pcDNA3-ERα, pcDNA3-hERβ were kindly provided by Dr. Kato (Institute of Molecular and Cellular Biosciences, University of Tokyo). JFH1 expression plasmid was provided by Dr. Wakita (National Institute of Infectious Diseases).

Luciferase Assay—A luciferase assay monitoring HCV replication activity was performed as described previously (22, 26). In Fig. 1, *A* and *F*, we used LucNeo#2 cells, stably carrying luciferase-containing subgenomic replicon (22). In Figs. 2 (*D* and *E*), 4*C*, and 6*A*, we transiently transduced luciferase-containing replicon LMH14 RNA together with each expression plasmid into cured MH-14 cells (26). A luciferase assay detecting the transcriptional activity driven from the ERE was performed as described previously (25).

Real Time RT-PCR Analysis—Real time RT-PCR analysis was performed as previously described (20).

Immunoblot Analysis—Immunoblot analysis was performed as previously described (25). The antibodies used in this study are anti-NS5A (kindly provided by Dr. Takamizawa (Osaka University)), anti-NS5B (anti-NS5B#14; a generous gift from Dr. Kohara (Tokyo Metropolitan Institute of Medical Science)), anti-NS5B (NS5B#6; a kind gift from Dr. Fukuya (Osaka University)), anti-tubulin (Oncogene), anti-FLAG (Sigma), anti-IκBα (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-calnexin (StressGen), and anti-caveolin-2 antibodies (BD Biosciences Pharmingen).

Indirect Immunofluorescence Analysis—Indirect immunofluorescence analysis was performed as described previously (25). The antibodies used were anti-NS5A and anti-protein-disulfide isomerase antibodies (StressGen).

siRNA—siRNA duplexes (5′-GUGUGCAAUGACUAUGCUUCA-3′ for si-ESRα and 5′-CGCAUCGGGAUAUCACUAUGG-3′ for si-ESRβ) were synthesized (Proligo). A randomized siRNA, si-control, was purchased from Dharmacon (nonspecific control duplex IX).

Enzyme-linked Immunosorbent Assay—HCV core was quantified in the culture medium of the cells transfected with JFH1 RNA (29) with an enzyme-linked immunosorbent assay according to the manufacturer's protocol (HCV antigen enzyme-linked immunosorbent assay test; Ortho-Clinical Diagnostics).

RT-PCR Analysis—RT-PCR analysis was performed as described (20) by using the following primer sets: 5′-CCTACTACCTGGAGAACG-3′ and 5′-GCTGGACACATATAGTCG-3′ for the detection of ESRα and 5′-AGCCATGACATTCTATAGC-3′ and 5′-CCACTTCGTAACACTTCC-3′ for ESRβ.

GST Pull-down Assay—The GST pull-down assay was conducted as described previously (25).

Immunoprecipitation Analysis—Immunoprecipitation analysis was performed as described previously (25). The antibodies

Downloaded from www.jbc.org at Kyoto University on March 25, 2008

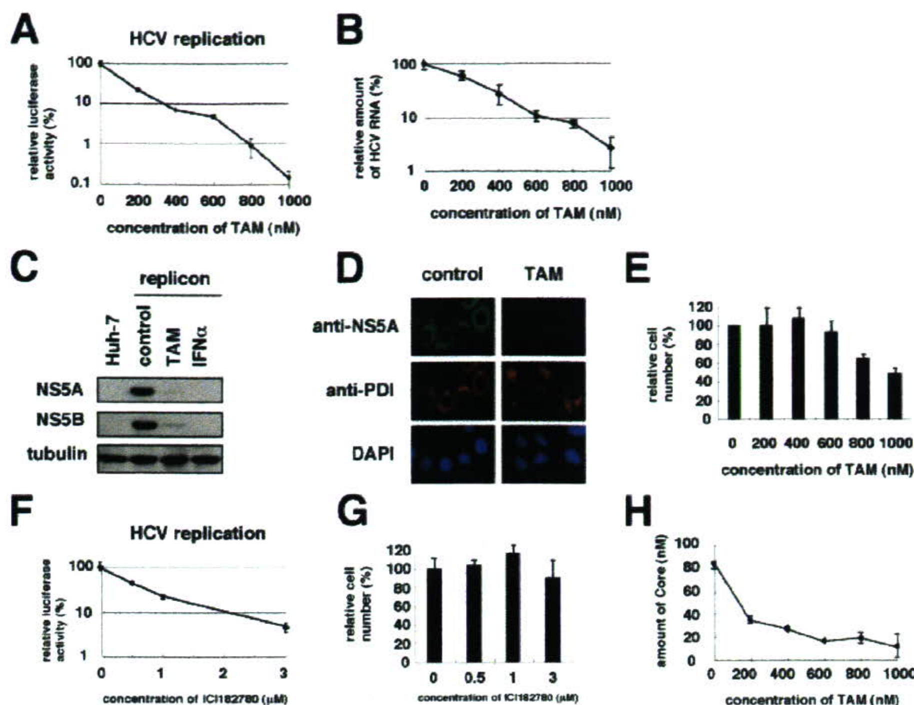


FIGURE 1. **TAM suppressed the replication of the HCV genome.** *A*, luciferase activities were measured using the LucNeo#2 cells, which carried a luciferase-containing replicon RNA, upon treatment with TAM at the indicated doses for 7 days. Relative luciferase activities are plotted against the concentrations of TAM. The data show the means of three independent experiments. The *error bars* are indicated. *B*, HCV RNA was quantified by real time RT-PCR analysis using the lysates from MH-14 cells, harboring the HCV subgenomic replicon, treated with the indicated doses of TAM for 7 days. Relative amounts of HCV RNA are shown. *C*, HCV NS5A and NS5B proteins as well as tubulin as an internal control were detected by immunoblot analysis in the lysates from MH-14 cells (replicon) treated without (control) or with 500 nM TAM or 100 IU/ml interferon-α as a positive control for 7 days and Huh-7 cells. *D*, HCV NS5A and protein-disulfide isomerase (*PDI*) as an internal control were detected by indirect immunofluorescence analysis in the cells treated without (control) or with 500 nM TAM for 7 days. 4′,6-Diamidino-2-phenylindole (*DAPI*) shows a nuclear staining. *E*, cell number was counted after 5 days upon treatment with various concentrations of TAM. Relative cell numbers are shown. *F*, luciferase activities with LucNeo#2 cells treated with various concentrations of ICI182780 were measured as described in *A*. *G*, cell number was counted under treatment with ICI182780 at the indicated concentrations. *H*, core in the culture medium of JFH1 RNA-transfected cells upon treatment with TAM was quantified as described under "Experimental Procedures."

used in this study were mouse normal IgG as a negative control (Zymed Laboratories), anti-NS5B (anti-NS5B#10; a generous gift from Dr. Kohara at the Tokyo Metropolitan Institute of Medical Science), anti-FLAG, and anti-caveolin-2 antibodies.

Fractionation of Cell Extracts—MH-14 cells transfected with the expression plasmid for FLAG-tagged ESRα were fractionated essentially as described previously (25).

HCV Replication Complex Assay—Isolation of HCV RC was done as described previously (16, 21).

In Vitro HCV Infection Experiment—*In vitro* HCV infection was conducted essentially as described (23). Briefly, HCV-infected serum (~2 × 10^5 copies) was inoculated into HuS-E7/DN24 cells (5 × 10^4 cells) for 24 h. After washes, cells were cultured in the medium supplemented with 10 μM PD98059 to stimulate HCV translation (27) (*scheme* in Fig. 6*B*). To observe HCV amplification, HCV RNA in the cells was quantified, since HCV RNA was hardly detected significantly in the culture medium (23).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide Assay—The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was performed to examine the cell viability using Cell Proliferation kit II, XTT (Roche Applied Science) according to the manufacturer's protocol.

RESULTS

Tamoxifen Suppressed HCV Genome Replication—We screened for agents that suppressed HCV genome replication using a HCV subgenomic replicon system (13, 16). Among the compounds tested, we observed that TAM inhibited HCV genome replication. HCV replication activity, monitored by luciferase activity (22), and the amount of HCV RNA were decreased with TAM treatment in a dose-dependent manner (Fig. 1, *A* and *B*). The expression of HCV proteins, NS5A and NS5B, detected by immunoblot (Fig. 1*C*) and indirect immuofluorescence analyses (Fig. 1*D*), also drastically decreased by treatment with TAM. A high concentration of TAM decreased cell proliferation (Fig. 1*E*). However, TAM suppressed HCV replication without any cytotoxicity in another cell line, HuS-E7/DN24 cells (Fig. 6, *C* and *D*). In addition, a pure anti-estrogen compound ICI182780, which had little cytotoxic effect, reduced HCV RNA (Fig. 1, *F* and *G*). Moreover, TAM inhibited the production of core in the culture medium of HCV JFH1-transfected cells, in a recently developed system of the production of infectious HCV particles (Fig. 1*H*) (28–30). The above data indicate that TAM suppresses HCV genome replication.

ESR Was Involved in HCV Genome Replication—Next, we investigated which cellular protein TAM targets to suppress HCV replication. It has been reported that TAM targets 1) ESR (31), 2) P-glycoprotein (32, 33), 3) calmodulin (34), 4) protein kinase C (35, 36), etc. Although other compounds targeting P-glycoprotein, calmodulin, and protein kinase C did not affect HCV replication in our screening (data not shown), ESR was suggested to play a role in HCV replication as shown below.

RNAi-mediated specific knockdown of endogenous ESRα and ESRβ (Fig. 2*A*) reduced HCV RNA in replicon-containing cells to ~20–40% and 60–70%, respectively (Fig. 2*B*). Transient transfection with ESRα and ESRβ expression plasmids, which activated ERE-driven transcription 4–5-fold (Fig. 2*C*), showed that ectopically expressed ESRα augmented HCV replication activity in a dose-dependent manner, whereas ESRβ did not (Fig. 2*D*). ESRα-induced augmentation of the replication was reversed upon TAM treatment (Fig. 2*D*). These results suggested a significant role of ESR, especially ESRα, in HCV

The Journal of Biological Chemistry
Downloaded from www.jbc.org at Kyoto University on March 25, 2008

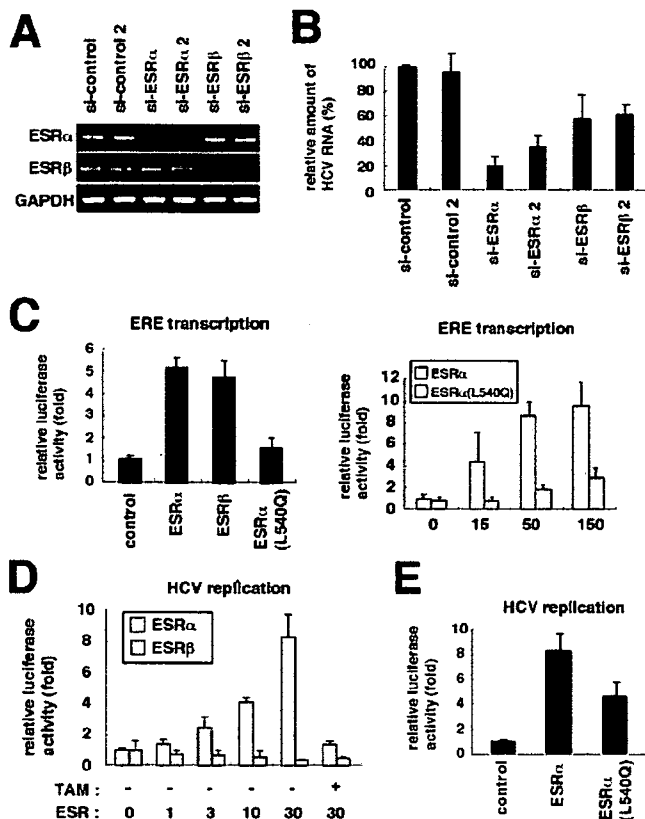


FIGURE 2. **ESR was involved in HCV genome replication.** *A*, specific knockdown of endogenous ESRα and ESRβ. RT-PCR analysis was performed to detect the expression of ESRα, ESRβ, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as an internal control in the cells transfected with siRNA recognizing ESRα (si-ESRα, si-ESRα2), ESRβ (si-ESRβ, si-ESRβ2), or randomized siRNA (si-control, si-control2). *B*, HCV RNA was quantified as shown in Fig. 1*B*, using the cells transfected with si-control, si-control2, si-ESRα, si-ESRα2, si-ESRβ, and si-ESRβ2 for 5 days. *C*, the ERE-mediated transcriptional activities were measured by a luciferase assay using the lysates from the cells transfected with pGL3-EREX3-TATA-Luc reporter plasmid together with pcDNA3-ERα (ESRα), pcDNA3-hERβ (ESRβ), pcDNA-ESRα(L540Q), or the empty vector (control) (*left*) or varying amounts (ng) of pcDNA3-ERα (ESRα) or pcDNA-ESRα(L540Q) (*right*) and treated with 100 nM estradiol for 36 h. *D* and *E*, HCV replication activities were examined by quantifying the luciferase activities using cured MH-14 cells transfected with the indicated doses (ng) of ESRα or ESRβ (*D*) or 30 ng of ESRα, ERα(L540Q), or the empty vector (control) (*E*) together with 0.125 μg of LMH14 RNA without or with 1 μM TAM for 4 days.

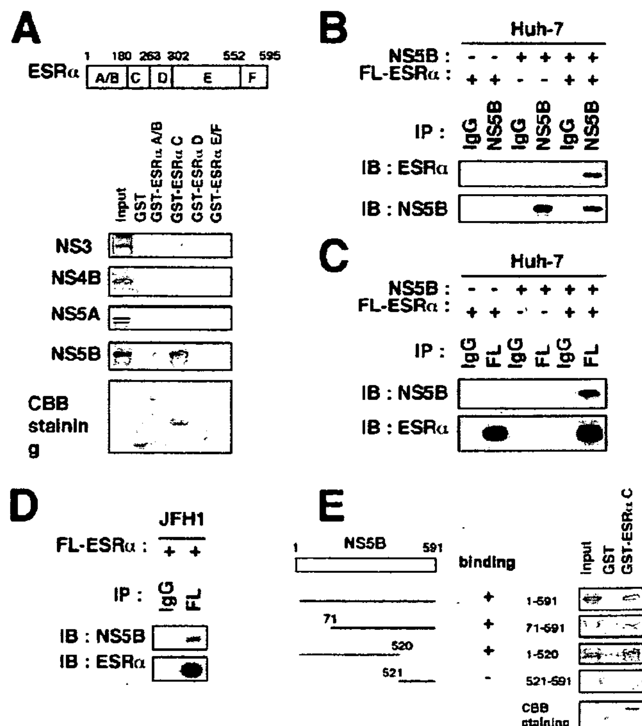


FIGURE 3. **ESRα interacted with HCV NS5B.** *A*, *top*, schematic representation of the primary structure of ESRα. ESRα consists of domains A–F. The amino acid numbers are also shown. *Bottom*, GST pull-down assays were performed using the recombinant proteins of the A/B, C, D, and E/F domain of ESRα fused with GST and *in vitro* translated HCV NS3, NS4B, NS5A, and NS5B protein. *Input*, the one-fifth amount of protein used for the pull-down assay. The Coomassie Brilliant Blue staining pattern of the precipitated fraction is also shown in the *bottom panel*. *B–D*, the lysates from the cells ectopically expressing NS5B (*B* and *C*) or the whole open reading frame of the HCV JFH1 strain (*D*) and/or FLAG-tagged ESRα were immunoprecipitated (*IP*) with anti-NS5B (*B*; NS5B), anti-FLAG antibody (*C* and *D*; FL), or mouse normal IgG as a negative control followed by the detection of ESRα and NS5B by immunoblot analysis (*IB*). *E*, deletion mutants of NS5B were subjected to a GST pull-down assay with GST-fused C domain of ESRα as described in *A*. The *left panel* shows a schematic representation of the full-length and truncated mutants of NS5B. The *numbers* indicate the amino acid numbers in NS5B.

genome replication. ESRα(L540Q), carrying a leucine to glutamine point mutation at aa 540 within the LXXLL motif (aa 536–540) of ESRα (37), had much lower transactivation activity driven from ERE (Fig. 2*C*). However, ESRα(L540Q) stimulated HCV replication activity ~5-fold, although the stimulation was less than that by wild-type ESRα (Fig. 2*E*). Thus, ESRα having lower transactivating capacity could still facilitate HCV replication.

ESRα Interacted with HCV NS5B—Thus, the chemical biology approach revealed the involvement of ESR in HCV genome replication. Then we investigated the molecular mechanism of ESR-induced HCV replication. A binding assay between ESRα and HCV proteins expressed in the HCV subgenomic replicon showed that the C domain of ESRα coprecipitated with NS5B but not NS3, NS4B, and NS5A (Fig. 3*A*). Other ESRα domains, A/B, D, and E/F, did not bind to any HCV proteins. A coimmunoprecipitation assay also indicated the presence of ESRα in the immunoprecipitate by anti-NS5B antibody (Fig. 3, *B* and *D*), and *vice versa* (Fig. 3*C*). Thus, ESRα specifically interacted with NS5B. Deletion analysis indicated that the region of 71–591 and 1–520 but not 521–591 of NS5B coprecipitated with the recombinant C domain of ESRα (Fig. 3*E*). This binding profile is different from that between cyclophilin B and NS5B, which we previously reported (21).

The ESRα-NS5B Interaction Was Important for the Regulation of HCV Genome Replication—To examine whether the interaction between ESRα and NS5B was essential for the ESRα-mediated regulation of HCV replication or not, we searched for a point mutant of ESRα that could not bind to NS5B by alanine-scanning mutation analysis. ESRα mutants, ESRα(255M) and ESRα(258M), in which IRK at aa 255–257 and DRR at aa 258–260 was replaced by TGT and AQT, respectively, had little affinity with NS5B (Fig. 4*A*) but still possessed the ERE-mediated transactivation capacity (Fig. 4*B*). However, both ESRα(255M) and ESRα(258M) caused only weak activations of HCV replication, compared with wild type ESRα (Fig. 4*C*). The data suggest that the interaction of ESRα with NS5B is

The Journal of Biological Chemistry

Downloaded from www.jbc.org at Kyoto University on March 25, 2008

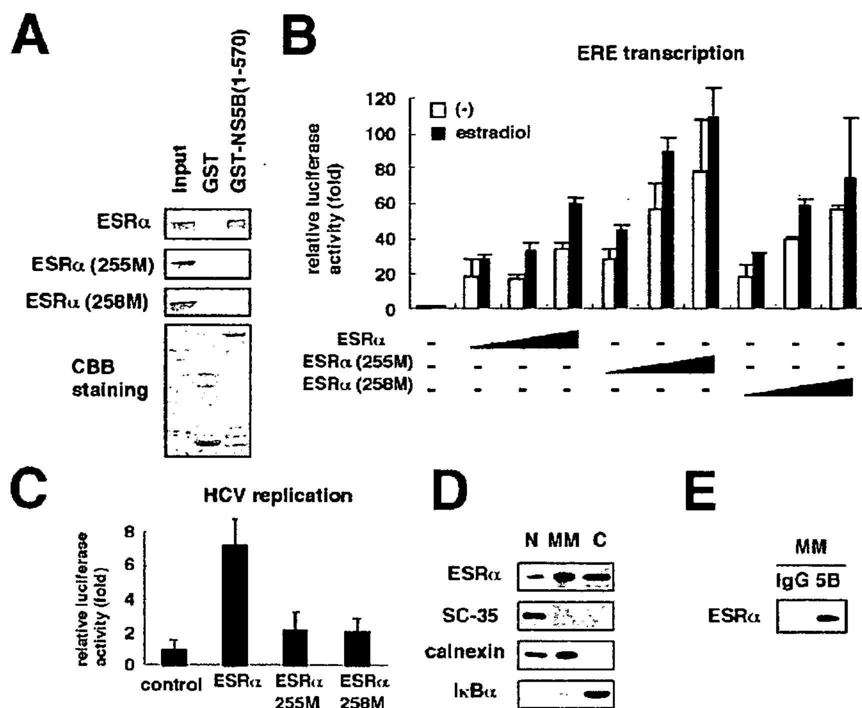


FIGURE 4. **The interaction of NS5B mediated the regulation of HCV genome replication by ESRα.** *A*, GST pull-down assays were performed as described in Fig. 3*A* using the wild type ESRα or point mutant of ESRα, ESRα(255M), and ESRα(258M). *B*, the mutation within ESRα(255M) and ESRα(258M) did not reduce the activation capacity of ERE-mediated transcription. Huh-7 cells were transfected with the expression plasmids for ESRα, ESRα(255M), or ESRα(258M) at doses of 10, 30, and 100 ng each together with pGL3-EREX3-TATA-Luc reporter plasmid and treated without (*white bar*) or with 100 nM estradiol (*black bar*) to quantify the luciferase activity. *C*, HCV replication activities were examined by quantifying the luciferase activities as described in the legend to Fig. 2*D* in the cells upon transfection with the expression plasmids for wild type ESRα, ESRα(255M), or ESRα(258M). *D*, the cells were fractionated into the nucleus (*N*), MM, and cytoplasm (*C*). Each fraction was detected for FLAG-tagged ESRα, SC-35, calnexin, and IκBα, respectively, by immunoblot analysis. Calnexin, an ER marker protein, was detected in the nucleus as well as MM, probably because of the existence of the nuclear membrane in the nuclear fraction. *E*, the MM fraction obtained in *D* was subjected to a coimmunoprecipitation assay using anti-NS5B or IgG followed by immunoblot analysis for the detection for ESRα.

critical for ESRα-mediated regulation of HCV genome replication.

Thus, ESRα interaction with NS5B regulates HCV replication. NS5B is mainly located on the cytoplasmic surface of the ER membrane (21, 38). On the other hand, ESRα as a nuclear hormone receptor is normally distributed in the cytoplasm and translocates into the nucleus upon ligand stimulation. In addition, a part of ESRα localizes on the membrane fraction. In our experiment, NS5B was mainly located around the ER, colocalized with the ER marker, protein-disulfide isomerase (data not shown) (21). Ectopically expressed ESRα showed diffuse distribution in the cells (data not shown). We fractionated cell homogenates and observed that a part of the ESRα resided in the microsomal membrane (MM) fraction (Fig. 4*D*). Moreover, ESRα in the MM fraction was coprecipitated with NS5B (Fig. 4*E*). It suggests the possibility that the interaction between NS5B and ESRα, at least in part of them, occurs on the ER membrane.

ESRα Promoted the Participation of NS5B in the HCV Replication Complex—It was reported that HCV proteins involved in the replication machinery was associated with the lipid raft on the ER and cofractionated with CAV2. A coimmunoprecipitation assay showed that NS5B associated with CAV2 (Fig. 5*A*). In the experiment investigating the role of ESRα in NS5B-CAV2 association, the coprecipitation of NS5B with CAV2 was decreased upon the knocking down of ESRα (Fig. 5*B*). Treatment with TAM abrogated the association of NS5B with CAV2 (Fig. 5*C*), although the total amount of NS5B in the cells is similar in the presence and absence of TAM for 24 h in this experiment (data not shown). Thus, ESRα was suggested to promote the association between NS5B and CAV2. Since a part of CAV2 resided on the lipid raft on the ER (18), ESRα-mediated binding between NS5B and CAV2 was possible to affect the localization of NS5B to the HCV RC. To see the consequential relevance of ESRα on NS5B function, we analyzed the HCV RC by treatment with digitonin/protease as described previously (16). HCV proteins involved in the RC and surrounded by the membrane structure are resistant to the treatment with digitonin followed by protease, whereas those unrelated to the replication outside the RC are digested by the treatment. By using this technique measuring the sensitivity to protease, HCV RC can be distinguished from the ER that is not related to the replication, although the RC and the nucleus cannot be separated. The experimental condition for fractionation was confirmed with the detection with IκBα and calnexin; a cytosolic protein IκBα was washed out following the treatment with digotinin (Fig. 5*D*, *lanes 1* and *2*), and ER protein calnexin, which did not accumulate in the RC, was digested by treatment with digotinin/protease (Fig. 5*D*, *lanes 2–4*). An ER lipid raft component, CAV2, was still detected under the digitonin/protease treatment (the RC-containing fraction) (Fig. 5*D*, *lanes 3* and *4*). Under this condition, a part of NS5B was detected in the digitonin/protease-resistant fraction, as described previously (16) (Fig. 5*D*, *lanes 3* and *4*). However, NS5B in this fraction was decreased upon treatment with TAM (Fig. 5*D*, *lanes 3*, *4*, *7*, and *8*). On the other hand, the amount of NS5A was not significantly changed by TAM treatment. Knocking down of ESRα also disrupted the association of NS5B with the RC-containing fraction (Fig. 5*E*). From the above results, it was suggested that ESRα promoted the participation of NS5B in the RC (also see "Discussion").

ESRα Could Serve as a Molecular Target of Anti-HCV Agents—Finally, we assessed the possibility that the association of ESRα with NS5B could serve as a target of anti-HCV agents. By introducing a decoy peptide against ESRα-NS5B interaction, consisting of the C domain of ESRα into replicon-bearing cells,

Downloaded from www.jbc.org at Kyoto University on March 25, 2008

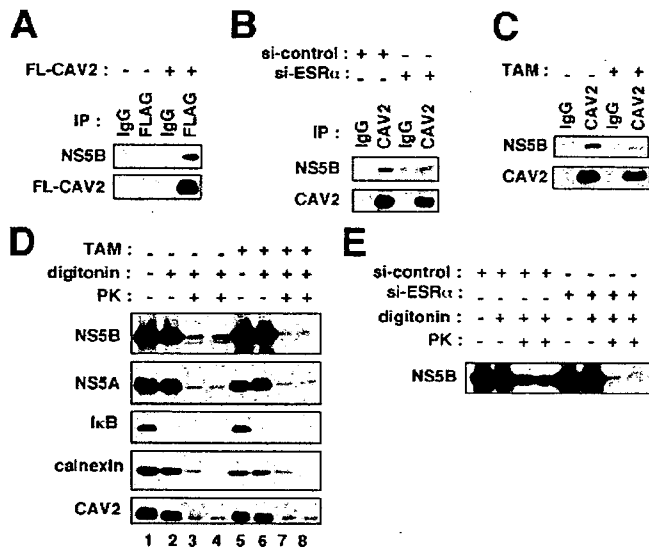


FIGURE 5. **ESRα promoted the participation of NS5B in HCV RC.** *A–C*, a coimmunoprecipitation assay (*IP*) was performed with anti-FLAG (*A*), anti-CAV2 (*B* and *C*) antibody, or mouse normal IgG from the lysates of the cells transfected without or with FLAG-tagged CAV2 (*A*), transfected with si-control or si-ESRα (*B*), or treated without or with 1 μM TAM (*C*). NS5B (*top*) and CAV2 (*bottom*) were detected by immunoblot analysis. *D*, detection of the amount of NS5B in the digitonin/protease-resistant fraction. MH-14 cells were treated without (*lanes 1–4*) or with 1 μM TAM (*lanes 5–8*) for 24 h. Cells were then treated without (*lanes 1* and *5*) or with digitonin (*lanes 2–4* and *6–8*), followed by digestion with proteinase K (0 μg/ml for *lanes 2* and *6*, 0.3 μg/ml for *lanes 3* and *7*, and 1 μg/ml for *lanes 4* and *8*). NS5B, NS5A, IκBα, calnexin, and CAV2 were detected by immunoblot analysis. *E*, HCV RC was isolated as described in *D* using the cells transfected with si-control or si-ESRα, and NS5B was detected. A similar result was obtained by using si-ESRα2.

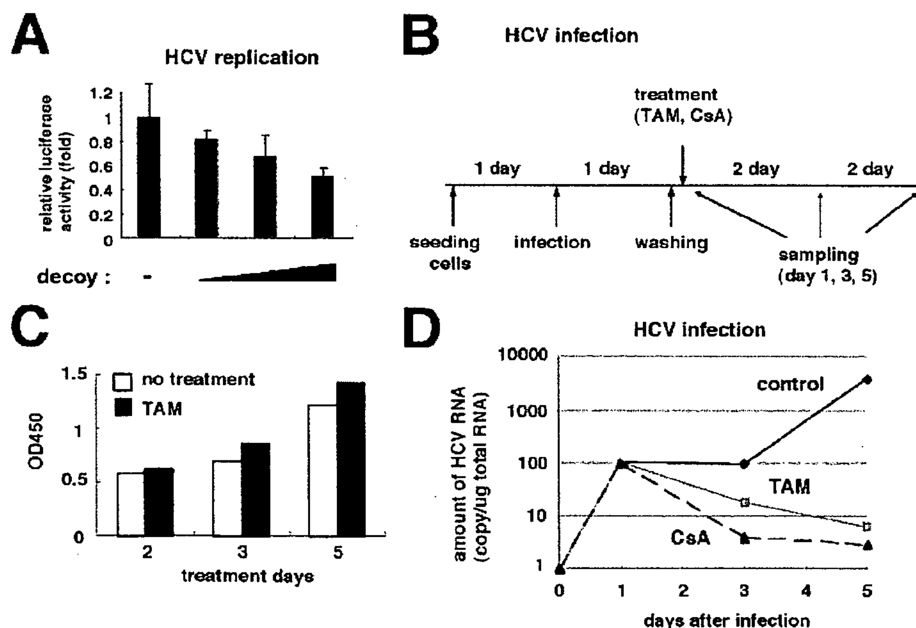


FIGURE 6. **ESRα could serve as a molecular target for anti-HCV agents.** *A*, HCV replication activity was measured by quantifying the luciferase activity as described in the legend to Fig. 2*D* in the cells overexpressing a decoy peptide consisting of the C domain of ESRα. *B*, experimental scheme of *in vitro* HCV infection experiment. After seeding the HuS-E7/DN24 cells, HCV-positive serum was inoculated for 24 h. After extensive washes, the cells were cultured with the medium supplemented without (control) or with 1 μM TAM or 3 μg/ml cyclosporin A. HCV genome RNA was quantified along with the time course (days 1, 3, and 5 postinoculation) by real time RT-PCR analysis. *C*, the treatment with 1 μM TAM did not show any cytotoxic effect on HuS-E7/DN24 cells. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were performed as described under "Experimental Procedures" to examine the viability of the cells at days 2, 3, and 5 postinoculation. *D*, HCV genome RNA was quantified as described in *B* and plotted against the time course.

HCV replication activity was reduced in a dose-dependent manner (Fig. 6*A*). To further observe the significance of ESRα in a physiological condition, we performed an *in vitro* infection experiment using serum from an HCV-infected patient as a nascent virus inoculum and nonneoplastic human hepatocytes as highly infection-permissive cells (Fig. 6*B*). Treatment with 1 μM TAM did not show a cytotoxic effect on these cells in any time course examined (Fig. 6*C*). However, treatment with TAM as well as cyclosporin A as a positive control inhibited the multiplication of viral genome RNA in the cells along with the time course (Fig. 6*D*). Thus, ESRα could serve as a potent molecular target of anti-HCV agents.

DISCUSSION

In general, viruses take advantage of host cell factors for their replication. So far, some factors have been shown to relevantly regulate HCV replication, including hVAP33 (39, 40), FBL2 (41), and cyclophilin B (21). Among these, FBL2 and cyclophilin B were identified by a chemical biological approach; FBL2 from the observation of an anti-HCV activity of lovastatin and an inhibitor of geranylgeranyl transferase (41–43); cyclophilin B from the inhibitory effect of cyclosporin A on HCV replication (20, 21). In this study, we found a suppressive capacity of TAM to HCV genome replication. Through further examination using TAM, we revealed ESRα as a host cell factor regulating HCV replication and suggested its regulation mechanism.

Currently, it is proposed that HCV RC that replicates the HCV genome is formed on the intracellular membrane, including the ER membrane (14–17). It was also reported that HCV genome replication was associated with the lipid raft on the intracellular membrane (18). Most HCV proteins are not related to the RC, whereas only a minor portion of HCV proteins take part in the RC to drive the viral replication (16). It has remained widely unknown, however, how HCV proteins are regulated to participate in the RC. It was reported that hVAP-33 binds to NS5A and NS5B, and this protein is related to the amount of NS5B in the lipid raft (40). hVAP-33 was speculated to recruit NS5B to the lipid raft, although its molecular mechanism has not been analyzed. This study suggested the interaction between ESRα and NS5B in the ER fraction, although we did not show the existence of ESRα in the RC, since the RC and the nucleus cannot be separated in the digitonin/protease treatment experiment. ESRα promoted the interaction of NS5B with CAV2. Previous papers reported that ESRα bound to CAV1 and CAV2 (6). From these observations, ESRα is

The Journal of Biological Chemistry

Downloaded from www.jbc.org at Kyoto University on March 25, 2008

likely to function as a bridging factor that connects NS5B to CAV2, although we cannot fully neglect the possibility that ESRα augments NS5B-CAV2 binding via another function, such as transcriptional activity. Because CAV2 resided on the lipid raft of the intracellular membrane (18), this action of ESRα may recruit NS5B to the lipid raft and the HCV RC. In fact, ESRα promoted the participation of NS5B in the HCV RC. Thus, ESRα is suggested to escort NS5B to the HCV RC, although it is also possible that ESRα augments the number of the RC itself. However, ESRα at least augments the amount of NS5B involved in HCV replication machinery to stimulate the replication. It was reported that the membrane-associated ESRα served as a platform where signalsomes, including receptor tyrosine kinase, nonreceptor tyrosine kinase Src, and G proteins, assembled and activated downstream signaling pathways (44–46). HCV may also take advantage of such platform characteristics of ESRα to form the RC for their efficient replication. Although the mechanisms of the nuclear receptor function of ESRα have been extensively elucidated, the functions of membrane-associated ESRα have not been widely characterized so far. This study suggested a novel physiological relevance of membrane-associated ESRα as a regulator of the viral replication.

Until now, there are no clinical studies that report a direct interaction of TAM treatment with HCV replication in patients infected with HCV. Given our results, examinations on the effect of TAM or other anti-estrogen drugs may be one of the useful approaches to develop a new anti-HCV strategy. On the other hand, we disclosed the mechanism of ESR-mediated regulation of HCV genome replication. Screening for compounds that inhibit this mechanism expectedly led to novel types of anti-HCV agents. Further analyses on ESR are needed to develop anti-HCV therapeutics as well as reveal the regulation mechanism of HCV replication.

Acknowledgments—We are grateful to Dr. T. Murata, T. Hishiki, and M. Hosaka for establishing the replicon-containing cells. We thank Dr. Aratake (Asahi Kasei Pharma) for helpful discussions. We also thank Dr. Kato, Dr. Takamizawa, Dr. Kohara, Dr. Fukuya, and Dr. Wakita for kindly providing the plasmids: pGL3-EREX3-TATA-Luc, pcDNA3-ERα, and pcDNA3-hERβ; anti-NS5A antibody; anti-NS5B antibody; anti-NS5B antibody; and JFH1 expression plasmid, respectively.

REFERENCES

1. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995) *Cell* **83**, 835–839
2. Acconcia, F., and Kumar, R. (2006) *Cancer Lett.* **238**, 1–14
3. Levin, E. R. (2005) *Mol. Endocrinol.* **19**, 1951–1959
4. Song, R. X., Zhang, Z., and Santen, R. J. (2005) *Trends Endocrinol. Metab.* **16**, 347–353
5. Razandi, M., Alton, G., Pedram, A., Ghonshani, S., Webb, P., and Levin, E. R. (2003) *Mol. Cell. Biol.* **23**, 1633–1646
6. Razandi, M., Oh, P., Pedram, A., Schnitzer, J., and Levin, E. R. (2002) *Mol. Endocrinol.* **16**, 100–115
7. Marquez, D. C., Chen, H. W., Curran, E. M., Welshons, W. V., and Pietras, R. J. (2006) *Mol. Cell. Endocrinol.* **246**, 91–100
8. Liang, T. J., Jeffers, L. J., Reddy, K. R., De Medina, M., Parker, I. T., Cheinquer, H., Idrovo, V., Rabassa, A., and Schiff, E. R. (1993) *Hepatology* **18**, 1326–1333
9. Grakoui, A., Wychowski, C., Lin, C., Feinstone, S. M., and Rice, C. M. (1993) *J. Virol.* **67**, 1385–1395
10. Hijikata, M., Kato, N., Ootsuyama, Y., Nakagawa, M., and Shimotohno, K. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 5547–5551
11. Bartenschlager, R., and Lohmann, V. (2001) *Antiviral Res.* **52**, 1–17
12. Tellinghuisen, T. L., and Rice, C. M. (2002) *Curr. Opin. Microbiol.* **5**, 419–427
13. Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L., and Bartenschlager, R. (1999) *Science* **285**, 110–113
14. Aizaki, H., Lee, K. J., Sung, V. M., Ishiko, H., and Lai, M. M. (2004) *Virology* **324**, 450–461
15. Egger, D., Wolk, B., Gosert, R., Bianchi, L., Blum, H. E., Moradpour, D., and Bienz, K. (2002) *J. Virol.* **76**, 5974–5984
16. Miyanari, Y., Hijikata, M., Yamaji, M., Hosaka, M., Takahashi, H., and Shimotohno, K. (2003) *J. Biol. Chem.* **278**, 50301–50308
17. Moradpour, D., Gosert, R., Egger, D., Penin, F., Blum, H. E., and Bienz, K. (2003) *Antiviral Res.* **60**, 103–109
18. Shi, S. T., Lee, K. J., Aizaki, H., Hwang, S. B., and Lai, M. M. (2003) *J. Virol.* **77**, 4160–4168
19. Watashi, K., and Shimotohno, K. (2007) *Rev. Med. Virol.* **17**, 245–252
20. Watashi, K., Hijikata, M., Hosaka, M., Yamaji, M., and Shimotohno, K. (2003) *Hepatology* **38**, 1282–1288
21. Watashi, K., Ishii, N., Hijikata, M., Inoue, D., Murata, T., Miyanari, Y., and Shimotohno, K. (2005) *Mol. Cell* **19**, 111–122
22. Goto, K., Watashi, K., Murata, T., Hishiki, T., Hijikata, M., and Shimotohno, K. (2006) *Biochem. Biophys. Res. Commun.* **343**, 879–884
23. Aly, H. H., Watashi, K., Hijikata, M., Kaneko, H., Takada, Y., Egawa, H., Uemoto, S., and Shimotohno, K. (2007) *J. Hepatol.* **46**, 26–36
24. Hino, H., Tateno, C., Sato, H., Yamasaki, C., Katayama, S., Kohashi, T., Aratani, A., Asahara, T., Dohi, K., and Yoshizato, K. (1999) *Biochem. Biophys. Res. Commun.* **256**, 184–191
25. Watashi, K., Hijikata, M., Tagawa, A., Doi, T., Marusawa, H., and Shimotohno, K. (2003) *Mol. Cell. Biol.* **23**, 7498–7509
26. Murata, T., Ohshima, T., Yamaji, M., Hosaka, M., Miyanari, Y., Hijikata, M., and Shimotohno, K. (2005) *Virology* **331**, 407–417
27. Murata, T., Hijikata, M., and Shimotohno, K. (2005) *Virology* **340**, 105–115
28. Lindenbach, B. D., Evans, M. J., Syder, A. J., Wolk, B., Tellinghuisen, T. L., Liu, C. C., Maruyama, T., Hynes, R. O., Burton, D. R., McKeating, J. A., and Rice, C. M. (2005) *Science* **309**, 623–626
29. Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H. G., Mizokami, M., Bartenschlager, R., and Liang, T. J. (2005) *Nat. Med.* **11**, 791–796
30. Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D. R., Wieland, S. F., Uprichard, S. L., Wakita, T., and Chisari, F. V. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102**, 9294–9299
31. Shang, Y. (2006) *Nat. Rev. Cancer* **6**, 360–368
32. Callaghan, R., and Higgins, C. F. (1995) *Br. J. Cancer* **71**, 294–299
33. Raderer, M., and Scheithauer, W. (1993) *Cancer* **72**, 3553–3563
34. Lopes, M. C., Vale, M. G., and Carvalho, A. P. (1990) *Cancer Res.* **50**, 2753–2758
35. O'Brian, C. A., Liskamp, R. M., Solomon, D. H., and Weinstein, I. B. (1985) *Cancer Res.* **45**, 2462–2465
36. O'Brian, C. A., Ward, N. E., and Anderson, B. W. (1988) *J. Natl. Cancer Inst.* **80**, 1628–1633
37. Leers, J., Treuter, E., and Gustafsson, J. A. (1998) *Mol. Cell. Biol.* **18**, 6001–6013
38. Schmidt-Mende, J., Bieck, E., Hugle, T., Penin, F., Rice, C. M., Blum, H. E., and Moradpour, D. (2001) *J. Biol. Chem.* **276**, 44052–44063
39. Evans, M. J., Rice, C. M., and Goff, S. P. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101**, 13038–13043
40. Gao, L., Aizaki, H., He, J. W., and Lai, M. M. (2004) *J. Virol.* **78**, 3480–3488
41. Wang, C., Gale, M., Jr., Keller, B. C., Huang, H., Brown, M. S., Goldstein, J. L., and Ye, J. (2005) *Mol. Cell* **18**, 425–434

Downloaded from www.jbc.org at Kyoto University on March 25, 2008

The Journal of Biological Chemistry

42. Kapadia, S. B., and Chisari, F. V. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 2561–2566
43. Ye, J., Wang, C., Sumpter, R., Jr., Brown, M. S., Goldstein, J. L., and Gale, M., Jr. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 15865–15870
44. Migliaccio, A., Piccolo, D., Castoria, G., Di Domenico, M., Bilancio, A., Lombardi, M., Gong, W., Beato, M., and Auricchio, F. (1998) *EMBO J.* **17,** 2008–2018
45. Razandi, M., Pedram, A., Greene, G. L., and Levin, E. R. (1999) *Mol. Endocrinol.* **13,** 307–319
46. Wyckoff, M. H., Chambliss, K. L., Mineo, C., Yuhanna, I. S., Mendelsohn, M. E., Mumby, S. M., and Shaul, P. W. (2001) *J. Biol. Chem.* **276,** 27071–27076

The Journal of Biological Chemistry

Downloaded from www.jbc.org at Kyoto University on March 25, 2008

Wiley InterScience DISCOVER SOMETHING GREAT
Reviews in Medical Virology
Rev. Med. Virol. 2007; 17: 245–252.
Published online 14 February 2007 in Wiley InterScience (www.interscience.wiley.com)
DOI: 10.1002/rmv.534

REVIEW

Chemical genetics approach to hepatitis C virus replication: cyclophilin as a target for anti-hepatitis C virus strategy

Koichi Watashi* and Kunitada Shimotohno
Department of Viral Oncology, Institute for Virus Research, Kyoto University, Kyoto, Japan

SUMMARY

Hepatitis C virus (HCV) is a major causative agent of liver diseases such as chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. Because the current standard therapy, interferon (IFN) or pegylated-IFN alone or in combination with ribavirin, is ineffective on approximately half of the HCV-infected patients, alternative therapeutics are greatly needed. The chemical genetics method is a useful strategy to elucidate molecular mechanisms of the viral life cycle and screen for anti-viral agents. This review focuses on the use of chemical genetics approach to virology, which could be called 'chemical virology', and introduces an example of such analysis. From a cell culture-based screening, an immunosuppressant cyclosporin A (CsA) was identified as an anti-HCV compound. Analysis using CsA as a bioprobe showed that cyclophilin (CyP) B, a cellular target of CsA, regulates the function of HCV RNA polymerase NS5B, which is essential for efficient viral genome replication. By targeting CyP, HCV genome replication was drastically suppressed. Thus, chemical genetics analysis identified CyPB as a cellular cofactor of HCV genome replication and a target for novel anti-HCV agents. Copyright © 2007 John Wiley & Sons, Ltd.

Received: 21 December 2006; Accepted: 22 December 2006

INTRODUCTION

Hepatitis C virus (HCV), a member of the flaviviridae family, has a positive strand RNA genome [1]. The genome encodes a precursor polyprotein, which is cleaved by host and viral proteases to generate at least 10 functional viral proteins: core, envelope (E)1, E2, p7, nonstructural protein (NS)2, NS3, NS4A, NS4B, NS5A and NS5B [2]. NS5B is an RNA-dependent RNA polymerase, which plays a central role in viral genome replication [1].

HCV is a major causative agent of liver diseases such as chronic hepatitis, liver cirrhosis and hepatocellular carcinoma [3]. HCV infection constitutes a serious health problem worldwide, affecting approximately 170 million individuals and causing approximately 280 000 deaths a year [4]. Currently, anti-HCV therapy has been restricted mainly to treatment with interferon (IFN) or pegylated-IFN alone or in combination with ribavirin (RBV) [4]. Since this treatment eliminates the virus persistently from only about half of HCV-infected patients, however, alternative approaches to eliminating HCV infection are greatly needed [5]. To develop new strategies against HCV, it is essential to analyse the mechanism of HCV replication as well as screening for anti-HCV compounds.

HCV subgenomic replicon system is a system to investigate HCV genome replication in cell culture [6,7]. From biochemical analyses using this replicon system, it is proposed that HCV genome replication occurs in the replication complex (RC), which includes the viral genome RNA and HCV NS proteins [8–12]. The RC is surrounded by a membrane structure and is protected from cellular proteases and nucleases, which seems to contribute to the increase of replication efficiency. The RC is formed on intracellular membranes, including the endoplasmic reticulum (ER). Electron

*Corresponding author: K. Watashi, Laboratory of Human Tumor Viruses, Institute for Virus Research, Kyoto University 53 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan.
E-mail: kwatashi@virus.kyoto-u.ac.jp

Abbreviations used
HCV, hepatitis C virus; IFN, interferon; CsA, cyclosporin A; CyP, cyclophilin; E, envelope; NS, nonstructural protein; RBV, ribavirin; RC, replication complex; ER, endoplasmic reticulum; HMG, 3-hydroxy-3-methyglutaryl; CN, calcineurin; P-gp, P-glycoprotein

Copyright © 2007 John Wiley & Sons, Ltd.

microscopy analyses have demonstrated that HCV proteins induce alterations in membrane structures around the ER, which is related to the formation of the RC [9,13]. Genetic analyses have shown that the amino acid substitutions that increase replication efficiency often occur in the viral genome [14–16]. Although the mechanisms responsible for the increase of replication efficiency by such adaptive mutations are not well understood, the S2204I mutation within the NS5A coding region, one of the most frequently observed adaptive mutations, has been found to suppress hyperphosphorylation of NS5A and be related to replication efficiency [17]. Moreover, molecular biological analyses have shown a series of cellular factors interacting with HCV proteins that may be involved in HCV genome replication [18]. Thus, the mechanisms of HCV genome replication can be better understood by different approaches.

CHEMICAL GENETICS FOR INVESTIGATING HCV REPLICATION

Chemical genetics, which has been proposed and developed during the late 1990s, is a research field in which chemical compounds are used to understand and control cellular and physiological functions of protein [19]. This technique involves analysis of phenotypic changes of a target cell that occur upon treatment with a compound that binds to and changes the function of the target molecule, in a manner similar to introducing a mutation into the protein when using classical genetics techniques (see below and Figure 1). In addition to biochemical, molecular biological and virological techniques, chemical genetics approaches can be applied and be useful to analysis of the regulatory mechanisms of viral life cycles, which are regulated by not only the virus itself, but also various cellular factors. The experimental flowchart for such analysis is summarised in Figure 1. First, screening is undertaken to identify compounds that affect the activity of a virological phenotype, such as viral genome replication, cytopathic effect, viral adhesion to cells, etc. (Fig. 1A). Second, the cellular target of the compound is identified by biochemical and molecular biological assays (Figure 1B). Finally, by examining the relationship between the 'phenotype' and the 'target', the molecular mechanism underlying the viral life cycle can be elucidated (Figure 1C).

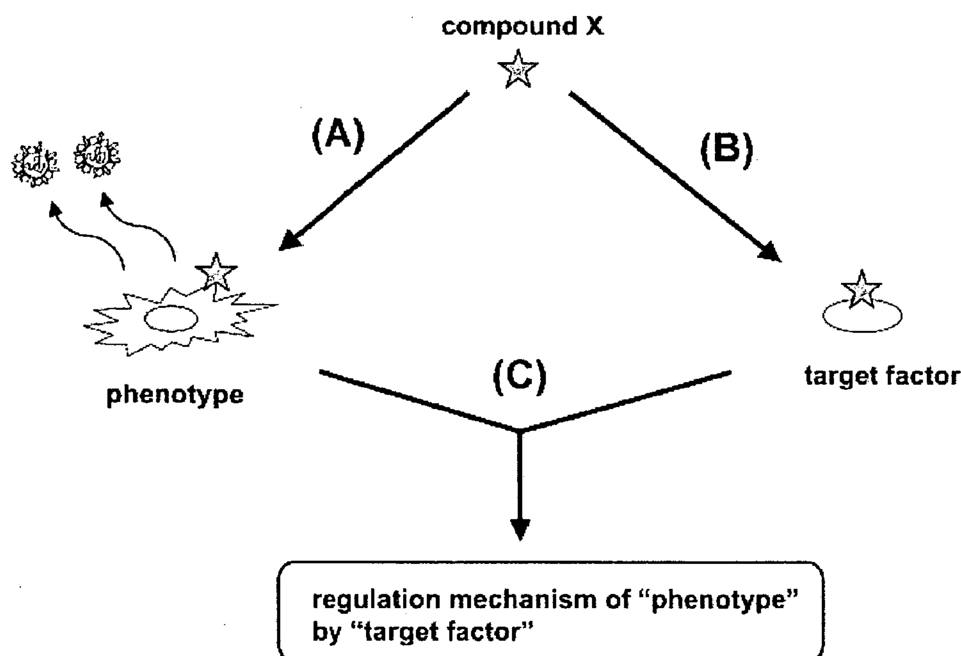


Figure 1. Schematic representation of chemical genetics analysis of viruses. (A) Screening for compounds that change viral phenotypes, such as viral genome replication. (B) Identification of the cellular target factor(s) of the compounds obtained in (A). (C) Analysis of the regulation of the 'phenotype' [observed in subpart (A)] by the 'target factor' [identified in subpart (B)]

Copyright © 2007 John Wiley & Sons, Ltd.

Mechanisms discovered using this method are expected to serve as targets for anti-viral strategies. Further, compounds found during screening may be useful as lead compounds for anti-viral drug development. Using such forward chemical genetics, cellular factors that play significant roles in HCV genome replication have been identified in at least three series of studies, as discussed below.

F-box and leucine-rich repeat protein 2 (FBL2)

The regulation of NS5A by FBL2 was demonstrated by studying the observation that lovastatin suppressed HCV genome replication. Ye *et al.* [20] reported that inhibitors of protein geranylgeranylation, including the 3-hydroxy-3-methyglutaryl (HMG) CoA reductase inhibitor lovastatin and an inhibitor of protein geranylgeranyl transferase I, suppressed HCV genome replication, accompanied by the dissolution of the RC. Kapadia and Chisari [21] also showed that lovastatin inhibited HCV genome replication. Addition of geranylgeraniol to lovastatin-treated cells restored viral replication [20]. From these results, the authors suggested that the geranylgeranylation of a host protein should be important for HCV genome replication. This geranylgeranylated protein has been identified as FBL2, a member of the F-box protein family [22]. FBL2 interacted with NS5A in a geranylgeranylation-dependent manner, and the knockdown of endogenous FBL2 or expression of a dominant negative form of FBL2 inhibited HCV genome replication. These results suggest that FBL2 regulates HCV genome replication through the geranylgeranylation-dependent interaction with NS5A. Further analysis is required to elucidate the mechanism by which FBL2 regulates the function of NS5A.

Sphingolipid

Screening using the subgenomic replicon system identified a lipophilic long-chain base compound, named NA255, from fungal metabolites as an inhibitor of HCV genome replication [23]. NA255 prevented the synthesis of sphingolipids, major components of the lipid rafts. It was suggested that sphingomyelin binds to NS5B and recruits it to the lipid rafts on the ER membrane, on which the HCV RC is assembled. NA255 was suggested to disrupt the association of HCV proteins to the RC.

Cyclophilin B

Through the forward chemical genetics approach, we found that an immunosuppressant cyclosporin A (CsA) possesses anti-HCV activity [24]. Analysis using CsA as a bioprobe suggested that cyclophilin (CyP) B, a cellular target of CsA, could play a critical role in HCV genome replication [25]. CyPB stimulated the RNA binding activity of NS5B via molecular interaction with NS5B and we showed that this regulatory mechanism could serve as a target for the development of anti-HCV agents. Our series of studies on CyPB are described below to illustrate the usefulness of the chemical genetics for analysing HCV life cycles.

CYCLOPHILIN B AS A REGULATOR OF HCV GENOME REPLICATION DEMONSTRATED BY CHEMICAL GENETICS

Suppression of HCV genome replication by cyclosporin A

Screening for compounds suppressing HCV genome replication was performed using the HCV subgenomic replicon system. Treatment with 100 IU/mL IFN-α for 7 days as a positive control decreased HCV RNA in the cells to around 1/400 (Figure 2A). Of many compounds tested, CsA, given at 1 µg/mL for 7 days, decreased HCV RNA to about 1/500 (Figure 2A) [24]. CsA also reduced the expression of HCV-encoded proteins to undetectable levels without affecting cellular protein expression (Figure 2B), and suppressed HCV RNA synthesis. These anti-viral effects were observed without cytotoxicity. CsA treatment inhibited the multiplication of HCV genomic RNA in a time course of *in vitro* infection experiment using the plasma derived from an HCV-infected patient [24]. These data indicate that CsA suppresses HCV genome replication in cell culture.

CyPB plays an important role in HCV genome replication

The next step was to identify the CsA target factor involved in HCV genome replication as shown in Figure 1B. CsA has three major cellular targets: CyP, the calcineurin (CN)/NF-AT pathway, and P-glycoprotein (P-gp) [25–27]. CsA binds

Copyright © 2007 John Wiley & Sons, Ltd.

DOI: 10.1002/rmv

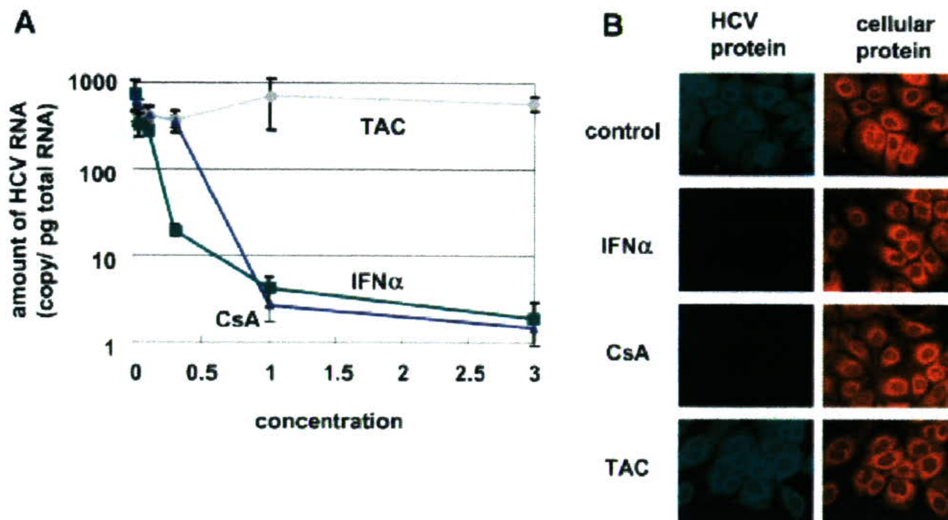


Figure 2. Suppression of HCV genome replication by cyclosporin A (CsA). (A) HCV RNA was quantified by real time RT-PCR analysis in cells treated with interferon (IFN)α (× 100 IU/mL), CsA (μg/mL), or tacrolimus (TAC) (μg/mL) at the indicated doses for 7 days. (B) HCV NS5A as an HCV protein (green) and PDI as a cellular protein (red) were detected by indirect immunofluorescence analysis in cells treated without (control) or with 100 IU/mL IFNα, 1 μg/mL CsA or 1 μg/mL TAC for 7 days. (Reprinted from reference [24] by American Association for the study of Liver Diseases, with permission from Elsevier)

directly to peptidyl prolyl cis–trans isomerase, CyP, and inhibits its enzymatic activity. The CsA/CyP complex subsequently interacts with and inhibits CN, a phosphatase involved in the activation of transcription factor NF-AT. The enzymatic inhibition of CN by CsA prevents the nuclear translocation and activation of NF-AT, which is essential for the T-cell immune response. The immunosuppressive function of CsA is mediated by the inhibition of the CN/NF-AT pathway. As the third action, CsA inhibits the activity of P-gp, a transporter on the plasma membrane. To determine which inhibitory action of CsA mediates the suppression of HCV genome replication, a series of CsA derivatives with activity against only some targets was used. Inhibition of CyP, but neither the inhibition of the CN/NF-AT pathway nor the P-gp activity, correlated with the suppression of HCV genome replication [25]. In support of this, other CyP inhibitors, sanglifehrins, also decreased HCV RNA in cells [25]. Thus, CyP plays a role in HCV genome replication.

CyP is a protein family consisting of at least 15 subtypes in mammals [28]. RNAi analysis showed that the specific knockdown of CyPA, CyPC, CyPE or CyPH did not affect HCV genome replication, while downregulation of CyPB decreased replication [25]. This indicates that CyPB specifically regulates HCV genome replication.

CyPB stimulates the RNA binding activity of NS5B

Finally, experiments were performed to elucidate the molecular mechanism by which CyPB associates with the viral life cycle (Figure 1C). First, we analysed binding between CyPB and HCV proteins. GST pull-down assays showed that recombinant CyPB interacted with NS5B but not with NS3, NS4B or NS5A protein [25]. In contrast, CyPA did not bind any HCV protein. Endogenous CyPB and NS5B were associated in co-immunoprecipitation analysis. This CyPB–NS5B interaction was dissociated following treatment with CsA. It is known that both NS5B and the HCV RNA are mainly localised to the cytoplasmic surface of the ER [25]. It was demonstrated that a fraction of CyPB was also localised on the cytoplasmic face of the ER membrane, where it co-localised with NS5B and HCV RNA [25]. Moreover, NS5B and CyPB formed a complex with HCV RNA in cells. Functionally, it was shown that CyPB regulated the RNA binding activity of NS5B. Specific knockdown of CyPB or treatment with CsA reduced the RNA-bound NS5B in the RC. Further, *in vitro*

Copyright © 2007 John Wiley & Sons, Ltd.

DOI: 10.1002/rmv

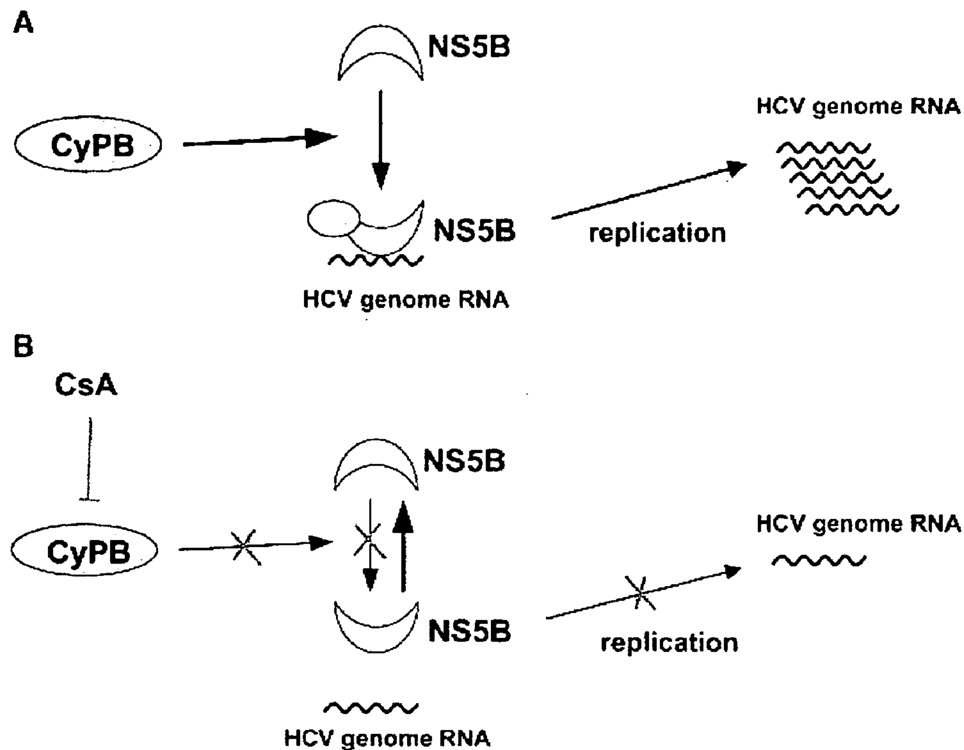


Figure 3. Schematic representation of the regulation of NS5B by cyclophilin (CyP) B. (A) In the normal state (in the absence of CsA), HCV RNA polymerase NS5B interacts with cellular CyPB. Through this interaction, the RNA binding activity of NS5B is increased, resulting in efficient replication of the HCV genome. (B) In the presence of CsA, the association of NS5B with CyPB is disrupted. The activity of NS5B is decreased and the level of HCV genome replication is reduced. Thus, CsA suppresses HCV genome replication

RNA binding assays demonstrated that the addition of recombinant CyPB augmented the RNA binding affinity of NS5B.

These findings are summarised in Figure 3. In the normal state (without CsA) (Figure 3A), NS5B interacts with cellular CyPB. This interaction augments the RNA binding activity of NS5B and drives efficient HCV genome replication. In the presence of CsA (Figure 3B), however, the association of CyPB and NS5B is disrupted. Without the stimulation of RNA binding activity by CyPB, NS5B functions less efficiently in replication, resulting in reduced HCV genome replication. Thus, regulation of the NS5B RNA binding activity by CyPB is essential for efficient replication of the HCV genome [25].

CyP as a target for the development of anti-viral agents

As described above, the regulation of NS5B by CyPB has now been revealed. Further studies were performed to determine whether this mechanism might be targeted for the development of anti-HCV agents. Although CsA had strong anti-HCV activity in hepatocytes, this compound simultaneously exerts immunosuppressive activity by acting on T cells. Therefore, CsA itself may be a double-edged sword in the settings of the clinical treatment of HCV. Ideally, compounds might be found that lack immunosuppressive activity and that more strongly inhibit the CyPB–NS5B interaction. NIM811, a CsA derivative in which MeLeu at Position 4 is replaced by MeIle, fulfils both of these criteria [29,30]. This compound completely lacks immunosuppressive function and has an approximately twofold stronger binding affinity to CyP [29]. The suppressive effect of NIM811 on HCV genome replication was greater than that of CsA, especially at relatively low doses as shown in Figure 4A [31,32]. Cotreatment with NIM811 and IFN-α achieved an approximately 2-log further reduction of replication compared with IFN-α treatment alone (Figure 4B). A 3-week treatment of the cells with NIM811 reduced the intra-

Copyright © 2007 John Wiley & Sons, Ltd.

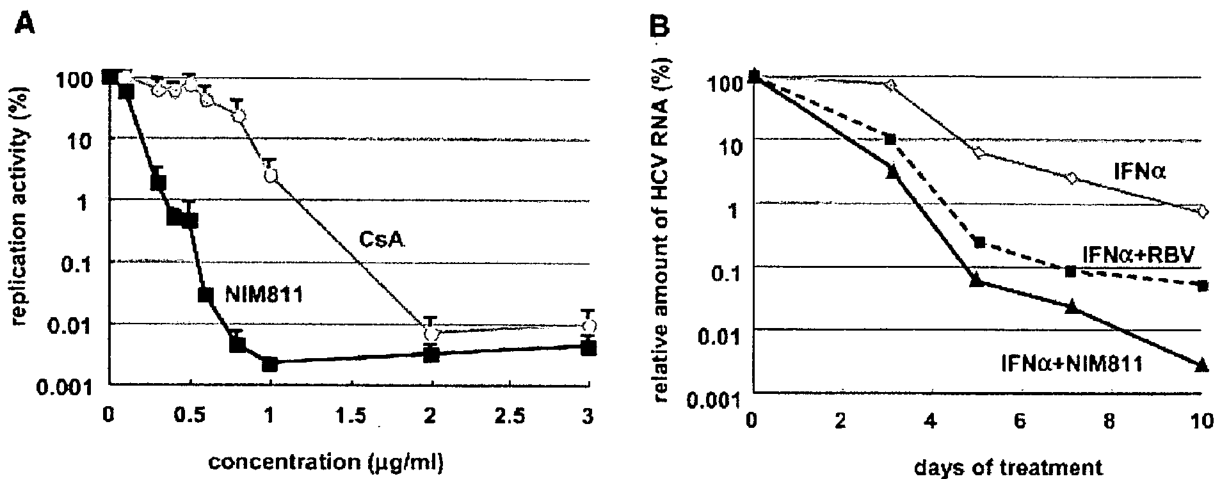


Figure 4. Suppression of HCV genome replication by NIM811. (A) Replication was monitored by the activity of luciferase expressed from a luciferase-containing replicon in cells treated with CsA or NIM811 at the indicated doses for 7 days. (B) HCV RNA was quantified in cells treated with 10 IU/mL IFNα alone, 10 IU/mL IFNα and 200 μM RBV or 10 IU/mL IFNα and 1 μg/mL NIM811 for the indicated number of days. (Reprinted from reference [31], with permission from Elsevier)

cellular HCV RNA below the detection level. Cotreatment with 1 μg/mL NIM811 and 10 IU/mL IFN-α for 10 days showed more than a 4-log reduction of HCV RNA, a far more dramatic reduction than that seen with IFN-α and RBV (Figure 4B) [31]. These results indicate that CyP can serve as a molecular target for the development of anti-HCV agents.

Effects of CyP inhibitors on HCV-infected patients

The anti-HCV effect of CsA has also been reported by several other groups [33–37]. Moreover, our study has elucidated the molecular mechanism of the regulation of HCV genome replication by CyPB. By targeting this mechanism, we identified NIM811 as a candidate anti-HCV agent. Clinical trials to evaluate NIM811 [31,35] as well as DEBIO-025, another non-immunosuppressive CsA derivative [37], are now underway. In regards to the effect of CsA itself on HCV-infected patients, it has been reported that cotreatment of IFN-α and CsA increases the sustained virological response compared with IFN-α monotherapy (55 vs. 32%) [38]. It was also reported that CsA treatment decreased HCV RNA to undetectable levels in 5/8 of patients who had HCV recurrence following liver transplantation and did not respond to IFN-α and RBV cotreatment [39]. Thus, the effects of cyclosporins on patients seem to be expectable, but these effects must be extensively investigated.

CONCLUSION

As discussed above, chemical compound-based virological analyses, which may be called 'chemical virology', can lead to novel findings regarding the mechanisms of the viral life cycle. Such analytical strategies can identify (1) viral replication mechanisms, (2) new function of cellular proteins, (3) molecular targets useful for the development of anti-viral compounds and (4) lead compounds for anti-viral agents. Among the many methods used to study viruses, chemical genetics may become a powerful tool to understand viral mechanisms at the molecular level.

ACKNOWLEDGEMENTS

This work was supported by Grants-in-Aid for cancer research and for the second-term comprehensive 10-year strategies for cancer control from the Ministry of Health, Labour and Welfare, by Grants-in-Aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology, by Grants-in-Aid for the Research for the Future program from the Japanese Society for the Promotion of Science and by Grants-in-Aid

Copyright © 2007 John Wiley & Sons, Ltd.

for the Program for Promotion of Fundamental Studies in Health Science from the Organization for Pharmaceutical Safety.

REFERENCES

1. Bartenschlager R, Lohmann V. Novel cell culture systems for the hepatitis C virus. *Antiviral Res* 2001; **52**: 1–17.
2. Hijikata M, Kato N, Ootsuyama Y, Nakagawa M, Shimotohno K. Gene mapping of the putative structural region of the hepatitis C virus genome by *in vitro* processing analysis. *Proc Natl Acad Sci USA* 1991; **88**: 5547–5551.
3. Liang TJ, Jeffers LJ, Reddy KR, *et al.* Viral pathogenesis of hepatocellular carcinoma in the United States. *Hepatology* 1993; **18**: 1326–1333.
4. Poynard T, Yuen MF, Ratziu V, Lai CL. Viral hepatitis C. *Lancet* 2003; **362**: 2095–2100.
5. Di Bisceglie AM, McHutchison J, Rice CM. New therapeutic strategies for hepatitis C. *Hepatology* 2002; **35**: 224–231.
6. Blight KJ, Kolykhalov AA, Rice CM. Efficient initiation of HCV RNA replication in cell culture. *Science* 2000; **290**: 1972–1974.
7. Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 1999; **285**: 110–113.
8. Aizaki H, Lee KJ, Sung VM, Ishiko H, Lai MM. Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts. *Virology* 2004; **324**: 450–461.
9. Egger D, Wolk B, Gosert R, *et al.* Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J Virol* 2002; **76**: 5974–5984.
10. El-Hage N, Luo G. Replication of hepatitis C virus RNA occurs in a membrane-bound replication complex containing nonstructural viral proteins and RNA. *J Gen Virol* 2003; **84**: 2761–2769.
11. Gosert R, Egger D, Lohmann V, *et al.* Identification of the hepatitis C virus RNA replication complex in Huh-7 cells harboring subgenomic replicons. *J Virol* 2003; **77**: 5487–5492.
12. Miyanari Y, Hijikata M, Yamaji M, Hosaka M, Takahashi H, Shimotohno K. Hepatitis C virus non-structural proteins in the probable membranous compartment function in viral genome replication. *J Biol Chem* 2003; **278**: 50301–50308.
13. Moradpour D, Gosert R, Egger D, Penin F, Blum HE, Bienz K. Membrane association of hepatitis C virus nonstructural proteins and identification of the membrane alteration that harbors the viral replication complex. *Antiviral Res* 2003; **60**: 103–109.
14. Krieger N, Lohmann V, Bartenschlager R. Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations. *J Virol* 2001; **75**: 4614–4624.
15. Lohmann V, Korner F, Dobierzewska A, Bartenschlager R. Mutations in hepatitis C virus RNAs conferring cell culture adaptation. *J Virol* 2001; **75**: 1437–1449.
16. Yi M, Lemon SM. Adaptive mutations producing efficient replication of genotype 1a hepatitis C virus RNA in normal Huh7 cells. *J Virol* 2004; **78**: 7904–7915.
17. Evans MJ, Rice CM, Goff SP. Phosphorylation of hepatitis C virus nonstructural protein 5A modulates its protein interactions and viral RNA replication. *Proc Natl Acad Sci USA* 2004; **101**: 13038–13043.
18. Tellinghuisen TL, Rice CM. Interaction between hepatitis C virus proteins and host cell factors. *Curr Opin Microbiol* 2002; **5**: 419–427.
19. Schreiber SL. Chemical genetics resulting from a passion for synthetic organic chemistry. *Bioorg Med Chem* 1998; **6**: 1127–1152.
20. Ye J, Wang C, Sumpter R Jr., Brown MS, Goldstein JL, Gale M Jr. Disruption of hepatitis C virus RNA replication through inhibition of host protein geranylgeranylation. *Proc Natl Acad Sci USA* 2003; **100**: 15865–15870.
21. Kapadia SB, Chisari FV. Hepatitis C virus RNA replication is regulated by host geranylgeranylation and fatty acids. *Proc Natl Acad Sci USA* 2005; **102**: 2561–2566.
22. Wang C, Gale M Jr., Keller BC, *et al.* Identification of FBL2 as a geranylgeranylated cellular protein required for hepatitis C virus RNA replication. *Mol Cell* 2005; **18**: 425–434.
23. Sakamoto H, Okamoto K, Aoki M, *et al.* Host sphingolipid biosynthesis as a target for hepatitis C virus therapy. *Nat Chem Biol* 2005; **1**: 333–337.
24. Watashi K, Hijikata M, Hosaka M, Yamaji M, Shimotohno K. Cyclosporin A suppresses replication of hepatitis C virus genome in cultured hepatocytes. *Hepatology* 2003; **38**: 1282–1288.
25. Watashi K, Ishii N, Hijikata M, *et al.* Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. *Mol Cell* 2005; **19**: 111–122.
26. Loor F, Tiberghien F, Wenandy T, Didier A, Traber R. Cyclosporins: structure–activity relationships for the inhibition of the human MDR1 P-glycoprotein ABC transporter. *J Med Chem* 2002; **45**: 4598–4612.
27. Wang P, Heitman J. The cyclophilins. *Genome Biol* 2005; **6**: 226.
28. Waldmeier PC, Zimmermann K, Qian T, Tintelnot-Blomley M, Lemasters JJ. Cyclophilin D as a drug target. *Curr Med Chem* 2003; **10**: 1485–1506.
29. Billich A, Hammerschmid F, Peichl P, *et al.* Mode of action of SDZ NIM 811, a nonimmunosuppressive

Copyright © 2007 John Wiley & Sons, Ltd.