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

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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

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[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.

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

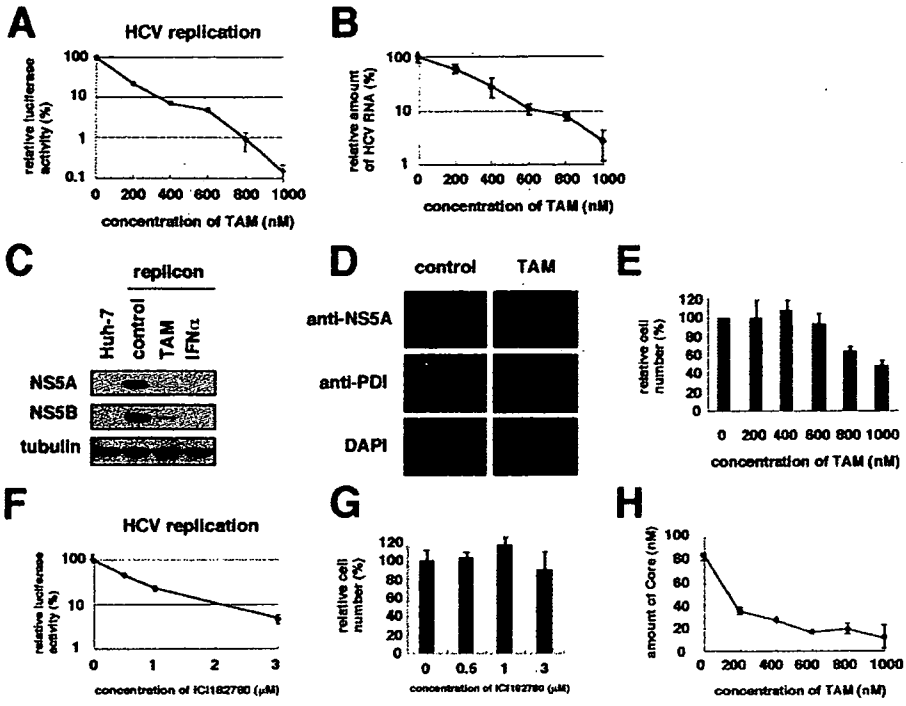

FIGURE 1. **TAM suppressed the replication of the HCV genome.** *A*, luciferase activities were measured using the LucNeo#2 cells, which carried a luciferase-containing replicon RNA, upon treatment with TAM at the indicated doses for 7 days. Relative luciferase activities are plotted against the concentrations 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

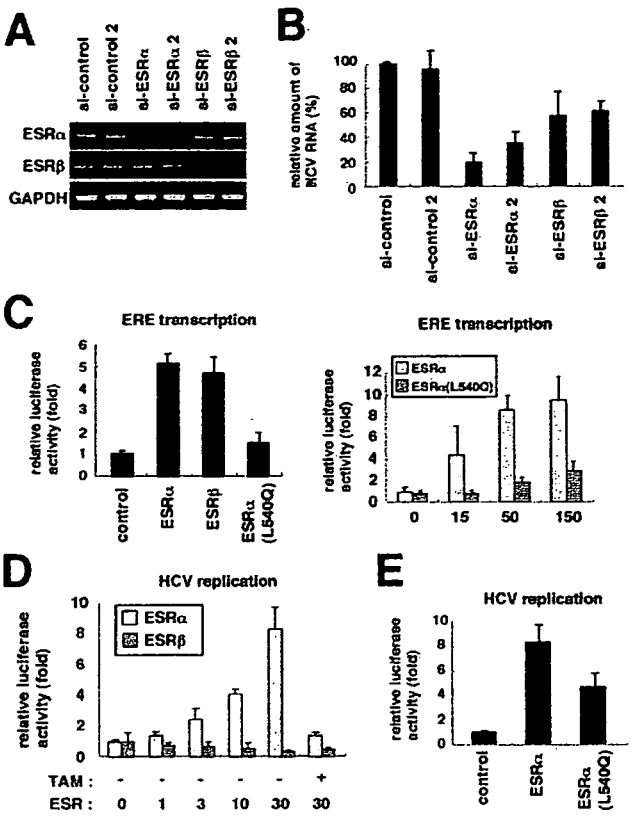

FIGURE 2. **ESR was involved in HCV genome replication.** *A*, specific knockdown of endogenous ESRα and ESRβ. RT-PCR analysis was performed to detect the expression of ESRα, ESRβ, and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as an internal control in the cells transfected with siRNA recognizing 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.

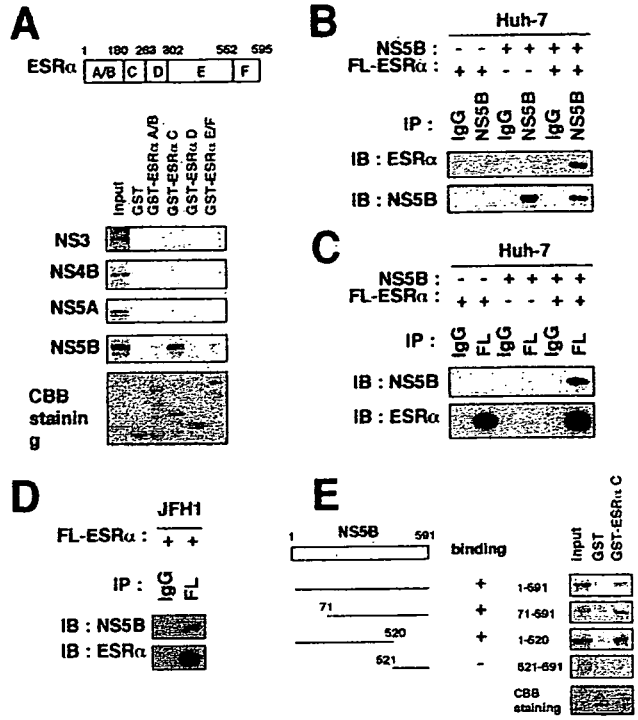

FIGURE 3. **ESRα interacted with HCV NS5B.** *A*, *top*, schematic representation of the primary structure of ESRα. ESRα consists of domains A–F. The amino acid numbers are also shown. *Bottom*, GST pull-down assays were performed using the recombinant proteins of the A/B, C, D, and E/F domain of ESRα 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

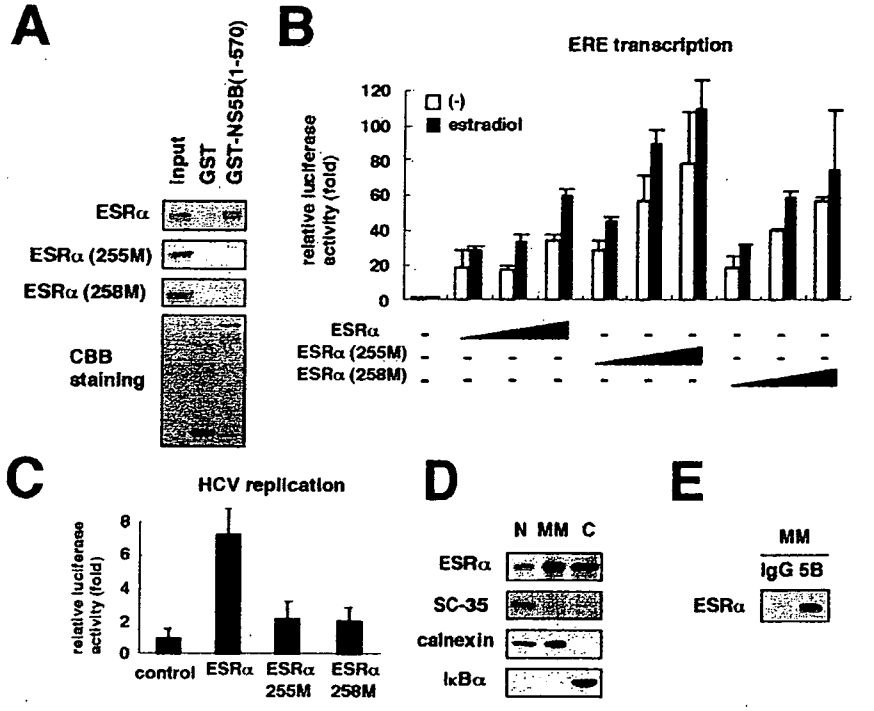

FIGURE 4. **The interaction of NS5B mediated the regulation of HCV genome replication by ESRα.** *A*, GST pull-down assays were performed as described in Fig. 3*A* using the wild type ESRα or point mutant of ESRα, ESRα(255M), and ESRα(258M). *B*, the mutation within ESRα(255M) and ESRα(258M) did not 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,

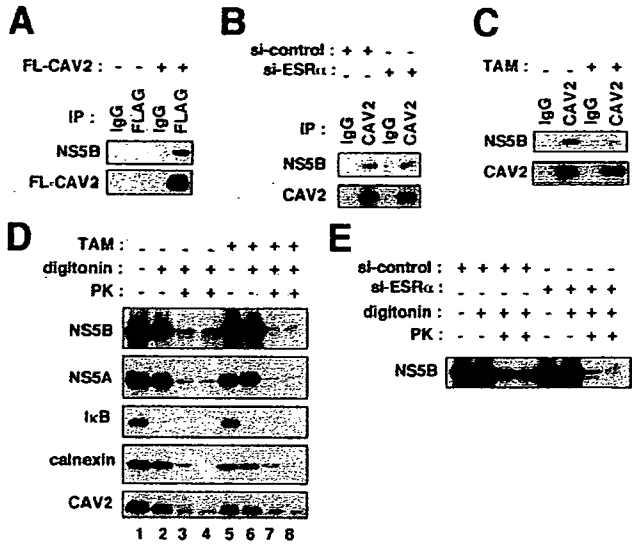

FIGURE 5. **ESRα promoted the participation of NS5B in HCV RC.** *A–C*, a coimmunoprecipitation assay (*IP*) was performed with anti-FLAG (*A*), anti-CAV2 (*B* and *C*) antibody, or mouse normal IgG from the lysates of the cells transfected without or with FLAG-tagged CAV2 (*A*), transfected with si-control 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.

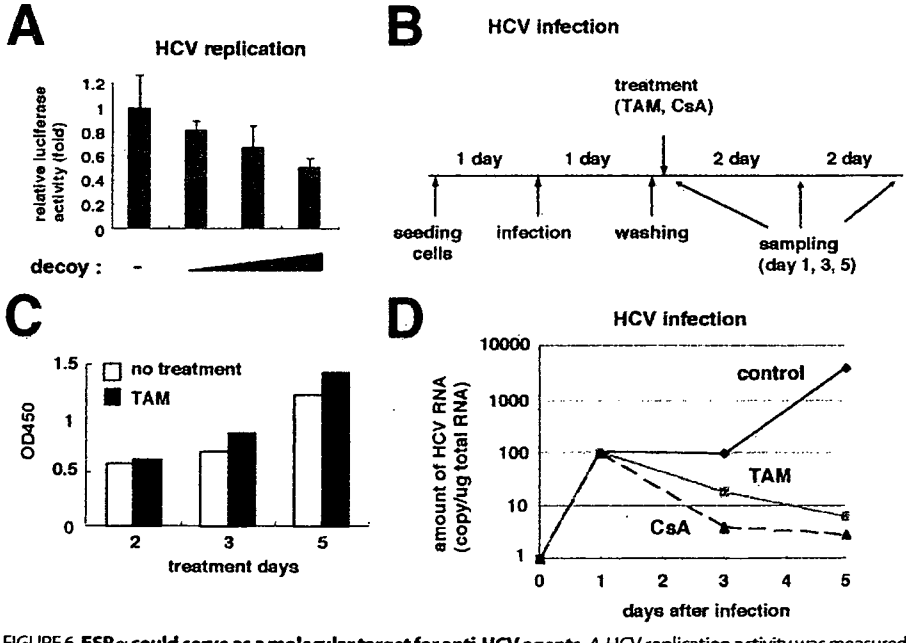

FIGURE 6. **ESRα could serve as a molecular target for anti-HCV agents.** *A*, HCV replication activity was measured by quantifying the luciferase activity as described in the legend to Fig. 2*D* in the cells overexpressing a decoy peptide consisting of the C domain of ESRα. *B*, experimental scheme 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

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.

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Cyclophilin B Is a Functional Regulator of Hepatitis C Virus RNA Polymerase

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Summary

Viruses depend on host-derived factors for their efficient genome replication. Here, we demonstrate that a cellular peptidyl-prolyl *cis-trans* isomerase (PPIase), cyclophilin B (CyPB), is critical for the efficient replication of the hepatitis C virus (HCV) genome. CyPB interacted with the HCV RNA polymerase NS5B to directly stimulate its RNA binding activity. Both the RNA interference (RNAi)-mediated reduction of endogenous CyPB expression and the induced loss of NS5B binding to CyPB decreased the levels of HCV replication. Thus, CyPB functions as a stimulatory regulator of NS5B in HCV replication machinery. This regulation mechanism for viral replication identifies CyPB as a target for antiviral therapeutic strategies.

Introduction

CyP was originally discovered as a cellular factor with high affinity for the immunosuppressant cyclosporin A (CsA) (Handschumacher et al., 1984; Schreiber, 1991). CyPs are a family of PPIases, which catalyze the *cis-trans* interconversion of peptide bonds amino-terminal to proline residues, facilitating changes in protein conformation (Fischer et al., 1998; Takahashi, 1999; Waldmeier et al., 2003). In mammals, CyPs include more than ten subtypes (Braaten and Luban, 2001; Takahashi, 1999; Waldmeier et al., 2003). CyP family members function in numerous cellular processes, including transcriptional regulation, immune response, protein secretion, and mitochondrial function (Braaten and Luban, 2001; Brazin et al., 2002; Colgan et al., 2004; Duina et al., 1996; Rycyzyn and Clevenger, 2002; Waldmeier et al., 2003). In this study, we report the involvement of CyPB in HCV genome replication and propose its molecular mechanism.

HCV is a major causative agent of liver diseases such as chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (Liang et al., 1993). HCV, a member of the Flaviviridae family, has a positive-strand RNA genome. The genome encodes a large precursor polyprotein, which is cleaved by host and viral proteases to generate at least ten functional viral proteins: core, envelope (E)1, E2, p7, nonstructural protein (NS)2, NS3, NS4A, NS4B, NS5A, and NS5B (Grakoui et al., 1993). NS5B is an RNA-dependent RNA polymerase (RdRp), which is crucial in viral genome replication (Bartenschlager and Lohmann, 2001; Tellinghuisen and Rice, 2002). Until recently, research into HCV genome replication has been hampered by the lack of a cell culture system that can efficiently reproduce HCV infection and proliferation. Even now, we do not have an in vitro system that efficiently produces infectious HCV viral particles. Lohmann et al. previously established the HCV subgenomic replicon system in which the HCV subgenomic RNA autonomously replicates in Huh-7 cells (Lohmann et al., 1999), which we refer to throughout this report as HCV replicon cells. This replicon system enables us to investigate HCV genome replication in a cell culture system. By using this system, several groups have recently reported that HCV genome replication occurs in a distinct, subcellular replication complex (RC), which includes viral genome RNA and HCV proteins (Aizaki et al., 2004; Egger et al., 2002; El-Hage and Luo, 2003; Gosert et al., 2003; Miyanari et al., 2003), in a manner similar to other RNA viruses (Noueiry and Ahlquist, 2003). The RC is formed on intracellular membranes, including the endoplasmic reticulum (ER) membrane. This membrane structure protects the RC from external nucleases and proteases, which seems advantageous for efficient viral genome replication (Aizaki et al., 2004; El-Hage and Luo, 2003; Miyanari et al., 2003). However, the role of cellular factors directly regulating the activity of the RC has remained unclear.

We and other groups have previously reported that CsA has the potential to suppress HCV genome replication (Nakagawa et al., 2004; Watashi et al., 2003a). In this study, we used CsA as a bioprobe to identify cellular factors involved in HCV genome replication. This strategy revealed that CyPB, one of the cellular targets of CsA, was required for the efficient replication of the HCV genome in the cells. Investigation into the molecular mechanism showed that CyPB interacted with NS5B and directly promoted its RNA binding activity. The CyPB-NS5B association is a potent target for the development of an antiviral therapeutics.

Results

Functional Inhibition of CyP Suppressed HCV Genome Replication

As HCV does not efficiently infect cultured cells, we studied viral replication by using HCV replicon cells. We have previously reported that CsA treatment suppressed HCV genome replication (Watashi et al., 2003a). A number of studies have shown that CsA has three major cellular targets: CyP, the calcineurin (CN)-NF-AT pathway, and P-glycoprotein (P-gp) (Loor et al., 2002; Takahashi, 1999) (Figure 1A). We initially determined which of these cellular targets of CsA were involved in HCV genome replication. We examined the effect of several CsA mutants (functional characteristics are shown in Figure 1A) on HCV genome replication by real-time RT-PCR and immunoblot analyses. CsA, (8′-OH-MeBmt[1])Cs,

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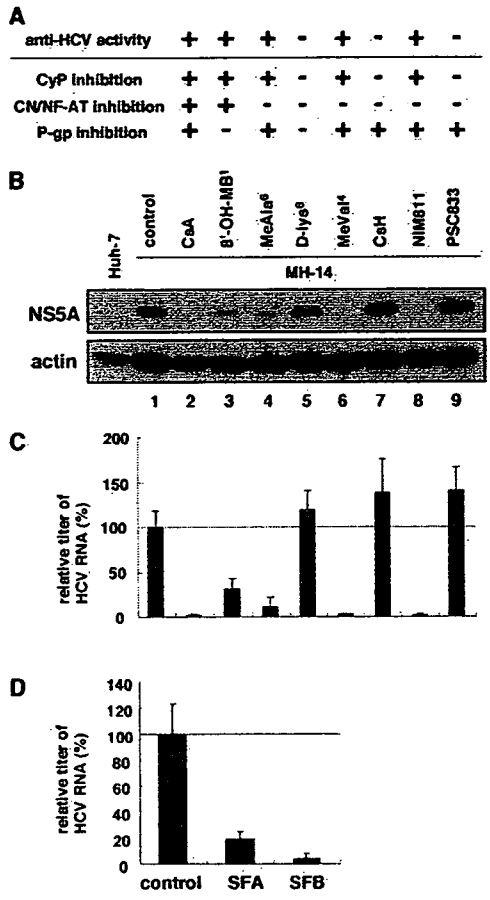

Figure 1. Functional Inhibition of Cyclophilin Suppressed HCV Genome Replication

(A) Characteristic features of cyclosporin A (CsA) and its derivatives, 8'-OH-MeBmt[1]-Cs (8'-OH-MB[1]), MeAla[6]-Cs (MeAla[6]), D-lys[8]-Cs (D-lys[8]), MeVal[4]-Cs (MeVal[4]), cyclosporin H (CsH), NIM811, and PSC833. "Anti-HCV activity" summarizes the potential for each derivative to decrease HCV replication, defined as + (effective), or – (not effective). The inhibition activity to cellular cyclophilin (CyP), calcineurin (CN)-NF-AT pathway, and P-glycoprotein (P-gp) is similarly defined by ±.

(B and C) MH-14 cells, carrying the HCV subgenomic replicon, were treated with 1 μg/ml CsA or its derivatives for seven days. Levels of NS5A (B, top) and actin as an internal control (B, bottom) in these cells were detected by immunoblot analysis. HCV RNA titers were quantified by real-time RT-PCR analysis, and relative titer is shown in (C). The data represent the means of three independent experiments. Error bars represent the SD.

(D) HCV RNA titers in MH-14 cells treated with 3 μg/ml sanglifehrin (SF)A and SFB for seven days were measured as described in (C). Error bars represent the SD.

(MeAla[6])Cs, (MeVal[4])Cs, and NIM811 had anti-HCV activity, decreasing both HCV protein production and HCV RNA titer in HCV replicon cells (Figures 1B and 1C, lanes 2, 3, 4, 6, and 8). In contrast, (D-lys[8])Cs, CsH, and PSC833 had little effect on HCV genome replication (Figures 1B and 1C, lanes 5, 7, and 9). A comparison of anti-HCV activity with the characteristics of these CsA derivatives (Figure 1A) indicates that the anti-HCV activity correlated with inhibition of CyP, but not the CN-NF-AT pathway or P-gp. Sanglifehrin (SF)A and SFB, additional CyP inhibitors (Sanglier et al., 1999), also decreased HCV RNA titers in replicon cells (Figure 1D), suggesting that CyP is linked to HCV genome replication.

CyPB Played a Significant Role in HCV Genome Replication

In mammalian cells, the CyP family contains more than ten subfamilies (Braaten and Luban, 2001; Takahashi, 1999; Waldmeier et al., 2003). CyPA and CyPB are the most abundant subtypes (Waldmeier et al., 2003). We applied RNAi methods to investigate the role of CyPs in HCV genome replication. By introducing small interfering RNA (siRNA) designed to recognize several CyP subtypes (si-CyP[broad]) (see Experimental Procedures), both endogenous CyPA and CyPB proteins, but not actin, were downregulated in replicon cells (Figure 2A, lane 2). The titer of HCV RNA in these cells was diminished to approximately one-fifth of the levels seen in the cells transfected with a randomized siRNA (si-control) (Figure 2B). Specific knockdown of endogenous CyPB also significantly reduced HCV RNA titer, whereas depletion of endogenous CyPA did not (Figure 2A, lanes 3 and 4, and Figure 2B). Depletion of CyPB did not affect cell proliferation (data not shown). A similar result was obtained in another HCV replicon cell clone, 50-1 (Watashi et al., 2003a) (data not shown). Introduction of siRNAs specific for other CyP family members, CyPC, CyPE, or CyPH (Figure 2C), had no effect on HCV RNA titer in replicon cells (Figure 2D). These data suggest that among the CyP family, CyPB is specifically linked to HCV genome replication. Also, in a system in which HCV genome replication can be monitored by luciferase activity (Murata et al., 2005) (Figure 2E), depletion of CyPB inhibited HCV genome replication (Figure 2F). This effect was reversed by the exogenous overproduction of FLAG-tagged CyPB (Figure 2G). These results further indicate that CyPB plays a significant role in HCV genome replication. A weak, nonspecific cross-silencing effect of si-CyPA on CyPB (Figure 2A, lane 3) did not affect HCV RNA titer, suggesting the possibility that the expression level of CyPB has a threshold to support HCV genome replication.

CyPB Interacted with HCV NS5B

To clarify the mechanisms underlying CyPB regulation of HCV genome replication, we analyzed the molecular interaction between CyPB and HCV proteins expressed in HCV replicon cells by GST pull-down. Human immunodeficiency virus (HIV)-1 Gag, which binds CyP family proteins (see Discussion) (Braaten and Luban, 2001; Luban et al., 1993), was used as a positive control (Figure 3A, panel e). As a result, the GST fusion of CyPB (GST-CyPB) coprecipitated only the NS5B viral protein (Figure 3A, panel d). The interaction between NS5B and CyPB was confirmed by immunoprecipitation of NS5B from replicon cells ectopically expressing CyPB (Figure 3B, lane 4). In addition, endogenous CyPB copurified with NS5B from replicon cells (Figure 3C, lane 2). Endogenous CyPB also associated with exogenously expressed NS5B in Huh-7 cells (Figure 3D, lane 4). In contrast, we could not detect the binding of CyPA to NS5B by using either a GST pull-down assay or an immuno-

precipitation assay (Figure 3A, panel d and Figure 3B, lane 8). These results suggest that CyPB specifically interacts with NS5B. CyPBΔPPI, which was previously shown to lose the catalytic activity (Rycyzyn and Clevenger, 2002), had a strong binding to NS5B (Figure 3B, lane 6).

GST pull-down assays demonstrated that the CyPB-NS5B interaction was reduced in a dose-dependent manner after treatment with CsA, which is an inhibitor of HCV genome replication (Figure 3E). Immunoprecipitations demonstrated that CyPB completely dissociated from NS5B in the presence of CsA (Figure 3F, lanes 2 and 4). We then determined the region of the NS5B molecule responsible for binding to CyPB by deletion analysis. The region comprising amino acids (aa) 71–591 of NS5B, which is deficient in RNA binding (Ishii et al., 1999), coprecipitated with GST-CyPB (Figure 3G, panel b). 401–591 and 521–591 aa, but not 201–400 aa, regions could be precipitated with GST-CyPB (Figure 3G, panels c–e). These results indicate that the carboxy (C)-terminal region of NS5B (521–591) interacts with CyPB. On the other hand, aa 1–570, which lack the C-terminal hydrophobic region, had little affinity for GST-CyPB (Figure 3G, panel f). Analysis of additional deletion mutants (Figure 3G, panels g–i) suggests that the whole 521–591 aa region rather than only the C-terminal 21 region (571–591 aa) of NS5B is likely to be important for the interaction with CyPB.

CyPB Associated with NS5B, which Is Functional for HCV Genome Replication

We next examined the subcellular localization of CyPB. In contrast to CyPA, which exhibited a diffuse distribution throughout the cell (Figure 4A, panel a), CyPB concentrated in the perinuclear region (Figure 4A, panel d). CyPB was colocalized with NS5B (Figure 4A, panels c–e), which reportedly resides on the cytoplasmic face of the ER membrane (Schmidt-Mende et al., 2001). To investigate whether CyPB is also located on the cytoplasmic face of the intracellular membrane, we next performed modified immunofluorescence analysis in which we pretreated the cells with digitonin to permeabilize the plasma membrane, but not intracellular membranes, followed by washing out the cytosol (see Experimental Procedures) (Watashi et al., 2001). PDI, a protein in the lumen of the ER, was not detected in this assay (Figure 4B, panel g), whereas CyPB as well as NS5B could be detected around the perinuclear region (Figure 4B, panels i and k). Also in Huh-7 cells, CyPB was observed in the perinuclear region (Figure 4B, panel o). This suggests that a fraction of CyPB is located on the cytoplasmic face of the intracellular membrane (see Discussion).

We labeled the site of newly synthesized HCV RNA by treating the cells with actinomycin D followed by addition of bromouridine (BrU) (El-Hage and Luo, 2003; Gosert et al., 2003; Restrepo-Hartwig and Ahlquist, 1996). In treated replicon cells, labeling was detected in the perinuclear region as previously reported (El-Hage and Luo, 2003; Gosert et al., 2003) (Figure 4A, panel q, shown by green), whereas no signal was observed in Huh-7 cells or replicon cells in the absence of BrU (Figure 4A, panels p and r). This signal merged with the localization of both NS5B and CyPB (Figure 4A, panels g–l).

In cells, a fraction of NS5B is involved in viral genome replication, but another fraction is not (Aizaki et al., 2004; El-Hage and Luo, 2003; Miyanari et al., 2003). These two fractions can be distinguished by digitonin/protease treatment; NS5B that does not function in HCV genome replication is sensitive to digitonin/protease treatment, whereas that participating in the viral RC and functioning for replication is protected from digitonin/protease digestion. After treatment with digitonin/proteinase K followed by washing and permeabilization with Triton X-100 (effective digitonin permeabilization and proteinase K digestion were confirmed by the complete loss of cytoplasmic protein IκBα detection [data not shown]), the signal intensities of NS5B and CyPB staining were reduced. Even under these conditions, a portion of NS5B, CyPB, and BrU was still detected merging in the perinuclear region (Figure 4A, panels m–o, and data not shown), although CyPB in the lumen of the ER could also be detected in this system (the localization of the NS5B-CyPB interaction is discussed further below). By using replicon cells treated with digitonin/proteinase K, NS5B and endogenous CyPB still coprecipitated (data not shown). Moreover, HCV RNA copurified with anti-CyPB as well as anti-NS5B antibody from the replicon cells crosslinked by formaldehyde (Figure 4C), suggesting the association between CyPB and NS5B-HCV RNA complex in the cells. These data cumulatively suggest that CyPB associates with a fraction of NS5B that is functional for viral genome replication.

CyPB Stimulated RNA Binding Activity of NS5B

To investigate which function of NS5B was modulated by CyPB, we first assumed the possibility that CyPB might alter formation of the RC like hVAP-33 does (see Discussion) (Gao et al., 2004). We estimated the amount of HCV proteins in the RC by examining the levels of digitonin/protease-resistant NS5A and NS5B as described previously (Miyanari et al., 2003). The amount of NS5A and NS5B resistant to digitonin/proteinase K digestion, however, was not affected by treatment with CsA, which inactivates CyPB function (Figure 5A). We did not observe any significant effect on the total protein levels of NS5A and NS5B after serial treatment with CsA for up to 24 hr (Figure 5A, lanes 1 and 6; Figure 5B, lower right panels; and Figure 5D, upper panel lanes 1 and 2). CsA treatment also had no effect on the subcellular localization of NS5B (data not shown). The RNA synthesis activity of the isolated RC, however, was significantly reduced after this CsA treatment condition (Figure 5B).

These results raised the possibility that CyPB directly regulated the function of NS5B within the RC. NS5B binds HCV RNA as a template to function as an RdRp. We therefore investigated the effect of CyPB on the RNA binding activity of NS5B in replicon cells by using poly-U Sepharose beads as a model RNA substrate, as described previously (Ishii et al., 1999; Lohmann et al., 1997; Qin et al., 2001). The association of NS5B with poly-U RNA was confirmed by using replicon cells (Fig-

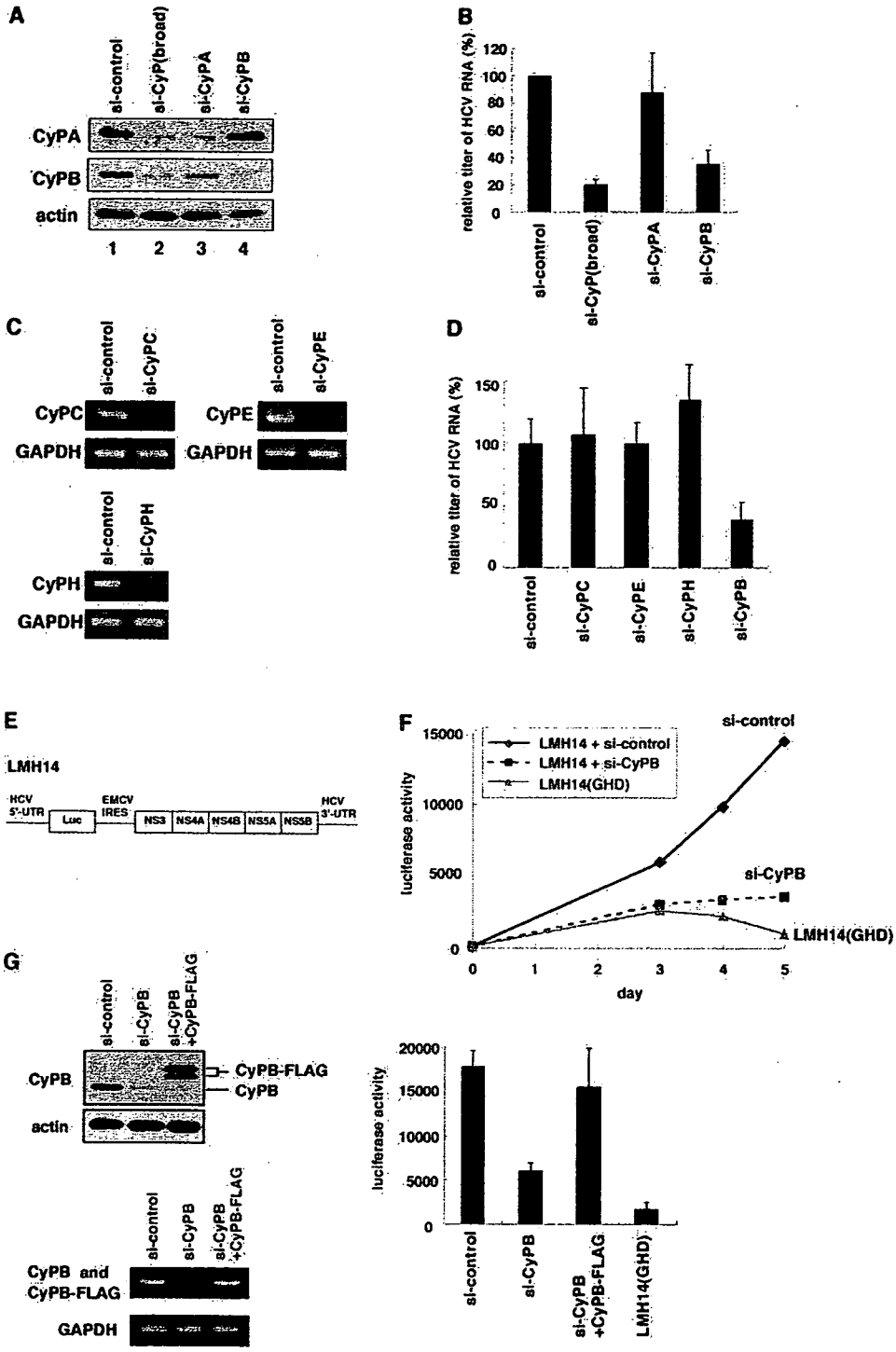

Figure 2. CyPB Regulated HCV Genome Replication

(A) Knockdown of endogenous CyPA and CyPB proteins. MH-14 cells were transfected with siRNA specific for CyPA (si-CyPA), CyPB (si-CyPB), or a broad range of CyP subtypes (si-CyP[broad]) or with a randomized siRNA (si-control). At 72 hr posttransfection, CyPA (top), CyPB (middle), and actin as an internal control (bottom) were detected in total cell lysates by immunoblot analysis.

(B) Depletion of CyPB decreased HCV RNA titers. At 5 days posttransfection of siRNA, HCV RNA titers were quantified by real-time RT-PCR analysis. The data represent the means of three independent experiments. Error bars represent the SD.

(C) siRNA constructs specific for CyPC (si-CyPC), CyPE (si-CyPE), CyPH (si-CyPH), or si-control were introduced into MH-14 cells. At 72 hr posttransfection, we analyzed the mRNA levels of CyPC, CyPE, CyPH, and GAPDH as an internal control by RT-PCR analysis.

(D) The experimental procedure is the same as described in (B). Error bars represent the SD.

(E) Schematic representation of the LMH14 RNA construct, which carries the luciferase gene driven by the HCV 5′-untranslated region (UTR)

ure 5C, upper panel lane 6). Endogenous CyPB, but not CyPA, also coprecipitated with poly-U RNA (Figure 5C, middle and lower panels lane 6). In Huh-7 cells, however, such CyPB-RNA binding was not observed (Figure 5C, lower panel lane 4), suggesting that CyPB binding to RNA is dependent on NS5B. The RNA binding affinity of NS5B isolated from the digitonin/protease-resistant fraction was largely abolished by CsA treatment (Figure 5D, upper panel lane 6), which abrogated the interaction of CyPB with NS5B (Figure 3F, lane 4). A similar result was obtained in another replicon cell clone, 50-1 (data not shown). siRNA-mediated knockdown of the endogenous CyPB also reduced the NS5B-poly-U RNA binding within the digitonin/protease-resistant fraction (Figure 5E, lane 6). These data suggest that CyPB promotes the binding of NS5B to RNA. This effect of CyPB was further documented by using an in vitro RNA binding assay. The binding of NS5B to either poly-U (Figure 5F) or poly-A (data not shown) RNA was increased by the addition of recombinant GST-CyPB. RNA binding, however, was unaffected by the addition of either recombinant GST-CyPA or GST-CyPBΔPPI, a PPIase-inactive point mutant of CyPB (Rycyzyn and Clevenger, 2002) that retained strong binding to NS5B (Figure 3B, lane 6) (Figure 5F). In contrast, CyPB did not augment the RNA binding activity of NS5B(1–570), which had little affinity for CyPB (Figure 3G, panel f) (Figure 5G). The stimulatory effect of CyPB on the RNA binding activity of NS5B was negated by treatment with CsA (Figure 5H). Although the conditions in this in vitro system differ from the cellular environment, these results clearly demonstrate that CyPB can augment RNA binding activity of NS5B even in vitro.

Association of CyPB with NS5B Was Critical for Efficient Replication of HCV Genome

To examine the physiological relevance of the association of CyPB with NS5B in HCV genome replication, we identified a point mutant of NS5B that was unable to bind to CyPB by alanine scanning mutation analysis on the 521–591 aa region of NS5B. In a GST pull-down assay, NS5B(P540A), which bore the replacement of the proline at 540 aa of NS5B by alanine, had little affinity for GST-CyPB (Figure 6A). We confirmed that NS5B(P540A) did not bind CyPB by immunoprecipitation (Figure 6B, lane 6). This P540A mutation decreased the RNA binding activity of NS5B in replicon cells (Figure 6C, lane 6), supporting our conclusion that CyPB plays a stimulatory role in RNA binding of NS5B. Because the P540A mutation may induce the overall conformational change of NS5B protein and destroy its function, we examined the basal function of NS5B(P540A). The levels of protein production and subcellular localization of exogenously produced NS5B(P540A) in Huh-7 cells were, however, similar to those seen in cells with wild-type (wt) NS5B (data not shown). This mutation did not cripple the molecular function of NS5B itself, including RNA polymerase activity and RNA binding activity in vitro (data not shown). The introduction of this point mutation into the NS5B sequence should not destroy the stem-loop RNA structure of the *cis*-acting replication element in the coding region for NS5B, which is present downstream of the P540 codon (Lee et al., 2004; You et al., 2004). The association of NS5B with the ER membrane via its 571–591 aa region is reportedly essential for HCV replication in cells (Moradpour et al., 2004). NS5B(P540A) still associated with intracellular membrane fractions (data not shown). Thus, NS5B(P540A) possessed basal functions of NS5B as far as we examined. However, the replication competency of HCV replicon RNA carrying this one point mutation was significantly decreased from levels observed for the wt in the luciferase assay system (Figure 6D). In this assay, we did not find any reversions at the second site or back to the wt within the NS5B-coding region in HCV replicon RNA of the transfected cells (data not shown). The colony formation assay also demonstrated a reduction in HCV genome replication with the NS5B P540A mutant (Figure 6E). These data suggest that the functional regulation of NS5B by CyPB is critical for the efficient replication of the HCV genome in the cells.

Discussion

Viruses exploit cellular factors for their efficient proliferation. Although it was recently reported that a SNARE-like protein, hVAP-33, augmented HCV replication by modulating RC formation (Gao et al., 2004), cellular factors directly regulating the activity of the RC have remained unknown. By using CsA, a compound exerting a strong anti-HCV potential, we determined that CyPB is required for efficient replication of the HCV genome. This cellular factor regulates HCV genome replication through modulation of the RNA binding activity of NS5B. CsA impaired this regulation of NS5B-RNA binding by CyPB to inhibit HCV genome replication. Previously, the CyP family was known to regulate the function of specific substrates such as calcineurin (Schreiber, 1992), prolactin (Rycyzyn and Clevenger, 2002), Itk (Brazin et al., 2002; Colgan et al., 2004), adenine nucleotide translocase (Waldmeier et al., 2003), and steroid receptors (Duina et al., 1996) by altering

and the coding region for HCV NS3 to NS5B whose expression is regulated by the EMCV IRES. By using this RNA construct, HCV replication can be monitored by measuring resultant luciferase activity as described previously (Murata et al., 2005).

(F) Suppression of HCV replication after CyPB knockdown. Cured MH-14 cells were transfected with LMH14 or LMH14(GHD) RNA together with si-control or si-CyPB. After 3, 4, or 5 days, luciferase activities were measured to plot against the time course. Solid line, transfected with LMH14 RNA and si-control; broken line, LMH14 RNA and si-CyPB; and faint line, LMH14(GHD) RNA, a replication-deficient mutant of LMH14 used as a negative control.

(G) On the right, cured MH-14 cells were transfected with LMH14 RNA plus si-control (si-control), LMH14 RNA plus si-CyPB (si-CyPB), LMH14 RNA plus si-CyPB and the expression plasmid for FLAG-tagged CyPB (si-CyPB + CyPB-FLAG), or LMH14(GHD) RNA (LMH14[GHD]). Luciferase activities were then quantified at 5 days posttransfection. Error bars represent the SD. Left panels show the immunoblot analysis (upper panels) by using anti-CyPB and anti-actin antibody and RT-PCR analysis (lower panels) by using the primer detecting CyPB and GAPDH upon the transfection of si-control, si-CyPB, and si-CyPB with CyPB-FLAG.

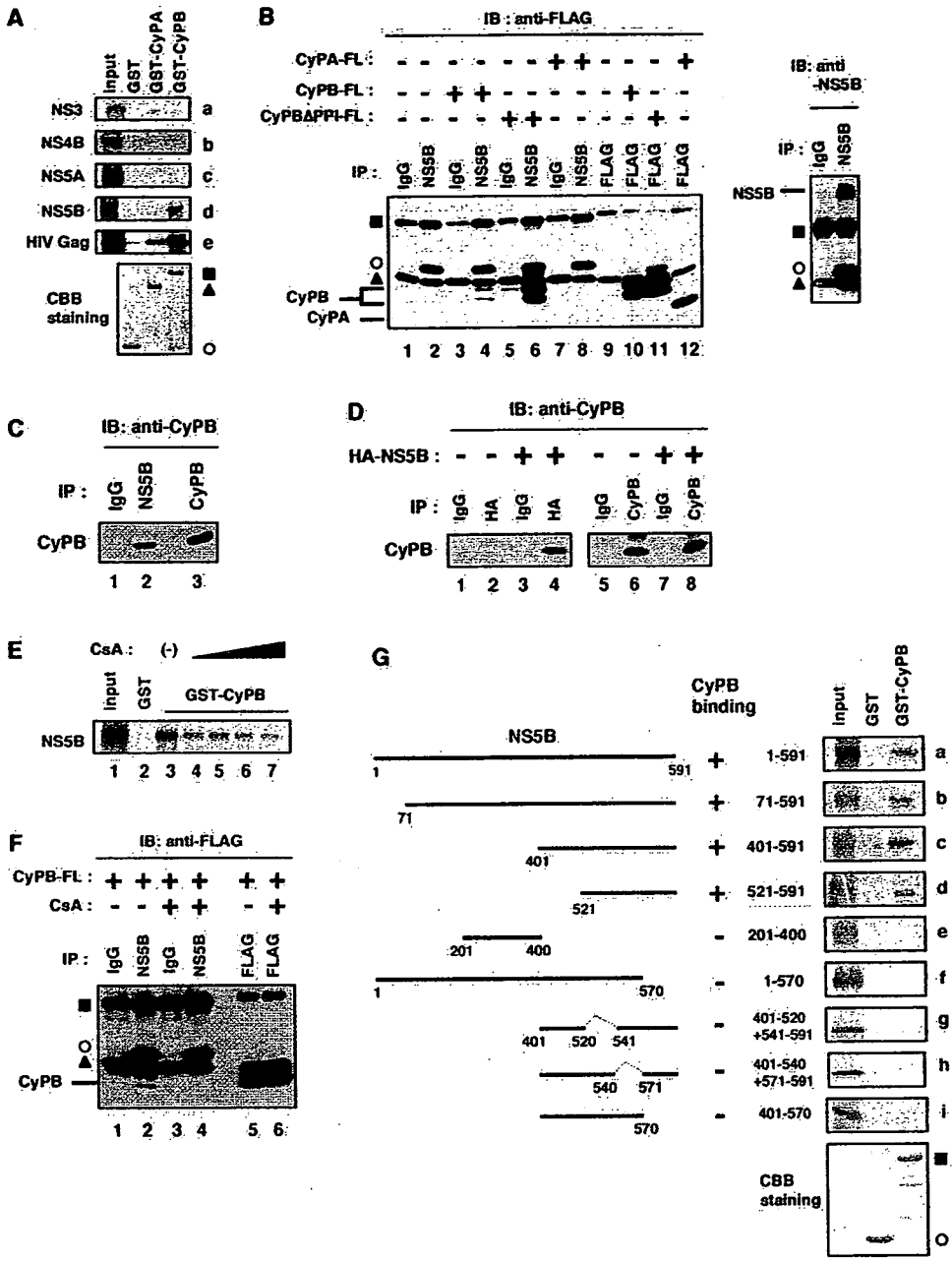

Figure 3. CyPB Interacted with HCV NS5B

(A) [^{35}S]-labeled in vitro translation products of HCV NS3 (panel a), NS4B (panel b), NS5A (panel c), NS5B (panel d), and HIV-1 Gag as a positive control (panel e) were incubated with recombinant GST fusions of CyPA or CyPB (GST-CyPA or GST-CyPB, respectively) or with GST as a negative control. "Input" designated the signal for 1/10 of the amount of [^{35}S]-labeled product used in the pull-down assay. CBB staining patterns for the pulled-down proteins are shown in the bottom panel. Open circle, GST; closed triangle, GST-CyPA; and closed square, GST-CyPB.

(B) Coimmunoprecipitation from lysates of MH-14 cells transfected with expression plasmids for FLAG-tagged CyPA (CyPA-FL) (lanes 7, 8, and 12), CyPB (CyPB-FL) (lanes 3, 4, and 10), CyPBΔPPI (CyPBΔPPI-FL) (lanes 5, 6, and 11), or empty vector (lanes 1, 2, and 9 and right panel). The plasmids used for transfection are indicated at the top of the panel by + or –. "IP" designates the antibodies used for immunoprecipitation. Coimmunoprecipitated proteins were detected by immunoblot analysis using either anti-FLAG (left) or anti-NS5B (right) antibodies, respectively. Closed square, Ig heavy chain; closed triangle, Ig light chain; and open circle, nonspecific band. CyPA, CyPB or CyPBΔPPI, and NS5B are indicated. Ectopically expressed CyPB showed a double band, consistent with the previous report (Price et al., 1994). The upper-slight band of CyPBΔPPI in lane 5 is likely to show nonspecific binding, because it was not precipitated with IgG in a buffer containing higher concentration of NP-40 (1% NP-40) (data not shown).

(C) Coimmunoprecipitation of endogenous CyPB with NS5B in MH-14 cells. The data are presented as in (B). The immunoprecipitates were detected with an anti-CyPB antibody.

(D) Coimmunoprecipitation of endogenous CyPB with exogenous NS5B in Huh-7 cells. Huh-7 cells were transfected with an expression

protein-protein interactions, nuclear import, enzymatic activity, or protein phosphorylation. In this study, we demonstrated that CyPB also modulated the activity of RNA-protein binding. This information will significantly impact our understanding of the molecular functions of CyP.

Regarding the role of CyP in virus proliferation, CyPA has been reported to be involved in the life cycle of HIV-1. CyPA binds HIV-1 Gag (Luban et al., 1993) and is incorporated into viral particles (Franke et al., 1994; Thali et al., 1994). This CyPA promotes viral infectivity (Braaten and Luban, 2001; Towers et al., 2003). HIV-1 Gag, however, binds to not only CyPA but also most other members of the CyP family (Braaten and Luban, 2001; Luban et al., 1993). In contrast to HIV-1 Gag, HCV NS5B bound CyPB, but not CyPA. Among the CyP family members examined, only CyPB regulated HCV genome replication. The subcellular localization of each CyP subtype varies; CyPA is found in the cytoplasm and nucleus, CyPD in the mitochondria, and CyPE in the nucleus (Takahashi, 1999). CyPB is located mainly in the lumen of the ER (Price et al., 1994; Takahashi, 1999). A subset of CyPB, however, was speculated to localize in the cytoplasm or in ER substructures on the cytoplasmic side (Bram et al., 1993). We found that a portion of CyPB localized on the cytoplasmic face of the ER irrespective of the presence of HCV replicon (Figure 4B), consistent with this previous speculation.

Almost all HCV proteins and genomic RNA are located around the ER (Bartenschlager and Lohmann, 2001). The majority of events in the HCV life cycle, including genome replication, protein maturation, assembly, and budding, occur around intracellular ER-like membranes (Bartenschlager and Lohmann, 2001). NS5B mainly localizes on the cytoplasmic side of the ER membrane through its C-terminal 571–591 aa hydrophobic region (Schmidt-Mende et al., 2001). A subset of the total NS5B participates in the RC, which is thought to have a cytoplasmic side membrane topology and is surrounded by a membrane structure (Aizaki et al., 2004). We cannot conclude clearly where CyPB-NS5B-RNA association occurs. The 521–591 aa region of NS5B, which is the binding region for CyPB, however, is thought to be inserted in the ER lipid bilayer membrane on the cytoplasmic face (Schmidt-Mende et al., 2001). A part of CyPB was also found on the cytoplasmic face of the membranes as described above. Moreover, crosslinking analysis (Figure 4C) suggests the interaction of CyPB with the HCV RNA-NS5B complex, which is likely to occur mainly in the RC. Thus, CyPB likely interacts with NS5B on the cytoplasmic face within the RC compartment (and in the lipid bilayer membrane).

Viral polymerases are promising targets for the development of antiviral agents. Several chemicals targeting NS5B have anti-HCV activity (Wu and Hong, 2003). These compounds directly inhibit the activity of recombinant NS5B in vitro. In addition to these typical anti-HCV compounds, inhibition of the association of CyPB with NS5B may serve as a strategy for the design of antiviral therapeutics. Our findings not only reveal one of the mechanisms of viral replication in the cells but also may lead to the development of antiviral therapeutics.

Experimental Procedures

Cell Culture and Transfection

Huh-7 and cured MH-14 cells (Murata et al., 2005) were cultured in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum, L-glutanime (Invitrogen), MEM nonessential amino acids (Invitrogen), and kanamycin (Meiji). MH-14 cells, carrying HCV subgenomic replicon (Miyanari et al., 2003), were cultured in the same medium supplemented with 300 μg/ml G418 (Invitrogen). Plasmid transfection was performed as described previously (Watashi et al., 2003b). RNA transfection for the reporter assay was achieved by using DMrie-C transfection reagent (Invitrogen) as recommended by the manufacturer. siRNA was transfected by using siLentFect (BIORAD) according to the manufacturer's protocol.

Reagents

CsA was purchased from Sigma. The CsA derivatives 8′-OH-MeBmt^{1}-Cs, MeAla^{6}-Cs, D-lys^{8}-Cs, MeVal^{4}-Cs, cyclosporin H, NIM811, and PSC833 and the macrolide type CyP inhibitors sanglifehrin A and B were kindly provided by Novartis (Basel, Switzerland). The characteristic feature of each CsA derivative is shown in Figure 1A (for details, see Billich et al. [1995], Loor et al. [2002], and Silverman et al. [1997]). (D-lys^{8})Cs binds CyP in vitro. In cells, however, the effective binding affinity to CyP is low because of inefficient cellular uptake (Billich et al., 1995).

Immunoblot Analysis

Immunoblot analysis was performed essentially as described previously (Watashi et al., 2003b).

Antibodies

The antibodies used in this experiment were anti-NS5A (a generous gift from Dr. Takamizawa, Osaka University), anti-NS5B (10 and 14; kindly provided by Dr. Kohara, Tokyo Metropolitan Institute of Medical Science), anti-actin (Sigma), anti-CyPA (Upstate Cell Signaling), anti-CyPB (Affinity BioReagents), anti-FLAG (M2; Sigma), anti-HA (3F10; Roche), anti-BrdU (Sigma), anti-IκBα (Santa Cruz), anti-PDI (StressGen), and anti-calnexin (StressGen) antibodies.

Real-Time RT-PCR Analysis

Real-time RT-PCR analysis was performed as described previously (Watashi et al., 2003a).

RNAi Technique

siRNA duplexes (si-CyPA, 5′-AAGCATACGGGTCCTGGCATC-3′; si-CyPB, 5′-AAGGTGGAGAGCACCAAGACA-3′; and si-CyP(broad), 5′-AAGCATGTGGTGTTTGGCAAA-3′) containing 3′dTdT over-

plasmid for HA-tagged NS5B (lanes 3, 4, 7, and 8) or an empty vector (lanes 1, 2, 5, and 6). The immunoprecipitates were detected by an anti-CyPB antibody.

(E and F) The interaction of CyPB with NS5B was disrupted by CsA treatment. In (E), a GST pull-down assay between GST-CyPB and NS5B was performed in the absence (lane 3) or presence (lanes 4–7) of CsA. The concentrations of CsA in lanes 4–7 are 0.8, 4, 8, and 16 μg/ml, respectively. In (F), coimmunoprecipitation between CyPB-FL and NS5B in MH-14 cells treated without (lanes 1, 2, and 5) or with 3 μg/ml CsA (lanes 3, 4, and 6) was analyzed.

(G) Mapping of the regions of NS5B responsible for the interaction with CyPB. At the left of the panel, schematic representation of the full-length and truncated mutants of NS5B is shown. The numbers indicate the amino acids residue numbers in NS5B. "CyPB binding" summarizes the results of a GST pull-down assay by ±; GST pull-down data are presented as described in (A).

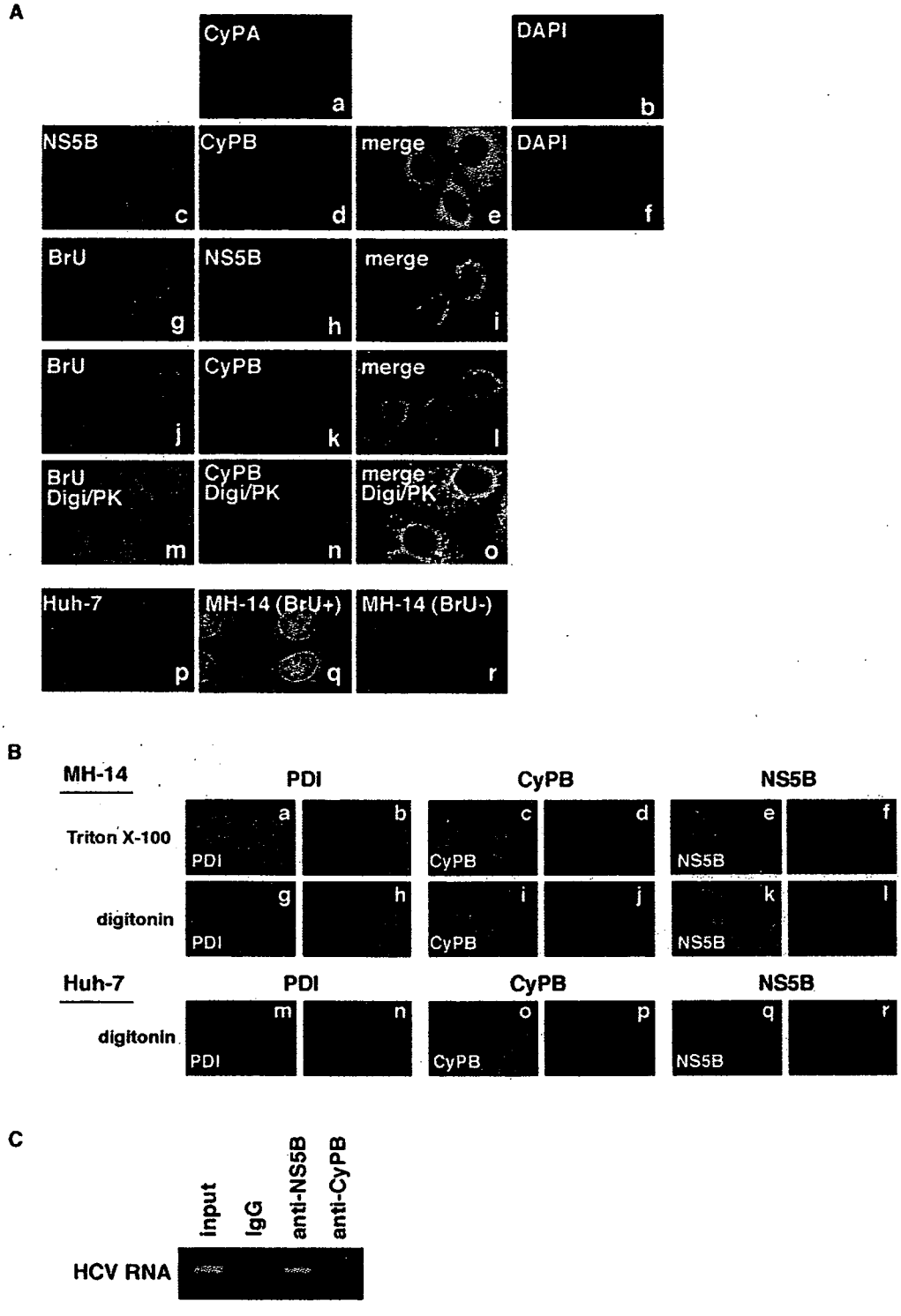

Figure 4. Colocalization of CyPB with NS5B around the Endoplasmic Reticulum

(A) CyPB colocalized with NS5B. Indirect immunofluorescence analysis was performed on MH-14 cells incubated in the absence (panels a–f and r) or presence of actinomycin D/BrU (panels g–o and q). Huh-7 cells were treated with actinomycin D/BrU as a negative control (panel p). Prior to immunofluorescence analysis with permeabilization by Triton X-100, cells were treated with digitonin (Digi) followed by digestion with 0.3 μg/ml proteinase K (PK) in panels m–o. The primary antibodies used were anti-CyPA (panel a, red), anti-CyPB (panels d, k, and n, red), anti-NS5B (panel c, green and panel h, red), and anti-BrdU (panels g, j, m, and p–r, green) antibodies. DAPI was used to visualize the nuclei (panels b, f, and p–r, blue). Panels a–b, c–f, g–i, j–l, and m–o show the same cells. Merged images of green and red signals are shown in panels e, i, l, and o. Panels p–r are the merged images of green (BrU) and blue (DAPI) signals.

(B) A portion of CyPB was located on the cytoplasmic face of the intracellular membrane. MH-14 cells were fixed and followed by permeabili-

hanging sequences were synthesized (QIAGEN). A control nucleotide, si-control, was purchased from Dharmacon (nonspecific control duplex IX). si-CyPC, si-CyPE, and si-CyPH were obtained from Ambion (predesigned siRNA). The sequence of si-CyP(broad) matches 100% with CyPA and CyPB mRNAs, a 20 bp match with CyPH, a 19 bp match with CyPC and CyP40, and a 17 bp match with CyP33. The sequence of si-CyPA and si-CyPB does not have significant identity to mRNA for CyPB and CyPA, respectively.

RT-PCR Analysis

RT-PCR analysis was performed as described (Watashi et al., 2003b) by using the following primer sets: 5′-GTTGGATCCATG GGCCCGGGTC-3′ and 5′-GTTCTCGAGTCACCAATCAGCGATC-3′ for the detection of CyPC, 5′-GTTGAATTCATGGCCACCACCAAG-3′ and 5′-GTTCTCGAGTCACACGTACTCCCCAC-3′ for CyPE, and 5′-GTTGGATCCATGGCGGTGGCAAATTC-3′ and 5′-GTTCTCGAG CTACATCTCCCCACAC-3′ for CyPH.

Luciferase Assay

A luciferase assay monitoring HCV replication levels was performed as previously described (Murata et al., 2005). The reporter RNAs used in this study were LMH14, LMH14(GHD), in which the GDD polymerase motif of NS5B was replaced by GHD to lose replication activity, and LMH14(P540A), in which the proline at position 540 of NS5B was replaced by alanine.

Plasmid Constructs

The CyPA and CyPB cDNAs were obtained by RT-PCR from a human liver cDNA library (Clontech) template by using the following primers: 5′-GTTGGATCCGCCATGGTCAACCCCACCG-3′ and 5′-GTT GAATTCTTCGAGTTGTCCACAGTC-3′ for CyPA and 5′-GTTGGA TCCGCCATGCTGCGCCTCTCC-3′ and 5′-GTTGAATTCCTCCTTG GCGATGGCAA-3′ for CyPB. pGEX-CyPA and pGEX-CyPB, which encode glutathione S-transferase (GST) fusions of CyPA and CyPB were constructed by insertion of the BamHI-EcoRI fragments into the appropriate site of the pGEX-6P1 vector (Clontech). The pCMV-CyPA-FL and pCMV-CyPB-FL plasmids expressing FLAG-tagged CyPA and CyPB, respectively, were obtained by inserting the CyPA and CyPB fragments into the BamHI-EcoRI site of the pCMV-FLAG(C) vector. CyPBΔPPI, of which arginine and phenylalanine at position 95 and 100 of CyPB were replaced by alanines, was an enzymatic-inactive mutant of CyPB (Rycyzyn and Clevenger, 2002). The subcloning of pCMV-CyPBΔPPI-FL, which encodes a FLAG-tagged CyPBΔPPI, was performed essentially as described (Rycyzyn and Clevenger, 2002). Expression plasmids for HCV NS3, NS4B, NS5A, and NS5B (pcDNA-NS3, pcDNA-NS4B, pcDNA-NS5A, and pcDNA-NS5B, respectively) were generated by insertion of PCR-amplified fragments encoding each HCV protein into the pcDNA3 vector (Invitrogen). The series of plasmids expressing deletion mutants of NS5B were constructed by inserting into the pcDNA3 vector various fragments amplified by PCR using appropriate synthetic oligonucleotides as primers and pLMH14 as a template. The primers for oligonucleotide-directed mutagenesis used to generate the NS5B(P540A) mutant were 5′-ACTCCAATTGCGG CTGCGTCC-3′ and 5′-GGACGCAGCCGCAATTGGAGT-3′. LMH14 and LMH14(GHD) have been described previously (Murata et al., 2005). The expression plasmid for HIV-1 Gag was kindly provided by Dr. Adachi, Institute of Health Biosciences, The University of Tokushima.

GST Pull-Down Assay

A GST pull-down assay was performed as described previously (Watashi et al., 2003b).

Immunoprecipitation Assay and RNA-Protein Binding Precipitation Assay

An immunoprecipitation assay was essentially performed as described (Watashi et al., 2003b). For the RNA-protein binding precipitation assay, either cells or digitonin/protease-treated cells were lysed in IP buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% NP-40, and 1 mM PMSF. After centrifugation, supernatants were incubated for 2 hr with poly-U or protein G Sepharose resin as a negative control (Amersham Biosciences). After four washes with IP buffer, precipitates were analyzed by immunoblot analysis.

Indirect Immunofluorescence Analysis

The normal indirect immunofluorescence analysis in Figures 4A and 4B, panels a–f, was performed as described previously (Watashi et al., 2003b). Briefly, the cells were fixed, and permeabilization with 0.1% Triton X-100 followed. These cells were subjected to the antibody reaction to detect each protein. In Figure 4A, panels g–r, de novo-synthesized HCV RNA was labeled with 5-BrU. Cells were treated with 10 μg/ml actinomycin D to block RNA transcription by cellular DNA-dependent RNA polymerases (Restrepo-Hartwig and Ahlquist, 1996). After a 30 min incubation, 40 mM BrU was added to the culture medium for labeling RNA (Fuchsova et al., 2002). After an additional 2 hr, the cells were subjected to immunofluorescence analysis. In the modified immunofluorescence analysis (Watashi et al., 2001) in Figure 4B, panels g–r, we treated the cells with 50 μg/ml digitonin at 27°C for 5 min to permeabilize the plasma membrane, but not the intracellular membrane. After washing out the cytosol, these cells were fixed and subjected to the antibody reaction.

Crosslinking Assay for the Detection of RNA-Protein Complex

Crosslinking and the subsequent immunoprecipitation were essentially performed as described (Watashi et al., 2003b). The immunoprecipitation was done in the presence of RNase inhibitor and polydI-dC. Recovered immunocomplexes were digested with proteinase K and treated with phenol/chloroform. RT-PCR was performed with the extracted RNA and 5′-TCCCGGTTGGACTTGTCC-3′ and 5′-GCCTATTGGCCTGGAGTG-3′ as a template and primer sets, respectively, by using a one-step RNA PCR kit.

Cell Permeabilization with Digitonin Followed by Digestion with Protease

Digitonin/protease treatment was performed as described previously (Miyanari et al., 2003). Briefly, cells were permeabilized by a 5 min incubation in buffer B containing 50 μg/ml digitonin at 27°C. After two washes in buffer B, cells were treated for 5 min with varying concentrations of proteinase K at 37°C.

RNA Synthesis with the Replication Complex

RNA synthesis reaction was performed as described (Miyanari et al., 2003), using reaction times of 0.5, 1, 2, 4, 6, 12, and 24 hr.

In Vitro RNA Binding Assay

An in vitro RNA binding assay was performed by using poly-U or poly-A Sepharose as a model RNA substrate, as described previously (Ishii et al., 1999). In vitro-translated [^{35}S]-labeled products and poly-U, poly-A, or protein G Sepharose resin as a negative

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zation with 0.1% Triton X-100 to detect PDI (panel a), CyPB (panel c), and NS5B (panel e) by normal immunofluorescence analysis as a control experiment (Triton X-100). In panels g–r (digitonin), MH-14 (panels g–l) and Huh-7 cells (panels m–r) were permeabilized with 50 μg/ml digitonin followed by extensive washes. These cells were then fixed and subjected to detection of PDI (panels g and m), CyPB (panels i and o), and NS5B (panels k and q). DAPI (panels b, d, f, h, j, l, n, p, and r) shows the nuclear staining in the same cells as that shown in panels a, c, e, g, i, k, m, o, and q.

(C) CyPB associated with HCV RNA-NS5B complex in the cells. Formaldehyde-crosslinked RNA-protein complexes in MH-14 cells were immunoprecipitated with anti-NS5B, anti-CyPB, or normal rabbit IgG (IgG). The RNA extracted from the immunoprecipitates was amplified by RT-PCR as described in the Experimental Procedures. "Input" designated the signal for 1/50 of the amount of cell lysate used in the immunoprecipitation assay.

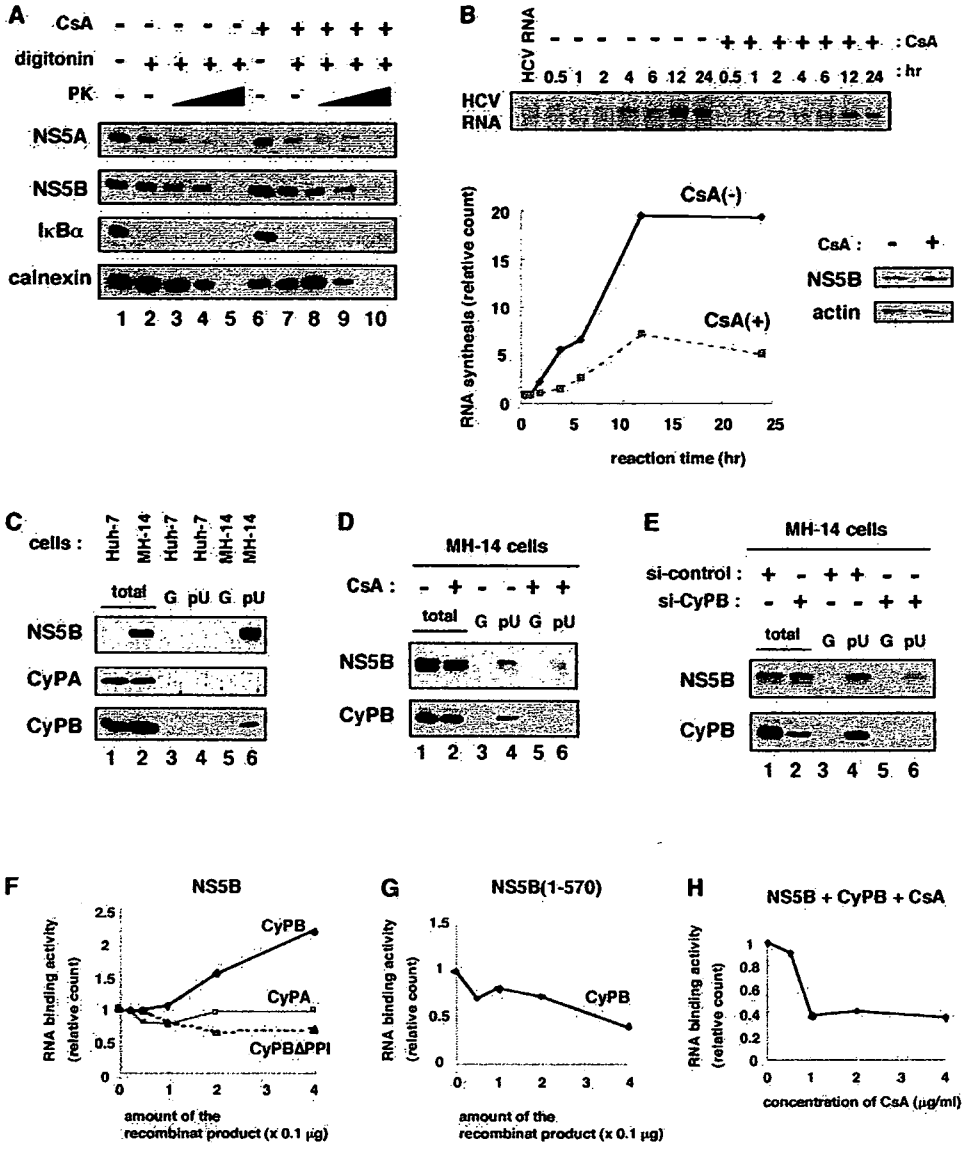

Figure 5. CyPB Stimulated RNA Binding Activity of NS5B

(A) Quantitation of the amount of HCV proteins in the digitonin/protease-resistant fraction. MH-14 cells were treated without (lanes 1–5) or with (lanes 6–10) 3 μg/ml CsA for 24 hr. Cells were then treated without (lanes 1 and 6) or with 50 μg/ml digitonin, followed by digestion with varying concentrations of PK (0 μg/ml for lanes 1, 2, 6, and 7, 0.03 μg/ml for lanes 3 and 8, 0.1 μg/ml for lanes 4 and 9, and 0.3 μg/ml for lanes 5 and 10). NS5A, NS5B, IκBα, and calnexin in whole-cell lysate were detected by immunoblot analysis.

(B) RNA synthesis activity in the HCV RC. MH-14 cells were treated without or with 3 μg/ml CsA for 24 hr. After isolating the RC by digitonin permeabilization, RNA synthesis reaction was performed with varying reaction times (0.5, 1, 2, 4, 6, 12, and 24 hr). At the top of the panel, treatment of CsA and reaction time is summarized. "HCV RNA" indicates the in vitro-synthesized HCV subgenomic replicon RNA. In the lower left panel, the radioactivity of synthesized RNA is plotted against the reaction time. The lower right panels show the protein expression levels of NS5B and actin.

(C) An RNA-protein binding precipitation assay was performed by using Huh-7 or MH-14 cells as described in the Experimental Procedures. The resultant precipitates were detected by immunoblot analysis with anti-NS5B (top), anti-CyPA (middle), and anti-CyPB (bottom) antibodies. "Total" indicates 1/6 of the amount of cell lysate used in the precipitation assay. "G" and "pU" designate the samples using protein G Sepharose and poly-U Sepharose as a resin, respectively.

(D) MH-14 cells treated with digitonin followed by digestion in 0.5 μg/ml PK were used for an RNA-protein binding precipitation assay with anti-NS5B antibody (top). Prior to the assay, CsA was treated (lanes 2, 5, and 6) or left untreated (lanes 1, 3, and 4) for 24 hr. CyPB levels were also examined in CsA-treated or untreated cells (bottom).

(E) MH-14 cells were transfected with si-control or si-CyPB. After 72 hr, cells were treated with digitonin/PK and analyzed as in (C).

(F–H) An in vitro binding assay was performed as described in the Experimental Procedures. [^{35}S]-labeled in vitro translation products of either the full-length NS5B (F and H) or the 1–570 aa region of NS5B (G) were incubated with poly-U Sepharose in the presence of either recombinant GST-CyPB (F–H), GST-CyPA (F), or GST-CyPBΔPPI (F). In (H), varying concentrations of CsA were also added to the reaction mixtures. The radioactivity of NS5B protein in the pulled-down fraction was counted to plot against either the amount of the recombinant product (F and G) or the concentration of CsA (H). In (F), the solid line represents being in the presence of GST-CyPB; the broken line, GST-CyPBΔPPI; and the faint line, GST-CyPA. These results were reproduced in three independent experiments.

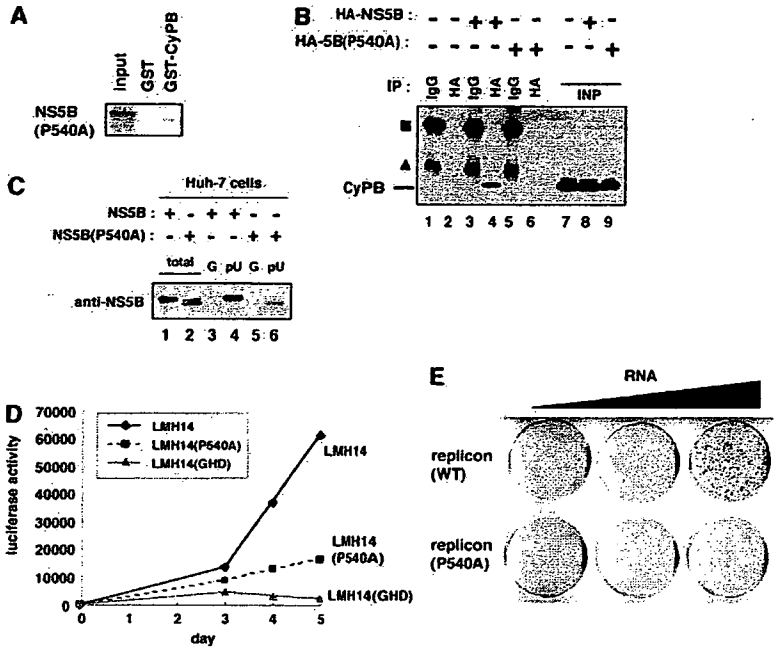

Figure 6. Association of CyPB with NS5B Was Critical for the Efficient Replication of the HCV Genome

(A) A GST pull-down assay was performed and presented as described in Figure 3A.

(B) An immunoprecipitation assay was performed as described in Figure 3B. The expression plasmid for HA-tagged NS5B (lanes 3, 4, and 8) and NS5B(P540A) (lanes 5, 6, and 9) and empty vector (lanes 1, 2, and 7) was used for the transfection. "INP" indicates 1/6 of the amount of cell lysate used for the immunoprecipitation.

(C) An RNA-protein binding precipitation assay was performed by using Huh-7 cells transfected with the expression vector for NS5B (lanes 1, 3, and 4) or NS5B(P540A) (lanes 2, 5, and 6) as described in Figure 5C.

(D) Luciferase assays were performed and are presented as in Figure 2F. Solid line, LMH14; broken line, LMH14(P540A); and faint line, LMH14(GHD).

(E) Colony formation assay. The subgenomic replicon RNA (replicon[wt]) and a replicon RNA carrying the P540A mutation in NS5B (replicon[P540A]) at various amount (2.5 μg in the left wells, 5 μg in the center wells, and 10 μg in the right wells) was transfected into Huh-7 cells. The colony formation assay was performed as described in the Experimental Procedures.

control were incubated in the presence of a varying amount of GST-fusion proteins, including GST-CyPB, GST-CyPA, and GST-CyPBΔPPI, at 4°C for 1–12 hr. In Figure 5H, CsA was added simultaneously. After five washes, resin bound radiolabeled proteins were fractionated, and its radioactivity was quantitated.

Colony Formation Assay

Plasmids were linearized with XbaI and transcribed into RNA in vitro by using a MEGAscript T7 kit (Ambion) according to the manufacturer's protocol. In vitro-transcribed RNA (2.5–10 μg) was transfected into Huh-7 cells by using DMrie-C transfection reagent. At 48 hr posttransfection, 1 mg/ml G418 was added to the medium. ~3 weeks later, cells were fixed and stained with crystal violet.

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