JOURNAL OF VIROLOGY, June 2008, p. 5269–5278 Vol. 82, No. 11
0022-538X/08/$08.00+0 doi:10.1128/JVI.02614-07

Cyclophilin A Is an Essential Cofactor for Hepatitis C Virus Infection and the Principal Mediator of Cyclosporine Resistance In Vitro▽

Feng Yang, Jason M. Robotham, Heather B. Nelson, Andre Irsigler, Rachael Kenworthy, and Hengli Tang*

Department of Biological Science, Florida State University, Tallahassee, Florida 32306-4370

Received 7 December 2007/Accepted 20 March 2008

Cyclosporine (CsA) and its derivatives potently suppress hepatitis C virus (HCV) replication. Recently, CsA-resistant HCV replicons have been identified in vitro. We examined the dependence of the wild-type and CsA-resistant replicons on various cyclophilins for replication. A strong correlation between CsA resistance and reduced dependency on cyclophilin A (CyPA) for replication was identified. Silencing of CyPB or CyPC expression had no significant effect on replication, whereas various forms of small interfering RNA (siRNA) directed at CyPA inhibited HCV replication of wild-type but not CsA-resistant replicons. The efficiency of a particular siRNA in suppressing CyPA expression was correlated with its potency in inhibiting HCV replication, and expression of an siRNA-resistant CyPA cDNA rescued replication. In addition, an anti-CyPA antibody blocked replication of the wild-type but not the resistant replicon in an in vitro replication assay. Depletion of CyPA alone in the CsA-resistant replicon cells eliminated CsA resistance, indicating that CyPA is the chief mediator of the observed CsA resistance. The dependency on CyPA for replication was observed for both genotype (GT) 1a and 1b replicons as well as a GT 2a infectious virus. An interaction between CyPA and HCV RNA as well as the viral polymerase that is sensitive to CsA treatment in wild-type but not in resistant replicons was detected. These findings reveal the molecular mechanism of CsA resistance and identify CyPA as a critical cellular cofactor for HCV replication and infection.

Hepatitis C virus (HCV), a member of the *Flaviviridae* family that includes other major human pathogens such as dengue and West Nile viruses, contains a positive-strand RNA genome of 9.6 kb encoding a single polyprotein, which is processed through proteolysis to become at least 10 viral proteins (18). Like other positive-strand RNA viruses, HCV replicates its genomic RNA in association with intracellular membranes (37). The nonstructural proteins, especially NS3, NS5A, and NS5B, directly participate in the replication process and determine replication efficiency from cognate 5′ and 3′ nontranslated regions (3). In addition, HCV replication is regulated by cellular proteins that either directly interact with viral proteins or modulate critical metabolic pathways essential for the virus (7, 11, 31, 40, 43).

Cyclophilins (CyPs) are a family of cellular enzymes possessing the peptidyl-prolyl isomerase activity. The prototypical member of the CyP family is CyPA, the main intracellular ligand of cyclosporine (CsA) (12). The CsA-CyPA complex binds to and inhibits calcineurin, a cellular phosphatase and a key mediator of T-cell activation (19). The role of human CyPs as cellular cofactors in HCV replication was first suggested by studies that showed that CsA is effective in suppressing HCV replication (27, 45). Subsequently, a correlation between the CyP-binding and anti-HCV activity was observed for derivatives of CsA (22, 46). Despite both protein binding and resistance mapping studies suggesting that NS5B is a viral target for CsA (8, 36, 46), the identities and relative contributions of the various CyPs implicated in this interaction remain controversial (28, 36, 46). Furthermore, although CsA and its derivatives efficiently inhibit the infection of JFH-1/HCVcc in vitro (32), the CyP involved has not been identified since CyPB, which has been reported to play a role in the replication of a genotype (GT) 1b replicon, is clearly dispensable for the replication of a JFH-1 replicon (14). Finally, the relationship between the dependency on CyPs and the observed CsA resistance has not been investigated. We report here that CyPA, and not CyPB or CyPC, is an essential cofactor for the replication of various HCV isolates and genotypes. Among these is JFH-1, the GT 2a isolate with the highest efficiency in producing infectious particles in cell culture (6, 17, 42, 47, 49). Our data further indicate that CyPA is the principal mediator of CsA resistance in vitro. Not only is the resistance to CsA correlated with resistance to CyPA suppression, but removal of CyPA from resistant replicon cells also eliminates resistance. Finally, CsA-resistant interaction between NS5B and CyPA contributes to the decreased drug sensitivity of the selected HCV replicons.

MATERIALS AND METHODS

Cells, compounds, and antibodies. GS5 and RS2 cells have been described previously (36). Huh-7.5 cells and the H77 replicon construct were provided by Charles Rice and Apath LLC. CsA was purchased from Alexis Corporation (San Diego, CA). We used the following antibodies: anti-CyPA (Biomol, Plymouth Meeting, PA), anti-CyPB (Affinity BioReagents, Golden, CO), anti-Ku80 and antiactin (Sigma-Aldrich), anti-NS5A and anti-NS5B (Virogen, Boston, MA), anti-NS3 (G. George Luo, University of Kentucky), and anticore (Affinity BioReagents, Golden, CO).

RNA interference. A human immunodeficiency virus (HIV)-based lentiviral vector was used to express all the short hairpin RNAs (shRNAs). The sh-Luc and sh-B710 RNAs have been described previously (36). Target sequences for the

* Corresponding author. Mailing address: Bio Unit I, Chieftain Way, Department of Biological Science, Florida State University, Tallahassee, FL 32306-4370. Phone: (850) 645-2402. Fax: (850) 644-0481. E-mail: tang@bio.fsu.edu.

▽ Published ahead of print on 2 April 2008.

other shRNAs are as follows: A-161, 5′-AAG GGT TCC TGC TTT CAC AGA-3′; A-285, 5′-AAG CAT ACG GGT CCT GGC ATC-3′; A-285′, 5′-AAG CAT ACA GGT CCT GGC ATC-3′; A-459, 5′-AAT GGC AAG ACC AGC AAG AAG-3′; C-454, 5′-AAG ACT GAA GGT GTG CTG GTA-3′; NTC, 5′-AAG GAG GTG ACA TCA CCA CTG-3′. Lentiviral vector production and transduction were performed as described previously (44). Stable cells expressing shRNAs were obtained by selection with 1 μg/ml of puromycin (MP Biomedicals, Solon, OH) for 3 weeks.

In vitro transcription, electroporation, colony formation assays, and quantitative RT-PCR. In vitro transcription, electroporation, colony formation assays, and quantitative reverse transcription-PCR (RT-PCR) have been described previously (36). The primers for detecting CyPA, CyPB, and CyPC have the following sequences: A-Forward, 5′-CGG GTC CTG GCA TCT TGT-3′, and A-Reverse, 5′-GCA GAT GAA AAA CTG GGA ACCA-3′; B-Forward, 5′-GGC CAA CGC AGG CAA A-3′, and B-Reverse, 5′-TCT AGC CAG GCT GTC TTG ACT GT-3′; C-Forward, 5′-GCT GAA GCA CTA TGG CAT TGG-3′, and C-Reverse, 5′-GAA CTG AGA GCC ATT GGT GTC A-3′.

Co-IP/RT-PCR. Replicon cells (5×10^6) were seeded into a T-75 flask (treated with 4 μg/ml CsA where indicated) 1 day before the immunoprecipitation (IP) experiment. Twenty-four hours later, cells were lysed in 1 ml of IP buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 0.5% NP-40). Two hundred units of RNaseOUT (Invitrogen, Carlsbad, CA) was added to the supernatant after centrifugation at 12,000 × *g* for 15 min. The supernatant was then added to 50 μl 75% protein G slurry containing either anti-CyPA or rabbit immunoglobulin G (IgG). The binding was allowed to proceed at 4°C overnight, after which the protein G beads were washed with IP buffer four times. RNA was extracted from the beads with an RNeasy kit (Qiagen, Valencia, CA). RT-PCR was then used to detect HCV internal ribosome entry site (IRES) with the following primers: IRES Forward, 5′-GTC TGC GGA ACC GGT GAG-3′; IRES Reverse, 5′-CGG GTT GAT CCA AGA AAG GAC-3′.

Recombinant protein production and GST binding assay. Recombinant protein expression and purification via glutathione Sepharose 4B beads were carried out according to the manufacturer's protocol (GE Healthcare, Piscataway, NJ). For the glutathione *S*-transferase (GST) pull-down assay, 20 μg of GST or GST-CyPA was brought to a final volume of 200 μl with binding buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM dithiothreitol [DTT], 0.5% NP-40, 0.5 mM PMSF, 5% glycerol). Replicon cells (4×10^5) were lysed in 300 μl of IP buffer supplemented with 1 mM DTT and 1 mM EDTA, and 40 μl of this lysate was added to each sample and allowed to rotate at 4°C for 30 min. Glutathione Sepharose 4B beads (25 μl of a 50% slurry per sample) were then added to each sample and allowed to rotate at 4°C for 30 min. Beads were washed and then sedimented at 500 × *g* for 5 min. Proteins bound to the beads were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. For the reactions that included CsA, the compound was added to the recombinant proteins prior to the addition of the cell lysates.

Co-IP of NS5B and CyPA. 293-T cells were transfected with cDNAs expressing Con1 NS5B and Flag-CyPA for 48 h. Cells were lysed in IP buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% NP-40,1 mM PMSF, 1 mM EDTA,1 mM DTT, 1× protease inhibitor cocktail) by rotating at 4°C for 30 min. The lysate was then clarified by centrifugation, and the supernatant was subjected to IP with EZview anti-Flag M2 affinity beads (Sigma-Aldrich, MO) according to the manufacturer's instructions. Proteins bounds to the beads following the IP protocol were eluted by boiling in SDS loading buffer and analyzed by SDS-PAGE followed by Western blotting using the indicated antibodies.

JFH-1/HCVcc production and infection. Full-length JFH-1 cDNA was provided by Takaji Wakita. Production of infectious HCVcc and infection of Huh-7.5 cells were performed as described previously (49). Western blotting and immunostaining of infected cells were carried out according to standard methods.

Electroporation of replicon RNA and cDNA expression plasmids. One microgram of replicon RNA was mixed with 9 μg of a pcDNA3.1-based plasmid containing no insert, CyPA cDNA, CyPB cDNA, or CyPC cDNA and used in a standard electroporation (36). RNA was extracted 7 h and 4 days postelectroporation and subjected to quantitative RT-PCR analysis to detect HCV RNA as described previously (36). All the CyP cDNAs were tagged with a Myc epitope at the C terminus, and the CyPA cDNA containing silent mutations in the sh-A161 recognition site was designated CyPA#.

In vitro replication assay. Replicon cells were washed with ice-cold wash buffer (30 mM HEPES [pH 7.4], 33 mM NH_4Cl, 7 mM KCl, 150 mM sucrose, 4.5 mM magnesium acetate) containing freshly added lysolecithin (250 μg/ml) for 2 min. After complete aspiration of the wash buffer, 125 μl of incomplete replication buffer (100 mM HEPES [pH 7.4], 50 mM NH_4Cl, 7 mM KCl, 1 mM spermidine) was added to each plate, and the cell lysates were collected with a cell scraper. We then centrifuged the lysate at 800 × *g* for 5 min to remove cell debris, and the recovered supernatant was stored at −80°C as replication lysates, 70 μl of which was used for each in vitro replication assay as described below. Before the replication assay was performed, NP-40 (1%, vol/vol) was added to each replication lysate and the mixture was rotated at 4°C for 1 h, after which time 7 μl rabbit anti-CyPA polyclonal antibody or normal rabbit IgG (negative control) was added, and the mixture was rotated at 4°C for an additional 4 h. In vitro replication was carried out for 4 h at 4°C in the presence of actinomycin D (10 μg/ml); RNaseOUT (800 U/ml); 25 μCi α-^{32}P-labeled CTP; 10 μM CTP; and 1 mM each of ATP, GTP, and UTP. RNA was immediately extracted after replication with TRIzol LS (Invitrogen) as described by the manufacturer. The products of the reactions were electrophoresed on 1.2% agarose gels for 2 to 3 h, dried on a gel dryer, and exposed to a phosphorimaging screen for at least 24 h.

RESULTS

A small interfering RNA (siRNA) directed at CyPA inhibits wild-type but not CsA-resistant HCV replicons. We recently isolated HCV subgenomic replicons resistant to CsA treatment in vitro (36). Cells containing the RS2 replicon were resistant to CsA at up to 2 μg/ml, while the cells expressing the GS5 cells were inhibited by CsA at even 0.25 μg/ml (Fig. 1A). To investigate the mechanism of this resistance, we examined the dependence of these replicons on the three CyPs (CyPA, -B, and -C) that have been implicated in HCV replication. We expressed shRNAs directed at each of these CyPs along with a control shRNA directed at firefly luciferase in either the GS5 or RS2 cells. All three CyP-directed shRNAs efficiently knocked down the expressions of their respective targets (Fig. 1B and C). The shRNA directed at CyPA (sh-A161) inhibited NS5A expression in GS5 cells but not in RS2 cells, despite similar knock-down levels of CyPA in the two cell lines. The shRNAs directed at CyPB (si-B710) or CyPC (sh-C454) had no effect on the NS5A level in either GS5 or RS2 cells. Fluorescence-activated cell sorting (FACS) results with green fluorescent protein (GFP) expression as a readout confirmed these results (data not shown). The same panel of shRNAs was also used in a colony formation assay designed to test their effects on HCV replication. We transduced the replicon cells with shRNA-expressing vectors that carry the puromycin *N*-acetyltransferase (*pac*) gene and then subjected the cells to a double selection with both puromycin and G418, which selects for cells that maintain replication. When GS5 cells were transduced with sh-A161, a significantly lower number of double-resistant colonies were observed, reflecting the inhibitory effect of sh-A161 on GS5 replication. This lower colony formation efficiency was not observed in RS2 cells (Fig. 1D). On the other hand, shRNAs directed at CyPB and CyPC had no significant effect on the number of colonies. Similar results were obtained with a CsA-resistant replicon single-cell clone, RS1-2 (data not shown).

To further validate a role of CyPA protein in HCV replication, we performed in vitro replication assays with cell extracts of replicon cells and then tested the ability of an antibody against CyPA to interfere with replication. Anti-CyPA effectively blocked replication when the assay was performed with GS5 lysate but failed to inhibit replication if RS2 lysate was used (Fig. 1E). These data corroborate the RNA interference results and suggest that CyPA protein is important for a step in

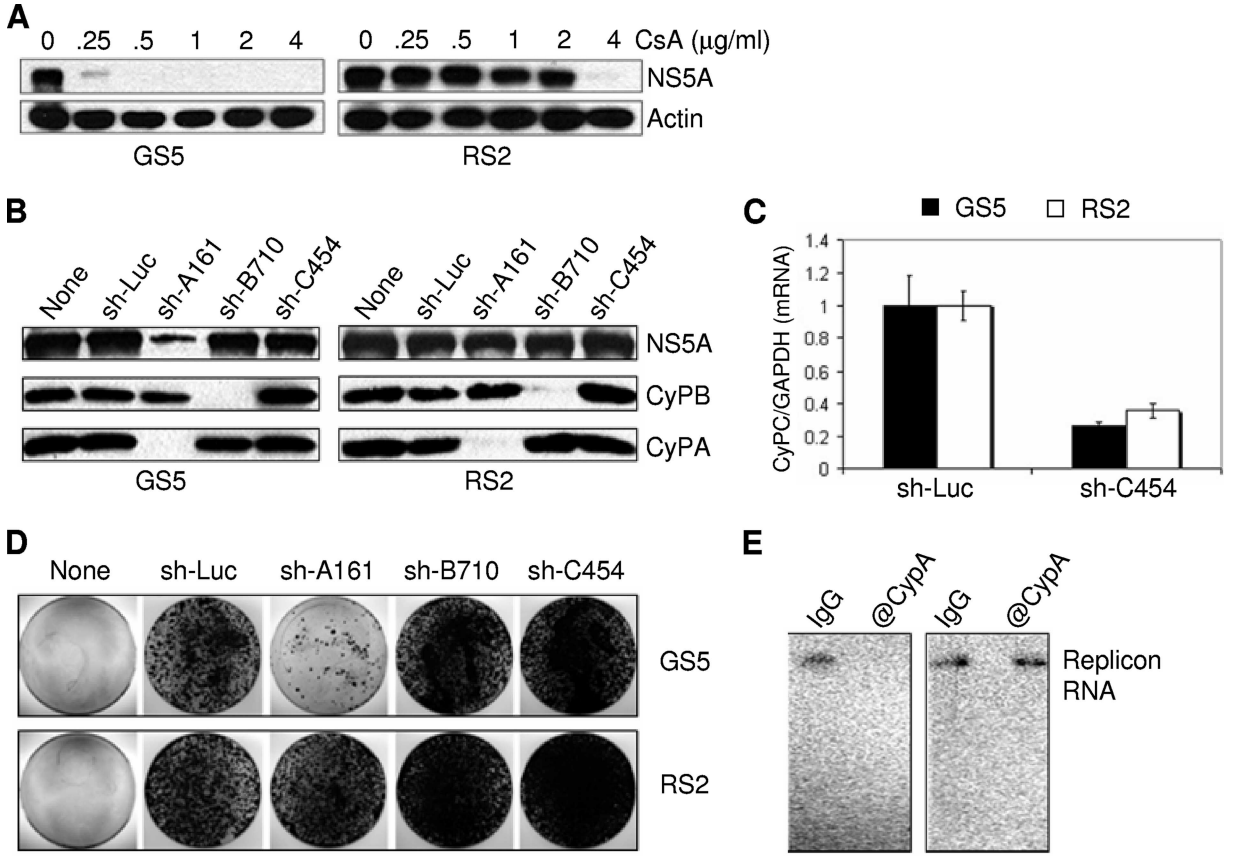

FIG. 1. An siRNA directed at CyPA inhibits replication of wild-type but not CsA-resistant replicons. (A) RS2 replicon is less sensitive than GS5 replicon to CsA. The two replicon cell lines were treated with CsA at the indicated concentrations for 4 days before being analyzed for NS5A expression by Western blotting. (B) Various shRNAs directed at three CyPs or firefly luciferase were introduced into the replicon cells and expressed for 7 days. The cells were then lysed and probed for the indicated proteins. (C) Suppression of CyPC RNA expression by sh-C454. Total RNA was then extracted 7 days posttransduction and subjected to real-time RT-PCR analysis to determine the level of CyPC mRNA. (D) After introduction of the shRNAs as in panel B, the cells were subjected to double selection with puromycin and G418. At the end of the selection, the cell colonies were stained with violet blue. (E) In vitro replication reaction of GS5 or RS2 lysate was allowed to progress in the presence of either IgG negative control or an anti-CyPA antibody for 4 h. The ^{32}P-labeled products of replication were detected as a single band of expected size on an RNA gel.

the HCV replication process that can be measured by the in vitro replication assay.

HCV replicon expression is correlated with CyPA expression level. Several other siRNAs directed at CyPA have been evaluated in vitro for anti-HCV effects and have yielded conflicting results. Two shRNAs directed at CyPA were reported by Nakagawa et al. to have both knocked down CyPA and inhibited replication of a GT 1b replicon (28). In contrast, Watashi et al. reported a CyPA siRNA that efficiently suppressed CyPA expression but failed to inhibit HCV replication (46). In an attempt to reconcile these results, we constructed shRNAs directed at the same CyPA mRNA sites as reported by these groups (Fig. 2A) and compared their effects on HCV replication to those of sh-A161. sh-285's target was the same 21-nucleotide sequence as Watashi's siRNA ("si-CyPA") and sh-459's was a 21-nucleotide sequence that was encompassed by Nakagawa's shRNA #441, which had a target sequence of 29 nucleotides. We also constructed sh-A285′, which is identical to sh-285 except for a mismatch of one nucleotide between the siRNA and the target sequence. Consequently, sh-285′ was expected to lack the ability to knock down CyPA. Finally, we constructed a negative-control shRNA (sh-NTC) that does not recognize any human mRNA in GenBank. When this panel of shRNAs was introduced into GS5 cells, three of them, sh-A161, sh-A285, and sh-A459, knocked down CyPA expression as expected (Fig. 2B). All three cell lines with CyPA knock-downs also had reduced NS5A levels. The shRNAs that did not inhibit CyPA expression also did not affect the replicon. Our original shRNA (sh-A161) was the most effective in both silencing CyPA expression and inhibiting NS5A synthesis (Fig. 2B). Because the different effects of sh-A285 and Watashi's si-CyPA could potentially be explained by the difference between shRNA and synthesized siRNA duplex, we tested a synthesized form of sh-A161 and sh-A285 in transient-transfection experiments. Both siRNAs (si-A161 and si-A285) inhibited CyPA and NS5A expression (Fig. 2C). In this form, si-A161 is no more potent than si-A285 in suppressing CyPA expression; accordingly, the abilities of these two siRNAs to inhibit HCV replicon were also comparable. The overall reduction of both CyPA and NS5A levels was less dramatic with these preformed siRNA duplexes, likely due to the lower efficiency of transfecting siRNA into cells in comparison to trans-

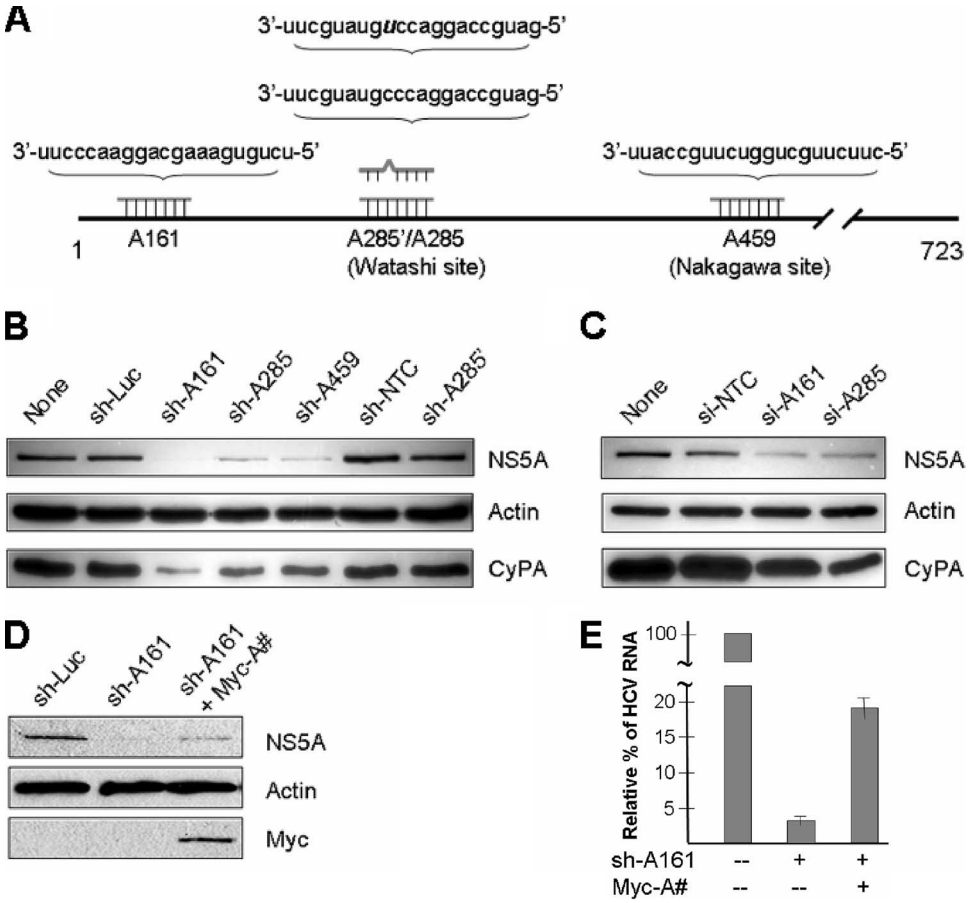

FIG. 2. HCV replicon expression is correlated with CyPA expression level. (A) Schematic representation of the target sites of anti-CyPA shRNAs used in this study. The target sites of previously reported siRNAs are also shown. (B) GS5 cells were transduced with the indicated shRNAs for 7 days. Total protein lysates were then subjected to SDS-PAGE and immunoblotting. (C) Effects of synthesized siRNA duplexes directed at either the 161 or the 285 site on HCV replication. The detection of NS5A, actin, and CyPA was performed 7 days after transfection of the siRNA duplexes into GS5 cells. (D) An siRNA-resistant CyPA cDNA rescued NS5A expression in the presence of sh-A161. A mammalian expression plasmid bearing a CyPA cDNA (Myc-A#) that contained silent mutations in the recognition site of sh-A161 was introduced into GS5 cells, which had been transduced with sh-A161, to examine the effect of restoring CyPA expression on HCV replication. (E) Expression of CyPA# cDNA in CyPA-knock-down cells partially rescues HCV RNA replication. In vitro-transcribed Rep1b RNA was coelectroporated into Huh-7.5/sh-A161 cells with the Myc-A# cDNA expression plasmid. RNA was extracted at 7 h and 4 days postelectroporation for analysis of HCV IRES and GAPDH RNA level. After normalization to GAPDH levels, the ratio of the day 4 HCV RNA versus the 7-h HCV RNA was calculated and plotted. A parallel electroporation of Rep1b RNA into Huh-7.5/sh-Luc cells was used as a positive control, which is set at 100%.

ducing the shRNAs using lentiviral vectors. To eliminate definitively the possibility that the target of all these siRNAs was a chimeric mRNA containing the entire CyPA mRNA as part of its sequence (38), we performed cDNA rescue experiments. A cDNA of CyPA that contained silent mutations in the recognition site of sh-A161 was constructed and cloned into a mammalian expression vector. A Myc tag was placed at the C terminus of the protein to allow specific detection. When this cDNA (Myc-A#) was introduced into replicon cells together with sh-A161, both NS5A expression (Fig. 2D) and HCV RNA replication (Fig. 2E) were partially rescued, indicating that sh-A161 indeed exerts its inhibitory effect by repressing CyPA expression.

CyPA mediates CsA resistance of RS2 cells. In contrast to the GS5 replicon, RS2 replicated efficiently in the presence of substantial CyPA knock-down (Fig. 1D). This result could be explained by either CyPA-independent replication or a replication strategy that requires much less CyPA protein because shRNAs normally cannot eliminate the gene product completely. To distinguish between these two possibilities, we treated the RS2/sh-A161 replicon cells, recovered after double selection of puromycin and G418 following shRNA transduction, with CsA. If RS2 replicates in a CyP-independent manner in these cells, one would expect no effect of the CsA on the RS2/sh-A161 cells. On the other hand, if the RS2 replicon was resistant to CsA and sh-A161 because it needed less CyPA to replicate (thus requiring a higher concentration of CsA to inhibit its replication), then one would expect a reduced resistance to CsA as the pool of CyPA is smaller in this case. Indeed, the latter appeared to be the case, as the RS2/sh-A161 replicon was six to eight times more sensitive to CsA than was the RS2/sh-Luc replicon (Fig. 3A), essentially reverting to the sensitivity level of the wild-type replicon GS5. Western blotting confirmed the substantial suppression of CyPA in the RS/sh-

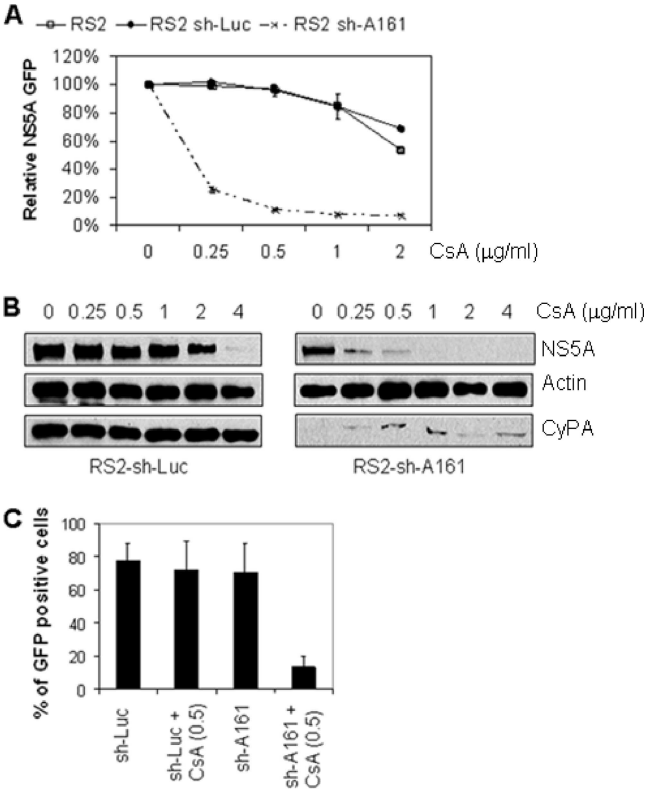

FIG. 3. CyPA is the key mediator of CsA resistance in RS2 cells. (A) RS2 cells and two derivative cell lines stably expressing sh-Luc and sh-A161 were treated with increasing amounts of CsA for 4 days before being fixed for FACS analysis. The percentage of GFP-positive cells at the end of the treatments is normalized to the value of the untreated sample. (B) Western blot results confirmed the substantial knockdown of CyPA in the RS2/sh-A161 cells and their heightened CsA sensitivity. (C) CsA treatment of RS2 cells transiently transduced with sh-A161. The RS2 cells were transduced with either sh-Luc or sh-A161 for 4 days; half of the cells were then treated with 0.5 μg/ml of CsA for 3 more days before being subjected to FACS analysis of NS5A-GFP expression.

A161 cells (Fig. 3B). To control for the possibility that the difference in sensitivity to CsA was caused by some unidentified differences in the antibiotic-selected cells, we applied the double treatment (sh-A161 and CsA) to RS2 cells without selection. When the RS2 cells were treated with 0.5 μg/ml CsA or transduced with sh-A161, no significant suppression was seen in either treatment, but when RS2 cells were first transduced with sh-A161 and then treated with the same concentration of CsA, a dramatic inhibition was achieved (Fig. 3C). Taken together, these data indicate that CyPA is the principal mediator of the CsA resistance observed in RS2 cells, the replication of which requires a much reduced level of CyPA.

CyPA is essential for the replication of multiple HCV isolates. GS5 and RS2 cells contain GT 1b replicons with the GFP gene inserted into the NS5A region (25, 29). We next examined the effect of CyPA knock-down on replicons without GFP. Both GT 1a and 1b replicons are tested. To this end, we established stable Huh-7.5 cell lines that expressed the various shRNAs and then challenged them, by electroporation, with in vitro-transcribed GT 1a (H77) and 1b (Con1) subgenomic replicon RNAs. The shRNAs effectively silenced the expression of their respective targets in the stable cells as expected (Fig. 4A). No defect in morphology or growth rate was detected for any of the stable cell lines (data not shown), confirming that these CyPs are dispensable for cell survival in vitro (5). Expression of sh-A161 completely inhibited the ability of either replicon RNA to form colonies, whereas sh-Luc had no effect (Fig. 4B). An inhibitory effect (~50%) of sh-B710 on the GT 1a replicon was sometimes observed, but the colony formation efficiency was still much higher than that in sh-A161 cells. No other shRNA showed any consistent inhibitory effect on any of the replicons. Transient-transduction experiments again confirmed the inhibitory effect of sh-A161 on the expression of NS5A of both GT 1a and 1b replicons (Fig. 4C).

The complete cycle of HCV infection can now be studied in cell culture with infectious viruses produced in vitro (6, 17, 42, 47, 49), so we next determined whether CyPA is required for HCV infection in vitro. Stable Huh-7.5 cells harboring control or sh-A161 were infected with HCVcc particles produced with the JFH-1 genome. Infection of Huh-7.5 cells was efficient; viral RNA and antigens became readily detectable in the target cells within a few days after infection. Expression of sh-Luc or sh-B710 had no effect on HCV infection, whereas the sh-A161 cells were highly refractory to infection (Fig. 5A). The protection provided by sh-A161 was observed by several methods: RT-PCR, fluorescence staining for core protein, or Western blot detection of NS3 (Fig. 5B and C). The sh-A161 cells remained fully susceptible to infection by vesicular stomatitis virus, a negative-strand RNA virus that is sensitive to nonspecific antiviral responses (Fig. 5D).

Last, to determine whether sh-A161 could repress an existing infection, we infected Huh-7.5 cells with JFH-1 virus for 10 days and then introduced sh-A161 by transduction. The expression of NS3 was measured 7 days after transduction. As shown in Fig. 5E, delivery of sh-A161 into infected cells suppressed viral replication, parallel to the results obtained with transient transduction of replicon cells.

The association of CyPA with HCV polymerase and RNA in replicon cells is correlated with CsA resistance. In vitro replication results suggested that CyPA is directly involved in the replication process (Fig. 1E). To determine whether CyPA is associated with HCV genome in replicon cells, we precipitated CyPA from replicon lysates and extracted the coprecipitated RNA, which was then subjected to RT-PCR analysis with primers complementary to the 5′ nontranslated region of HCV. HCV RNA was found to be precipitated by an anti-CyPA antibody but not by an IgG control antibody (Fig. 6A). This association was inhibited by CsA treatment when the experiment was performed with the GS5 replicon (Fig. 6B, left). For the RS2 replicon, association with CyPA was resistant to CsA treatment (Fig. 6B, right). We next examined the interaction between CyPA and the HCV polymerase NS5B. GST-CyPA specifically bound NS5B while GST protein alone was not able to bind (Fig. 6C). The interaction between GS5 NS5B and GST-CyPA was reduced by CsA treatment and became abolished at 2.4 μg/ml CsA (Fig. 6D, left). NS5B from the RS2 cells, however, retained binding to GST-CyPA even at this concentration of the drug (Fig. 6D, right). These results suggest that the association of CyPA with the HCV replication machinery is targeted by CsA and the CsA-resistant interaction

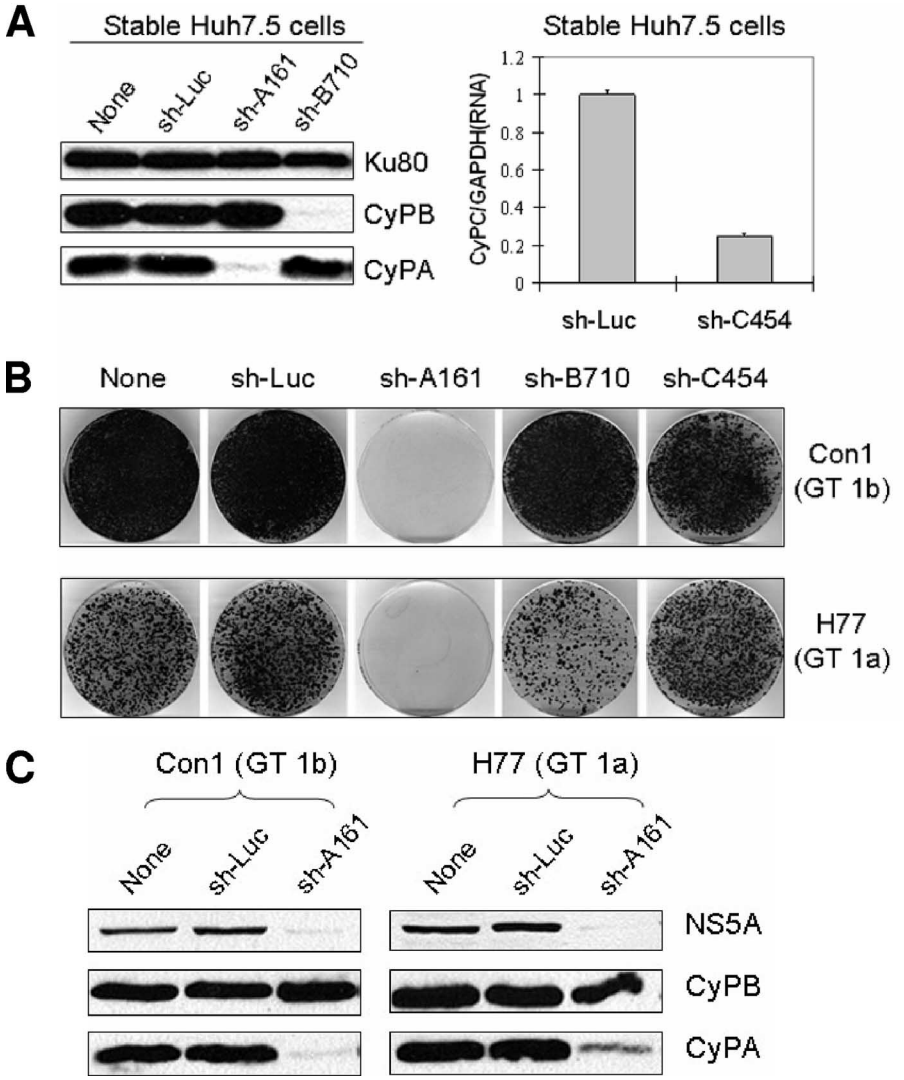

FIG. 4. CyPA is essential for replication of both GT 1a and GT 1b replicons. (A) Silencing of CyP targets in stable Huh-7.5 cells transduced with shRNAs directed at CyPA, CyPB, and CyPC. (B) sh-A161 blocks the ability of GT 1a and 1b replicons to replicate and form colonies in stable Huh-7.5 cells. One microgram of replicon RNA was used in each electroporation, and the selection was allowed to proceed for 4 weeks before the plates were stained. (C) Inhibition of Con1 and H77 replication by transiently expressed sh-A161. The Con1 and H77 replicon cells were transduced with either the sh-Luc or the sh-A161 vector for 7 days. The cells were lysed, and total proteins were subjected to immunoblotting for the detection of viral proteins NS5A and the two CyPs shown.

between NS5B and CyPA contributes to the CsA-resistant replication of the RS2 replicon. To examine if NS5B could interact with CyPA in vivo in the absence of any other HCV proteins and viral RNA, we performed co-IP experiments with NS5B and Flag-tagged CyPA transiently expressed in 293-T cells. NS5B coprecipitated with CyPA in this setting (Fig. 6E), indicating that the CyPA-NS5B interaction in vivo is not mediated by any other viral protein or RNA. Only a fraction of the total NS5B was precipitated by the anti-Flag beads as expected because the expressed NS5B proteins were expected to interact with both Flag-tagged and untagged, endogenous CyPA.

Different expression levels of CyP isoforms in replicon cells. Consistent with the critical role of CyPA in mediating CsA's action in regulating a variety of biological activities such as immunosuppression (12), HIV infection (5), and HCV replication (this study), it has been shown that the expression level of CyPA is 10 to 100 times higher than that of other CyPs in various tissues (2). Here we examined the endogenous expression level of the three CyP isoforms in the replicon cells. Using quantitative RT-PCR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as an internal control, we discovered a significant difference in the expression levels of CyPA, CyPB, and CyPC in the replicon cells. The CyPA mRNA was expressed approximately 10 and 150 times higher than CyPB and CyPC mRNAs, respectively (Fig. 7). Moreover, the expression levels of the CyPs were found to be very similar in GS5 and RS2 cells, ruling out the possibility that RS2 cells are more resistant to CsA because of higher endogenous level of CyPs.

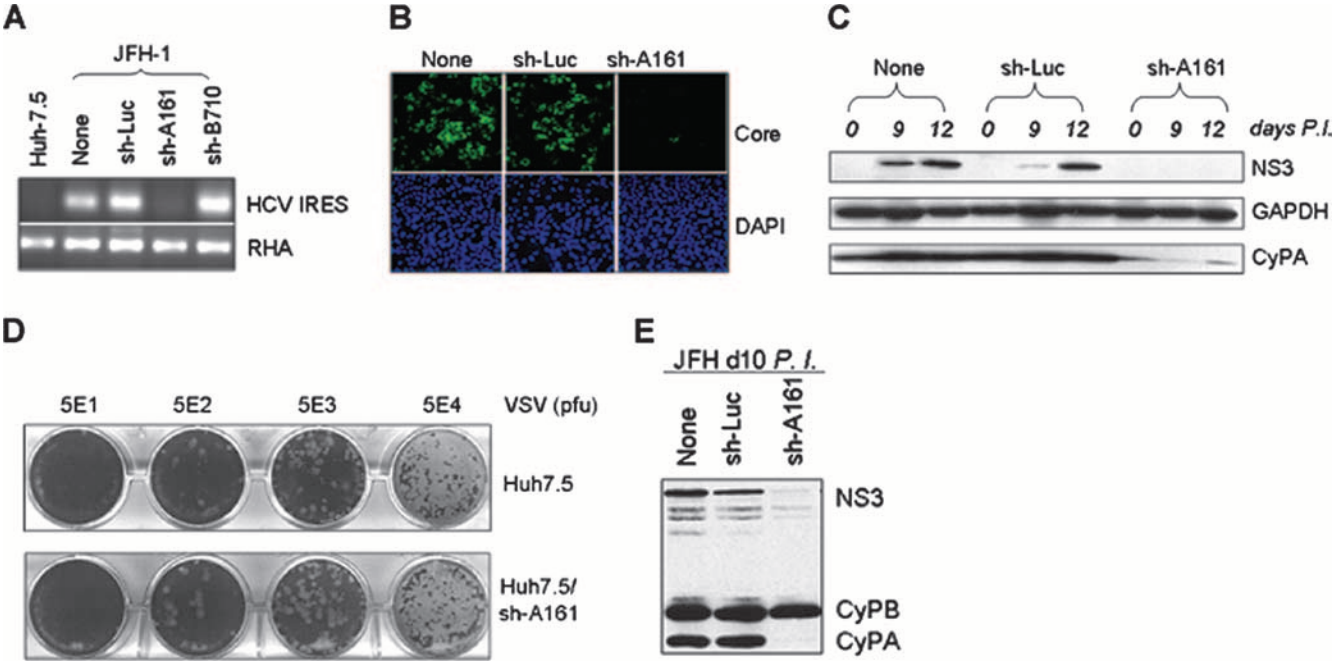

FIG. 5. Suppression of CyPA expression prevents infection by HCVcc/JFH-1. (A to C) The indicated stable cell lines were infected with JFH-1 for 9 to 12 days (9 days for panels A and B and up to 12 days for panel C), at which time (A) total RNA was isolated and subjected to RT-PCR detection of either HCV RNA or a cellular gene, RNA helicase A (RHA). (B) The cells were fixed with paraformaldehyde and stained with an anticore antibody. DAPI, 4′,6′-diamidino-2-phenylindole. (C) Total proteins were extracted and subjected to immunoblotting with an anti-NS3 antibody. (D) Infection of Huh-7.5/sh-A161 cells by vesicular stomatitis virus (VSV). Huh-7.5 cells with or without sh-A161 were subjected to infection by serial dilutions of VSV, ranging from 50 to 5×10^4 PFU. A plaque assay was then performed to quantify infection results. (E) Inhibition of an existing infection by sh-A161. Huh-7.5 cells were first infected with HCVcc/JFH-1 for 10 days and then transduced with either sh-Luc or sh-A161 vector. One week after transduction, total protein was extracted for immunoblotting for detection of the indicated proteins.

DISCUSSION

CsA-resistant HCV replicons have been isolated and characterized in vitro. Viral mutations, particularly those in the NS5B coding region, are correlated with CsA resistance (8, 36). In the study reported here, we demonstrated that the mechanism of this resistance lies in a reduced dependency on a specific target protein of CsA, CyPA, by the mutant replicons. Replication of wild-type replicons (GS5, Con1/SGR, and H77/SGR) was readily inhibited when CyPA expression was silenced, whereas the CsA-resistant replicons (RS2 and RS1-2) remained replication competent even in those cells with substantial CyPA knock-down. Although the correlated resistance to either CsA treatment or CyPA knock-down immediately suggested that RS2 cells are able to replicate independently of CyPs, in actuality, the RS2/sh-A161 replicon reverted to being CsA sensitive compared to RS2 and RS2/sh-Luc replicons. Moreover, treating the RS2 cells with a combination of 0.5 μg/ml CsA and sh-A161 inhibited replication, although neither treatment alone was effective. These results are best explained by a model in which CsA directly competes with HCV for binding to CyPA and an increase in affinity for CyPA as a result of the mutations in the RS2 replicon. The wild-type replicons are sensitive to CsA because they require a particular level of interaction with CyPA, which is disrupted upon CsA or siRNA treatment. If the mutations in the resistant replicons confer increased affinity for CyPA, these replicons would then require a lower amount of CyPA to achieve the same level of interaction. This trait is manifested as resistance to CyPA depletion and to the otherwise inhibitory concentrations of CsA. This hypothesis is supported by in vitro binding experiments involving NS5B and CyPA. NS5B from RS2 cells bound to GST-CyPA in a CsA-resistant manner while the interaction between the GS5 NS5B protein and CyPA was readily disrupted by the compound (Fig. 6C). It is also consistent with the finding that RS2's resistance to CsA is relative in that high concentrations of the compound can still inhibit the replication of the RS2 replicon, although high concentrations of CsA may have pleiotropic effects that have not been monitored by our experiments. Finally, in addition to enhancing binding affinity, the mutations may also alter the NS5B conformation in a way that requires less CyPA to properly function.

Our data also show that CyPA is an essential cofactor for both the subgenomic replicon and the infectious virus of wild-type HCV. Silencing of CyPA not only protected naïve Huh-7.5 cells from challenges by replicon RNA or JFH/HCVcc but also suppressed replication in cells with a preexisting replicon or viral infection, indicating a continuing requirement for CyPA in replicon or infected cells. When multiple shRNAs against CyPA were tested, a direct correlation was observed between the ability of a particular shRNA to knock down CyPA and the level of HCV inhibition. The replication efficiency of the GS5 replicon appeared to be exquisitely sensitive to the expression level of CyPA, as even small changes in the latter had a pronounced effect on the former (Fig. 2B). The

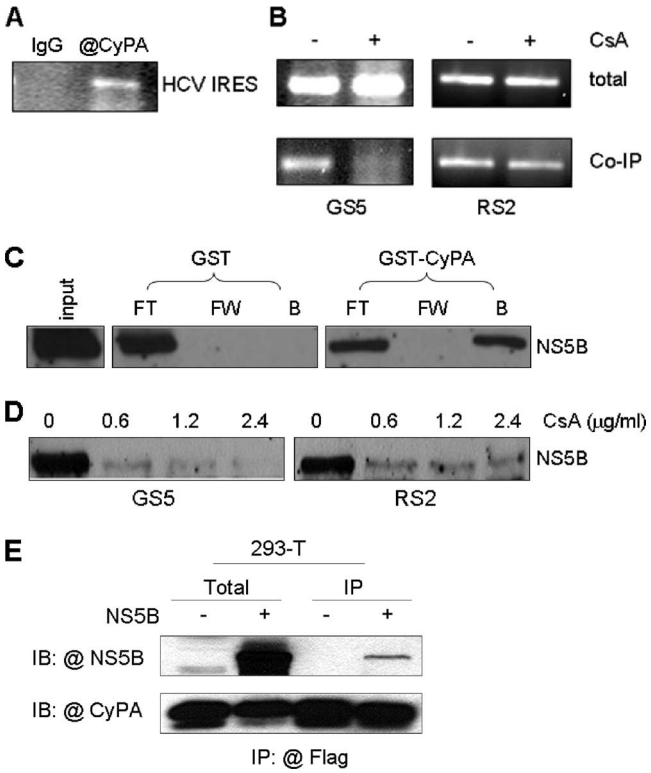

FIG. 6. CyPA interacts with NS5B and HCV RNA. (A) HCV RNA from replicon cells could be coprecipitated with CyPA. GS5 replicon cells were used, and HCV IRES was detected by RT-PCR. (B) Interaction between CyPA and HCV replicon RNA is sensitive to CsA treatment in GS5 but not in RS2 cells. The co-IP experiments were performed with anti-CyPA in the presence or absence of 4 μg/ml CsA. (C) CyPA interacts with NS5B in vitro. A GS5 replicon lysate was incubated with equal amounts of GST or GST-CyPA in vitro. The binding proteins were then purified with glutathione beads and subjected to Western blot assays to detect NS5B. FT, flowthrough; FW, final wash; B, bound. (D) CsA abolishes CyPA's interaction with GS5 NS5B but not with RS2 NS5B. The GST pull-down assay in panel C was performed in the presence of increasing amounts of CsA. Lysates from GS5 or RS2 cells were used. (E) NS5B interacts with GST-CyPA in the absence of other HCV proteins or RNA. 293-T cells were cotransfected with plasmids expressing Con1 NS5B and Flag-tagged CyPA for 48 h. The cells were lysed and subjected to IP by anti-Flag monoclonal antibody-conjugated beads.

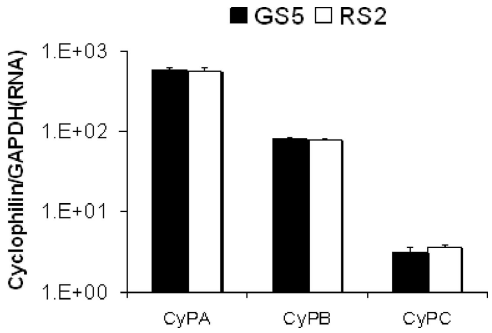

FIG. 7. Relative expression levels of CyPA, CyPB, and CyPC in HCV replicon cells. (A) CyPA is expressed at a much higher level than is CyPB or CyPC. Total RNA was extracted from GS5 and RS2 cells and subjected to reverse transcription with oligo(dT) primers. The cDNA was then divided into four equal parts for the detection of CyPA, CypB, CyPC, and GAPDH mRNA with real-time PCR.

requirement for CyPA was also observed with HCV of the GT 1b, 1a, and 2a genomes. This result contrasts with the reported role of CyPB in HCV replication, which appeared to be restricted to GT 1b replicons (14), and perhaps even to a specific subset of GT 1b replicons (36). A putative role of CyPC in the replication of a GT 1b replicon was also suggested (28). In our experiments, silencing of either of these CyPs did not have any consistent effect on HCV replication or infection in the various assays and viral genomes that we used. The structures of these CyPs, especially in complex with CsA, are quite similar (15, 23, 30, 34). In fact, the CsA-binding pocket in CyPB has the same structure as that in CyPA (23, 30). Since CsA competes with NS5B for CyP binding, one might expect all these CyPs to be able to bind NS5B and support HCV replication. However, the CyPA-directed shRNAs and the anti-CyPA antibody, all of which affected only CyPA and not CyPB or CyPC, were sufficient to suppress and in some cases completely abolish replication or infection, suggesting that other CyPs were not able to fulfill CyPA's essential role in HCV replication. An analogous observation has been made for HIV replication in T cells. Although both CyPA and CyPB bind to HIV Gag in vitro (4, 21), disrupting the CyPA gene alone by homologous recombination in a T-cell line completely abolished any effect of CsA on HIV, indicating that no other CyP could substitute for CyPA in these PPIA$^{-/-}$ cells (5). Subtle differences in structure and/or subcellular localization between CyPA and other members of the CyP family may contribute to this phenomenon. More importantly, the expression levels of these CyPs differ significantly both in the replicon cells (Fig. 7) and in liver tissue (2) in that CyPA is expressed 8 to 10 times more highly than CyPB and CyPC combined. As a result, even if CyPB or CyPC can interact with HCV in biochemical assays, neither comprises a large enough fraction of the total CyP pool in vivo to make significant contributions to HCV replication. In fact, CyPB has been found to interact with the NS5B protein of JFH-1 but its knock-down failed to suppress JFH-1 replication (14). Since JFH-1 is still inhibited by CsA, the authors of that report speculated that another member of the CyP family mediates the effect of the compound. Here we identify CyPA as the cellular factor that mediates the CsA inhibition of JFH-1 in addition to GT 1a and 1b replicons.

Biochemical experiments including in vitro replication and binding assays reveal an interaction between CyPA and the replication complex of HCV. An antibody against CyPA blocked replication directed by lysates from GS5 but not from RS2 replicon; moreover, CyPA was found to associate with HCV RNA in replicon cells in a CsA-sensitive manner. This association is mediated by an interaction between CyPA and NS5B. The CyPA-NS5B interaction appears to be direct and independent of other nonstructural proteins since recombinant NS5B produced from *Escherichia coli* also binds CyPA (data not shown). A direct interaction between CyPA and HIV capsid (CA) protein has also been demonstrated (21). The CyPA-CA complex not only constitutes the molecular target of CsA-mediated modulation of HIV infection in T cells (21) but also participates in innate immunity pathways that restrict HIV replication (20). CyPA binding either shields HIV capsid from

an endogenous restriction factor or renders the viral core vulnerable to the TRIM-5α-mediated inhibition, depending on the host cell type (39). Similar to the CyPA-NS5B interaction, binding of CyPA to HIV CA or its precursor protein, Gag, is also sensitive to CsA. The well-characterized crystal structure of the CyPA-CA complex (9, 41, 48) may provide relevant insight and guidance for further characterization of the CyPA-NS5B interface.

Precisely why HCV requires CyPA to replicate is not yet understood. CsA has been shown to interrupt RNA binding by NS5B synthesized in replicon cells (36, 46). In addition, CsA treatment induces the unfolded protein response, resulted from protein misfolding and accumulation in the endoplasmic reticulum (16, 26, 28, 33). CsA's effect on protein folding is consistent with the proposed chaperone role of immunophilins (1) and results of recent research on CyPA's role in modifying the retroviral capsid protein (39). Extensive misfolding of nonstructural proteins would certainly affect the assembly or the integrity of the HCV replication complex, which presumably assembles on endoplasmic reticulum-derived or related intracellular membranes (10, 24, 35). These proposed mechanisms of action for CsA are not mutually exclusive as the folding status of NS5B or the integrity of the replication complex could well affect NS5B's ability to bind to RNA inside the cells. Additional experiments designed to pinpoint the detailed mechanism by which CyPA serves as a HCV cofactor are under way in the authors' lab, and the CsA-resistant replicon cells and stable cells with various CyPs knocked down should provide important tools for these studies.

Derivatives of CsA that lack immunosuppressive function are being pursued as candidate antiviral drugs (13, 22, 32). We demonstrate here that manipulating the expression level of CyPA alone in target cells can produce substantial HCV inhibition. Agents affecting CyPA but not other CyPs, such as the sh-A161 described here, may have advantages over CsA as candidate drugs because of their better discrimination of the member of the CyP family that seems to be most acutely required for the replication of HCV.

ACKNOWLEDGMENTS

This work was supported by the James and Esther King Biomedical Research Program and the American Heart Association National Center.

We thank Takaji Wakita, Charlie Rice, Timothy Tellinghuisen, Guangxiang Luo, and Fanxiu Zhu for reagents; we also thank Ruth Didier for assistance with flow cytometry, the molecular cloning core facility of the FSU Department of Biological Science, and Anne B. Thistle for proofreading.

REFERENCES

1. **Barik, S.** 2006. Immunophilins: for the love of proteins. Cell. Mol. Life Sci. **63:**2889–2900.
2. **Bergsma, D. J., C. Eder, M. Gross, H. Kersten, D. Sylvester, E. Appelbaum, D. Cusimano, G. P. Livi, M. M. McLaughlin, K. Kasyan, et al.** 1991. The cyclophilin multigene family of peptidyl-prolyl isomerases. Characterization of three separate human isoforms. J. Biol. Chem. **266:**23204–23214.
3. **Binder, M., D. Quinkert, O. Bochkarova, R. Klein, N. Kezmic, R. Bartenschlager, and V. Lohmann.** 2007. Identification of determinants involved in initiation of hepatitis C virus RNA synthesis by using intergenotypic replicase chimeras. J. Virol. **81:**5270–5283.
4. **Braaten, D., H. Ansari, and J. Luban.** 1997. The hydrophobic pocket of cyclophilin is the binding site for the human immunodeficiency virus type 1 Gag polyprotein. J. Virol. **71:**2107–2113.
5. **Braaten, D., and J. Luban.** 2001. Cyclophilin A regulates HIV-1 infectivity, as demonstrated by gene targeting in human T cells. EMBO J. **20:**1300–1309.
6. **Cai, Z., C. Zhang, K. S. Chang, J. Jiang, B. C. Ahn, T. Wakita, T. J. Liang, and G. Luo.** 2005. Robust production of infectious hepatitis C virus (HCV) from stably HCV cDNA-transfected human hepatoma cells. J. Virol. **79:**13963–13973.
7. **Evans, M. J., C. M. Rice, and S. P. Goff.** 2004. Phosphorylation of hepatitis C virus nonstructural protein 5A modulates its protein interactions and viral RNA replication. Proc. Natl. Acad. Sci. USA **101:**13038–13043.
8. **Fernandes, F., D. S. Poole, S. Hoover, R. Middleton, A. C. Andrei, J. Gerstner, and R. Striker.** 2007. Sensitivity of hepatitis C virus to cyclosporine A depends on nonstructural proteins NS5A and NS5B. Hepatology **46:**1026–1033.
9. **Gamble, T. R., F. F. Vajdos, S. Yoo, D. K. Worthylake, M. Houseweart, W. I. Sundquist, and C. P. Hill.** 1996. Crystal structure of human cyclophilin A bound to the amino-terminal domain of HIV-1 capsid. Cell **87:**1285–1294.
10. **Gosert, R., D. Egger, V. Lohmann, R. Bartenschlager, H. E. Blum, K. Bienz, and D. Moradpour.** 2003. Identification of the hepatitis C virus RNA replication complex in Huh-7 cells harboring subgenomic replicons. J. Virol. **77:**5487–5492.
11. **Hamamoto, I., Y. Nishimura, T. Okamoto, H. Aizaki, M. Liu, Y. Mori, T. Abe, T. Suzuki, M. M. Lai, T. Miyamura, K. Moriishi, and Y. Matsuura.** 2005. Human VAP-B is involved in hepatitis C virus replication through interaction with NS5A and NS5B. J. Virol. **79:**13473–13482.
12. **Handschumacher, R. E., M. W. Harding, J. Rice, R. J. Drugge, and D. W. Speicher.** 1984. Cyclophilin: a specific cytosolic binding protein for cyclosporin A. Science **226:**544–547.
13. **Inoue, K., T. Umehara, U. T. Ruegg, F. Yasui, T. Watanabe, H. Yasuda, J. M. Dumont, P. Scalfaro, M. Yoshiba, and M. Kohara.** 2007. Evaluation of a cyclophilin inhibitor in hepatitis C virus-infected chimeric mice in vivo. Hepatology **45:**921–928.
14. **Ishii, N., K. Watashi, T. Hishiki, K. Goto, D. Inoue, M. Hijikata, T. Wakita, N. Kato, and K. Shimotohno.** 2006. Diverse effects of cyclosporine on hepatitis C virus strain replication. J. Virol. **80:**4510–4520.
15. **Ke, H., Y. Zhao, F. Luo, I. Weissman, and J. Friedman.** 1993. Crystal structure of murine cyclophilin C complexed with immunosuppressive drug cyclosporin A. Proc. Natl. Acad. Sci. USA **90:**11850–11854.
16. **Kozutsumi, Y., M. Segal, K. Normington, M. J. Gething, and J. Sambrook.** 1988. The presence of malfolded proteins in the endoplasmic reticulum signals the induction of glucose-regulated proteins. Nature **332:**462–464.
17. **Lindenbach, B. D., M. J. Evans, A. J. Syder, B. Wolk, T. L. Tellinghuisen, C. C. Liu, T. Maruyama, R. O. Hynes, D. R. Burton, J. A. McKeating, and C. M. Rice.** 2005. Complete replication of hepatitis C virus in cell culture. Science **309:**623–626.
18. **Lindenbach, B. D., and C. M. Rice.** 2005. Unravelling hepatitis C virus replication from genome to function. Nature **436:**933–938.
19. **Liu, J., J. D. Farmer, Jr., W. S. Lane, J. Friedman, I. Weissman, and S. L. Schreiber.** 1991. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell **66:**807–815.
20. **Luban, J.** 2007. Cyclophilin A, TRIM5, and resistance to human immunodeficiency virus type 1 infection. J. Virol. **81:**1054–1061.
21. **Luban, J., K. L. Bossolt, E. K. Franke, G. V. Kalpana, and S. P. Goff.** 1993. Human immunodeficiency virus type 1 Gag protein binds to cyclophilins A and B. Cell **73:**1067–1078.
22. **Ma, S., J. E. Boerner, C. TiongYip, B. Weidmann, N. S. Ryder, M. P. Cooreman, and K. Lin.** 2006. NIM811, a cyclophilin inhibitor, exhibits potent in vitro activity against hepatitis C virus alone or in combination with alpha interferon. Antimicrob. Agents Chemother. **50:**2976–2982.
23. **Mikol, V., J. Kallen, and M. D. Walkinshaw.** 1994. X-ray structure of a cyclophilin B/cyclosporin complex: comparison with cyclophilin A and delineation of its calcineurin-binding domain. Proc. Natl. Acad. Sci. USA **91:**5183–5186.
24. **Miyanari, Y., M. Hijikata, M. Yamaji, M. Hosaka, H. Takahashi, and K. Shimotohno.** 2003. Hepatitis C virus non-structural proteins in the probable membranous compartment function in viral genome replication. J. Biol. Chem. **278:**50301–50308.
25. **Moradpour, D., M. J. Evans, R. Gosert, Z. Yuan, H. E. Blum, S. P. Goff, B. D. Lindenbach, and C. M. Rice.** 2004. Insertion of green fluorescent protein into nonstructural protein 5A allows direct visualization of functional hepatitis C virus replication complexes. J. Virol. **78:**7400–7409.
26. **Munro, S., and H. R. Pelham.** 1986. An Hsp70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. Cell **46:**291–300.
27. **Nakagawa, M., N. Sakamoto, N. Enomoto, Y. Tanabe, N. Kanazawa, T. Koyama, M. Kurosaki, S. Maekawa, T. Yamashiro, C. H. Chen, Y. Itsui, S. Kakinuma, and M. Watanabe.** 2004. Specific inhibition of hepatitis C virus replication by cyclosporin A. Biochem. Biophys. Res. Commun. **313:**42–47.
28. **Nakagawa, M., N. Sakamoto, Y. Tanabe, T. Koyama, Y. Itsui, Y. Takeda, C. H. Chen, S. Kakinuma, S. Oooka, S. Maekawa, N. Enomoto, and M. Watanabe.** 2005. Suppression of hepatitis C virus replication by cyclosporin a is mediated by blockade of cyclophilins. Gastroenterology **129:**1031–1041.
29. **Nelson, H. B., and H. Tang.** 2006. Effect of cell growth on hepatitis C virus (HCV) replication and a mechanism of cell confluence-based inhibition of HCV RNA and protein expression. J. Virol. **80:**1181–1190.

30. **Neri, P., G. Gemmecker, L. D. Zydowsky, C. T. Walsh, and S. W. Fesik.** 1991. NMR studies of [U-^{13}C]cyclosporin A bound to human cyclophilin B. FEBS Lett. **290:**195–199.
31. **Okamoto, T., Y. Nishimura, T. Ichimura, K. Suzuki, T. Miyamura, T. Suzuki, K. Moriishi, and Y. Matsuura.** 2006. Hepatitis C virus RNA replication is regulated by FKBP8 and Hsp90. EMBO J. **25:**5015–5025.
32. **Paeshuyse, J., A. Kaul, E. De Clercq, B. Rosenwirth, J. M. Dumont, P. Scalfaro, R. Bartenschlager, and J. Neyts.** 2006. The non-immunosuppressive cyclosporin DEBIO-025 is a potent inhibitor of hepatitis C virus replication in vitro. Hepatology **43:**761–770.
33. **Paslaru, L., M. Pinto, and M. Morange.** 1994. GRP78 induction by cyclosporin A in human HeLa cells. FEBS Lett. **350:**304–308.
34. **Pflugl, G., J. Kallen, T. Schirmer, J. N. Jansonius, M. G. Zurini, and M. D. Walkinshaw.** 1993. X-ray structure of a decameric cyclophilin-cyclosporin crystal complex. Nature **361:**91–94.
35. **Quinkert, D., R. Bartenschlager, and V. Lohmann.** 2005. Quantitative analysis of the hepatitis C virus replication complex. J. Virol. **79:**13594–13605.
36. **Robida, J. M., H. B. Nelson, Z. Liu, and H. Tang.** 2007. Characterization of hepatitis C virus subgenomic replicon resistance to cyclosporine in vitro. J. Virol. **81:**5829–5840.
37. **Salonen, A., T. Ahola, and L. Kaariainen.** 2005. Viral RNA replication in association with cellular membranes. Curr. Top. Microbiol. Immunol. **285:** 139–173.
38. **Sayah, D. M., E. Sokolskaja, L. Berthoux, and J. Luban.** 2004. Cyclophilin A retrotransposition into TRIM5 explains owl monkey resistance to HIV-1. Nature **430:**569–573.
39. **Sokolskaja, E., and J. Luban.** 2006. Cyclophilin, TRIM5, and innate immunity to HIV-1. Curr. Opin. Microbiol. **9:**404–408.
40. **Stone, M., S. Jia, W. D. Heo, T. Meyer, and K. V. Konan.** 2007. Participation of rab5, an early endosome protein, in hepatitis C virus RNA replication machinery. J. Virol. **81:**4551–4563.
41. **Vajdos, F. F., S. Yoo, M. Houseweart, W. I. Sundquist, and C. P. Hill.** 1997. Crystal structure of cyclophilin A complexed with a binding site peptide from the HIV-1 capsid protein. Protein Sci. **6:**2297–2307.
42. **Wakita, T., T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H. G. Krausslich, M. Mizokami, R. Bartenschlager, and T. J. Liang.** 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. **11:**791–796.
43. **Wang, C., M. Gale, Jr., B. C. Keller, H. Huang, M. S. Brown, J. L. Goldstein, and J. Ye.** 2005. Identification of FBL2 as a geranylgeranylated cellular protein required for hepatitis C virus RNA replication. Mol. Cell **18:**425–434.
44. **Waninger, S., K. Kuhen, X. Hu, J. E. Chatterton, F. Wong-Staal, and H. Tang.** 2004. Identification of cellular cofactors for human immunodeficiency virus replication via a ribozyme-based genomics approach. J. Virol. **78:**12829–12837.
45. **Watashi, K., M. Hijikata, M. Hosaka, M. Yamaji, and K. Shimotohno.** 2003. Cyclosporin A suppresses replication of hepatitis C virus genome in cultured hepatocytes. Hepatology **38:**1282–1288.
46. **Watashi, K., N. Ishii, M. Hijikata, D. Inoue, T. Murata, Y. Miyanari, and K. Shimotohno.** 2005. Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. Mol. Cell **19:**111–122.
47. **Yi, M., R. A. Villanueva, D. L. Thomas, T. Wakita, and S. M. Lemon.** 2006. Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. Proc. Natl. Acad. Sci. USA **103:**2310–2315.
48. **Zhao, Y., Y. Chen, M. Schutkowski, G. Fischer, and H. Ke.** 1997. Cyclophilin A complexed with a fragment of HIV-1 gag protein: insights into HIV-1 infectious activity. Structure **5:**139–146.
49. **Zhong, J., P. Gastaminza, G. Cheng, S. Kapadia, T. Kato, D. R. Burton, S. F. Wieland, S. L. Uprichard, T. Wakita, and F. V. Chisari.** 2005. Robust hepatitis C virus infection in vitro. Proc. Natl. Acad. Sci. USA **102:**9294–9299.