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# Interaction with a Ubiquitin-Like Protein Enhances the Ubiquitination and Degradation of Hepatitis C Virus RNA-Dependent RNA Polymerase

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**To identify potential cellular regulators of hepatitis C virus (HCV) RNA-dependent RNA polymerase (NS5B), we searched for cellular proteins interacting with NS5B protein by yeast two-hybrid screening of a human hepatocyte cDNA library. We identified a ubiquitin-like protein, hPLIC1 (for human homolog 1 of protein linking intergrin-associated protein and cytoskeleton), which is expressed in the liver (M. F. Kleijnen, A. H. Shih, P. Zhou, S. Kumar, R. E. Soccio, N. L. Kedersha, G. Gill, and P. M. Howley, Mol. Cell 6: 409-419, 2000). In vitro binding assays and in vivo coimmunoprecipitation studies confirmed the interaction between hPLIC1 and NS5B, which occurred through the ubiquitin-associated domain at the C terminus of the hPLIC1 protein. As hPLICs have been shown to physically associate with two E3 ubiquitin protein ligases as well as proteasomes (Kleijnen et al., Mol. Cell 6: 409-419, 2000), we investigated whether the stability and posttranslational modification of NS5B were affected by hPLIC1. A pulse-chase labeling experiment revealed that overexpression of hPLIC1, but not the mutant lacking the NS5B-binding domain, significantly shortened the half-life of NS5B and enhanced the polyubiquitination of NS5B. Furthermore, in Huh7 cells that express an HCV subgenomic replicon, the amounts of both NS5B and the replicon RNA were reduced by overexpression of hPLIC1. Thus, hPLIC1 may be a regulator of HCV RNA replication through interaction with NS5B.**

Hepatitis C virus (HCV), a member of the *Flaviviridae* family, is the major causative agent of non-A, non-B hepatitis (3). Its 9.6-kb positive-sense, single-stranded RNA genome encodes a single polyprotein of about 3,010 amino acids, which is proteolytically processed by a combination of host- and virus-encoded proteases into at least 10 distinct structural and nonstructural protein products (6, 11). Among the nonstructural proteins (NS2 to NS5B), NS5B is an RNA-dependent RNA polymerase (12). Recombinant NS5B proteins purified from insect cells (1) or *Escherichia coli* (25) can synthesize RNA by using various natural or artificial RNAs as templates in vitro. Indeed, NS5B alone is capable of copying in vitro-transcribed full-length HCV RNA (16) through either primer-dependent elongation (1) or de novo initiation (14, 16, 26) mechanisms. In the cells, however, NS5B is anchored to the membrane through the C-terminal domain of 21 hydrophobic amino acids (19). It may form a complex with certain cellular proteins, such as a vesicle-associated protein, hVAP33 (20), and other HCV nonstructural proteins, including NS3, NS4A, and NS5A, through direct or indirect interactions (8, 20). These interactions likely modulate the activity of RNA-dependent RNA polymerase in vivo.

Although no efficient cell culture system is available for studying HCV RNA replication in natural infections, high-level replication of an HCV subgenomic RNA, which is composed of an HCV internal ribosome entry site-directed neomycin phosphotransferase gene and encephalomyocarditis virus internal ribosome entry site-directed NS3 to NS5B, can be achieved in a human hepatoma cell line, Huh7 (13). However, the low frequency of selectable cell clones supporting HCV subgenomic RNA replication suggests that some cellular factors may be critical for efficient replication of HCV RNA. Furthermore, only a single cell line (Huh7) can support HCV subgenomic RNA replication. The level of RNA replication also varies with cell growth conditions (17). All of these findings suggest that HCV RNA replication is regulated by cellular factors. Furthermore, in the livers of chronically HCV-infected patients, viral proteins and RNA are typically difficult to detect because of low quantity. Thus, there likely are mechanisms, through either inhibition of synthesis or enhancement of degradation, to modulate HCV RNA and protein expression. The modulation of viral replication will be advantageous for the virus to escape from the host's defense mechanism.

A major pathway to degrade cellular proteins is the ubiquitin-dependent proteasomal mechanism, which involves a cascade reaction catalyzed by the E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and E3 ubiquitin protein ligase (2). The E3 ubiquitin ligase provides critical selectivity in determining which proteins will be degraded (2). Proteins

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modified by polyubiquitin chains are recognized by proteasome, probably by using a ubiquitin-like protein as an adaptor (10). A family of ubiquitin-like proteins, whose members contain a type 2 ubiquitin-like domain (UBL domain) at their N-terminal regions, was shown to bind the S5a proteasome subunit with a hydrophobic surface of the UBL domain (21). Yeast protein Dsk2p and its human homologs hPLIC1 and hPLIC2 belong to this type 2 ubiquitin-like protein family. Dsk2p binds to polyubiquitin via its C-terminal ubiquitin-associated domain (UBA domain) (5). hPLIC proteins not only interact with proteasome but also physically associate with at least two ubiquitin E3 ligases, E6AP and βTrCP (10). Therefore, hPLICs functionally connect the ubiquitination machinery to the proteasome (10).

In the infected host, ubiquitination of viral proteins may enhance cytotoxic T-lymphocyte induction (18). In a DNA immunization study, cotranslational ubiquitination of a plasmid-encoded lymphocytic choriomeningitis virus nucleoprotein resulted in rapid degradation of the protein, leading to enhanced antigen presentation and more efficient sensitization of target cells in a cytotoxicity assay (18).

To understand the mechanism of regulation of HCV replication, we set out to investigate potential cellular molecules that may interact with HCV proteins and thereby regulate their synthesis, turnover, or function. In this study, we identified a ubiquitin-like protein, hPLIC1, as an NS5B-binding protein. We showed that overexpression of hPLIC1 decreased the NS5B protein level through ubiquitin-dependent degradation and, in turn, decreased the viral RNA level. This is the first cellular protein that has been shown to affect NS5B turnovers.

## MATERIALS AND METHODS

**Plasmid construction.** To construct the plasmids used in the yeast two-hybrid screening, vector pGBT9, which encodes the GAL4 DNA-binding domain, and vector pGAD10, which encodes the GAL4 activation domain, were employed (Clontech, Palo Alto, Calif.). HCV NS5B (genotype 1a) was amplified by PCR from a full-length HCV cDNA (3) and cloned into GBT9 at *Eco*RI and *Sal*I sites to generate the pGBT9/NS5B construct, which can express a GAL4 DNA-binding domain–NS5B fusion protein in yeast. The cDNA library was custom made by Clontech using mRNAs from HepG2 cells that had been treated with 1000 U of alpha interferon (Sigma, St. Louis, Mo.) per ml at 37°C for 20 h. The cDNA was cloned into the *Eco*RI site of the pGAD10 vector to generate GAL4 activation domain-cDNA fusion proteins.

To construct the full-length hPLIC1, 5 μg of total RNA from HepG2 cells was annealed with a specific primer and incubated with avian myeloblastosis virus reverse transcriptase at 42°C for 90 min. The reaction mixture was then boiled for 5 min and used as the template in a PCR to amplify full-length hPLIC1, which was then cloned into the *Bam*HI and *Eco*RI sites of pcDNA3.1-TOPO-TA (Invitrogen).

The plasmid used for expressing glutathione *S*-transferase (GST)-hPLIC1 fusion protein in *E. coli* was constructed by inserting the hPLIC1 cDNA fragment into the *Bam*HI and *Eco*RI sites of the pGEX-4T-1 vector (Novagen, Madison, Wis.). To construct the mammalian expression vector for the full-length or truncated hPLIC1 tagged with a Flag epitope, a PCR-generated hPLIC1 fragment containing the Flag sequence (GAT TAC AAG GAT GAC GAC GAT AAG) at its 5′ end was cloned into the *Bam*HI and *Eco*RI sites of pcDNA3.1-TOPO-TA (Invitrogen).

Plasmid pCDNA3.1-NS5B, used to express HCV NS5B protein in Huh7 cells for in vitro translation of the NS5B protein, was described previously (20). It contains a T7 promoter as well as a cytomegalovirus immediate-early promoter. The PCR-generated NS5B fragment containing the Flag sequence was cloned into the *Bam*HI site of pcDNA3.1 (Invitrogen). The PCR-generated NS5A fragment (genotype 1a) containing the Flag sequence was cloned into the *Kpn*I and *Xba*I sites of pcDNA3.1 (Invitrogen).

**Yeast two-hybrid screening.** Yeast two-hybrid screening was performed with *Saccharomyces cerevisiae* Y190 according to the instructions of the manufacturer (Clontech). Yeast cells were first transformed with plasmid pGBT9/NS5B and then sequentially transformed with 100 μg of pGAD10/cDNA by the lithium acetate method. Double transformants were selected on $Trp^-$ $Leu^-$ $His^-$ glucose plates supplemented with 25 mM 3-amintriazole. Seven days after transformation, the surviving cells were tested for β-galactosidase (β-Gal) activity by using a filter lift assay. After being frozen in liquid nitrogen and thawed at room temperature, filter replicas of yeast transformants were then overlaid on Whatman 3 MM paper saturated with 6-bromo-4-chloro-3-indolyl-β-D-galactosidase solution (0.033% in Z buffer) and incubated at 30°C for 1 h. Blue colonies were isolated, plated, and tested again for β-Gal activity. Yeast clones containing plasmid pGAD10/cDNA were isolated from the positive clones as a result of the spontaneous loss of plasmid pGBT9/NS5B on $Trp^+$-SD medium plates. The isolated pGAD10/cDNA plasmid was verified by cotransformation with either pGBT9/NS5B or the parental plasmid pGBT9. Only the pGAD10/cDNA clone that developed blue color when cotransformed with pGBT9/NS5B, but not with pGBT9 vector, was considered a true positive.

**Sequence analysis.** The positive pGAD10/cDNA clone was sequenced with a Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham, Piscataway, N.J.). The DNA sequences were translated and compared with the nonredundant sequence database by using a family of BLAST programs through the National Center for Biotechnology Information network service.

**GST pull-down assay.** The GST fusion protein binding assay was performed as described previously (20). pGEX-4T-1 or pGEX-4T-1/hPLIC1 plasmids were transformed and expressed in *E. coli* BL21(DE3) (Novagen). GST or GST fusion proteins were purified with glutathione–Sepharose-4B beads. The amounts of purified proteins were estimated by Coomassie blue staining of sodium dodecyl sulfate (SDS)-polyacrylamide gels. $^{35}$S-labeled NS5B protein was in vitro translated in the TNT T7 Quick coupled transcription/translation system (Promega, Madison, Wis.) with pcDNA3.1/NS5B plasmid DNA as a template. Five micrograms each of GST and GST fusion proteins was incubated with 2 μl of in vitro-translated NS5B for 2 h at 4°C in binding buffer (40 mM HEPES [pH 7.5], 100 mM KCl, 0.1% Nonidet P-40, 20 mM 2-mercaptoethanol). After being washed four times in wash buffer (40 mM HEPES [pH 7.5], 100 mM KCl, 0.4% Nonidet P-40, 20 mM 2-mercaptoethanol), the beads were boiled in Laemmli sample buffer for 5 min. The dissociated proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and detected by autoradiography.

**Antibodies.** HCV patient serum and a monoclonal antibody were used to detect NS5B. To generate an NS5B-specific monoclonal antibody, Sf9 cells infected with recombinant baculoviruses expressing His-tagged NS5B were harvested 3 days after transfection. NS5B protein was purified with Ni-nitrilotriacetic acid agarose (Qiagen, Hilden, Germany). Six-week-old female BALB/c mice were immunized intraperitoneally with 50 μg of the purified NS5B per injection, which had been mixed with an equal volume of Titer Max adjuvant (CytRx, Norcross, Ga.). Mice were boosted three times at 2-week intervals thereafter. Spleen cells were isolated from the mice 3 days after the final injection and fused to the myeloma cell line HL-1 (Ventrex, Portland, Maine) with polyethylene glycol 1500 (Boehringer Mannheim). Hybridoma culture supernatants were screened for anti-NS5B antibody by Western blotting. NS5B-positive clones were subcloned twice by limiting dilution.

Monoclonal NS5A antibody was purchased from Biodesign International (Saco, Maine). Polyclonal NS5A antibody was described previously (20). Polyclonal Flag antibody and monoclonal tubulin and ubiquitin (P4D1) antibodies were purchased from Sigma. Tetramethyl rhodamine isocyanate (TRITC)-conjugated antibody against mouse immunoglobulin G was purchased from American Qualex (La Mirada, Calif.).

**Cell lines, transfection, immunoprecipitation, and immunoblotting.** Huh7 cells were transfected with various plasmids by using FuGENE6 (Roche) in 100-mm-diameter plates. For the immunoprecipitation study, the transfected cells were harvested at 48 h posttransfection and washed with cold phosphate-buffered saline (PBS). The samples were then collected in 1 ml of radioimmunoprecipitation assay buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 12 mM deoxycholate sodium salt, 0.1% SDS, 1% Triton X-100, 0.2 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride). After the cells were passed through a 25-gauge needle 10 times, the cell lysates were centrifuged at maximum speed in a microcentrifuge for 5 min at 4°C and the supernatant was collected. Immunoprecipitation was performed at 4°C in 1× TM10 buffer (50 mM Tris-HCl [pH 7.9], 100 mM KCl, 12.5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride). Cell lysates (50 μl) were incubated with 3 μl of anti-Flag M2-agarose affinity gel (Sigma) in a total volume of 300 μl of reaction buffer. After incubation overnight, the beads were washed four

times with 1× TM10 buffer. The precipitates were then boiled for 5 min in Laemmli sample buffer and run on a 10% polyacrylamide gel. Proteins were transferred to a nitrocellulose membrane (Hybond-ECL Plus) and analyzed by the ECL PLUS detection method (Amersham).

An HCV subgenomic replicon derived from plasmid 1bneo/delS (7) was kindly provided by C. Seeger of Fox Chase Cancer Center, Philadelphia, Pa. A stable cell line, Huh7-replicon, which contains the actively replicating RNA replicon, was selected from G418-resistant colonies after electroporation of Huh7 cells with the RNA transcribed from the plasmid. The control Huh7 cells were selected with G418 after electroporation with a plasmid encoding neomycin phosphotransferase.

**Pulse-chase labeling of NS5B.** Huh7 cells transfected with pCDNA3.1/Flag-NS5B and the full-length or truncated hPLIC1-expressing constructs were incubated at 48 h after transfection with Dulbecco's modified Eagle's medium (DMEM) without methionine, cysteine, and L-glutamine (Sigma) for 30 min and were pulse-labeled with 100 μCi of [$^{35}$S]methionine-cysteine protein labeling mix (NEN) per ml for 15 min. The cells were then washed twice with cold PBS and twice with complete DMEM and incubated in complete DMEM for various periods of time. The cells were lysed in 1 ml of radioimmunoprecipitation assay buffer, and the protein extract was immunoprecipitated with 5 μl of anti-Flag M2-agarose affinity gel (Sigma). The precipitates were then boiled for 5 min in Laemmli sample buffer and run on a 10% polyacrylamide gel before autoradiography.

**Immunofluorescence staining.** Huh7-replicon cells were seeded on eight-well chamber slides and incubated at 37°C under 5% $CO_2$ overnight before transfection with the hPLIC1 plasmid at 50% confluence. At 48 h after transfection, cells were fixed with 4% formaldehyde in PBS and permeabilized by incubation with 0.1% Triton X-100 in PBS for 30 min. The cells were then incubated with the primary antibody and TRITC-conjugated secondary antibody.

**Northern blot analysis.** Total cellular RNA was extracted with Trizol reagent (Gibco-BRL). Ten micrograms of total RNA was denatured by glyoxal-dimethyl sulfoxide treatment, electrophoresed through a 1% agarose gel in 10 mM sodium phosphate (pH 7.0), and then transferred to a nylon membrane and immobilized by UV cross-linking. RNA was glyoxalated before being electrophoresed on the gel. An in vitro-transcribed, $^{32}$P-labeled negative-strand 3′-untranslated region RNA was used as a probe to detect positive-strand HCV replicon RNA. A plasmid containing the GAPDH (glyceraldehyde-3-phosphate dehydrogenase)-coding sequence was used to generate in vitro-transcribed, $^{32}$P-labeled negative-strand GAPDH RNA. The membrane was hybridized in Rapid-hyb buffer (Amersham Pharmacia Biotech) for 2 h at 70°C, washed once in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS for 20 min at room temperature and twice in 1× SSC–0.1% SDS first and then 0.1× SSC–0.1% SDS for 20 min at 65°C, and then exposed to X-ray film or to a phosphorimaging screen for quantitative analysis.

## RESULTS

**Identification of a human cellular protein interacting with HCV NS5B protein.** To study the possible regulators of NS5B in the HCV life cycle, we performed a yeast two-hybrid screening of the HepG2 cDNA library by using the full-length NS5B of HCV genotype 1a as the bait to identify cellular proteins that can interact with the NS5B protein. Of $2 \times 10^6$ transformants screened, a single positive clone was obtained. The isolated pGAD10/cDNA plasmid was verified in a colony lift filter assay of β-Gal by cotransformation with either pGBT9/NS5B or the parental plasmid pGBT9. Double transformants containing control plasmids were selected on $Trp^-$ $Leu^-$ glucose plates without 3-amintriazole. Only the transformants containing the isolated pGAD10/cDNA and pGBT9/NS5B yielded blue colonies in the β-galactosidase assay (Fig. 1A). The cDNA sequence of this clone revealed that it encodes hPLIC1 (10), which is a 66-kDa ubiquitin-like protein, except for the N-terminal 90 amino acids (aa) (Fig. 2). hPLIC1 is one of the two human homologs of the yeast Dsk2 protein, which is a polyubiquitin-binding protein (5). hPLICs have been shown to form a link between the ubiquitination machinery and the proteasome (10). We further cloned a full-length hPLIC1 cDNA by reverse transcription-PCR of total RNA from HepG2 cells.

To investigate whether the full-length hPLIC1 can interact with HCV NS5B directly, we first performed an in vitro GST pull-down assay. *E. coli*-expressed GST-hPLIC1 fusion protein was incubated with in vitro-translated, $^{35}$S-labeled NS5B. The results showed that NS5B was pulled down by GST-hPLIC1 (Fig. 1B, lane 2) under the in vitro binding conditions used. In contrast, NS5B did not bind GST protein under the same conditions (Fig. 1B, lane 3). To demonstrate that interaction of hPLIC1 with NS5B could occur in the cells, we performed coimmunoprecipitation experiments with Huh7 cells expressing both NS5B and Flag-tagged hPLIC1. Cell lysates were immunoprecipitated with an anti-Flag antibody, and the immunoprecipitates were analyzed by immunoblotting with an HCV patient serum to detect NS5B. The results showed that NS5B was coimmunoprecipitated with hPLIC1 (Fig. 1C, lane 2), whereas in the absence of hPLIC1, the anti-Flag antibody could not precipitate NS5B (Fig. 1C, lane 4). Coomassie blue staining of the immunoprecipitates showed that the same amounts of cellular proteins were precipitated in both lysates (Fig. 1C). However, NS5B was not visible by the staining (Fig. 1C, lanes 12 and 13). The control experiment showed that the polyclonal anti-Flag antibody efficiently precipitated Flag-hPLIC1 (Fig. 1C, lanes 5 and 6). These results indicate that the full-length hPLIC1 interacts with NS5B both in vitro and in vivo.

**hPLIC1 interacts with NS5B at its C-terminal UBA domain.** To map the NS5B-interacting domains of hPLIC1, five truncated constructs of hPLIC1, each with a Flag tag at the N terminus (Fig. 2A), were coexpressed with NS5B in Huh7 cells and precipitated with anti-Flag antibody. The expression and levels of these truncated hPLIC1 were comparable, and all of them could be precipitated with a monoclonal anti-Flag antibody (Fig. 2B lower panel, lanes 1 to 10). The results showed that, among these truncated hPLICs, only the C-terminal aa 488 to 589 could precipitate NS5B (Fig. 2B, lane 2), indicating that NS5B bound primarily to the C-terminal region (aa 488 to 589) of hPLIC1, which contains the UBA domain. The N-terminal 138 or 198 aa of hPLIC1, which contain the UBL domain, did not bind NS5B (lanes 3 to 6). The N-terminal 542 aa or aa 194 to 487 of hPLIC1 did not pull down NS5B but precipitated some proteins reactive with the human serum (lanes 10 and 8). The nature of these proteins is not clear. These results indicate that the C-terminal UBA domain of hPLIC1 contains the critical residues for binding NS5B.

**Overexpression of hPLIC1 enhances the degradation of NS5B in vivo.** Recent studies have shown that Dsk2p binds to polyubiquitin via its C-terminal UBA domain and may deliver the polyubiquitinated proteins to the proteasome (5). Therefore, we investigated whether hPLIC1 can mediate the degradation of NS5B. We pulse-labeled, with [$^{35}$S]methionine, Huh7 cells transfected with the Flag-tagged NS5B and either Flag-hPLIC1 or vector plasmid and chased for various periods of time to examine the stability of NS5B protein. The NS5B and hPLIC1 proteins were precipitated by anti-Flag antibodies. The results showed that the half-life of the Flag-NS5B protein was approximately 4 to 5 h in the absence of hPLIC1 (Fig. 3A, left panel, and B). In the presence of hPLIC1, however, the half-life of Flag-NS5B was shortened to less than 2 h (Fig. 3A,

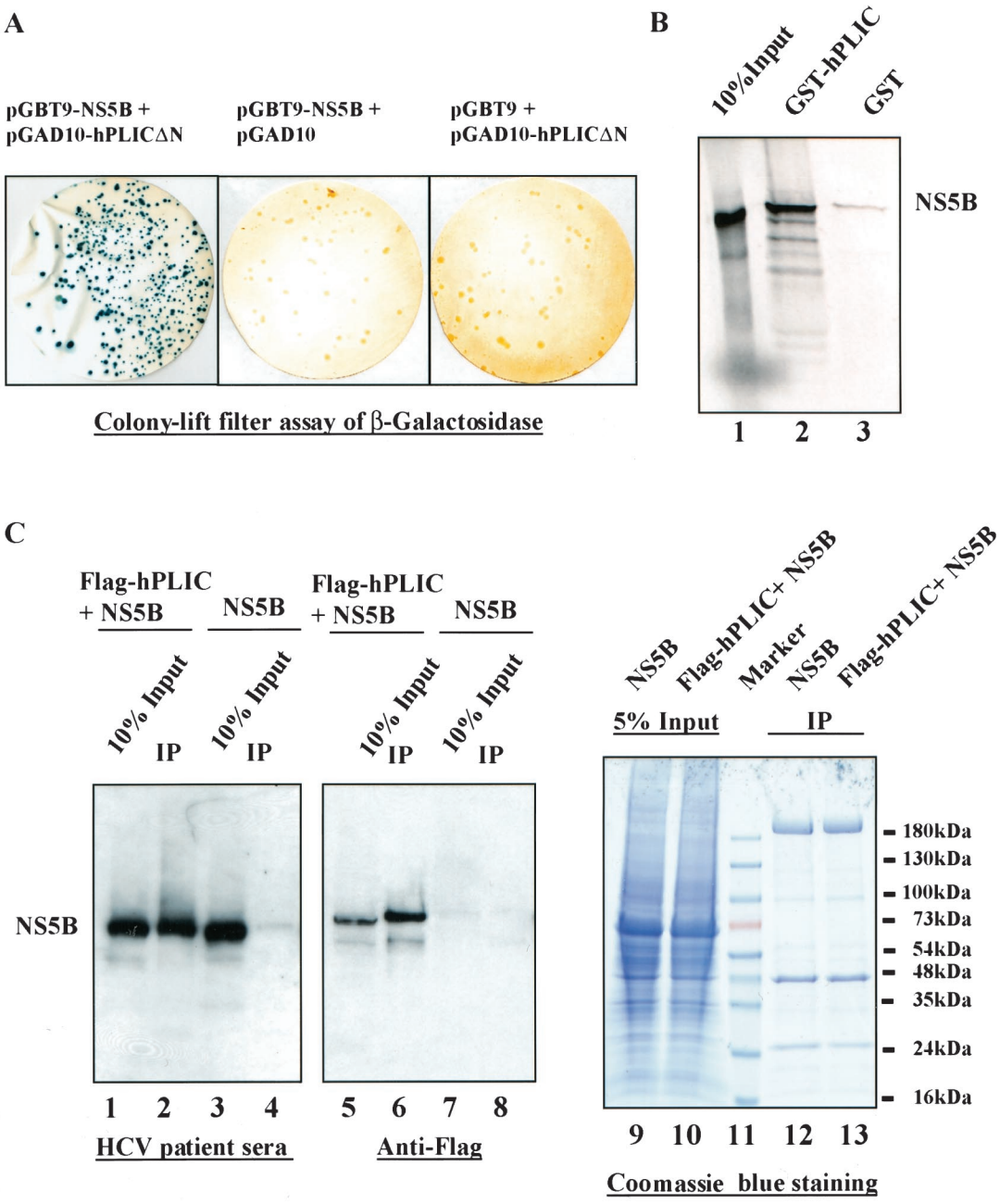



FIG. 1. Interaction of hPLIC1 with NS5B. (A) Interaction of NS5B with the N-terminally deleted hPLIC1 in yeast. The original cDNA clone from the two-hybrid screening was used. A colony lift filter assay of β-Gal is shown. (B) In vitro interaction of the full-length hPLIC1 with NS5B in GST-fusion protein binding assay. GST-hPLIC1 was incubated with in vitro-translated $^{35}$S-labeled NS5B and precipitated with glutathione beads (lane 2). GST protein alone was used as a negative control (lane 3). Lane 1 represents 10% of the input NS5B protein used in lanes 2 and 3. (C) In vivo coimmunoprecipitation of hPLIC1 with NS5B. The Flag-tagged full-length hPLIC1 was cotransfected with NS5B into Huh7 cells. Cell lysates were immunoprecipitated (IP) with anti-Flag-cross-linked Sepharose 4B beads. An HCV patient serum was used to detect NS5B (lanes 1 to 4) in Western blotting. Polyclonal anti-Flag antibody was used to detect Flag-hPLIC1 (lanes 5 to 8). Huh7 cells transfected with NS5B alone (lanes 3, 4, 7, and 8) were used as the negative control. Lanes 1, 3, 5, and 7 represent 10% of lysates prior to immunoprecipitation. A duplicate gel loaded with similar samples was stained with Coomassie blue (lanes 9 to 13). Lanes 9 and 10 represent 5% of lysates prior to immunoprecipitation.

middle panel, and Fig. B). Flag-NS5B became almost undetectable after 3 h of chase. In contrast, the overexpression of a truncated hPLIC1 without the C terminus (aa 1 to 542), which does not bind to NS5B (Fig. 2B), did not alter the stability of NS5B (Fig. 3A, right panel, and B). It should be noted that both the full-length (Fig. 3A, arrow) and C-terminally deleted hPLIC1 (Fig. 3A, arrowhead) were much more stable than NS5B (Fig. 3A, circle). Two nonspecific bands of 190 and 45 kDa, which were also seen in the mock-transfected cells and also correspond to those seen in Fig. 1C (lanes 12 and 13),

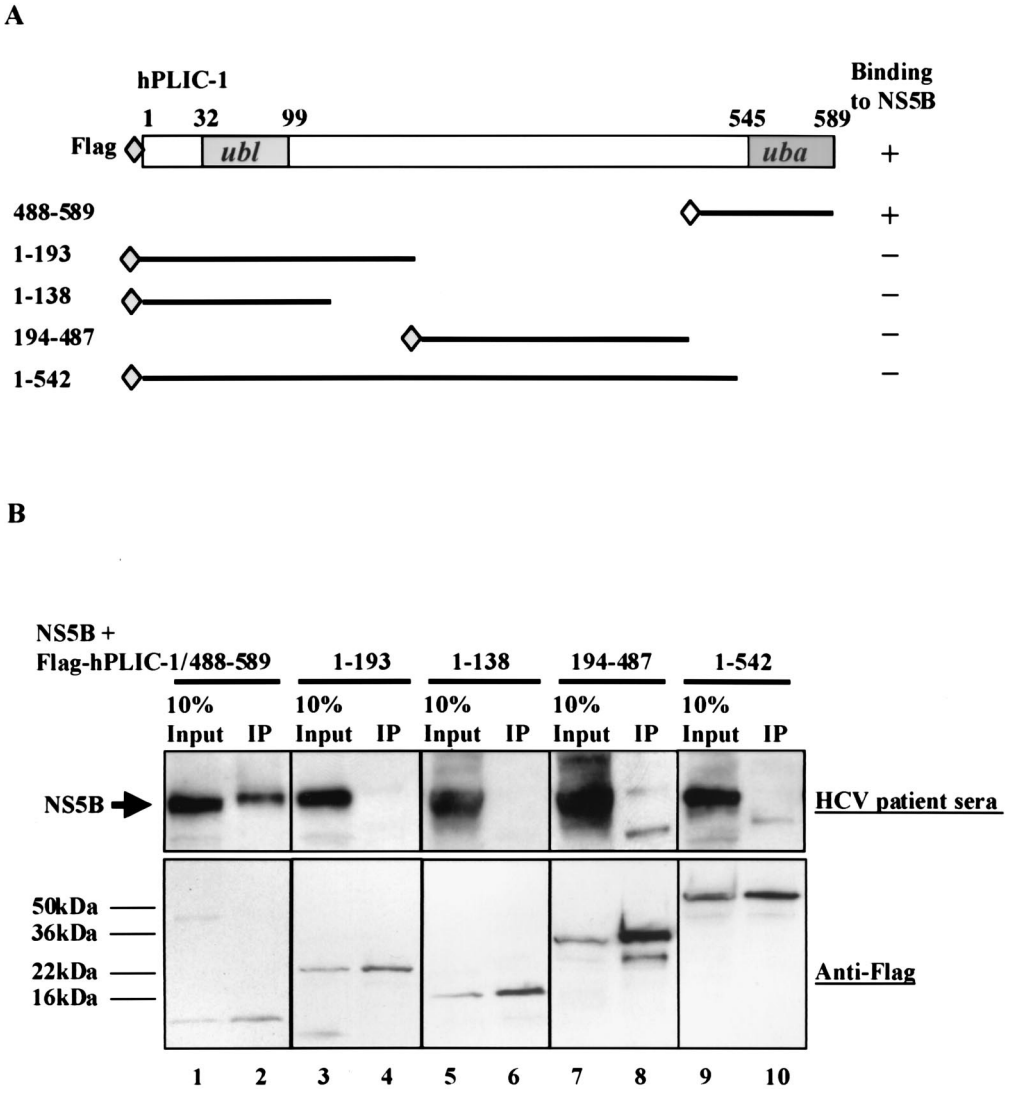



FIG. 2. Mapping the NS5B-binding domain on hPLIC1. (A) A schematic drawing indicates the structure of the Flag-tagged, truncated hPLIC1 constructs used and the results of binding. (B) In vivo coimmunoprecipitation of the truncated hPLIC1 with NS5B. Various Flag-tagged truncated hPLIC1 proteins were cotransfected with NS5B into Huh7 cells. Cell lysates were immunoprecipitated (IP) with anti-Flag-cross-linked Sepharose 4B beads. In the upper panels, an HCV patient serum was used to detect NS5B by immunoblotting. In the lower panels, polyclonal anti-Flag antibody was used to detect truncated hPLIC1 proteins. Lanes 1, 3, 5, 7, and 9 represent 10% of lysates prior to immunoprecipitation.

showed increased radioactivities, partially upon degradation of NS5B. Their increased intensity was likely the result of decreased competition from NS5B for binding to the beads and hPLIC1. These results suggest that hPLIC1 promoted the degradation of NS5B in an interaction-dependent manner.

**Overexpression of hPLIC1 enhances the ubiquitination of NS5B.** To establish that the enhanced degradation of NS5B by hPLIC was due to increased ubiquitination of NS5B, we measured the ubiquitination level of NS5B protein. In this experiment, Huh7 cells were transfected with Flag-NS5B and hPLIC1 or vector. Flag-NS5B was immunoprecipitated by anti-Flag antibody and probed with antiubiquitin monoclonal antibody in Western blotting to detect ubiquitinated NS5B. Only a nonspecific band of 60 kDa was detected in the cells transfected with Flag-NS5B and vector (Fig. 4A, lane 5). When NS5B was cotransfected with hPLIC1, a high-molecular-weight smear was detected by the antiubiquitin antibody (Fig. 4A, lane 6), indicating that polyubiquitination of NS5B was enhanced by hPLIC1. The addition of the proteasome inhibitor lactacystin also enhanced the multiubiquitinated NS5B (Fig. 4A, lane 8), although the addition of lactacystin did not further increase the level of ubiquitinated NS5B in cells cotransfected with hPLIC1 (Fig. 4A, lane 9). In the absence of NS5B, lactacystin treatment did not yield any ubiquitinated protein in the immunoprecipitate (lane 10), indicating that the ubiquitinated proteins detected were indeed ubiquitinated NS5B. As a control, Huh7 cells were transfected with Flag-NS5A and hPLIC1 or vector. Flag-NS5A was immunoprecipitated by anti-Flag antibody and probed with antiubiquitin monoclonal antibody in Western blotting to detect ubiquitinated NS5A. Only a nonspecific band of 60 kDa and two other upper bands were detected (Fig. 4B, lanes 5 to 10). However, neither increased ubiquitination of

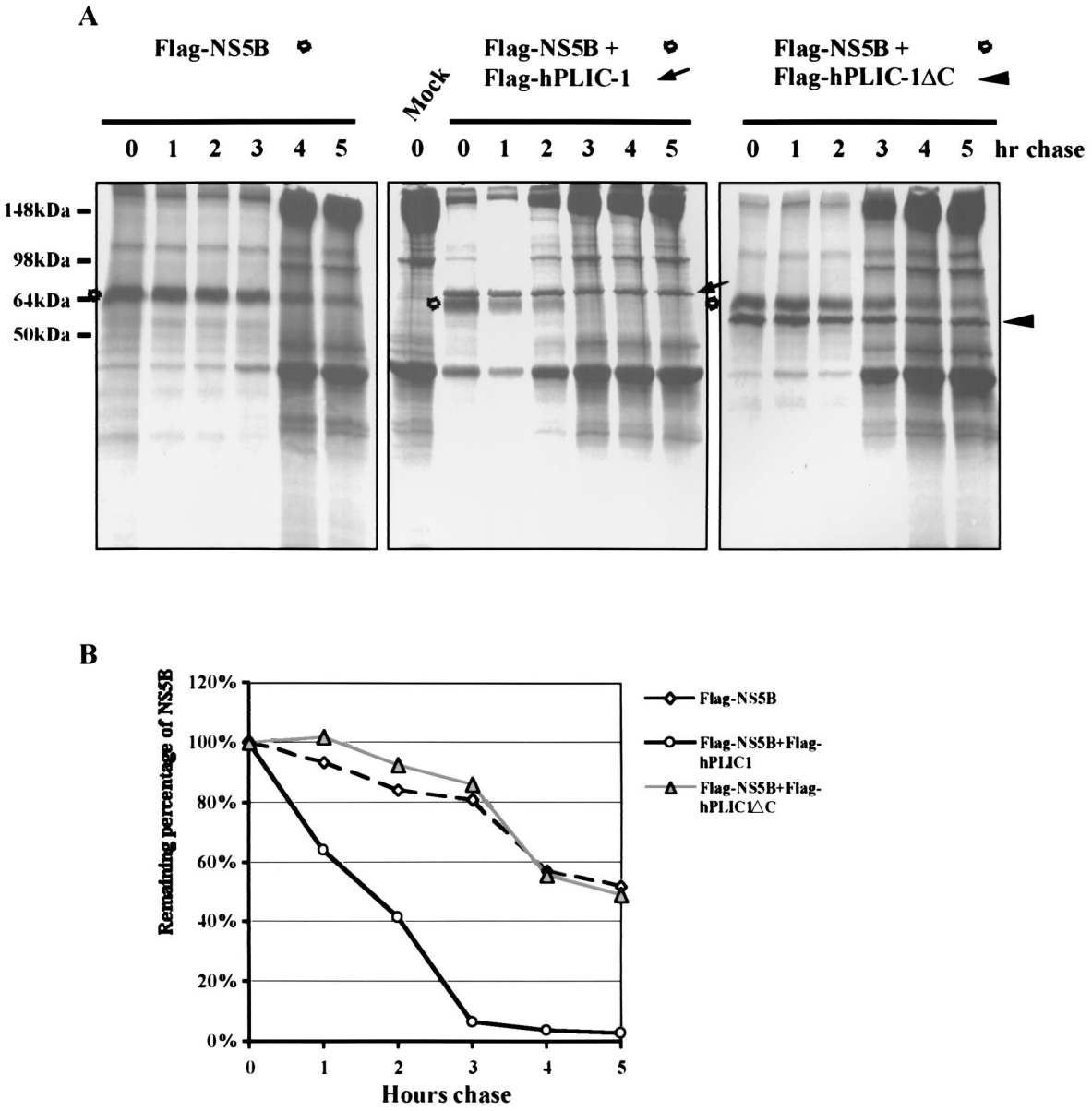



FIG. 3. Detection of in vivo degradation of NS5B by pulse-chase labeling. (A) The transfected Huh7 cells were pulse-labeled with $^{35}S$ protein labeling mix for 15 min, followed by chase in medium containing excessive cold amino acids for 0 to 5 h. Flag-tagged proteins were immunoprecipitated on an anti-Flag M2-agarose gel prior to SDS-PAGE. The positions of Flag-NS5B, Flag-hPLIC-1, and Flag-hPLIC-1ΔC (aa 1 to 542) are indicated by a circle, arrow, and arrowhead, respectively. This is a representative gel from three independent experiments. Lane mock, untransfected cells precipitated with anti-Flag antibody. (B) Quantification of the intensity of each band from the gel shown in panel A by Alpha Innotech software.

these proteins nor a high-molecular-weight smear could be detected, with or without addition of lactacystin, indicating that hPLIC1 did not facilitate ubiquitination of NS5A, in contrast to NS5B. Taken together with the finding that NS5B was destabilized by hPLIC1, these results suggest that hPLIC1 promotes ubiquitination of NS5B and that NS5B is degraded by a ubiquitin-mediated mechanism.

**Overexpression of hPLIC1 decreases the protein level of NS5B in an HCV subgenomic replicon cell line.** To study whether the steady-state level of NS5B in HCV RNA-replicating cells can be modulated by hPLIC1, a Huh7 cell line expressing a subgenomic HCV replicon (7) was analyzed. The cells were transfected with hPLIC1 or vector plasmids and harvested at 48 h posttransfection for immunofluorescence staining and Western blot analysis of NS5B and NS5A (as a control). The staining patterns of NS5B and NS5A in all Huh7-replicon cells were similar and appeared as perinuclear speckles. However, the staining level of NS5B was significantly lower in the Huh7-replicon cells transfected with hPLIC1 than in the control Huh7-replicon cells (Fig. 5A, upper row). In contrast, no significant difference in the staining level of NS5A was observed in these cells (Fig. 5A, lower row). Similar results

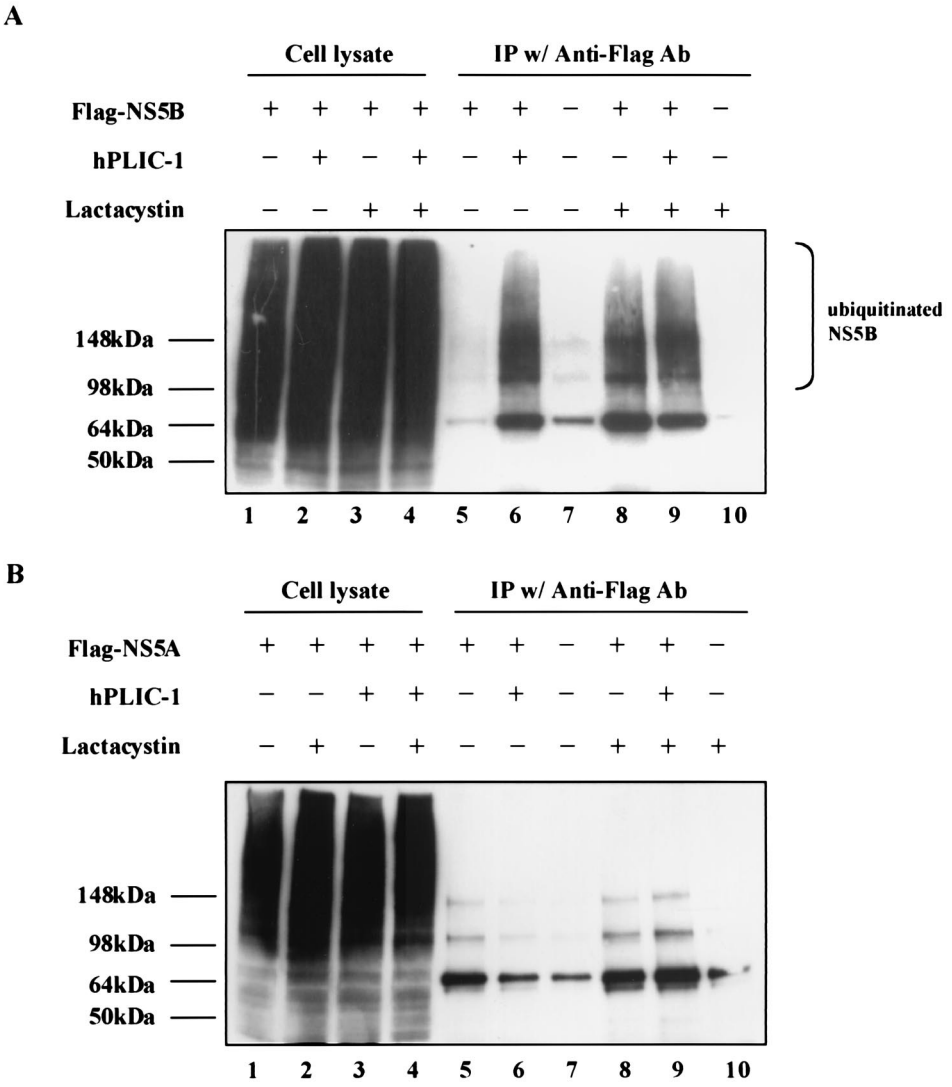



FIG. 4. In vivo ubiquitination of NS5B in the presence of hPLIC1. (A) Huh7 cells were cotransfected with Flag-NS5B and hPLIC1 or vector. Flag-NS5B was immunoprecipitated with anti-Flag M2-agarose affinity gel, separated by SDS-PAGE, transferred to nitrocellulose membrane, and probed with antiubiquitin monoclonal antibody (Ab). Cells used for lanes 3, 4, 8, 9, and 10 were treated with 5 μM lactacystin for 1 h before harvesting. Vector-transfected cells (lanes 7 and 10) served as negative control. (B) Experiments similar to those in panel A were performed except that Huh7 cells transfected with Flag-NS5A were used.

were also obtained in Western blotting. In the hPLIC1-transfected cells, the amount of NS5B was approximately 70% of that in the control cells, whereas NS5A remained at the same level as in control cells (Fig. 5B). Tubulin in each sample was determined as a loading control (Fig. 5B). Taken together, these results indicated that overexpression of hPLIC1 could reduce the amount of NS5B in Huh7-replicon cells. Considering that the transfection efficiency of hPLIC1 was approximately 50% (data not shown), this level of reduction of NS5B was likely significant.

To establish that the decrease of NS5B level correlated with the overexpression of hPLIC1, Huh7-replicon cells were transfected with green fluorescent protein (GFP)-fused hPLIC1 or GFP alone and stained with either NS5B or NS5A antibodies. In Huh7-replicon cells expressing GFP-hPLIC1, NS5B was barely detectable, whereas in the cells not expressing GFP-hPLIC1, NS5B was strongly positive (Fig. 5C, left panels). This result is probably a reflection of the increased degradation of NS5B, although we could not eliminate the possibility that the interaction with hPLIC1 may shield the NS5B epitope. In contrast, no negative correlation between the NS5A staining and GFP-hPLIC1 expression was observed in these cells (Fig. 5C, right panels). As a control, GFP alone did not affect the staining pattern of NS5B (Fig. 5C, middle panels). These results indicated that the expression of hPLIC1 reduced the expression of NS5B but not NS5A.

**Overexpression of hPLIC1 decreases the RNA level of the HCV subgenomic replicon.** To further determine whether hPLIC1 could regulate HCV RNA replication as a result of accelerated degradation of NS5B, which is RNA-dependent RNA polymerase, we performed Northern blot analysis of HCV RNA in these replicon cells. At 24 h posttransfection

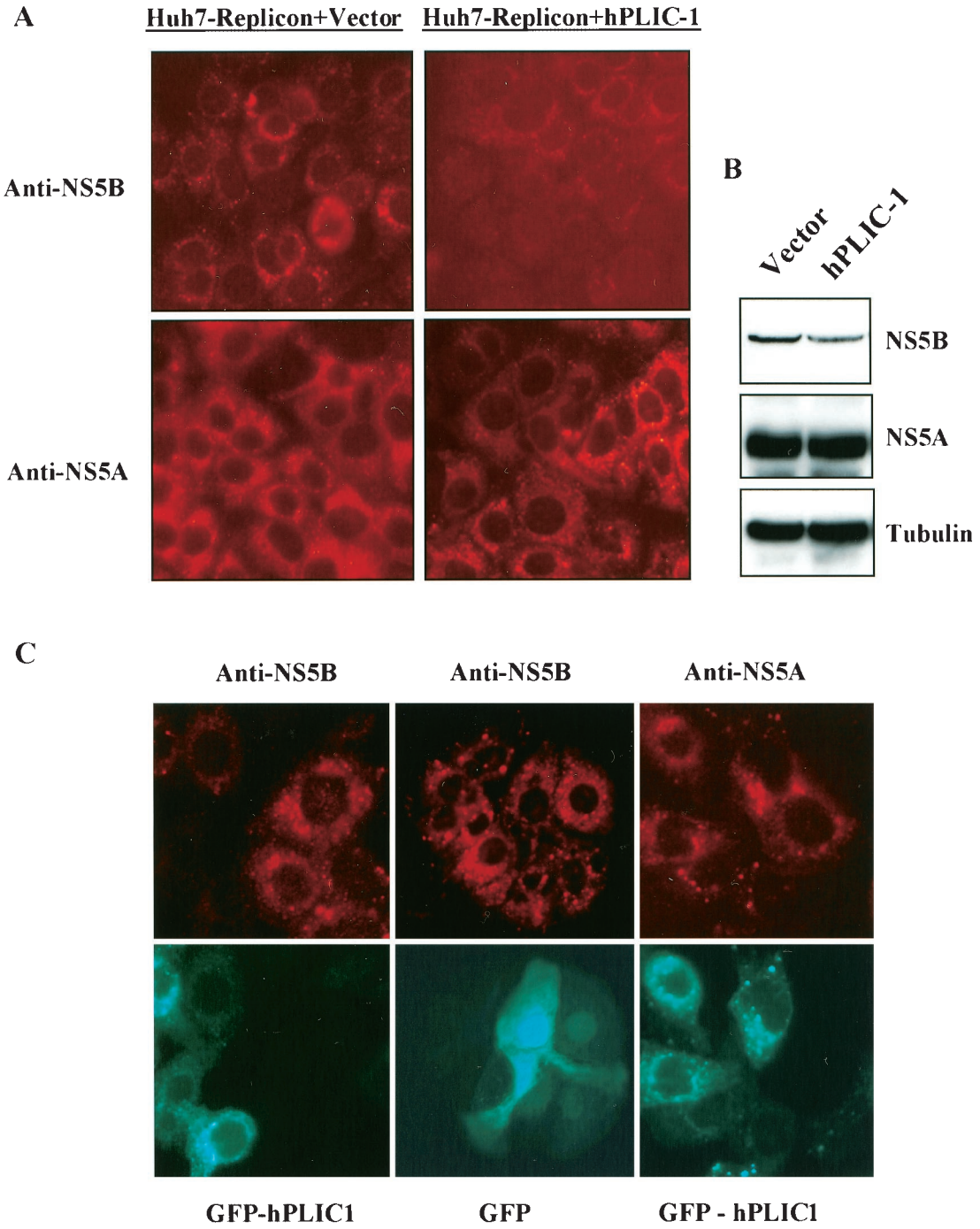
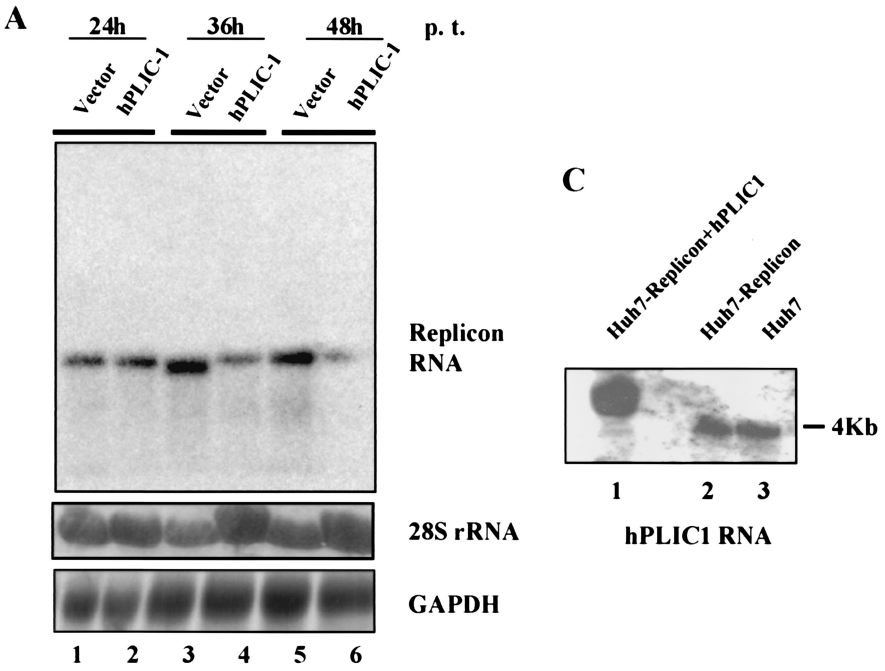



FIG. 5. Steady-state level of NS5B protein in the presence of hPLIC1. Huh7-replicon cells transfected with hPLIC1 or vector were harvested at 48 h posttransfection. (A) Immunofluorescence staining of NS5B in Huh7-replicon cells after transfection with hPLIC1. Cells seeded on chamber slides were fixed in 4% formaldehyde–PBS, permeabilized by 0.1% Triton X-100–PBS, and immunostained with monoclonal antibodies against NS5A or NS5B, followed by secondary antibodies conjugated with TRITC. Images were taken under fluorescence microscopy with the Zeiss program for the same length of exposure time. (B) Western blot analysis of protein expression in transfected Huh7-replicon cells. Thirty micrograms of postnuclear cell lysate from each transfection was separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with a monoclonal antibody against either NS5B or NS5A. The same membrane was reprobed with antitubulin antibody as loading control. (C) Immunofluorescence staining of NS5B in Huh7-replicon cells transfected with pEGFPC1-hPLIC1. Huh7-replicon cells on chamber slides were transfected with pEGFPC1-hPLIC1 or pEGFPC1 vector. Cells were immunostained with monoclonal antibodies against NS5A or NS5B, followed by secondary antibodies conjugated with TRITC at 48 h posttransfection. The green cells indicate GFP-hPLIC1 or GFP expression.



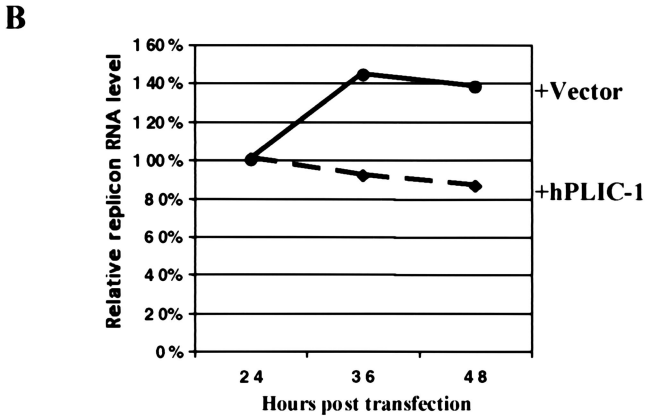




FIG. 6. Steady-state level of HCV subgenomic replicon RNA in the presence of hPLIC1. (A) Northern blot analysis of total RNA extracted from Huh7-replicon cells transfected with hPLIC1 or vector at various times posttransfection (p. t.). This is a representative gel from three experiments. Ten micrograms of total RNA from each plate was separated by electrophoresis on a 1% agarose gel and transferred to a nylon membrane. A specific RNA probe complementary to the HCV 5′-untranslated region RNA was used in hybridization at 70°C. The 28S rRNA was visualized by staining the membrane with 0.03% methylene blue in 0.3 M sodium acetate, pH 5.2. (B) Quantification of the gel shown in panel A by phosphorimaging screen. (C) hPLIC1 mRNA expression determined by Northern blot analysis. Lane 1 contained 5 μg of total RNA from Huh7 replicon cells transfected with hPLIC1. The strong band in lane 1 probably represents plasmid cDNA. Twenty micrograms of total RNA was loaded in both lanes 2 and 3.

with hPLIC1 or vector plasmid, the replicon RNA (8 kb) levels were similar (Fig. 6A, lanes 1 and 2). However, at 36 to 48 h posttransfection, the HCV RNA level was significantly lower in the hPLIC1-transfected replicon cells than in the vector control cells (Fig. 6A, lanes 3 to 6). The cellular 28S rRNA and GAPDH RNA were measured as loading controls. Quantification of the RNA showed that the amount of the replicon RNA was approximately 40% lower in the hPLIC1-transfected cells than in the control cells at later time points (Fig. 6B). The reduction of the replicon RNA level by hPLIC1 correlated with the reduction of the protein level of NS5B. These results combined suggest that hPLIC1 protein binds to HCV NS5B protein and regulates the amount of NS5B and HCV RNA synthesis.

Finally, to determine whether hPLIC1 could play a physiological role in regulating HCV RNA replication, we examined whether hPLIC1 was expressed in Huh7 cells. Northern blot analysis showed that hPLIC1 mRNA was indeed expressed in

both Huh7 cells and those supporting HCV subgenomic replicon (Fig. 6C). No difference in the level of hPLIC1 was noted. The expression of hPLIC in hepatocyte cell lines suggests that it may function in HCV replication under physiological conditions.

## DISCUSSION

In this study, we identified a ubiquitin-like protein, hPLIC1, as an HCV NS5B-binding protein by yeast two-hybrid screening of a human hepatocyte cDNA library. Our data indicate that hPLIC1 binds to NS5B and promotes its ubiquitination. In pulse-chase labeling experiments, we showed that the half-life of NS5B was significantly decreased by hPLIC1 binding, leading to a reduction of NS5B protein. The reduction of NS5B by hPLIC1 was further demonstrated in Huh7 cells expressing an HCV subgenomic replicon. Since NS5B catalyzes HCV RNA replication, the reduction of the amount of NS5B led to a reduction of the subgenomic replicon RNA replication. Our findings provide a novel mechanism for regulating HCV RNA replication through NS5B degradation.

The degradation of NS5B by hPLIC1 is mediated through a ubiquitin-dependent pathway; however, the precise mechanism by which hPLIC1 mediates its degradation remains to be studied. hPLIC1 may recruit ubiquitin ligases to NS5B, as hPLIC1 associates with at least two ubiquitin E3 ligases (10), E6AP and βTrCP. There are many lysine residues in NS5B that may serve as targets of ubiquitination. A stretch of CSSNVS sequence at aa 366 to 371 of NS5B, which is conserved among different HCV genotypes, is similar to the βTrCP recognition motif DSGΨXS (where Ψ is a hydrophobic residue and X is any residue) (23). Whether NS5B could be a substrate of βTrCP or other ubiquitin ligases using hPLIC1 as an adaptor remains to be determined. We have shown that hPLIC significantly enhanced the polyubiquitination level of NS5B (Fig. 4A), which serves as the signal for degradation. The binding domain of NS5B on hPLIC1 is localized at the C-terminal UBA domain (Fig. 2). The truncated hPLIC1 without the UBA domain neither associated with nor promoted degradation of NS5B (Fig. 2 and 3). However, the UBA domain alone was not sufficient to cause the degradation of NS5B (data not shown), probably because the UBL domain of hPLIC is also required for interaction with the proteasome (21).

Several cellular proteins in various organisms have been shown to interact with and be regulated by PLIC1. PLICs were originally identified as integrin-associated protein (IAP)-interacting proteins from a mouse cDNA library and were named PLICs for proteins linking IAP and cytoskeleton (22). Transfection of PLICs induces redistribution of vimentin and cell spreading in IAP-expressing cells (22). The physiological effects of interaction with PLICs vary dramatically. For example, hPLIC1 (also named ubiquilin) has been shown to enhance the synthesis of presenilin, which has been linked to early-onset Alzheimer disease, without altering its half-life (15). hPLIC-1 also interacts with mTOR (also known as RAFT or FRAP [FKBP-rapamycin-associated protein]) but does not modify mTOR abundance (24). A *Xenopus* homolog of Dsk2p and hPLIC1, XDRP1, was reported to promote the degradation of cyclin B (5), whereas the degradation of cyclin A was inhibited by XDRP1 (4). It is not clear whether proteasomal activities are involved in all these interactions. In some cases, hPLIC1 was suggested to serve as a molecular chaperone. Indeed, hPLIC2 (also known as Chap1 and ubiquilin2) has been shown to bind the ATPase domain of the Hsp70-like Stch protein (9). Overexpression of the yeast DSK2p caused accumulation of lysine 48-linked ubiquitin chains, and its disruption caused inhibition of their degradation (5). The effect of hPLIC1 on NS5B, as we reported here, is consistent with the reported functions of DSK2p.

hPLIC1 is widely expressed in various tissues, most abundantly in skeletal muscle, heart, brain, kidney, and liver (22, 24). In rat, the brain, spleen, and liver have the highest levels of PLIC protein expression (24). We found that hPLIC1 mRNA was detected in Huh7 and several other cell lines (Fig. 6C and data not shown). Thus, the hPLIC1-mediated degradation of NS5B is physiologically relevant and may take place in HCV-infected liver as well. In contrast, the expression of murine PLIC2 mRNA is limited to the smooth muscle and brain tissue and is undetectable in the liver (22). Thus, we did not investigate further whether or not hPLIC2 may interact with NS5B. It will be interesting to determine whether hPLIC1 levels vary with growth conditions and cell cycles, which have been shown to affect HCV RNA replication.

Since hPLIC1 regulates the steady-state level of NS5B and consequently the HCV RNA level, it will also be interesting to determine whether there are adaptive mutations in the subgenomic replicon that disable the interaction of NS5B with hPLIC1 and render a high replication efficiency to the replicon. The hPLIC1-binding sequences on NS5B are currently being mapped. It appears that the binding is independent of HCV genotype, as NS5B of both genotypes 1a and 1b can bind to hPLIC1 (data not shown).

In summary, hPLIC1 is the first cellular NS5B-interacting protein that has been found to regulate the protein level of NS5B. This interaction is mediated via the UBA domain of hPLIC1 and promotes degradation of NS5B through a ubiquitin-dependent pathway. It may play a role in regulating HCV RNA replication.

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