

RESULTS

Suppression of HCV replication by purified herbal extracts, isoliquiritigenin and glycycomarin

TO SCREEN THE herbal drugs and these purified extracts (Table 1) for their antiviral effects against HCV replication, Huh7/Rep-Feo cells were cultured with various concentrations of 5 herbal extracts; *Glycyrrhizae radaix*, *Rhemanniae radix*, *Paeoniae radix*, *Artemisiae capillari spica*, and *Rhei rhizoma*, and 13 compounds purified from these herbal extracts. Levels of HCV replication were quantified by internal luciferase assay after 48 h. None of the herbal extracts showed any effects on HCV replication (data not shown). On the other hand, among the 13 purified compounds, isoliquiritigenin and glycycomarin, which were purified from *Glycyrrhizae radix*, suppressed replication of HCV replicon in a dose-dependent manner. The EC50s were 6.2 ± 1.0 and

$15.5 \pm 0.8 \mu\text{g/mL}$ for isoliquiritigenin and glycycomarin, respectively (Figs 2a,3a). The MTS assay did not show any effect on cell growth and viability (Fig. 2b), indicating that the antiviral action of the two compounds is not due to cytotoxic or antiproliferative effects. Huh7/Rep-Feo cells were cultured with various concentrations of isoliquiritigenin and glycycomarin, and the dose-effect correlation and time courses of replicon expression were measured by luciferase assay. After addition of each compounds, suppressive effect of the HCV replicon lasted for 48 h in a dose and time-dependent manner (Fig. 3b).

Realtime-RT-PCR and Western blotting analyses

In the realtime RT-PCR analysis and Northern blot analyses, levels of the replicon RNA decreased in a dose-dependent manner following treatment with isoliquir-

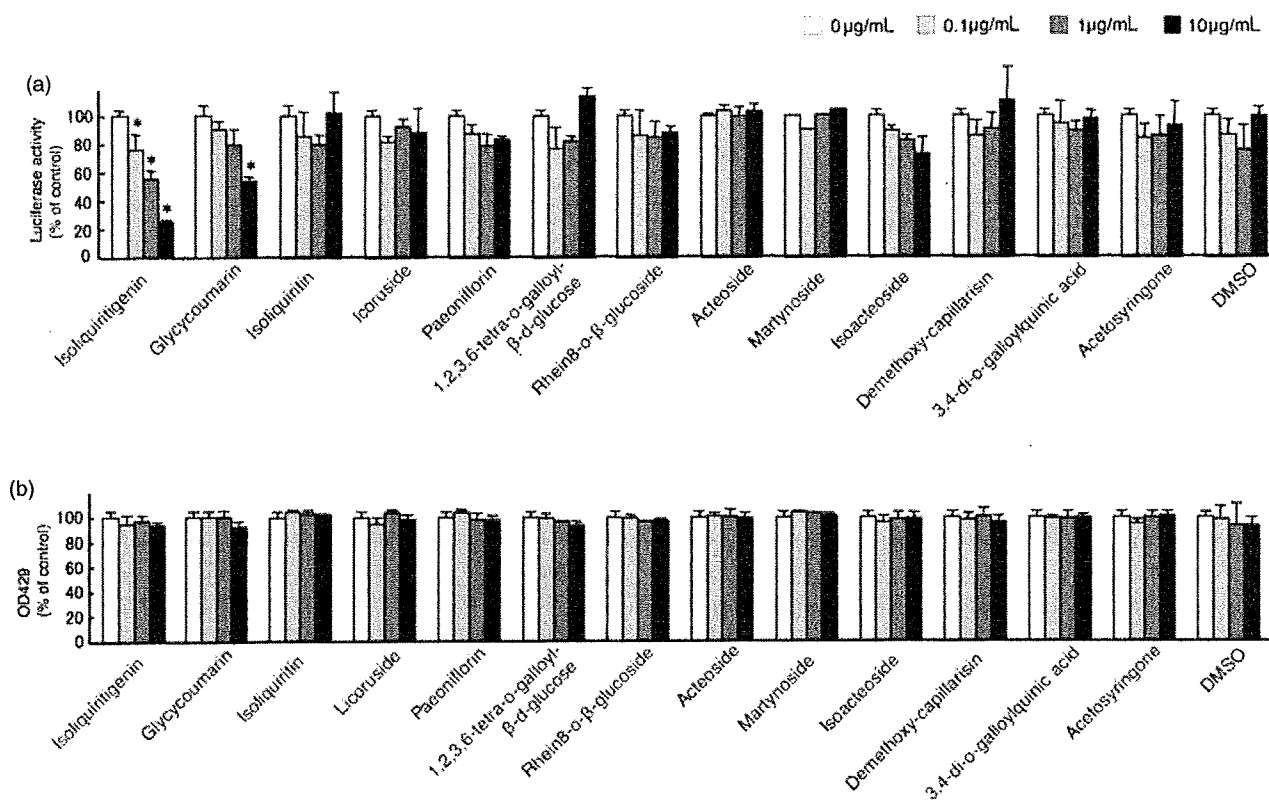


Figure 2 Effects of purified extracts from herbal drugs on expression of HCV replicon. (a) Huh7/Rep-Feo cells, which constitutively express the HCV Feo replicon, were cultured in the presence of 13 compounds at concentrations of 0, 0.1, 1, and 10 µg/mL. The internal luciferase activities were measured after 48 h of culture. Assays were performed in triplicate. Error bars indicate mean ± SD. Asterisks indicate p-values of less than 0.05. (b) MTS assay of Huh7/Rep-Feo cells cultured with the concentrations of 13 compounds indicated. Error bars indicate mean ± SD.

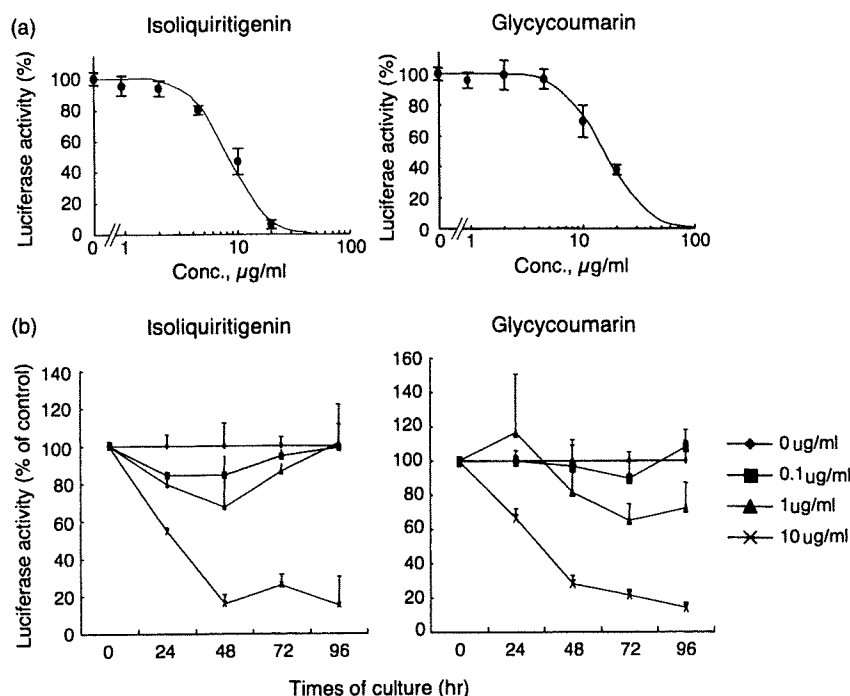


Figure 3 Dose- and time-dependent suppression of HCV replication by isoliquiritigenin and glycycomarin. (a) Relative log (dose)-response plots for isoliquiritigenin or glycycomarin. Error bars indicate mean \pm SD of triplicate analyses. Calculated probit curves are overlaid in each plot. (b) Huh7/Rep-Feo cells were cultured with the concentrations of isoliquiritigenin and glycycomarin indicated. The internal luciferase activities were measured at times of culture indicated. Assays were performed in triplicate. Error bars indicate mean \pm SD.

itigenin and glycycomarin (Fig. 4a,b). Similarly, in Western blot analysis, the HCV non-structural protein, NS5A, which was translated from the HCV replicon, decreased by corresponding amounts in response to treatment with isoliquiritigenin and glycycomarin (Fig. 4c). Densitometric analysis of NS5A protein showed that the intracellular levels of the virus protein in Huh7/Rep-Feo cells correlated well with the luciferase activities.

Absence of synergistic anti-HCV effects of interferon-alpha with isoliquiritigenin or glycycomarin

To determine whether IFN and these two compounds have a synergistic inhibitory effect on the replicon, Huh7/Rep-Feo cells were cultured with combinations of IFN α -2b and isoliquiritigenin or glycycomarin at various concentrations. The relative dose-inhibition curves of IFN were plotted under each fixed concentrations of isoliquiritigenin or glycycomarin of 0, 0.1, 1, 10 μ g/mL, respectively (Fig. 5). The curves did not show synergy of the two compounds and IFN against the HCV replicon. To see whether the action of isoliquiritigenin and glycycomarin involve interferon-Jak/STAT-ISRE pathway, we conducted ISRE reporter assays. We transfected the p-55C1BLuc plasmid in Huh7 cells and cultured the cells in the presence of isoliquiritigenin or

glycycomarin. After 12 h of incubation, those drugs did not activate ISRE-promoter activities (data not shown). These results suggested that the action of the compounds on the intracellular replication of HCV replicon was independent of the IFN-ISRE pathway.

Isoliquiritigenin and glycycomarin do not suppress the HCV IRES-dependent translation

We next determined whether these two compounds suppress HCV IRES-dependent translation, we used Huh7 cell line that had been stably transfected with pCneo-Rluc IRES-Fluc (Huh7/CRIF; Fig. 1b). Treatment of these cells with isoliquiritigenin or glycycomarin resulted in no significant change of the internal luciferase activities at concentrations of these two compounds that suppressed expression of the HCV replicon (Fig. 6a). The MTS assay did not show any effect on cell growth and viability at concentrations used in this assay (Fig. 6b).

Isoliquiritigenin and glycycomarin suppress HCV-JFH1 virus cell culture

The demonstrated inhibitory effects isoliquiritigenin and glycycomarin on HCV subgenomic replication were validated further by using HCV-JFH1 cell culture system.²⁵ As shown in Figure 7a, treatment of the cells with the two compounds suppressed time-dependent

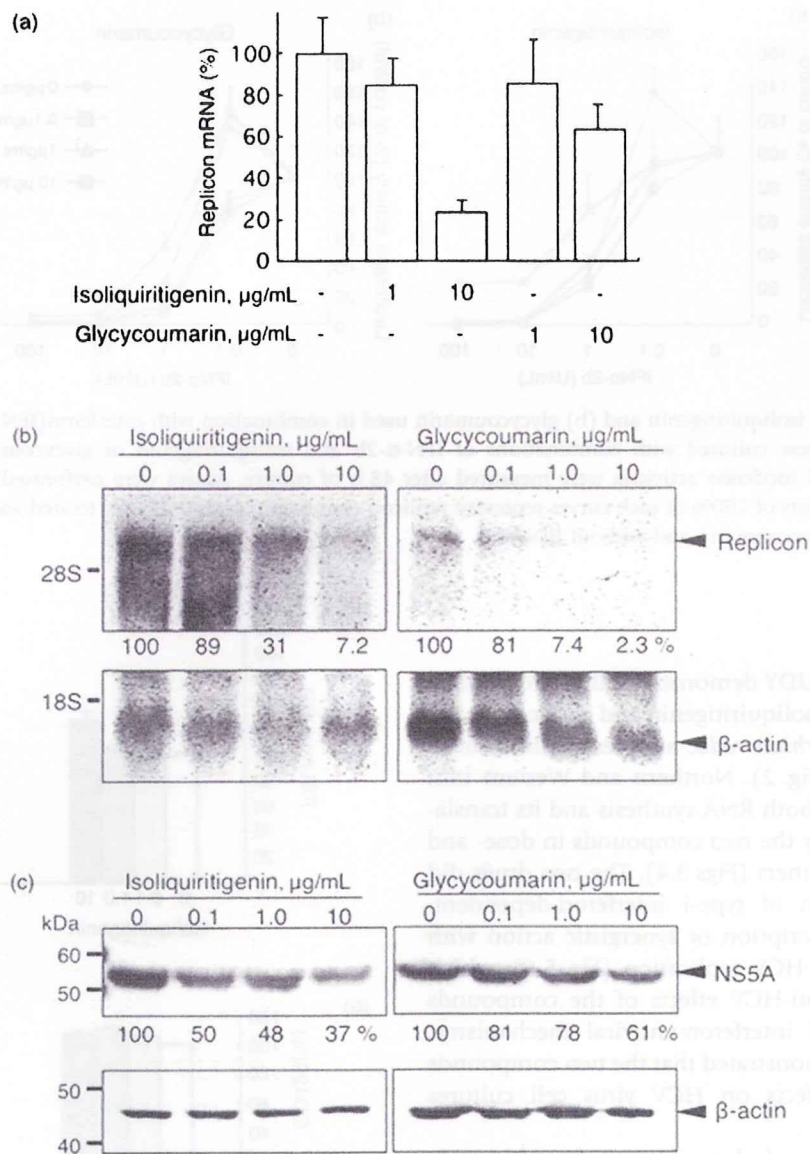


Figure 4 Suppression of replicon RNA and NS5A synthesis by isoliquiritigenin and glycy coumarin. Huh7/Rep-Feo cells were cultured with indicated concentrations of two compounds, isoliquiritigenin and glycy coumarin, and harvested at 48 hr after exposure. (a) Real-time RT-PCR analyses. (b) Northern-blot hybridization. Fifteen micrograms of total cellular RNA was electrophoresed in each lane. The upper part of the membrane containing the hepatitis C virus replicon RNA was hybridized with a digoxigenin-labeled probe specific for the replicon sequence, and the lower part was hybridized with beta-actin probe. Densitometry for replicon RNA was performed and indicated as percents of drug-negative control. (c) Western blotting. Thirty micrograms of total cellular protein was electrophoresed in each lane. Densitometry of NS5A protein was performed and indicated as percents of drug-negative control.

increase of HCV core antigen in the medium. In all time points, core antigen levels were significantly lower in culture that were treated with isoliquiritigenin and glycy coumarin than the untreated culture. The effect of glycy coumarin was partly reversed on day six probably

due to chemical instability of the compound. Consistently, the Western blot showed that the cellular HCV core protein expression was substantially suppressed by treatment with isoliquiritigenin and glycy coumarin (Fig. 7b).

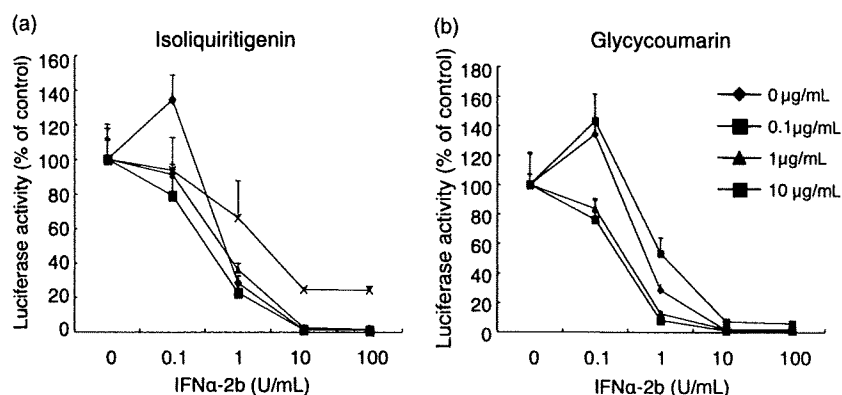


Figure 5 Effects of (a) isoliquiritigenin and (b) glycycomarin used in combination with interferon(IFN)- α on HCV replication. Huh7/Rep-Feo cells were cultured with combinations of IFN- α -2b and isoliquiritigenin or glycycomarin at concentrations indicated. The internal luciferase activities were measured after 48 h of culture. Assays were performed in triplicate. Error bars indicate mean \pm SD. Plots of 100% in each curves represent replicon expression levels that were treated with indicated amounts of isoliquiritigenin or glycycomarin and without IFN.

DISCUSSION

THE PRESENT STUDY demonstrates that two purified herbal extracts, isoliquiritigenin and glycycomarin, isolated from *Glycyrrhizae radix*, suppress replication of an HCV replicon (Fig. 2). Northern and Western blot analyses reveal that both RNA synthesis and its translation were reduced by the two compounds in dose- and time-dependent manners (Figs 3,4). The two drugs did not show activation of type-I interferon-dependent, ISRE-mediated transcription or synergistic action with interferon-alpha on HCV replication (Fig. 5,6), which suggests that the anti-HCV effects of the compounds are independent of interferon-antiviral mechanisms. Finally, we have demonstrated that the two compounds show inhibitory effects on HCV virus cell cultures (Fig. 7).

Flavonoid is a class of plant pigment, found in wide range of green vegetables and fruits. They are classified into flavon, flavonol, flavanone, flavanol, isoflavone, chalcone, anthocyanin and catechin, according to their molecular structures. Many flavonoids have various biological functions such as antibacterial,²⁸ antioxidative and anticarcinogenic activities.²⁹ Isoliquiritigenin is a simple chalcon derivative and found in licorice and vegetables including shallots and bean sprouts. Isoliquiritigenin has several biochemical activities similar to other flavonoids. It has various biochemical activities such as antioxidative and superoxide scavenging activities,³⁰ an antiplatelet aggregation effect,³¹ an inhibitory effect on aldose reductase activity,³² estrogenic properties³³ and selective inhibition of H2 receptor-mediated signaling.³⁴

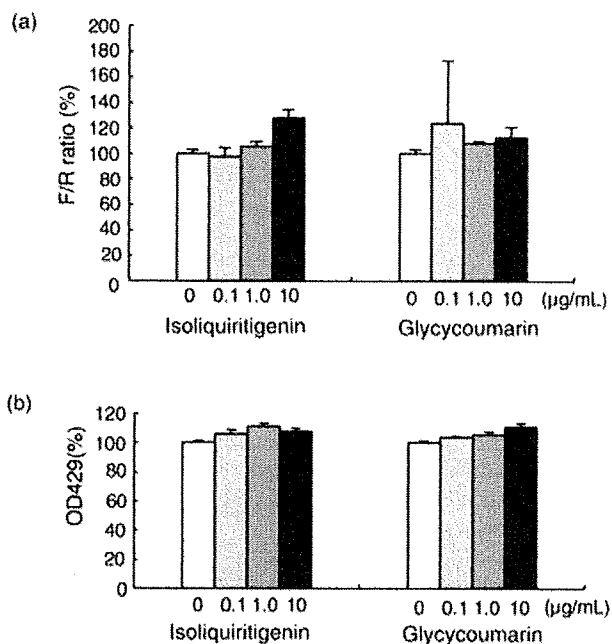


Figure 6 Isoliquiritigenin and glycycomarin do not influence the HCV IRES-mediated translation. A bicistronic reporter gene plasmid, pCIneo-Rluc-IRES-Fluc, was stably transfected into Huh7 cells (Huh7/CRIF, *see* the Methods). (a) Dual luciferase assay. The cells were cultured with isoliquiritigenin or glycycomarin at the concentrations indicated, and dual luciferase activities were measured after 48 h of treatment. Values are displayed as ratios of Fluc to Rluc. Error bars indicated mean \pm SD. (b) MTS assay of Huh7/neo-Rluc-IRES-Fluc cells cultured with isoliquiritigenin or glycycomarin at the concentrations indicated. MTS assays at 48 h after treatment with each drug were performed in triplicate. Error bars indicate mean \pm SD.

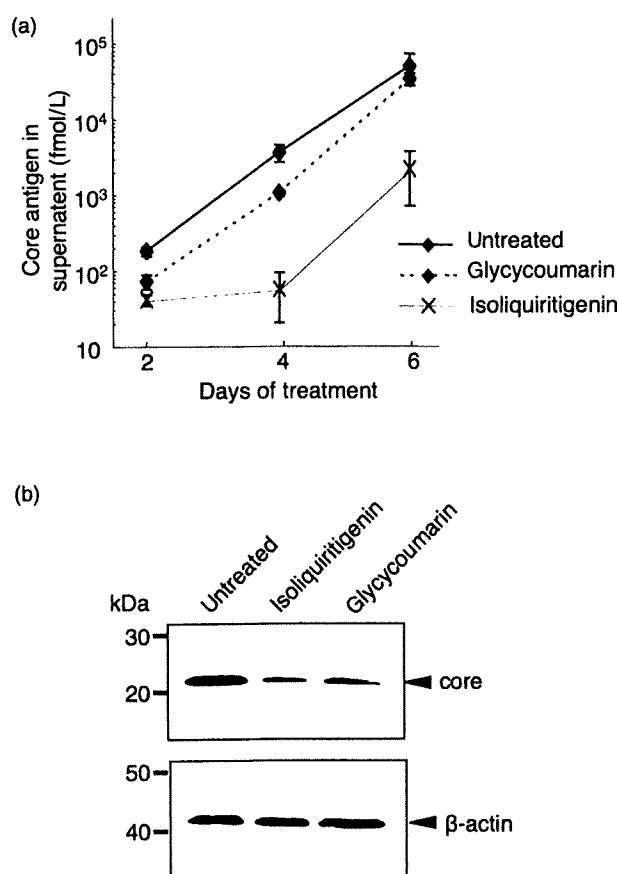


Figure 7 Suppression of HCV-JFH1 virus expression by *isoliquiritigenin* and *glycycomarin*. (a) Naïve Huh 7.5.1 cells were infected with culture supernatant of HCV-JFH1-infected cells and were subjected to culture in the presence of indicated drugs. Culture supernatants were collected at indicated days, and HCV core antigen was measured. Assays were done in triplicate and indicated as mean \pm SD. (b) Cells were harvested at day 6, and Western blotting was performed using anti-core and anti-beta-actin antibodies.

Extracts of a licorice root, *Glycyrrhizae radix*, show anti-inflammatory properties in chronic and acute liver inflammation,³⁵ and are widely and extensively prescribed in Japan as Strong Neominophagen C (SNMC). A major ingredients of *Glycyrrhizae radix* are glycyrrhizin and liquiritin. However, glycyrrhizin and liquiritin did not suppress HCV replication, suggesting that the commercially available SNMC will not elicit antiviral effects against HCV. On the other hand, there have been reports on the pharmacological action of glycycomarin. Glycycomarin displays antibacterial properties in the upper respiratory tract in infections such as *Streptococcus pyogenes*, *Haemophilus influenzae* and *Moraxella*

catarrhalis,³⁶ and methicillin-resistant *Staphylococcus aureus*,³⁷ but the mechanisms of action is unclear.

To our knowledge, there have been no reports on the serum concentration of glycycomarin and isoliquiritigenin in patients taking medicines or dietary supplements containing *Glycyrrhizae radix*. However, therapeutic doses of 3–12 g per day of powdered root have been suggested for pathological conditions including chronic hepatitis, muscle cramp, acute gastritis, and urolithiasis. Thus, further studies are required to assess the human exposure to these flavonoids, the pharmacological dose-dependent properties and the tissue distribution and drug kinetics.

Considering the current status of limited therapy options for HCV infection and their unsatisfactory outcomes, large scale screening of anti-HCV molecules for the development of novel antiviral therapies is called for. In the present study, we have screened Chinese herbal extracts for the ability to suppress HCV replication, and identified two extracts, isoliquiritigenin and glycycomarin, which specifically suppressed HCV replication. These results suggest that these agents will be a promising for use in the stabilization of HCV replication and active liver inflammation. In addition, further investigations of the action of these drugs on the expression, processing or maturation of HCV proteins may elucidate new aspects of the viral infection and replication and may constitute novel molecular targets for anti-HCV chemotherapeutics.

ACKNOWLEDGEMENTS

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Cochaperone Activity of Human Butyrate-Induced Transcript 1 Facilitates Hepatitis C Virus Replication through an Hsp90-Dependent Pathway[∇]

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Hepatitis C virus (HCV) nonstructural protein 5A (NS5A) is a component of the replication complex consisting of several host and viral proteins. We have previously reported that human butyrate-induced transcript 1 (hB-ind1) recruits heat shock protein 90 (Hsp90) and FK506-binding protein 8 (FKBP8) to the replication complex through interaction with NS5A. To gain more insights into the biological functions of hB-ind1 in HCV replication, we assessed the potential cochaperone-like activity of hB-ind1, because it has significant homology with cochaperone p23, which regulates Hsp90 chaperone activity. The chimeric p23 in which the cochaperone domain was replaced with the p23-like domain of hB-ind1 exhibited cochaperone activity comparable to that of the authentic p23, inhibiting the glucocorticoid receptor signaling in an Hsp90-dependent manner. Conversely, the chimeric hB-ind1 in which the p23-like domain was replaced with the cochaperone domain of p23 resulted in the same level of recovery of HCV propagation as seen in the authentic hB-ind1 in cells with knockdown of the endogenous hB-ind1. Immunofluorescence analyses revealed that hB-ind1 was colocalized with NS5A, FKBP8, and double-stranded RNA in the HCV replicon cells. HCV replicon cells exhibited a more potent unfolded-protein response (UPR) than the parental and the cured cells upon treatment with an inhibitor for Hsp90. These results suggest that an Hsp90-dependent chaperone pathway incorporating hB-ind1 is involved in protein folding in the membranous web for the circumvention of the UPR and that it facilitates HCV replication.

Hepatitis C virus (HCV) is the major causative agent of non-A, non-B hepatitis in humans and infects approximately 170 million people worldwide (64). HCV belongs to the genus *Hepacivirus* of the family *Flaviviridae* and is classified into six major genotypes (39). The virus forms small, round, enveloped particles and possesses a genome consisting of a single positive-stranded RNA with a nucleotide length of 9.6 kb. The viral genome encodes a single precursor polyprotein consisting of approximately 3,000 amino acids, which in turn is posttranslationally processed into 10 viral proteins by host and viral proteases. The structural proteins are cleaved from the N-terminal one-fourth of the polyprotein by the host signal peptidase and signal peptide peptidase (36, 43, 44), resulting in the maturation of capsid protein, two envelope proteins, and viroporin p7. The nonstructural protein 2 (NS2) protease cleaves its own carboxyl terminus, and then NS3 cleaves the appropriate downstream positions to produce NS3, NS4A, NS4B, NS5A, and NS5B (24, 60), which form the replication complex, together with several host proteins (14, 35).

NS5A is a membrane-anchored zinc-binding phosphoprotein that appears to possess diverse functions, including the suppression of host defense and the regulation of virus replication (1, 15, 58), but its biological function remains unclear.

Several groups, including ours, have suggested that the molecular chaperone, heat shock protein 90 (Hsp90), and several cochaperones participate in the replication complex of HCV through interaction with NS5A or other NS proteins (45, 56, 65). Hsp90 is the highly conserved and ubiquitously expressed protein that acts as a key regulator for the turnover and the activities of more than 200 signaling proteins, including steroid receptors and cell-signaling kinases (66). The chaperone activity of Hsp90 contributes to the refolding of an unfolded protein in an ATP-dependent manner, and the execution of Hsp90-dependent protein folding requires the formation of a multi-chaperone complex containing other chaperones (e.g., Hsp70, Hsp104, and Hsp40) and cochaperones (e.g., p23, Hop, and immunophilins) (4, 18, 48). Geldanamycin or its derivatives, which are represented as specific inhibitors of Hsp90, can destabilize and then degrade client proteins (41, 55).

The host chaperone mechanism is involved in the folding of viral polymerase to support viral replication (6, 27). Moreover, host chaperones have been reported to play roles in the assembly of viral particles and the sorting of virus proteins (9, 32, 38). We have previously reported that Hsp90 chaperone activities and chaperone-associated proteins are required for the efficient propagation of HCV (45, 56) and that human butyrate-induced transcript 1 (hB-ind1) is involved in the propagation of HCV through interactions with NS5A and Hsp90 via the coiled-coil domain and the FXXW motif, respectively (56). hB-ind1 was first reported to be a multiple-membrane-spanning protein consisting of 362 amino acids that possesses a significant homology with a cochaperones, p23, that regulates

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Hsp90 function by its cochaperone activity (11). However, the roles of hB-ind1 in the life cycle of HCV have not been precisely clarified. In this study, we investigated the role of the Hsp90-related chaperone system, including hB-ind1, in the regulation of the RNA replication and particle production of HCV.

MATERIALS AND METHODS

Plasmids. The plasmids encoding hB-ind1, NS5A, Hsp90, and FKBP8 were prepared by methods described previously (45, 56). The DNA fragments encoding hB-ind1 mutants were prepared by PCR with the introduction of a silent mutation that is resistant to the short hairpin RNA in the hB-ind1 knockdown cells, as described previously (56). The human p23 gene and glucose-regulated protein 78 (GRP78) promoter region (-151 to +22) were amplified by PCR from the total cDNA and genomic DNA of Huh7 cells, respectively. The DNA fragments encoding mutants of hB-ind1 and p23 were prepared by the method of splicing by overlap extension (26) and introduced into pEF FLAGGS pGKpuro (28). The GRP78 promoter region was introduced between the KpnI and HindIII sites of pGL3-basic (Promega, Madison, WI) and designated pGRP78-luc. The reporter plasmid carrying a firefly luciferase gene under the control of the GR promoter (pGR-luc) was purchased from Panomics (Fremont, CA). The internal-control plasmid encoding a *Renilla* luciferase (pRL-TK) was purchased from Promega. The plasmid pFK-I₃₈₉ neo/NS3-3'/NK5.1 (47) was kindly provided by R. Bartenschlager. The plasmids used in this study were confirmed by sequencing them with an ABI Prism 3130 genetic analyzer (Applied Biosystems, Tokyo, Japan).

Cells and virus infection. All cell lines were cultured at 37°C under a humidified atmosphere and 5% CO₂. The human embryonic kidney 293T and hepatocellular carcinoma Huh7 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal calf serum (FCS). The human hepatocellular carcinoma cell line Huh7.5.1 was kindly provided by F. Chisari (70) and was maintained in DMEM containing nonessential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% FCS. The Huh9-13 cell line, which is a Huh7 cell line harboring a subgenomic HCV RNA replicon (35), was maintained in DMEM containing 10% FCS, nonessential amino acids, and 1 mg/ml G418 (Nakalai Tesque, Kyoto, Japan). The hB-ind1 knockdown cell line Huh-KD and control cell line Huh-ctrl were described previously (56). Huh-KD cells were transfected with each of the expression plasmids encoding wild-type or mutant hB-ind1 and cultured for 1 week in the presence of 10 µg/ml of puromycin. The remaining cells were used for the experiments described below. The viral RNA of JFH1 was introduced into Huh7.5.1 cells according to the method of Wakita et al. (62) for preparation of the infectious HCV particles in cell culture.

Antibodies. The rabbit anti-hB-ind1 antibody was prepared as described previously (56). Mouse monoclonal antibodies to HCV NS5A, influenza virus hemagglutinin (HA) and FLAG tags, and β-actin were purchased from Austral Biologicals (San Ramon, CA), Covance (Richmond, CA), and Sigma, respectively. Mouse anti-protein disulfide isomerase (PDI) immunoglobulin G2a (IgG2a) was from Affinity Bioreagents (Golden, CO). Mouse anti-double-stranded RNA (dsRNA) IgG2a (J1 and K2) antibodies were from Biocenter Ltd. (Szirak, Hungary). Alexa Fluor 488 (AF488)-conjugated anti-mouse IgG1, AF647-conjugated anti-rabbit IgG, and AF594-conjugated anti-mouse IgG2a and IgG2b antibodies were from Invitrogen (San Diego, CA).

Transfection, immunoblotting, and immunoprecipitation. Transfection and immunoprecipitation analyses were carried out as described previously (25, 45). Immunoprecipitates boiled in loading buffer were subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and were reacted with the appropriate antibodies. The immune complexes were visualized with Super Signal West Femto substrate (Pierce, Rockford, IL) and detected by an LAS-3000 image analyzer system (Fujifilm, Tokyo, Japan). The protein bands of GRP78 and β-actin were quantified by Multi Gauge software (Fujifilm), and the values of GRP78 expression were normalized with those of β-actin.

Quantitative reverse transcriptase PCR. HCV RNA was estimated by the method described previously (56). Total RNA was prepared from cells by using an RNeasy minikit (Qiagen, Tokyo, Japan). First-strand cDNA was synthesized using an RNA LA PCR in vitro cloning kit (Takara Bio Inc., Shiga, Japan) and random primers. Each cDNA was estimated with Platinum SYBR green qPCR SuperMix UDG (Invitrogen) according to the manufacturer's protocol. Fluorescent signals were analyzed with an ABI Prism 7000 (Applied Biosystems). The

internal ribosomal entry site regions of HCV and mRNAs of GAPDH (glyceraldehyde-3-phosphate dehydrogenase), GRP78, and growth arrest- and DNA damage-inducible gene 153 (GADD153) were amplified using the primer pairs 5'-GAGTGTCTGTCAGCCTCCA-3' and 5'-CACTCGCAAGCACCTATC A-3', 5'-GAAGGTGAAGGTGGAGTC-3' and 5'-GAAGGTGAAGGTGG AGTC-3', 5'-CGCCAAGCGGCTCATC-3' and 5'-AACCACCTTGAACGGC AAGA-3', and 5'-AGCTGGAACCTGAGGAGAGA-3' and 5'-TGGATCAGT CTGGAAAAGCA-3', respectively. The values of the HCV genome or each mRNA were normalized with those of GAPDH mRNA. Each PCR product was detected as a single band of the correct size on agarose gel electrophoresis (data not shown).

In vitro transcription and RNA transfection. The plasmid pFK-I₃₈₉ neo/NS3-3'/NK5.1 was linearized by treatment with ScaI and then transcribed in vitro using the MEGAscript T7 kit (Applied Biosystems) according to the manufacturer's protocol. The in vitro-transcribed RNA was electroporated into cells at 4 million cells/0.4 ml under conditions of 270 V and 960 µF using a Gene Pulser (Bio-Rad, Hercules, CA). The colony formation assay was carried out by a method described previously (45).

Indirect immunofluorescence assay. Cells cultured on glass slides were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 30 min. After being washed twice with PBS, the cells were permeabilized for 20 min at room temperature with PBS containing 0.25% saponin and blocked with PBS containing 0.2% gelatin (gelatin-PBS) for 60 min at room temperature. The cells were incubated with gelatin-PBS containing rabbit anti-hB-ind1 antibody, mouse anti-NS5A IgG1, mouse anti-PDI IgG2a, mouse anti-FKBP8 IgG2b, or mouse anti-dsRNA IgG2a (J1 and K2) at 37°C for 60 min; washed three times with PBS containing 1% Tween 20; and incubated with gelatin-PBS containing AF488-conjugated anti-mouse IgG1 or AF647-conjugated anti-rabbit or AF594-conjugated anti-mouse IgG2a or IgG2b antibodies at 37°C for 60 min. Finally, the cells were washed three times with PBS containing 1% Tween 20 and observed with a FluoView FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan).

Correlative FM-EM. Correlative fluorescence microscopy-electron microscopy (FM-EM) allows individual cells to be examined both in an overview with FM and in a detailed subcellular-structure view with EM (51). The endogenous hB-ind1 and NS5A were stained and observed in the HCV replicon cells by the correlative FM-EM method as described previously (45).

Luciferase assay. Each plasmid was transfected into Huh7, Huh9-13, and interferon (IFN)-cured cells seeded in a 12-well plate, and the cells were treated with 1 µM dexamethasone (Sigma) for 12 h or with 17-dimethylamino-ethylamino-17-demethoxygeldanamycin (DMAG) (Sigma) for 6 h at 36 h posttransfection and lysed in 200 µl of passive lysis buffer (Promega). Luciferase activity was measured in 20-µl aliquots of the cell lysates using a Dual-Luciferase Reporter Assay System (Promega). Firefly luciferase activity was standardized with that of *Renilla* luciferase cotransfected with the internal-control plasmid pRL-TK. The resulting values were expressed as the increase in relative light units (RLU).

Statistical analysis. Results were expressed as the mean ± standard deviation. The significance of differences in the means was determined by Student's *t* test.

RESULTS

The p23-like domain of hB-ind1 has cochaperone activity. Although we had previously reported that hB-ind1 regulates HCV RNA replication through interaction with NS5A and Hsp90, the molecular mechanisms underlying the regulation of HCV replication remained to be clarified. To gain more insights into the potential cochaperone activity of hB-ind1 in the Hsp90 chaperone system, we prepared expression plasmids encoding a wild-type p23 and three p23 mutants—one in which the FXXW motif was replaced with AXXA (p23AxxA), one in which the cochaperone domain of p23 was replaced with the p23-like domain of hB-ind1 (cp23), and one in which both substitutions were made (cp23AxxA) (Fig. 1A). HA-tagged Hsp90 was coexpressed with FLAG-tagged p23 or the FLAG-tagged p23 mutants in 293T cells (Fig. 1B). Hsp90 was coimmunoprecipitated with wild-type p23 and a cp23 mutant, but not with the p23AxxA or cp23AxxA mutants, indicating that the FXXW motif of hB-ind1, as is the case with that of p23

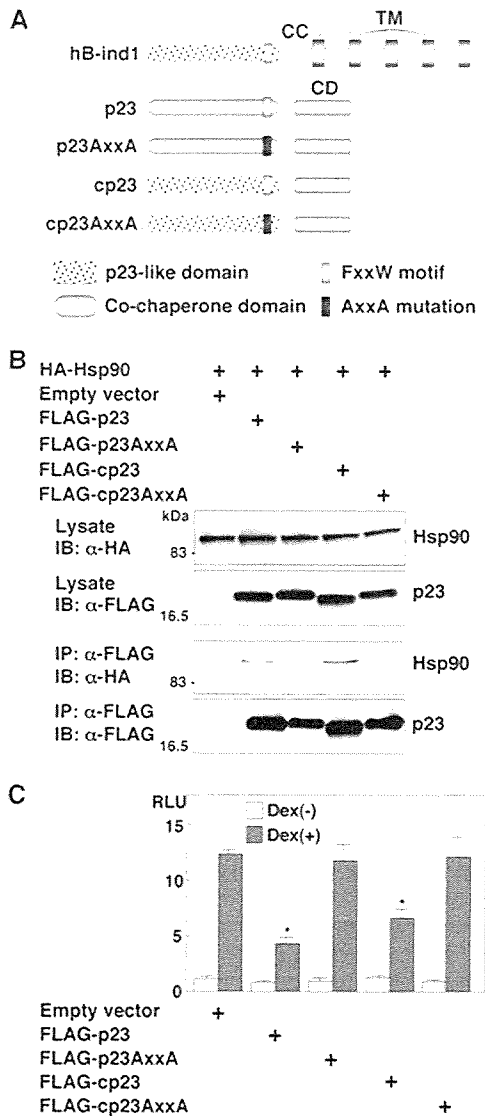


FIG. 1. Construction and characterization of p23 mutants. (A) Structures of hB-ind1, p23, and the three p23 mutants. hB-ind1 consists of a p23-like domain, an FXXW motif, a coiled-coil domain (CC), and a transmembrane domain (TM). p23 consists of a co-chaperone domain, an FXXW motif, and a chaperone domain (CD). The three p23 mutants, p23AxxA, cp23, and cp23AxxA, were constructed by replacing the FXXW motif with AXXA, the co-chaperone domain of p23 with the p23-like domain of hB-ind1, and both of the regions, respectively. (B) FLAG-tagged p23, p23AxxA, cp23, or cp23AxxA was coexpressed with HA-tagged Hsp90 in 293T cells and immunoprecipitated (IP) with anti-FLAG antibody. The immunoprecipitates were subjected to immunoblotting (IB). (C) The expression plasmid encoding FLAG-tagged p23, cp23, p23AxxA, or cp23AxxA was cotransfected with pGR-luc and pRL-TK plasmids into 293T cells and treated with 1 mM dexamethasone [Dex(+)] at 36 h posttransfection or untreated [Dex(-)], and the luciferase activities were determined at 12 h of incubation. The firefly luciferase activity was normalized with that of *Renilla* luciferase, and the GR-responsive promoter activity was indicated as the RLU. The error bars indicate standard deviations. The asterisks indicate significant differences ($P < 0.01$) versus the control value. The data shown are representative of three independent experiments.

(67), is also involved in binding to Hsp90. Hsp90 participates in the folding and stabilization of the ligand-binding domain of the glucocorticoid receptor (GR), together with p23 and other cofactors (49). p23 was shown to act not only in the activation (30), but also in the inhibition, of GR signaling (67). To examine whether hB-ind1 has the ability to work as a cochaperone in an Hsp90-dependent manner, each of the plasmids encoding p23 or the p23 mutants was cotransfected with a reporter plasmid carrying a firefly luciferase gene under the control of the GR promoter (pGR-luc), together with an internal-control plasmid (pRL-TK), and GR-mediated transcriptional activity was determined at 12 h after treatment with dexamethasone, a ligand of GR. Expression of the p23 or cp23 mutant, but not of the AXXA mutants, significantly inhibited GR-mediated transcription (Fig. 1C). These results indicate that the p23-like domain of hB-ind1 possesses cochaperone activity comparable to that of p23.

The p23-like domain of hB-ind1 is interchangeable with the p23 co-chaperone domain during complex formation with NS5A, Hsp90, and FKBP8. Previous reports have suggested that HCV NS5A interacts with several host proteins, including FBL2 (63), vesicle-associated membrane protein-associated protein subtype A (VAP-A) (61), VAP-B (25), FKBP8 (45), and hB-ind1 (56), and that these interactions participate in the replication of HCV. We have shown that hB-ind1 interacts with NS5A and Hsp90 through the coiled-coil domain and the FXXW motif in the p23-like domain, respectively, and that coexpression of FKBP8 enhances the interaction of Hsp90 with hB-ind1 (56). To determine the effect of the mutation in the p23-like domain of hB-ind1 on interaction with Hsp90, NS5A, and FKBP8, we prepared an expression plasmid encoding wild-type hB-ind1 and three hB-ind1 mutants, one in which the p23-like domain was replaced with the co-chaperone domain of p23 (chB-ind1), one in which the FXXW motif was replaced with AXXA (hB-ind1AxxA), and one in which both replacements were made (chB-ind1AxxA) (Fig. 2A). The FLAG-tagged wild-type or mutant hB-ind1 was coexpressed with HA-tagged Hsp90 (Fig. 2B, left) or HA-tagged NS5A (Fig. 2B, right) in 293T cells and immunoprecipitated with anti-FLAG antibody. Hsp90 was coprecipitated with wild-type hB-ind1 and the chB-ind1 mutant, but not with the hB-ind1AxxA and chB-ind1AxxA mutants (Fig. 2B, left), confirming that the FXXW motif is crucial for the interaction with Hsp90. In contrast, NS5A was coprecipitated with each of the hB-ind1 proteins, suggesting that mutation in the p23-like domain of hB-ind1 has no effect on the binding of hB-ind1 to NS5A through the coiled-coil domain (Fig. 2B, right). To determine the effect of FKBP8 expression on the interaction between hB-ind1 and Hsp90, FLAG-tagged wild-type hB-ind1 or the chB-ind1 mutant was coexpressed with HA-tagged FKBP8 and/or Hsp90 in 293T cells and immunoprecipitated with anti-FLAG antibody. The amounts of Hsp90 coprecipitated with hB-ind1 or chB-ind1 were increased by coexpression of FKBP8 (Fig. 2C). To further examine the interaction of hB-ind1 with Hsp90 and NS5A at an endogenous expression level in Huh9-13 cells harboring an HCV subgenomic RNA replicon, lysates of the replicon cells were subjected to immunoprecipitation analysis. Endogenous Hsp90 and NS5A were specifically coimmunoprecipitated with endogenous hB-ind1 (Fig. 2D). These results suggest that the p23-like domain of hB-ind1 is inter-

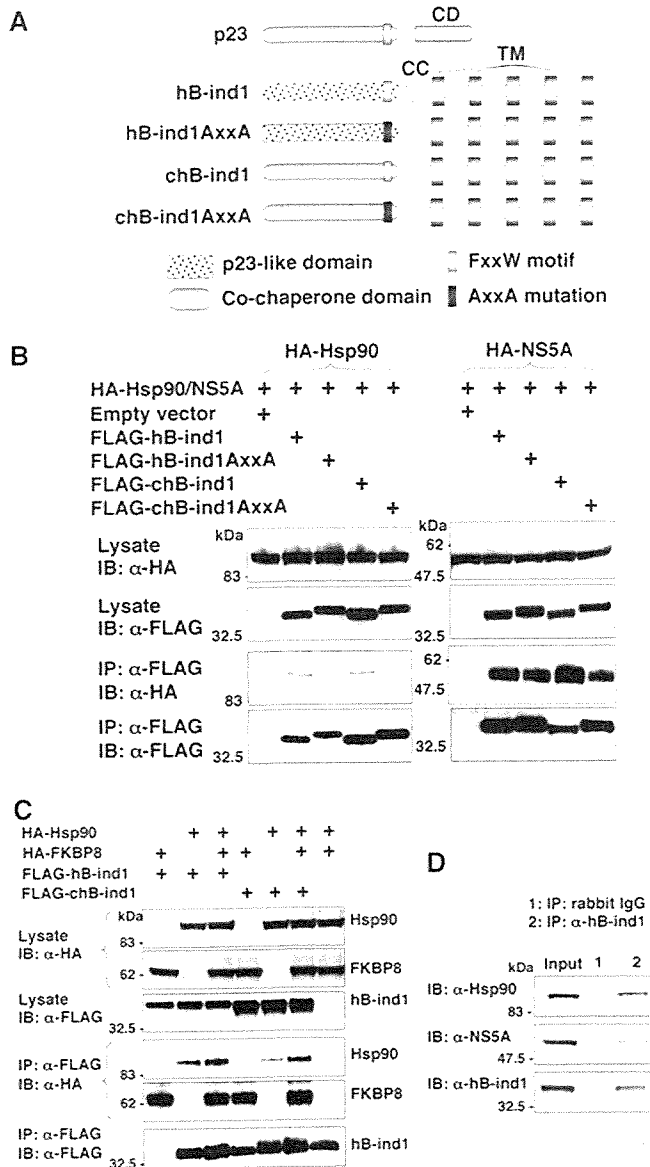


FIG. 2. Construction and characterization of hB-ind1 mutants. (A) Structures of p23, hB-ind1, and the three hB-ind1 mutants. The three hB-ind1 mutants, hB-ind1AxxA, chB-ind1, and chB-ind1AxxA, were constructed by replacing the FXXW motif with AXXA, the p23-like domain of hB-ind1 with the co-chaperone domain of p23, and both of the regions, respectively. (B) FLAG-tagged hB-ind1, hB-ind1AxxA, chB-ind1, or chB-ind1AxxA was coexpressed with either HA-tagged Hsp90 (left) or NS5A (right) in 293T cells and immunoprecipitated (IP) with anti-FLAG antibody. The immunoprecipitates were subjected to immunoblotting (IB). (C) HA-tagged Hsp90 and HA-FKBP8 were expressed with FLAG-tagged hB-ind1 and chB-ind1 in various combinations in 293T cells and immunoprecipitated with anti-FLAG antibody, and the immunoprecipitates were detected by immunoblotting. (D) Endogenous hB-ind1 in Huh9-13 cells harboring subgenomic HCV replicon RNA was immunoprecipitated with anti-hB-ind1 rabbit IgG (lane 2). The cell lysate was mixed with normal rabbit IgG as a negative control (lane 1). The immunoprecipitates were analyzed by immunoblotting with an antibody to Hsp90, NS5A, or hB-ind1. The data shown are representative of three independent experiments.

changeable with the co-chaperone domain of p23 during complex formation with NS5A, Hsp90, and FKBP8.

Cochaperone activity in the p23-like domain of hB-ind1 is required for propagation of HCV. The p23-like domain of hB-ind1 has been suggested to be required for HCV propagation (56). However, the involvement of the co-chaperone activity of hB-ind1 in HCV propagation has not been examined. To assess the effect of co-chaperone activity in the p23-like domain of hB-ind1 on the RNA replication and particle production of HCV, each of the expression plasmids encoding the FLAG-tagged wild-type or mutant hB-ind1 carrying the silent mutations resistant to small interfering RNA was transfected into hB-ind1 knockdown (Huh-KD) cells and cultured for a week in the presence of puromycin. The expressions of FLAG-tagged hB-ind1 and the mutants in the Huh-KD cells were comparable to that of the endogenous hB-ind1 in the control (Huh-ctrl) cells transfected with an empty vector (Fig. 3A). Subgenomic HCV replicon RNA transcribed from pFK-I₃₈₉ neo/NS3-3'/NK5.1 was transfected into these cells and cultured for 4 weeks in the presence of G418. Although the number of colonies was reduced in the Huh-KD cells compared with the Huh-ctrl cells after transfection with an empty vector, as described previously (56), the colony numbers were recovered by the expression of the hB-ind1 or chB-ind1 mutant, but not by that of the hB-ind1AxxA or chB-ind1AxxA mutants (Fig. 3B). Similarly, intracellular HCV RNA and infectious viral titers in the culture supernatants of Huh-KD cells infected with JFH1 virus were partially recovered by the expression of the hB-ind1 or chB-ind1 mutant, but not by that of the hB-ind1AxxA or chB-ind1AxxA mutant (Fig. 3C). These results suggest that co-chaperone activity in the p23-like domain of hB-ind1 is required for HCV propagation and that the co-chaperone domain of p23 can substitute for the p23-like domain of hB-ind1.

hB-ind1 colocalizes with NS5A, FKBP8, and dsRNA on the membranous web. Our previous report revealed the interplay among hB-ind1, Hsp90, FKBP8, and NS5A and showed that these interactions play an important role in HCV replication (56). However, the subcellular localization of the endogenous hB-ind1 in the replicon cells and JFH1 virus-infected cells has not been precisely assessed. To determine the subcellular localization of hB-ind1 in the context of HCV replication, the expression of hB-ind1 and NS5A in the replicon cells and JFH1 virus-infected cells was examined by immunofluorescence analyses (Fig. 4A). Endogenous hB-ind1 was colocalized with the endoplasmic reticulum (ER)-marker PDI and NS5A as dot-like structures in the Huh9-13 replicon cells (Fig. 4A, top) and in cells infected with JFH1 virus (Fig. 4A, bottom), and these dot-like structures disappeared in concert with the loss of NS5A expression by treatment with IFN- α in the replicon cells and was not observed in the mock-infected Huh7.5.1 cells. Furthermore, FKBP8 (Fig. 4B, top) and dsRNA (Fig. 4B, bottom) were colocalized with hB-ind1 and NS5A in the dot-like structures in Huh9-13 replicon cells. These results indicate that HCV replicating RNA is localized with hB-ind1, FKBP8, and NS5A in the dot-like compartments. HCV RNA replication or expression of viral proteins leads to formation of the convoluted membranous structures designated the membranous web (14, 23). The large structures of the replication complexes in the replicon cells indicate membranous webs with

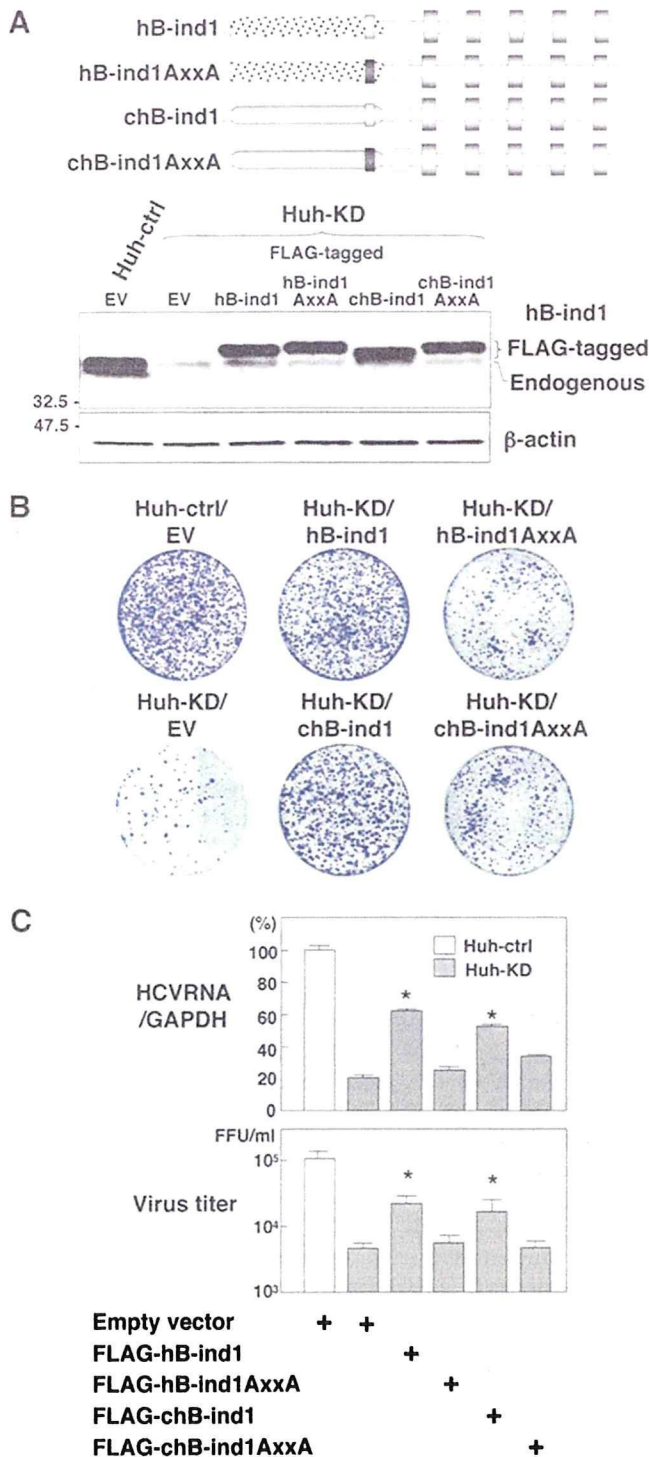


FIG. 3. Effects of the cochaperone activity of hB-ind1 on the propagation of HCV. (A) Huh-KD cells were transfected with either an empty vector or an expression plasmid encoding FLAG-tagged hB-ind1, hB-ind1AxxA, chB-ind1, or chB-ind1AxxA, which are resistant to small interfering RNA due to the introduction of silent mutations, and cultured for a week in the presence of 10 μg/ml of puromycin. The surviving cells were used in the subsequent experiments. The endogenous and exogenous expression of hB-ind1 and the mutants was detected by immunoblotting. The control cell line (Huh-ctrl) or the Huh-KD cell line transfected with an empty vector (EV) was used as a control. (B) Huh-KD cells were transfected with the plasmids and

restricted motility (68). To further analyze the subcellular compartments, including hB-ind1 and NS5A, the same field of the Huh9-13 replicon cells was observed under FM and EM by using the correlative FM-EM technique (Fig. 5A, upper two rows). The large structures that included hB-ind1 and NS5A in the replicon cells were observed under FM and EM (white-boxed areas) and further magnified (black-boxed areas). Convoluted membranous structures that consisted of small vesicles and that were similar to the membranous web were observed. Another field of view yielded similar results (Fig. 5A, lower two rows). The membranous web resembling the convoluted structures was not observed in the Huh9-13 cells depleted of viral RNA by IFN treatment (Fig. 5B). Together, these results suggest that hB-ind1 interacts with NS5A on the membranous web in cells replicating HCV RNA.

Hsp90 is involved in the circumvention of the UPR during HCV replication. Hsp90 regulates the folding and stability of proteins in all eukaryotes (59), and inhibition of the chaperone pathway suppresses correct protein folding, which leads to induction of proteasome-mediated degradation of the unfolded proteins and the unfolded protein response (UPR). Our previous (46) and present studies (Fig. 4 and 5) showed that several cochaperone components are recruited in the membranous web, suggesting that the Hsp90 chaperone system participates in the replication complex to circumvent the induction of the UPR and to maintain the folding of the host and viral proteins in a replication-competent state. To determine the induction of the UPR by HCV replication, Huh9-13 replicon cells were transfected with a reporter plasmid carrying a firefly luciferase gene under the control of the GRP78 promoter, which is activated by the induction of the UPR, together with an internal-control plasmid. Although the GRP78 promoter activity was slightly enhanced in the Huh9-13 cells compared to that in the parental cells, a fourfold increase of GRP78 promoter activity in the replicon cells was observed after treatment with an Hsp90 inhibitor, DMAG, in contrast to the twofold increase in similarly treated parental Huh7 cells, and the activation of the GRP78 promoter was canceled by treatment with IFN-α despite DMAG treatment (Fig. 6A), suggesting that the Hsp90 chaperone system participates in the circumvention of the UPR induced by the replication of HCV RNA. In addition, activation of GRP78 at transcriptional and translational levels after treatment with DMAG was higher in the

then selected with puromycin. The resulting cells were further transfected with a replicon RNA transcribed from pFK-1₃₈₉ neo/NS3-3'/NK5.1, cultured for 4 weeks in the presence of 1 mg/ml of G418, and stained with crystal violet after fixation with 4% paraformaldehyde. The Huh-KD cell line transfected with an empty vector (EV) was used as a positive control. (C) The cells prepared as described above were infected with JFH1 virus and harvested at 3 days postinfection. The amount of intracellular HCV RNA was estimated by quantitative reverse transcriptase PCR and normalized with that of GAPDH mRNA. The values of HCV RNA are presented as percentages versus those of Huh-ctrl cells transfected with an empty vector. The culture supernatants were subjected to a focus-forming assay. Virus titers are presented as focus-forming units (FFU) per ml. The error bars indicate standard deviations. The asterisks indicate significant differences (P < 0.01) versus the value of the control. The data shown are representative of three independent experiments.

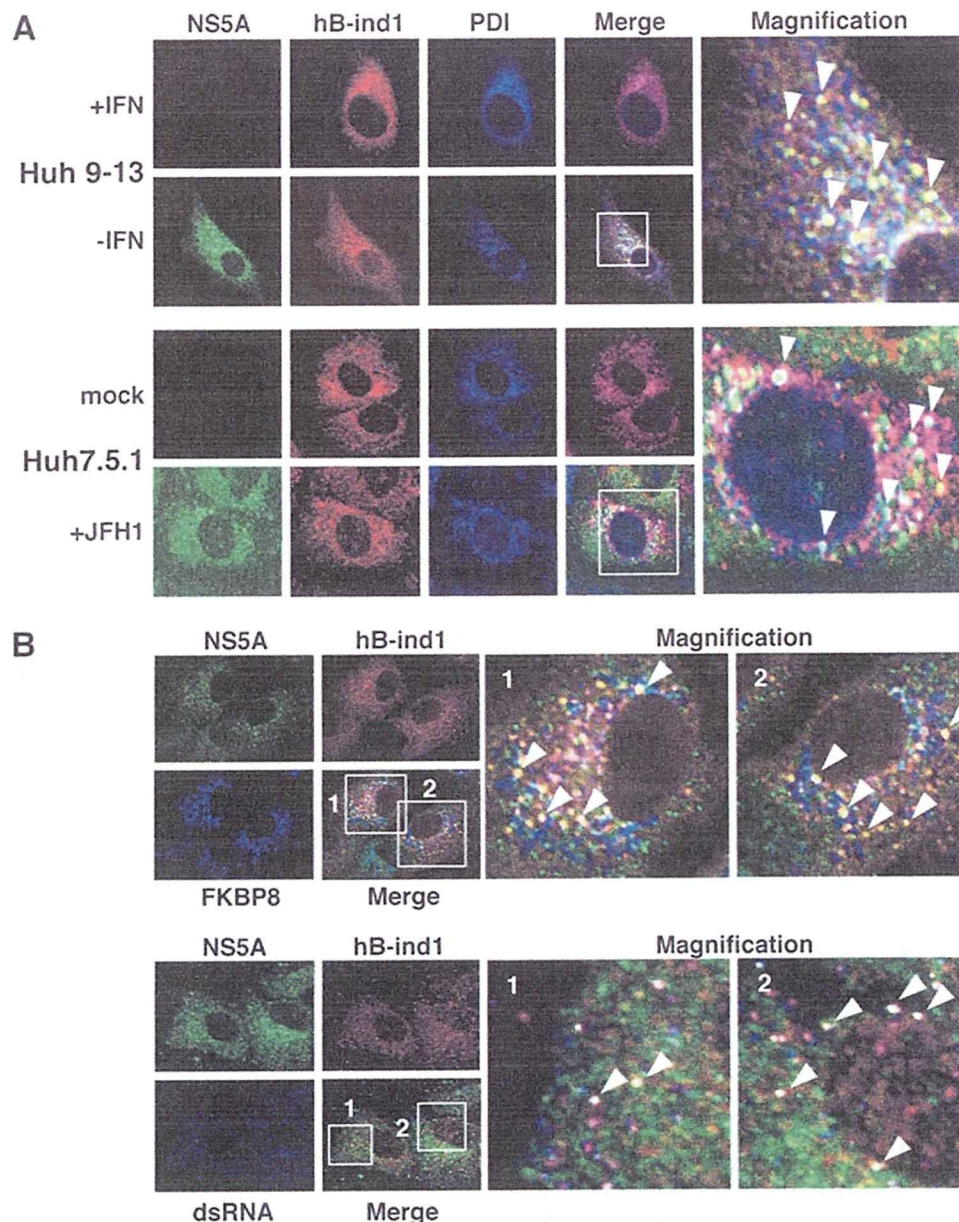


FIG. 4. Intracellular localization of hB-ind1 in replicon cells and infected cells. (A) Huh9-13 replicon cells with IFN- α or untreated and Huh7.5.1 cells infected with JFH1 virus or naïve cells were stained with antibodies against NS5A, hB-ind1, or PDI and examined by immunofluorescence assay. The boxed areas in the merged images are magnified and displayed on the right. The arrowheads indicate intracellular positions colocalized with NS5A, hB-ind1, and PDI. (B) Huh9-13 replicon cells were fixed, permeabilized, and stained with appropriate antibodies to NS5A, hB-ind1, and FKBP8 (top) or dsRNA (bottom). The boxed areas in the merged images are magnified and displayed on the right. The arrowheads indicate intracellular positions colocalized with NS5A, hB-ind, and FKBP8 or dsRNA. The images shown are representative of three independent experiments.

HCV replicon cells than in the parental cells or in cured cells, which were depleted of HCV RNA by treatment with IFN- α (Fig. 6B). Furthermore, DMAG treatment enhanced the transcription of the UPR marker protein GADD153 at a higher level in the replicon cells than in the parental Huh7 or the cured cells (Fig. 6C). These results suggest that the Hsp90-dependent chaperone system plays a crucial role in the folding of the host and viral proteins involved in HCV replication and in the regulation of UPR induction.

DISCUSSION

Studies of the relationship between Hsp90 and steroid receptors, such as GR, have revealed the activities of cochaperones (52, 67). Cochaperones, such as p23, appear to interact with and dissociate from Hsp90 and the client protein complex in a defined order. These cochaperones participate in the chaperone complex in a late step and promote the dissociation of the client proteins from Hsp90 to facilitate formation of the

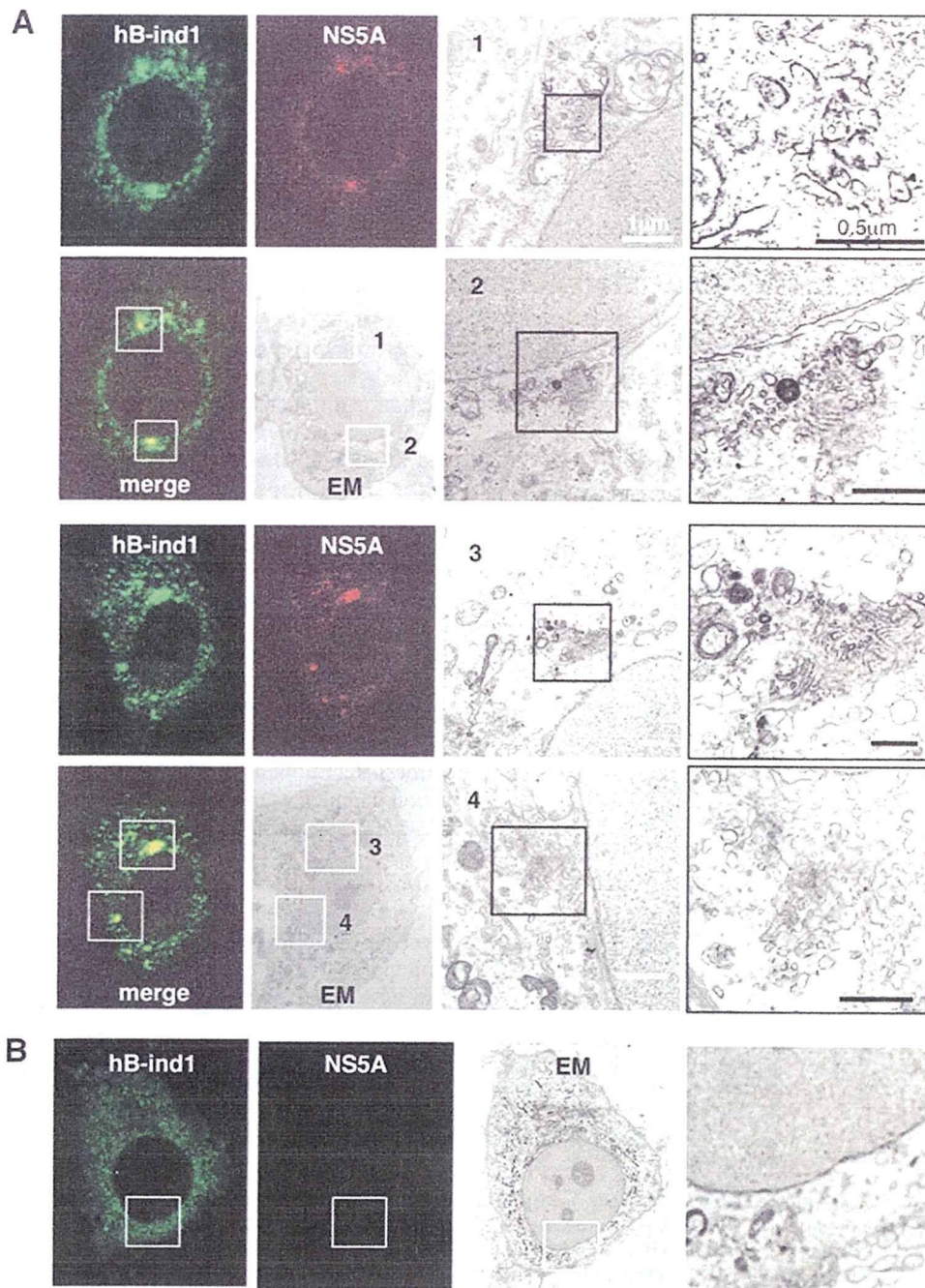


FIG. 5. hB-ind1 interacts with NS5A in the membranous web. Huh9-13 replicon cells were stained with specific antibodies to hB-ind1 and NS5A. Identical fields of Huh9-13 (A) or the cured cells (B) were observed under EM by using the correlative FM-EM technique. The white-boxed areas indicate the colocalized areas of hB-ind1 with NS5A. Magnified views of the white-boxed areas are displayed in the third column from the left. The right column contains further-magnified images of each of the black-boxed areas. Another field of view is presented in the lower two rows.

chaperone complex in the next chaperone cycle (16–18). In this study, we have shown that hB-ind1 participates in HCV replication and that the p23-like domain of hB-ind1 possesses co-chaperone activity comparable to that of the co-chaperone domain of p23, suggesting that hB-ind1 is involved in the recycling of the chaperone complex in the membranous web to maintain the function of the replication complex of HCV.

Previous studies have indicated that HCV proteins rear-

range the ER membrane into the small convoluted membranous vesicles that are collectively known as the membranous web, and these vesicles have been suggested to be the intracellular compartments in which HCV replication takes place (14, 23, 68). In the living replicon cells, two forms of replication complexes, small and large vesicles, are detected, both of which include the viral replication complexes (68). Large vesicles, corresponding to membranous webs, exhibit restricted motil-

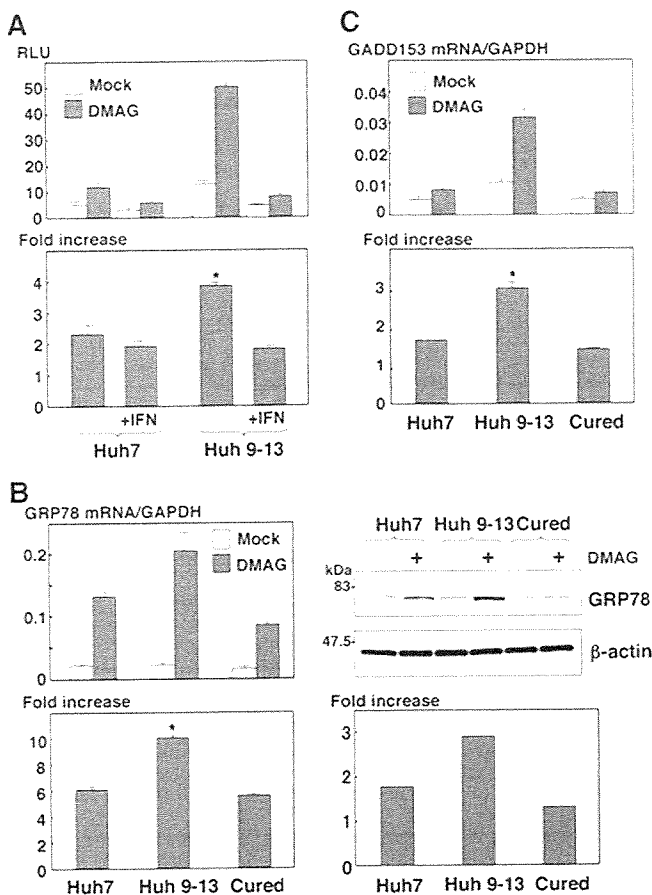


FIG. 6. Effect of Hsp90 inhibitor on the induction of the UPR in HCV replicon cells. (A) Huh7 and Huh9-13 replicon cells were transfected with a reporter plasmid, pGRP78-luc, and an internal-control plasmid, pRL-TK. The transfected cells were treated with IFN- α (+IFN) from 6 to 36 h posttransfection or left untreated and then further incubated for 6 h in the presence or absence of 1 μ M DMAG. The resulting cells were harvested and subjected to a dual-luciferase assay. The firefly luciferase activity is indicated as the RLU (top) after standardization with that of *Renilla* luciferase. The enhancement of promoter activity by treatment with DMAG is presented as the increase (bottom). (B) Huh7 cells, Huh9-13 cells, and Huh9-13 cells cured by IFN- α treatment (Cured) were cultured for 6 h in the presence or absence of 1 μ M DMAG, and the amount of GRP78 mRNA was measured by real-time PCR. The value of the mRNA was normalized with the amount of GAPDH mRNA (upper left), and the transcriptional enhancement by treatment with DMAG is presented as the increase (lower left). The expression levels of GRP78 and β -actin in the cells were determined by immunoblotting (upper right) and are presented as the increase (lower right). (C) The amounts of GADD153 mRNA in Huh7 cells, Huh9-13 cells, and the cured cells cultured for 6 h in the presence or absence of 1 μ M DMAG were measured by real-time PCR. The values of the mRNA were normalized with the amount of GAPDH mRNA (top), and the transcriptional enhancement by treatment with DMAG is presented as the increase (bottom). The error bars indicate standard deviations. The asterisks indicate significant differences ($P < 0.01$) versus the control value. The data shown are representative of three independent experiments.

ity, while small vesicles show fast movement (68), and FM and EM have revealed that NS5A is colocalized with hB-ind1, as well as FKBP8 (45), in the membranous webs. hB-ind1 was first identified as a regulator of Rac1 that activates JNK and NF- κ B (11). Rac1 is a member of the Rho GTPase family and plays

crucial roles in cytoskeletal dynamics, membrane ruffling, and gene transcription through the effectors of the Rho GTPase family members. IQGAP1 and PAK1 are Rac1 effectors that bind to Rac proteins and are also involved in the replication of HCV (5, 7, 19, 31, 50). The tetratricopeptide repeat domain of immunophilin family members, such as FKBP8, has been shown to interact with Hsp90 (12, 45) and the GR-Hsp90 complex that leads to association with dynein for retrograde transport, along with microtubules (12). Hsp90 has been shown to play an important role in the interaction of transcriptase with genomic RNA of hepatitis B virus (27) and the nuclear transportation of the polymerase of influenza virus (40). Flock house virus also recruits Hsp90 in the polymerase synthesis in the early step of infection (9). Hsp90 may be involved in the regulation of the movement and arrangement of the HCV replication complexes through interaction with Rac1, hB-ind1, and FKBP8. Further investigation is needed to clarify the role of the Hsp90 chaperone system in the life cycle of HCV.

The surrounding membranes, including the membranous web, may protect the viral replication complex and RNA genome against digestion by the host proteases and nucleases (69). The replication complex is composed of viral nonstructural proteins and host proteins, including chaperone and co-chaperone proteins. HCV NS5A has been shown to interact with various host proteins, including cochaperones, such as FKBP8 and hB-ind1, and to recruit a chaperone, Hsp90, into the replication complex through interaction with these cochaperones. Recruitment of the chaperone complex into the replication complex is crucial for the correct folding of newly synthesized viral proteins to maintain the efficient replication of the viral genome. HCV replication has been shown to be improved by the adaptive mutations suppressing the phosphorylation status of NS5A in the replicon cells (3). Although suppression of the hyperphosphorylation of NS5A by treatment with kinase inhibitors improves the replication of the replicons that have no adaptive mutations (42), several kinase inhibitors have been shown to suppress the replication of the HCV replicon carrying the adaptive mutations (29), and phosphorylation of NS5A by casein kinase II was shown to improve virus production but not HCV RNA replication (57). Hsp90 is capable of directly modulating the activities of several kinases (37, 53, 54), and thus, it might be feasible that cochaperones, including hB-ind1 and FKBP8, participate in the propagation of HCV by regulating the phosphorylation status of NS5A in cooperation with Hsp90.

The host chaperone system regulates the quality of client proteins, and impairment of the chaperone activity induces accumulation of misfolded proteins and affects the natural cellular function and viability (20, 21, 33). In this study, DMAG treatment induced a higher level of UPR in HCV replicon cells than in parental and cured cells, indicating that the Hsp90 chaperone system participates in the maintenance of correct folding of the viral and host proteins in the replication complex in the membranous web and in the circumvention of the UPR induced by HCV replication. Treatment with geldanamycin or its derivatives has been shown to inhibit GRP94, which is the Hsp90 paralog located in the ER (10), and to disrupt the ER chaperone pathway, leading to the induction of ER-associated protein degradation, transcriptional attenuation, and eventually induction of apoptosis (34). ER chaperones, such as

GRP94, may also participate in the correct folding of the viral and host proteins in the replication complex for efficient replication of the HCV genome.

Geldanamycin and its derivatives have been reported to remarkably inhibit poliovirus replication *in vivo* without any emergence of drug-resistant escape mutants (22), suggesting that an inhibitor of the chaperone system may be a promising candidate for the treatment of viral infectious diseases with low risk of the emergence of drug-resistant viruses. In addition, Hsp90 inhibitors exhibit anticancer activities through the suppression of various cell signals essential for cancer growth and the enhancement of radiation sensitivity (2, 8, 13). In conclusion, our data indicate that hB-ind1 is included within the HCV replication complex and regulates HCV RNA replication through its own cochaperone activity. Hsp90 and cochaperones, including hB-ind1 and FKBP8, which are required for efficient HCV replication, should be ideal targets for the treatment of chronic hepatitis C with a low frequency of emergence of drug-resistant breakthrough viruses.

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Biological and immunological characteristics of hepatitis E virus-like particles based on the crystal structure

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Hepatitis E virus (HEV) is a causative agent of acute hepatitis. The crystal structure of HEV-like particles (HEV-LP) consisting of capsid protein was determined at 3.5-Å resolution. The capsid protein exhibited a quite different folding at the protruding and middle domains from the members of the families of *Caliciviridae* and *Tombusviridae*, while the shell domain shared the common folding. Tyr-288 at the 5-fold axis plays key roles in the assembly of HEV-LP, and aromatic amino acid residues are well conserved among the structurally related viruses. Mutational analyses indicated that the protruding domain is involved in the binding to the cells susceptible to HEV infection and has some neutralization epitopes. These structural and biological findings are important for understanding the molecular mechanisms of assembly and entry of HEV and also provide clues in the development of preventive and prophylactic measures for hepatitis E.

capsid | HEV | VLP

Hepatitis E is an acute viral hepatitis caused by infection with hepatitis E virus (HEV) that is transmitted primarily by a fecal-oral route (1, 2). Numerous epidemic and sporadic cases have occurred in developing countries of Asia, the Middle East, and North Africa, where sanitary conditions are not well-maintained. Hepatitis E affects predominantly young adults, and HEV infection in pregnancy is one of the risk factors for severe disease and death (3). Recent epidemiological studies show that significant prevalence of HEV and anti-HEV antibody is found in humans and several animals worldwide, even in developed countries (4–8).

HEV is the sole member of the genus *Hepevirus* within the family *Hepeviridae* and has a 7.2-kb positive-sense RNA genome (9). Five major genotypes have been identified so far (2). The viruses in the genotypes 1 and 2 are maintained among only humans, while those in the genotypes 3 and 4 are found in pigs or wild animals (4–6). However, infections of human with genotypes 3 and 4 via zoonotic transmission or blood transfusion were reported in the developed countries, such as Japan and the United States (7, 8, 10), suggesting that hepatitis E caused by infection with genotypes 3 and 4 of HEV is an important emerging infectious disease. The viruses in the genotype 5 are of avian origin and are thought to be untransmissible to humans (11). The genomic RNA contains three ORFs (ORFs) encoding nonstructural proteins (ORF1), the viral capsid protein composed of 660 amino acids (ORF2) and a small phosphorylated protein of unidentified function (ORF3) (1, 9). The viral capsid protein induces neutralizing antibodies by its immunization (12–15) or during the course of infection (16, 17). A typical signal sequence at the N terminus and 3 potential *N*-glycosylation sites (Asn-X-Ser/Thr) are well-conserved in the capsid protein de-

rived from all mammalian genotypes (18, 19), but the glycosylation status of this protein is still controversial and the biological significance of the modification in the viral life cycle remains unknown. Although propagation of HEV in the cell culture systems reported in earlier studies was not efficient (20–23). Tanaka et al. succeeded in the establishment of a persistent infection system of HEV genotype 3 in human hepatoma (PLC-PRF/5) and human carcinomic alveolar epithelial (A549) cell lines (24). However, sufficient amounts of viral particles cannot be obtained for studies of the structure, life cycle, and pathogenesis of HEV.

Electron microscopy of human stool specimens showed that HEV is a nonenveloped spherical particle with a diameter of approximately 320 Å (25). As an alternative to in vitro propagation of HEV, the baculovirus expression system opens the prospect of studying HEV capsid assembly, since HEV-like particles (HEV-LP) with protruding spikes on the surface can be formed in insect cells infected with a recombinant baculovirus expressing the capsid protein of a genotype 1 strain (26–28). Cryo-electron microscopic (cryoEM) analysis has revealed that HEV-LP is a $T = 1$ icosahedral particle composed of 60 copies of truncated products of ORF2 (27, 28). The HEV-LP appeared to be empty due to a lack of significant density containing RNA inside and was 270 Å in diameter (26–28), which is smaller than the diameter of the native virions. However, the HEV-LP retained the antigenicity and capsid formation of the native HEV particles.

The crystal structures of the recombinant or native $T = 3$ viral particles derived from structurally related mammalian and plant viruses, such as recombinant Norwalk virus (rNV; PDB accession code 1IHM) (29), San Miguel sea lion virus (SMSV; PDB accession code 2GH8) (30), the members of the family *Caliciviridae*, and Carnation mottle virus (CARMV; PDB accession code 1OPO) (31), a member of the family *Tombusviridae*, have

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Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 2ZTN).

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been determined at resolutions of 3.4 Å, 3.2 Å, and 3.2 Å, respectively. In this study, to understand the structural basis on HEV, we solved the crystal structure of HEV-LP derived from a genotype 3 strain at 3.5-Å resolution and found differences in the folding of the capsid protein among these viruses. On the other hand, we found several structural similarities of shell formation. In particular, it was revealed that aromatic amino acids (Tyr-288 in the case of HEV-LP) at the 5-fold axis play a crucial role in the hydrophobic interaction required for particle formation and are well conserved among these viruses. Furthermore, mutational analyses depicted the putative cellular receptor-binding regions and epitopes for neutralizing of binding (NOB) antibodies on the 3D structure of HEV-LP. The availability of the 3D structure of HEV-LP at high resolution will provide valuable information not only for analyses of the entry and assembly of HEV, but also for the development of a vaccine for hepatitis E.

Results

Preparation of HEV-LP of a Genotype 3. Upon infection with a recombinant baculovirus possessing a genome of the truncated capsid protein (amino acids 112–608) from a genotype 3 strain under the control of polyhedrin promoter, a large amount of HEV-LP was secreted into the culture supernatant as described in the case of HEV-LP of genotype 1 strain (26–28). The purified HEV-LP of genotype 3 was used for further structural and biological analyses.

Overall Structure of HEV-LP. The crystal structure of HEV-LP derived from the genotype 3 strain was determined at 3.5-Å resolution by the molecular replacement method by using a cryoEM map of HEV-LP derived from the genotype 1 strain (27, 28) as an initial phasing model (Fig. 1A). As shown in the previous papers (27, 28), HEV-LP shows a $T = 1$ icosahedral symmetry with an external diameter of 270 Å. This particle is composed of 60 subunits of the truncated capsid proteins, forming the icosahedral 2-, 3-, and 5-fold axes. It has 30 protrusions at the 2-fold axis of the surface with large depressions at the 3- and 5-fold axes.

Structure of the HEV Capsid Protein. The truncated HEV capsid protein has 3 definite domains designated as S (shell), M (middle), and P (protruding) composed of the amino acid residues 129–319, 320–455, and 456–606, respectively (Fig. 1B). Because the N- and C-terminally truncated capsid proteins were used for the characterization, the typical signal sequence (amino acids 1–22) and following arginine-rich domain (amino acids 23–111) and the C-terminal domain removed by cleavage in insect cells (amino acids 609–660) were not determined in this study. Additionally, the amino acid residues 112–128, 486–487, 555–560, and 607–608 were disordered in this study. The S domain, which forms an internal scaffold structure of the particle, folds into a classical anti-parallel jelly roll-like β -sandwich structure with 8 β -strands (designated as B to I) and 4 short α -helices that are conserved among many viral capsids (Fig. 1B and Fig. S1) (29–33). The M domain, which is one of the characteristic domains, has a twisted anti-parallel β -barrel structure composed of 6 β -strands and 4 short α -helices. This domain is tightly associated with the S domain and located on the surface around the icosahedral 3-fold axis (Fig. 1A and B). The M and P domains are linked with a long proline-rich hinge (amino acids 445–467). Previous studies on the structures of rNV (29) and SMSV (30) revealed that the P domains of the viruses are composed of 2 subdomains, P1 and P2, and the P2 subdomain is located as a large protrusion of the P1 subdomain (Fig. S1). In contrast, the P domain of HEV-LP is composed of a single individual domain forming a twisted anti-parallel β -sheets structure (Fig. 1B and Fig. S1), demonstrating that the capsid protein

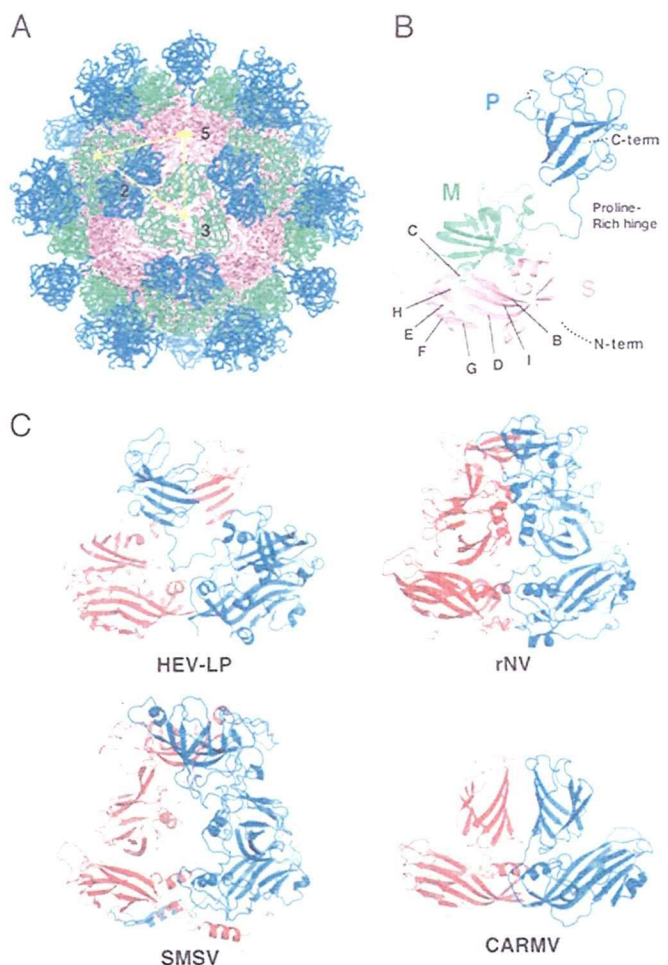


Fig. 1. Crystal structure of HEV-LP and comparison of capsid protein dimers of HEV-LP, rNV, SMSV, and CARMV. The S, M, and P domains of the HEV capsid protein are indicated by pink, green, and blue, respectively. (A) HEV-LP is composed of sixty capsid subunits forming icosahedral 2-, 3-, and 5-fold axes and indicating a $T = 1$ symmetry. (B) The ribbon diagram of a capsid subunit of HEV-LP (PDB accession code: 2ZTN) shows P, M, and S domains at the top, middle, and bottom, respectively. The disordered regions are shown with dashed lines. The S domain shows a jelly roll-like β -barrel structure conserved in some viruses. The conserved anti-parallel β -strands are indicated (B to I). (C) The ribbon diagrams of crystal structures of capsid protein dimers of HEV-LP and those of rNV (PDB accession code 1IHM), SMSV (PDB accession code 2GH8), and CARMV (PDB accession code 1OPO) are indicated. Each capsid protein monomer is colored in red and blue.

of HEV-LP has a significantly different fold from those of caliciviruses, except for the S domain. Although we have no evidence of glycosylation of HEV-LP prepared in insect cells, the HEV capsid protein has 3 potential *N*-glycosylation sites, Asn-137-Leu-Ser, Asn-310-Leu-Thr and Asn-562-Thr-Thr (19). In the dimer structure, the former 2 sites are mapped on the horizontal surface of the S domain, as shown in Fig. S2A. However, Asn-137 and Asn-310 are located in the interfaces of the pentamer and trimer structures, respectively (Fig. S2B and C), suggesting that, if it occurs at all, *N*-glycosylation in these sites may inhibit assembly of HEV-LP. Indeed, Graff et al. (18) reported that HEV carrying mutations in Asn-137 or Asn-310 to Glu lost infectivity to cells or rhesus macaques due to a defect in the virion assembly. On the other hand, Asn-562 is mapped in the central region in the top of the P dimer and exposed in the surface of HEV-LP.

The Dimer Structure at the 2-Fold Axis. It is noteworthy that the HEV-LP dimer at the icosahedral 2-fold axis shows a crossing

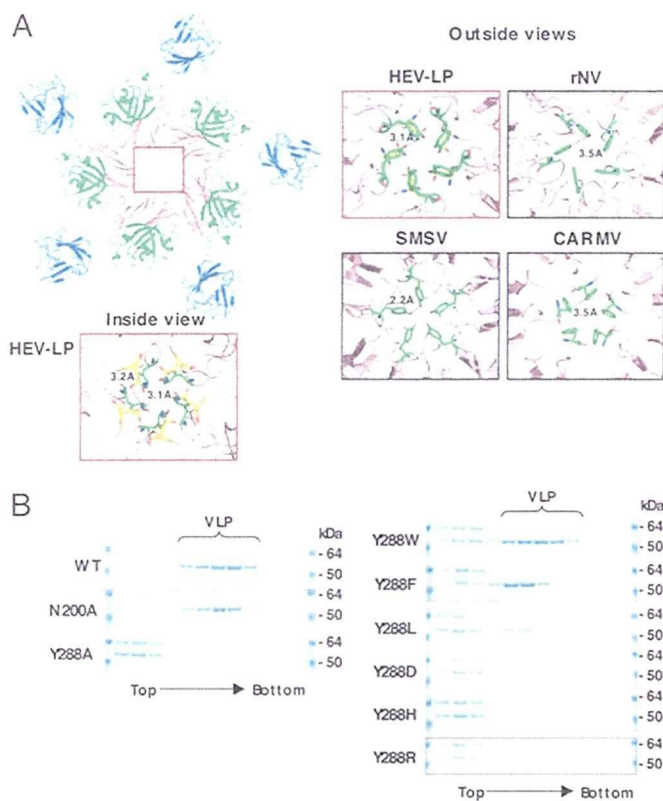


Fig. 2. Interaction of capsid protein subunits of HEV-LP around the 5-fold axis. (A) The pentamer of the capsid protein of HEV-LP. The close-up surface diagram of the 5-fold axis showed from outside and inside of HEV-LP. Amino acid residues Asn-200 and Tyr-288 are shown in yellow and green, respectively. The close-up surface diagram of the 5-fold axis showed from outside of rNV, SMSV, and CARMV. The aromatic amino acids Phe-118 of rNV, Tyr-330 of SMSV, and Phe-145 of CARMV are indicated in green. The deduced interacting atoms are connected with dashed lines, and the distances are indicated. (B) Sucrose density fractionation assay using the wild-type or mutant capsid proteins (53 kDa) in which the amino acids composing the 5-fold axis were substituted. The capsid protein composing HEV-LP was found in the 5–9th fractions from the top, while that which failed to form particles was found in the top fractions. The molecular mass of approximately 64 kDa was a non-specific protein.

topology of the P versus M and S domains, while that of the other viruses with protrusions at the 2-fold axis, containing rNV, SMSV, and CARMV, exhibits a parallel topology of each domain (Fig. 1C). The flexibility of the long proline-rich hinge region between the M and P domains allows this unique topology of HEV-LP. The P domain of HEV-LP interacts with not only the P domain but also the M domain of the counterpart to stabilize the dimer structure. Despite these topological differences, the overall structure of the protrusion dimeric structure at the 2-fold axis is similar to that of rNV and SMSV. The disordered residues 486–487 and 555–560 are located in the apical region of the protrusion, suggesting that this region is flexible to take advantage of the interaction with other molecules.

Five-Fold Axis Packaging. The inter-molecule-interface of the capsid pentamer at the icosahedral 5-fold axis is composed of only S domains, and these interaction regions are narrower than those of the dimer and trimer at the 2-fold and 3-fold axes, respectively (Fig. 2A), suggesting that the pentamer formation is a key step of HEV-LP assembly. There are 4 loops between the β -sheets in the S domain, designated as loops B–C (amino acids 139–152), D–E (amino acids 196–206), F–G (amino acids 236–241), and H–I (amino acids 281–296), around the center of the

pentamer structure. Among them, the loops B–C and F–G are not in close proximity to the next subunits, suggesting they are not implicated in the inter-molecular interaction. In contrast, loops D–E and H–I do interact with the next subunits. In particular, the side chains of Asn-200 and Tyr-288 in loops D–E and H–I, respectively, interact with those of the next subunits, from which they are separated by a distance of approximately 3.2 Å, filling in the central pore (Fig. 2A). These observations led us to hypothesize that these amino acid residues are important for assembly and stability of the particles. To examine this hypothesis, we constructed 2 mutant capsid proteins in which Asn-200 was replaced with alanine (N200A) or Tyr-288 was replaced with alanine (Y288A), and the effect of these mutations on the particle formation was determined by a density-fractionation assay (Fig. 2B). Comparative amounts of the mutant proteins to the wild-type capsid were expressed and released into the supernatants of cells infected with the recombinant baculoviruses. N200A but not Y288A formed VLP as the wild-type, indicating that Tyr-288 plays a more crucial role in particle formation than Asn-200. The aromatic amino acids, Phe-118, Tyr-330, and Phe-145, are also found in the icosahedral 5-fold axis of rNV, SMSV, and CARMV, respectively (Fig. 2A). To examine the role of the aromatic side chain in Tyr-288 in the particle formation, a series of mutants in which Tyr-288 was replaced with tryptophan, phenylalanine, leucine, asparatic acid, histidine, or arginine (Y288W, Y288F, Y288L, Y288D, Y288H, or Y288R) were generated. All of them were expressed and released into the culture medium, as well as was the wild type. The mutants with aromatic amino acids, Y288W and Y288F, were able to form HEV-LP, whereas other mutants produced no or very few particles (Fig. 2B). These results suggest that the aromatic side chain of Tyr-288 plays a crucial role in the HEV-LP formation by shutting off the central pore of the pentamer, and that the aromatic amino acids in the positions corresponding to Tyr-288 of HEV are functionally conserved among the structurally related viruses.

Binding of HEV-LP to Cultured Cells. The early steps of HEV entry remain unclear because of the lack of a robust cell culture system for HEV, despite recent progress in the *in vitro* propagation of HEV in the cell lines PLC/PRF/5 and A549 (24). HEV-LP was able to bind to several cell lines, including PLC/PRF/5 and A549 cells, but not to mouse myeloma P3 × 63Ag8U.1 (P3U1) cells (Fig. S3), suggesting that a binding assay using HEV-LP is useful to examine the first step of receptor-binding of HEV to the target cells. Among the cell lines examined, the human hepatoma cell line Huh7, exhibited a greater ability to bind to HEV-LP than the cell lines PLC/PRF/5 and A549. Therefore, Huh7 cells were used for the following binding experiments of HEV-LP.

Three-Dimensional Mapping of Epitopes for NOB Antibodies. We examined the ability of the 10 newly produced anti-HEV-LP monoclonal antibodies to inhibit the binding of HEV-LP to Huh7 cells (Fig. 3A). Two of the monoclonal antibodies, MAB1323 and MAB272, exhibited NOB of HEV-LP to Huh7 cells and recognized the P domain by immunoblotting using the GST (GST)-fused HEV capsid proteins (Fig. S4). However, further truncation of the C-terminal 28 or N-terminal 24 amino acids from the GST-fused P domain abrogated the binding with the antibodies, indicating that it is difficult to determine the epitopes of the antibodies in more detail using a series of truncated mutants of the P domain. A competitive enzyme-linked immunosorbent assay (ELISA) suggested that MAB1323, MAB272, and MAB161, but not MAB358, which was used as a detector in the binding assay, recognized the same or adjacent epitopes (Fig. S5). The P domains of rNV and feline calicivirus were suggested to be involved in the binding to the receptor molecules (34–36), and we therefore hypothesized that the P