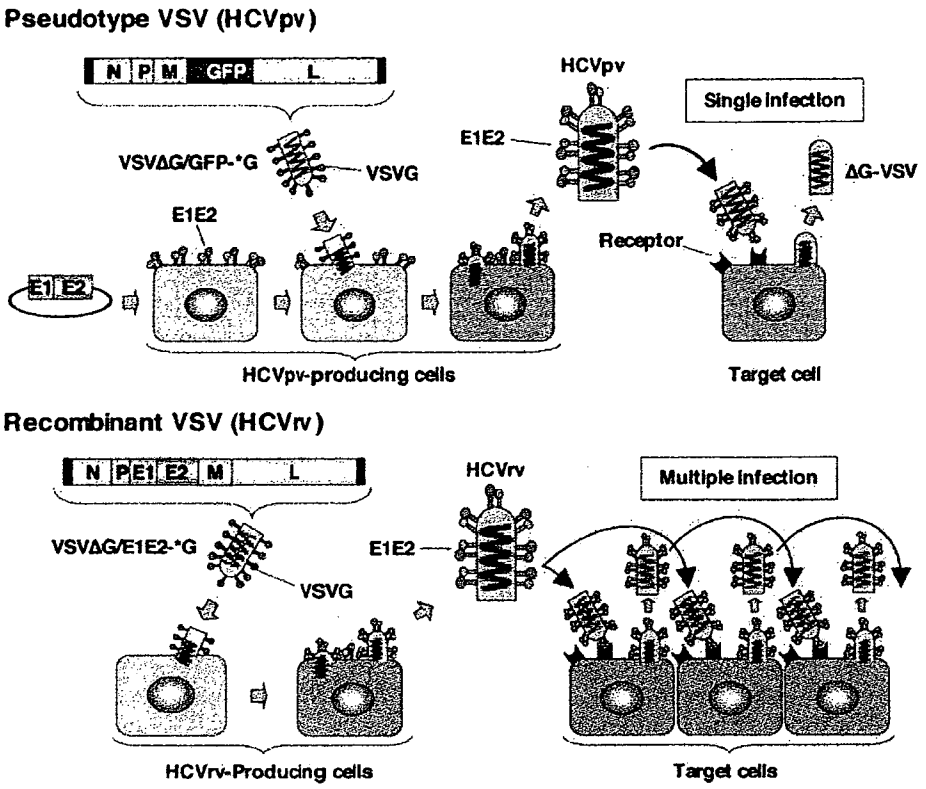

FIG. 1. Schematic representation of the production of HCVpv and HCVrv. Pseudotype VSV (HCVpv): producer cells (Huh7 or 293T) were transfected with an expression plasmid encoding the HCV E1 and E2 genes and then infected with a VSVG-complemented pseudotype virus (VSVΔG/GFP-*G). The HCVpv released from the producer cells infected target cells but was not able to produce infectious progeny virus. Recombinant VSV (HCVrv): various mammalian producer cells were inoculated with a VSVG-complemented recombinant virus (VSVΔG/E1E2-*G) encoding the HCV E1 and E2 genes instead of VSVG. HCVrv was capable of undergoing a fully productive infection generating infectious progeny virus that could be passaged into naïve cells.

titers of HCVrv were determined by a focus-forming assay as described below. To examine the effects of oligosaccharide modification of the E1 or E2 envelope proteins on the infectivity of the HCVpv and HCVrv, the cell lysates and the purified virions were digested with endoglycosidase H (Endo H) or peptide-*N*-glycosidase F (PNGase F) (Boehringer Mannheim, Mannheim, Germany), following a protocol provided by the manufacturer, and analyzed by immunoblotting. Pseudotype VSVs bearing VSVG (VSVpv) and MLV RD114 envelope protein (MLVpv) were produced in 293T cells transfected with pCAGVSVG and pFBASALF (provided by Miyazawa), respectively, and used as controls.

Immunofluorescence and focus-forming assay. The cells infected with HCVpv, HCVrv, VSV, or HCVcc were cultured at 30°C with 0.8% methylcellulose in 10% FBS DMEM for the indicated periods and fixed with 4% paraformaldehyde solution for 1 h. Cells were washed once with PBS, treated with 0.5% Triton X-100 for 20 min for permeabilization, and then incubated with mouse monoclonal antibody to VSV N (10G4) (HCVpv, HCVrv, and VSV) or rabbit polyclonal antibody to NS5A (22) (HCVcc) for 1 h. Then, the cells were visualized by staining with Alexa 488-conjugated anti-mouse immunoglobulin G (IgG) or anti-rabbit IgG (Molecular Probes, Eugene, OR) for the immunofluorescence assay. The nuclei were counterstained with Hoechst 33258 (Molecular Probes). For the focus-forming assay, cells were treated with secondary antibodies and stained by using a VECTASTAIN Elite ABC anti-mouse IgG kit with a VIP substrate (Vector Laboratories, Burlingame, CA), following a protocol provided by the manufacturer. The infectious titers of the viruses were expressed as focus-forming units.

Inhibition of HCVpv or HCVrv infection by treatment with antibodies against hCD81, E1 and E2, HCV patient sera, and siRNA. To determine the involvement of hCD81 in infection, Huh7 or HepCD81 cells were pretreated with 5 μg/ml of anti-hCD81 for 1 h at 37°C and inoculated with HCVpv or HCVrv. In addition, Huh7 cells on six-well plates were transfected with 80 nM of siRNAs targeted to hCD81 by using Nucleofector II (Amaxa GmbH, Cologne, Germany) according to the manufacturer's protocol. The hCD81 siRNAs (sc-35030) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). At 24 h posttransfection, cells were trypsinized, seeded at 8×10^3 cells/well into 96-well plates, and cultured for 48 h at 37°C. HCVpv or HCVrv was inoculated into the target cells, and infectivity was determined at 24 h postinfection. To characterize the infection with HCVpv and HCVrv, viruses were preincubated with 20 μg/ml of anti-E1 (AP21.010) (13) or anti-E2 (AP33) monoclonal antibodies, 1:50 diluted anti-E1 (R852) or anti-E2 (R646) polyclonal rabbit sera, and sera from chronic HCV patients or healthy donors for 1 h at 37°C and then inoculated into Huh7 cells. Informed consent was obtained from the patients and the donors. After 1 h of adsorption at 37°C, the cells were washed three times with DMEM containing 10% FBS, and infectivity was determined after 24 h of incubation at 37°C.

Effects of chemicals on HCVpv, HCVrv, and HCVcc infection. To determine the entry pathways of the viruses, Huh7 cells were preincubated with various concentrations of bafilomycin A_1 (Sigma) for 1 h at 37°C followed by infection with HCVpv, HCVrv, VSVpv, or MLVpv. The residual infectivity was determined as described above. *N*-(*n*-Nonyl) deoxynojirimycin (Nn-DNJ) and 1-deoxymannojirimycin hydrochloride (DMJ) were purchased from Toronto Research Chemicals Inc. (Downsview, ON, Canada). Nn-DNJ and DMJ were dissolved in ethanol and PBS, respectively, and diluted with medium before use. HCVcc was generated as previously described (47). Huh7 cells were inoculated with the viruses for 2 h at 37°C, replaced with medium containing either Nn-DNJ or DMJ, and cultured for 24 h (VSV), 72 h (HCVrv), or 96 h (HCVcc). The effects of Nn-DNJ or DMJ on the incorporation of the envelope proteins and generation of infectious particles were analyzed by immunoblotting and Coomassie staining. For the focus-forming assay, 0.8% methylcellulose in 10% FBS DMEM containing the reagents was overlaid on the cells. The infectious titers of VSV and HCVrv were determined by a focus-forming assay as described below. The infectious titers of HCVcc were evaluated by a quantitative core enzyme-linked immunosorbent assay as described previously (37).

RESULTS

Production and characterization of HCVrv. HCVrv was recovered from plasmids using established methods for the recovery of recombinant VSV in BHK cells. To ensure that infectious virus was produced, the recoveries were performed in cells transiently expressing VSV G protein. To determine if the HCV envelope proteins could mediate infection, the G-pseudotyped viruses were used to infect either Huh7 or 293T cells, and then the supernatants were titered on Huh7 cells. VSV lacking an envelope protein (VSVΔG) was used as a negative control. Infectivities of HCVrv generated in either 293T cells or Huh7 cells were dependent on the combination of incubation temperature and period (data not shown). The highest infectivity was constantly recovered in either cell line when cultured at 30°C for 48 h rather than when cultured at 37°C. Thus, HCVrv was prepared at 30°C for the remaining experiments. To determine whether the cell line used affected the generation of HCVrv, the infectivity in Huh7 cells and incorporation of HCV proteins into particles of HCVrv generated in various cell lines were examined (Fig. 2). HCVrv generated in Huh7 and 293T cells exhibited the highest infectivity in Huh7 cells, followed by Hep3B, PLC/PRF/5, and HepG2 cells. Significant infectivity was not observed for virus produced in HeLa, Vero, or BHK cells. Incorporation of the E1 and E2 proteins varied among the particles produced in the different cell lines. Although incorporation of E1 and E2 proteins into the particles was high in HCVrv generated in HepG2, BHK, and Huh7 cells, HCVrv produced in BHK cells exhibited the lowest infectivity to Huh7 cells. On the other hand, incorporation of the E1 and E2 proteins into HCVrv particles generated in 293T and Hep3B cells was low, whereas these viruses exhibited substantial infectivity to Huh7 cells. These results indicate that there is no clear correlation between the quantity of incorporation of HCV envelope proteins and the infectivity of HCVrv in Huh7 cells, although the producer cell type is important.

Characterization of HCVrv and HCVpv. To examine the properties of the HCV envelope proteins incorporated into the recombinant and pseudotype VSV particles, E1 and E2 proteins of the H77 strain (genotype 1a) expressed in 293T cells and incorporated into the viral particles were examined by immunoblotting with anti-E1 (BDI198) and anti-E2 (AP33) monoclonal antibodies (Fig. 3A). The E1 and E2 proteins of the cell lysates and virions of HCVpv or HCVrv were sensitive to both Endo H and PNGase F, suggesting that both HCVrv and HCVpv possess E1 and E2 proteins with high-mannose glycans, as reported for the E1 and E2 proteins of HCVpp (48). Next, to examine the infectivity of HCVrv and HCVpv to the target cells, viruses bearing HCV envelope proteins of genotypes 1a (H77 strain) and 1b (Con1 strain) were generated in 293T or Huh7 cells and inoculated into Huh7 cells. The infectivities in Huh7 cells of HCVrv carrying E1 and E2 proteins of the H77 or Con1 strains were 10- to 20-fold higher ($\sim 1 \times 10^6$ IU/ml) than those of HCVpv ($\sim 1 \times 10^5$ IU/ml) (Fig. 3B). HCVpv generated in 293T cells exhibited higher infectivity than that generated in Huh7 cells. No difference in the infectivities of HCVpv and HCVrv between the 1a and 1b genotypes was observed. To determine the relationship between the incorporation of E1 and E2 proteins into HCVrv

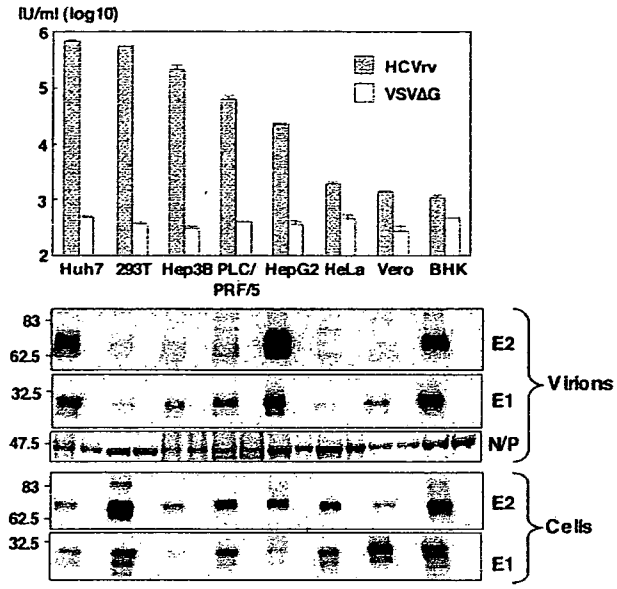

FIG. 2. Production and characterization of HCVrv. (Top) The infectivity of HCVrv of the H77 strain produced in the indicated cell lines at 30°C for 48 h was determined in Huh7 cells at 37°C for 24 h postinfection by counting VSV N-positive cells. The results shown are from three independent assays, with the error bars representing the standard deviations. (Bottom) Expression and incorporation of the HCV E1 and E2 proteins in cells and purified viral particles. HCV E1 or E2 proteins were detected by immunoblotting with anti-E1 or anti-E2 monoclonal antibodies. An envelope-less pseudotype virus, VSVΔG, was used as a negative control.

particles and their infectivities, the culture supernatants of 293T cells infected with HCVrv (H77 strain) were subjected to CsCl equilibrium gradient centrifugation, and each fraction was analyzed by immunoblotting and titration of infectivity in Huh7 cells (Fig. 3C). Immunoblot analyses revealed that incorporation of E1 and E2 proteins into HCVrv particles was detected in fractions 4 to 8 (Fig. 3C, top). These fractions exhibited the highest infectious titers (5×10^5 to 1×10^6 IU/ml), corresponding to buoyant densities of 1.2 to 1.3 g/ml (Fig. 3C, bottom).

Propagation of HCVrv. To examine the propagation of HCVrv in the target cells, Huh7 cells were infected with HCVrv at an MOI of 0.01 and incubated for up to 120 h. As a negative control, HCVpv was employed (Fig. 4A). A visible cytopathic effect was observed in Huh7 cells infected with HCVrv but not with HCVpv after 48 h incubation (data not shown). Immunofluorescence staining of Huh7 cells infected with HCVrv with antibody against VSV N revealed that VSV N protein was present from 24 h postinfection and had infected all cells at 120 h postinfection. In contrast, VSV N protein staining was decreased in cells infected with HCVpv at 120 h postinoculation. Focus formation of HCVrv in Huh7 cells was visualized by immunostaining under a methylcellulose overlay (Fig. 4B). Although the focus sizes of HCVrv were smaller than those of wild-type VSV, focus formation of HCVrv was clearly detected in a dose-dependent manner. In contrast, no focus formation was detected in cells infected with HCVpv. These results indicate that HCVrv is replication competent in

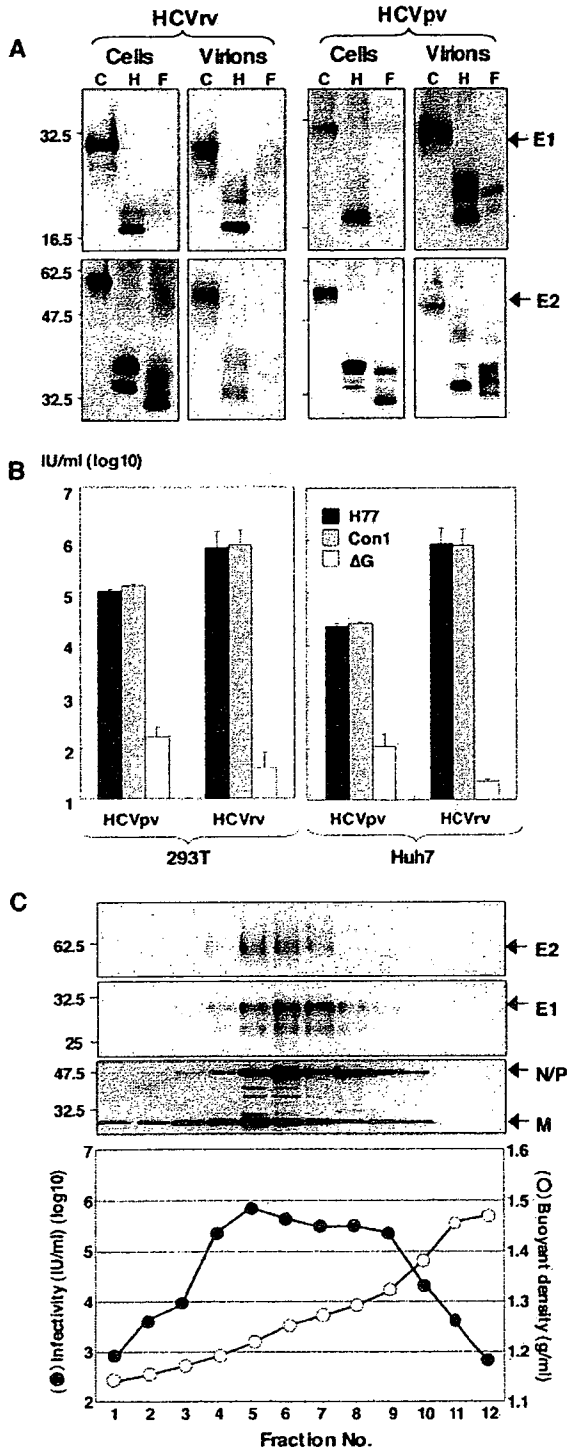

FIG. 3. Characterization of HCVrv and HCVpv. (A) The E1 and E2 proteins of the H77 strain expressed in 293T cells and incorporated into the particles of HCVrv and HCVpv were either untreated (C) or treated with endoglycosidase H (H) or peptide-*N*-glycosidase F (F). Following fractionation on sodium dodecyl sulfate-polyacrylamide gel gels, the glycoproteins were detected by immunoblotting with anti-E1 (BDI198) and anti-E2 (AP33) monoclonal antibodies. (B) The infectivities of HCVrv and HCVpv bearing HCV envelope proteins of genotypes 1a (H77 strain) and 1b (Con1 strain) generated in 293T or Huh7 cells were determined with Huh7 cells. The envelopeless VSV (ΔG) was used as a control. (C) (Top) CsCl gradient sedimentation of HCVrv produced in 293T cells. The supernatant was fractionated from the top of the gradient and analyzed by immunoblotting with anti-E2, anti-E1, and anti-VSV antibodies. (Bottom) The infectivity (filled circles) of each fraction was determined after the removal of CsCl with column purification. Fraction densities (open circles) are expressed in grams/milliliter.

Huh7 cells. To further determine the cell tropism for virus propagation, HCVrv was generated in various cell lines, and replication was assessed during incubation for up to 6 days (Fig. 4C, left). The growth kinetics of the wild-type VSV revealed an efficient replication of VSV in all the cell lines examined (Fig. 4C, right). Huh7 cells exhibited the highest susceptibility to propagation of HCVrv, followed by Hep3B cells, and no propagation was detected in the other cell lines. These results indicate that various human cell lines are capable of producing HCVrv that is infectious to Huh7 cells and that Huh7 cells are highly permissive to the propagation of HCVrv.

Involvement of hCD81 in the infection with HCVpv and HCVrv. Among the candidates for entry receptor of HCV, hCD81 was shown to be most essential for the infection with HCVpp (5, 23) and HCVcc (27, 56, 60). The infection of Huh7 cells with HCVpv and HCVrv was inhibited by anti-hCD81 antibody, whereas no inhibition of VSVpv infection was observed (Fig. 5A). Treatment with siRNA targeted to hCD81 induced a reduction of hCD81 expression on the surface of Huh7 cells (Fig. 5B), and the susceptibility of hCD81-knockdown cells to infection with HCVpv and HCVrv, but not to that with VSVpv, was clearly reduced (Fig. 5C). To further determine the involvement of hCD81 in the infectivity of HCVpv and HCVrv, hCD81-negative HepG2 cells stably expressing hCD81 (HepCD81) were established, and fluorescence-activated cell sorter (FACS) analysis revealed that expression of hCD81 on the cell surface was higher than that of Huh7 cells (Fig. 5D). Although HCVpv and HCVrv are not infectious in HepG2 cells, HepCD81 cells were permissive to both HCVpv and HCVrv infection, and pretreatment with the anti-hCD81 antibody inhibited the infection of HepCD81 cells with HCVpv and HCVrv (Fig. 5E). These results indicate that hCD81 plays a crucial role in infection with HCVpv and HCVrv, as it has been reported to play in infection with HCVpp and HCVcc.

Infectivity of HCVpv and HCVrv in various cell lines. To further examine the cell tropism of the viruses, HCVpv and HCVrv of the H77 and Con1 strains generated in 293T or Huh7 cells and HCVpp of the H77 strain generated in 293T cells were inoculated into various cell lines and primary Hc (Table 1). As expected, the control VSVΔG exhibited no infectivity in any of the cells examined (data not shown). The HCVpv and HCVrv derived from both genotypes were highly infectious in Huh7 cells, followed by HepCD81 and Hep3B cells, and weakly infectious in PLC/PRF/5, 293T, and Vero cells. No infectivity was detected in the other cell lines examined. The cell tropisms of the HCVpp were similar to those of HCVpv and HCVrv. Although the ectopic expression of hCD81 in Chinese hamster ovary cells (CHOCD81) did not confer susceptibility to HCVpv, HCVrv, or HCVpp infection,

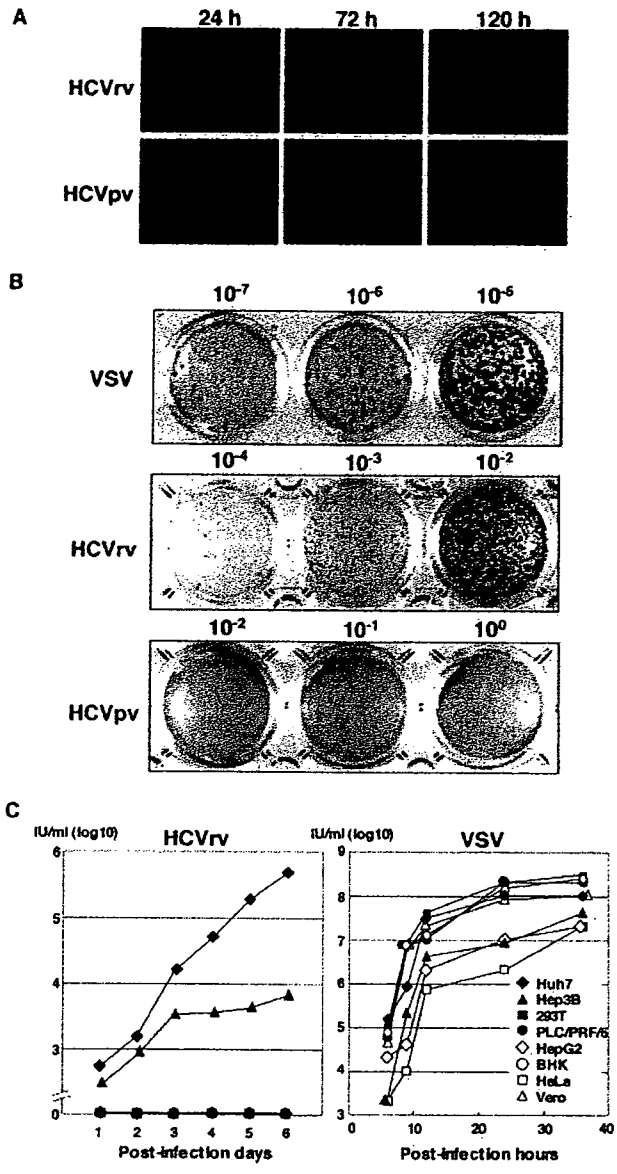

FIG. 4. Propagation of HCVrv. (A) Detection of viral proteins in Huh7 cells infected with HCVpv or HCVrv. Huh7 cells were infected with HCVpv or HCVrv at an MOI of 0.01. Twenty-four, 72, and 120 h after infection, cells were fixed and stained with monoclonal antibody to VSV N protein and Alexa 488-conjugated secondary antibody. Cell nuclei were stained by Hoechst 33258. Pictures were taken using a fluorescence microscope by double exposure of the same fields with filters for Alexa 488 or Hoechst 33258. (B) Focus formation of HCVpv, HCVrv, or VSV in Huh7 cells. Huh7 cells were infected with serial 10-fold dilutions of HCVpv, HCVrv, or VSV and incubated at 30°C for 72 h for HCVpv and HCVrv or 24 h for VSV in a culture medium containing 0.8% methylcellulose. Foci of infected cells were detected by immunohistochemical staining. (C) Kinetics of HCVrv (left) and VSV (right) propagation in various cell lines. HCVrv and VSV generated in Huh7 cells were used to infect cells at an MOI of 0.01. The culture supernatant was collected at the indicated time points and titrated by a focus-formation assay. Infectious titers are expressed in IU/milliliter.

the expression of hCD81 in HepG2 cells (HepCD81) (Fig. 5A and D) rendered them permissive to infection with all of the viruses. Furthermore, Hc were not susceptible to the infection with HCVpv, HCVrv, or HCVpp, despite the expression of hCD81. These results suggest that expression of hCD81 is essential for the infection with HCVpv and HCVrv, as reported for infection with HCVpp and HCVcc, but conditions with a lack of hCD81 are insufficient for the infection with HCVpv, HCVrv, and HCVpp.

Neutralization of HCVpv and HCVrv infection by antibodies to HCV envelope proteins and sera of HCV patients. It has been reported that HCVpp can be neutralized by several well-characterized E2-specific monoclonal and polyclonal antibodies (5, 23, 49). The neutralization activity of anti-E1 (AP21.010) and anti-E2 (AP33) monoclonal antibodies (49) and anti-E1 (R852) and anti-E2 (R646) rabbit polyclonal antibodies raised against the E1 and E2 proteins of the H77 strain on the infection with HCVpv and HCVrv was determined (Fig. 6A). The infections with both HCVpv and HCVrv bearing E1 and E2 proteins of the H77 strain were clearly inhibited by anti-E2 (AP33) antibody or anti-E2 (R646) rabbit serum, consistent with a previous report on the effect of these antibodies on HCVpp infection (49), whereas no neutralization by AP21.010 and R852 antibodies was observed. The infections with HCVpv and HCVrv bearing E1 and E2 proteins of the Con1 strain were also inhibited by AP33 and R646 antibodies (data not shown), suggesting that the infectivity of HCVpv and HCVrv was cross-neutralized by anti-E2 antibody, as reported for HCVpp (49). These results indicate that the E2 protein plays a crucial role in the infectivity of both HCVpv and HCVrv. Although the addition of naïve human sera (HD) inhibited infection with VSVpv, infection with HCVpv or HCVrv was clearly enhanced, as reported for HCVpp infection of Huh7 cells (28, 42). To assess the neutralization ability of these antibodies in patients, HCVpv and HCVrv were incubated with a 2% concentration of the sera of chronic HCV patients infected with genotype 1b HCV (Fig. 6B). All of the sera of patients of genotype 1b showed high levels of neutralization activity against infection with HCVpv and HCVrv bearing envelope proteins of genotype 1a, whereas they had no effect on the infectivity of VSVpv, in contrast to the inhibition achieved by the naïve sera. These results indicate that HCV patients elicit high levels of antibodies that are likely to cross-neutralize the infectivity of HCVpv and HCVrv.

Inhibition of HCVpv and HCVrv infection by bafilomycin A_1. Enveloped viruses enter target cells through two different pathways: one is a pH-independent direct fusion at the plasma membrane, and the other is a pH-dependent receptor-mediated endocytosis (58). Previous studies have revealed that both HCVpp and HCVcc were sensitive to the inhibitors of vacuolar acidification, such as ammonium chloride, concanamycin A, or bafilomycin A_1, suggesting that these viruses enter via a pH-dependent endocytosis into target cells (23, 61). To determine the entry pathway of HCVpv and HCVrv, Huh7 cells were pretreated with various concentrations of bafilomycin A_1, and then the cells were inoculated with HCVpv, HCVrv, VSVpv, and MLVpv (Fig. 7). As expected, the treatment did not affect the infection with MLVpv bearing an envelope protein of MLV that enters cells via a pH-independent pathway. In contrast, infection with VSVpv bearing the G protein of VSV,

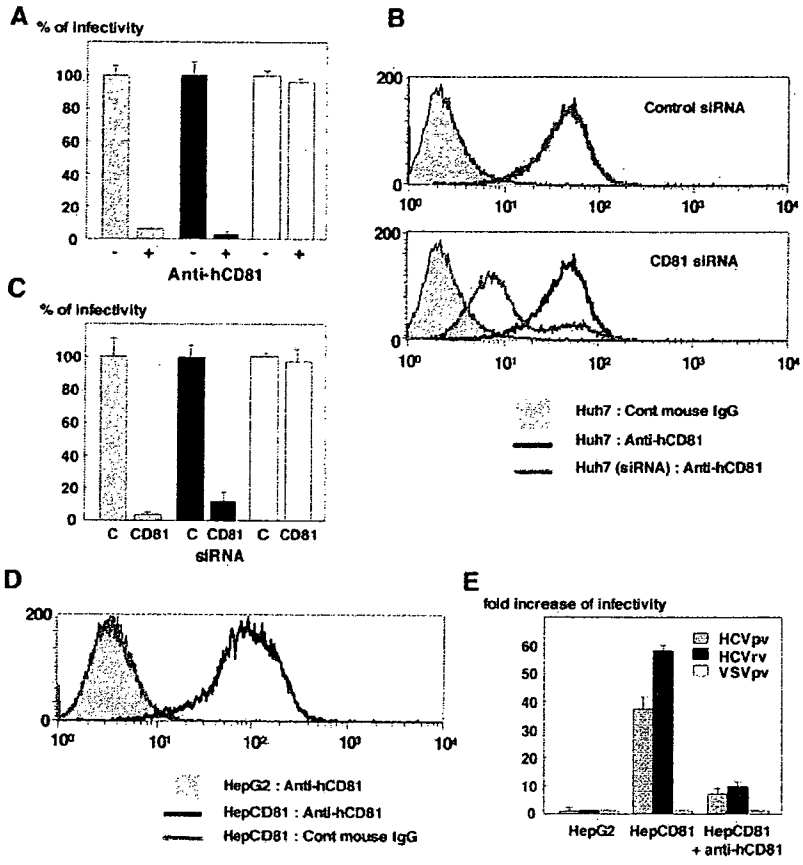

FIG. 5. Involvement of hCD81 in the infection of HCVpv and HCVrv. (A) Effect of anti-hCD81 antibody on the infectivity of HCVpv (gray-filled bars), HCVrv (black-filled bars), or VSVpv (open bars) in Huh7 cells. (B) Cell surface expression of hCD81 on Huh7 cells transfected with siRNA targeted to hCD81 or control siRNA was examined by FACS analysis after staining with anti-hCD81 antibody. (C) Effect of knockdown of hCD81 in Huh7 cells by siRNA targeted to hCD81 on the infection of HCVpv, HCVrv, or VSVpv. (D) Cell surface expression of hCD81 on HepG2 and HepCD81 cells was examined by FACS analysis after staining with anti-hCD81 antibody. (E) Infectivity of HCVpv, HCVrv, or VSVpv to HepG2 or HepCD81 cells and the effect of anti-hCD81 antibody on the infection of the viruses to HepCD81 cells. The results shown are from three independent assays, with the error bars representing the standard deviations.

which enters cells through pH-dependent endocytosis, was inhibited by the treatment with bafilomycin A_1 in a dose-dependent manner. Infection with HCVpv and HCVrv was also clearly inhibited by the treatment with bafilomycin A_1 in a dose-dependent manner, as with VSVpv. This suggests that low pH exposure is essential for the entry of HCVpv and HCVrv.

Effects of ER α-glucosidase inhibitors on HCVrv infection. Previous studies have shown that deoxynojirimycin (DNJ) and Nn-DNJ, a long-alkyl-chain iminosugar derivative of DNJ, inhibit the infection of flaviviruses such as Japanese encephalitis virus (JEV) and dengue virus in a dose-dependent manner (15, 64). Although the effects of glycosylation inhibitors on the folding and assembly of HCV envelope proteins in the N-glycosylation steps and the binding properties of HCV-LP produced in insect cells have been reported (11, 12), glycobiological analyses of HCV envelope proteins involved in virus infectivity have not been reported yet. To determine the effects of the inhibitor of Golgi mannosidase (DMJ) and of ER α-glucosidase (Nn-DNJ) on the infectivity of HCVrv, Huh7 cells were treated with these inhibitors. Treatment of Huh7 cells with Nn-DNJ but not with DMJ reduced the infectivity of HCVrv in a dose-dependent manner, and this reduction was more efficient than that in the infectivity of VSV (Fig. 8A, top). Although immunoblotting and Coomassie staining of the particles revealed that incorporation of the envelope proteins and generation of HCVrv and VSV particles recovered from cells treated with 100 μM of Nn-DNJ were severely impaired by the cytotoxic effects of Nn-DNJ (Fig. 8A, bottom left), treatment with 10 μM of Nn-DNJ selectively reduced the infectivity of HCVrv but not of VSV without any cytotoxic effect (Fig. 8A, top left). In contrast, Huh7 cells treated with more than 0.5 mM of DMJ exhibited a slight reduction of molecular sizes of E1 or VSVG proteins incorporated into the particles (Fig. 8A, bottom right); no effect on the incorporation of the envelope proteins into the viral particles and the infectivity was observed (Fig. 8A, top right). Next, we assessed the effects of the inhibitors on the propagation of the viruses. Focus formation of HCVrv was also inhibited by the treatment with Nn-DNJ but not with DMJ (Fig. 8B). To further confirm the effect of modification of the envelope glycoproteins by ER α-glucosidase on the infectivity of HCV, Huh7.5.1 cells were treated with the

TABLE 1. Infectivity of HCVpv, HCVrv, or HCVpp in various cells

Target cells	Cell surface expression of[a]:		Virus, producer cells, and strain (genotype)[b]								HCVpp virus produced in 293T cells and of strain H77 (genotype 1a)[b]
			HCVpv				HCVrv				
			293T		Huh7		293T		Huh7		
	hCD81	SR-BI	H77 (1a)	Con1 (1b)	H77 (1a)	Con1 (1b)	H77 (1a)	Con1 (1b)	H77 (1a)	Con1 (1b)	
Huh7	++	++	+++	+++	++	++	+++	+++	+++	+++	+++
HepG2	−	++	−	−	−	−	−	−	−	−	−
HepCD81	++	++	++	++	+	+	+++	+++	+++	+++	++
Hep3B	++	+	++	++	+	+	+++	+++	+++	+++	++
PLC/PRF/5	++	+	+	+	−	−	+	+	+	+	−
FLC4	−	++	−	−	−	−	−	−	−	−	−
Hc	++	−	−	−	−	−	−	−	−	−	−
HeLa	+	+	−	−	−	−	−	−	−	−	−
293T	++	+	+	+	−	−	+	+	+	+	−
Vero	−	−	+	+	−	−	+	+	+	+	−
BHK	−	−	−	−	−	−	−	−	−	−	−
CHOK1	−	−	−	−	−	−	−	−	−	−	−
CHOCD81	++	−	−	−	−	−	−	−	−	−	−

[a] Cell surface expression of receptor candidates was examined by FACS analyses with specific antibodies. Mean fluorescence intensity shifts of less than 1, between 1 and 2, and between 2 and 3 are indicated as −, +, and ++, respectively.

[b] Infectious titers higher than 5×10^4 IU/ml, between 5×10^3 and 5×10^4 IU/ml, between 5×10^2 and 5×10^3 IU/ml, and lower than 5×10^2 IU/ml are indicated as +++, ++, +, and −, respectively. The results were derived from at least three independent experiments, and the standard deviations did not exceed 30% of the mean values.

inhibitors, and infectivity of HCVcc was determined (Fig. 8C, top). Treatment with Nn-DNJ clearly inhibited the infection with HCVcc in a dose-dependent manner, as it did the infection with HCVrv. Focus formation of HCVcc was also inhibited by the treatment of Huh7.5.1 cells with Nn-DNJ (Fig. 8C, bottom). These results indicate that modification of the glycans of HCV E1 and E2 proteins in the ER by α-glucosidase rather than that in the Golgi is crucial for the infectivity of both HCVrv and HCVcc.

DISCUSSION

In general, enveloped viruses attach to host target cells and enter into cells through the interaction between viral envelope proteins and cell surface receptors and coreceptors. Due to the lack of a robust cell culture system to support the replication of various HCV genotypes, surrogate systems have been developed to examine the mechanisms of HCV infection. Although in vitro binding assays have identified several candidate receptors for HCV (4), the final determination of a true entry receptor or coreceptor capable of internalizing HCV particles has to be made by an infection assay. Toward this end, pseudotype virus systems based on VSV (27, 39) and retroviruses (5, 23) have been established. Both VSV and retroviruses normally bud from the plasma membrane, and therefore foreign envelope proteins expressed on the cell surface have been believed to incorporate into the pseudotype particles. HCV E1 and E2 proteins form heterodimers that have static ER retention signals in their C-terminal transmembrane region (17) and pulse-chase experiments and endoglycanase treatment of the intracellular forms of the proteins or those incorporated into the HCVpp have revealed that only a small fraction of the HCV envelope proteins are translocated to the plasma membrane and modified to the complex-type glycans (48). In addition, it was demonstrated that recruitment of the foreign envelope proteins by MLV and the lentivirus core protein does not occur at the cell surface but takes place intracellularly in the endosomal pathway (55, 56). Production of pseudotype VSVs bearing unmodified envelope glycoproteins of bunyaviruses has also been reported, in spite of the static retention of the envelope glycoproteins in the intracellular compartment and the lack of translocation into the plasma membrane (46). Therefore, cell surface expression of HCV envelope glycoproteins may not necessarily be a prerequisite for generation of pseudotype particles based on VSV or retroviruses.

Recombinant VSV encoding foreign viral envelope proteins in place of the G protein has been shown to be a powerful tool for the investigation of viral infection and the development of vaccines for diseases caused by infection with viruses such as influenza virus, human immunodeficiency virus, respiratory syncytial virus, human papillomavirus, and filoviruses (20, 31). Although recombinant VSV encoding HCV envelope proteins has been generated as a surrogate model for HCV infection and a vaccine vector (9, 35), recombinant VSV generated in rodent cells possessing the chimeric E1 and/or E2 proteins has been shown to be noninfectious in a human hepatoma cell line that is susceptible to HCVpp infection (9). In this study, we successfully generated infectious recombinant and pseudotype VSVs incorporating unmodified E1 and E2 proteins in hepatic and nonhepatic human cell lines. The previously observed lack of infectivity of the recombinant VSV carrying the chimeric HCV envelope proteins might be attributable to the production of viral particles in rodent (BHK) cells (9), because in this study the HCVrv generated in BHK cells exhibited no infectivity in the target cells in spite of a sufficient amount of incorporation of the HCV envelope proteins. These results suggest that posttranslational modification or host factor(s) specific to human cells might be involved in the endowment of infectivity of recombinant VSVs. Furthermore, HCVrv can be produced in various cell lines upon infection with the G-complemented particles, which are known to exhibit infectivity in several cell lines, in contrast to the pseudotype viruses, infec-

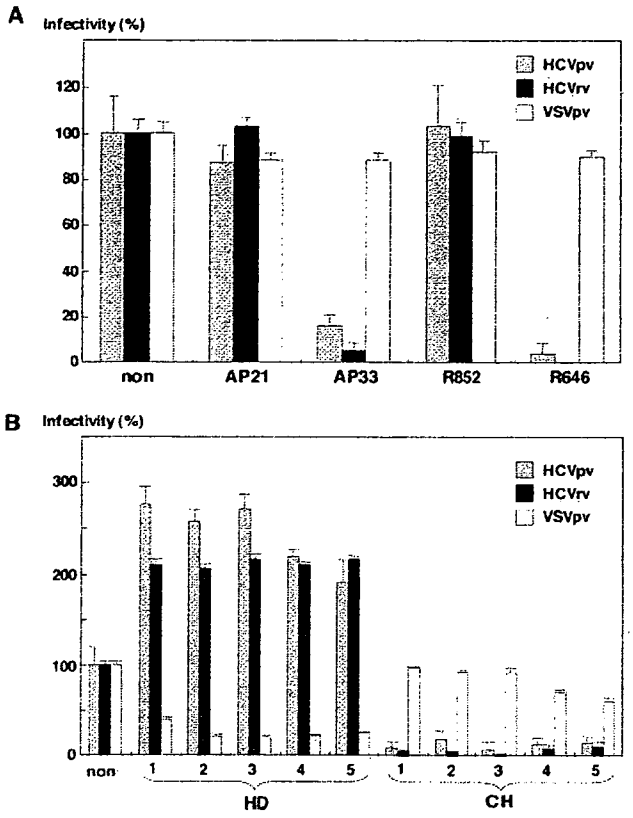

FIG. 6. Neutralization of HCVpv and HCVrv infection by antibodies to HCV envelope proteins and sera of HCV patients. (A) Effect of anti-E1 (AP21.010) and anti-E2 (AP33) monoclonal antibodies and anti-E1 (R852) and anti-E2 (R646) rabbit sera on the infectivity of HCVpv (gray-filled bars), HCVrv (black-filled bars), or VSVpv (open bars) to Huh7 cells. The viruses were preincubated for 1 h at room temperature with the antibodies before infection of Huh7 cells. (B) Effects of human sera from healthy donors and HCV patients on the infection of HCVpv, HCVrv, or VSVpv. The viruses were preincubated for 1 h at room temperature with five different healthy human sera (HD) or chronic HCV patient sera (CH) diluted 1:50 before infection of Huh7 cells.

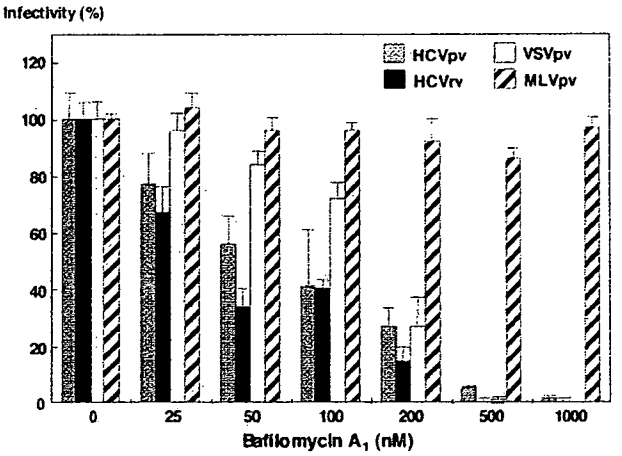

FIG. 7. Inhibition of HCVpv and HCVrv infection by bafilomycin A_1. HCVpv (gray-filled bars), HCVrv (black-filled bars), VSVpv (open bars), or MLVpv (striped bars) were inoculated to Huh7 cells after treatment with various concentrations of bafilomycin A_1. The results shown are from three independent assays, with the error bars representing the standard deviations.

tious particles of which were recovered only in cells exhibiting a high competency of transfection, such as 293T cells. Therefore, generation of HCVrv in various human cells, including nonhepatic cells such as B cells, might be useful for investigating the cell-specific modification and/or factors determining the cell tropism of HCV infection.

Overwhelming evidence that hCD81 facilitates the entry of HCV into Hc via interaction with the E2 protein has been accumulated not only by surrogate models, such as purified E2 proteins, HCV-LP, and HCVpp, but also by authentic HCV particles and HCVcc of genotype 2a (4). In this study, both HCVpv and HCVrv were shown to be infectious in Huh7 cells, and this infectivity was shown to be mediated through the interaction with hCD81. Although overexpression of hCD81 in HepG2 cells which lack endogenous expression of hCD81 renders them susceptible to infection by surrogate viruses, primary human Hc and HeLa cells expressing hCD81 and the rodent CHO cells stably expressing hCD81 (CHOCD81 cells) were resistant to infection by HCVrv and HCVpv (Table 1) (5, 14, 67), suggesting that hCD81 is one of the important factors for HCV entry but is not sufficient for infectivity of HCV in target cells. Recently, it was shown that participation of hCD81 in the infection of HCVpp or HCVcc bearing HCV envelope proteins isolated during chronic HCV infection was reduced, suggesting that the affinity of HCV envelope proteins to hCD81 was reduced and HCV utilizes receptors other than hCD81 (62, 69). HCVrv is useful for studies of the generation of various genotypes of escape variants under pressure of neutralization antibody or antagonist against HCV receptor candidates. Further studies of the functional relevance of hCD81 and other receptor candidates in the entry steps of HCV, such as binding, endocytosis, and membrane fusion, are needed.

Bafilomycin A_1, an H^+-ATPase inhibitor, was shown to reduce the infectivities of both HCVpv and HCVrv in a dose-dependent manner, as it did for the infectivities of both HCVpp and HCVcc (6, 23, 29, 61), suggesting that these viruses require low-pH-induced conformational changes of the envelope proteins upon entry. Furthermore, as with HCVcc (40, 61), preexposure of HCVpv and HCVrv to acidic pH did not reduce their infectivity (data not shown), indicating that additional factors are required for the internalization of the viruses. Recently, entry of HCVpp was shown to depend on the clathrin-mediated endocytosis through the knockdown of clathrin heavy chain by siRNA or chlorpromazine (8, 40), and dominant-negative mutants of Rab5 or Rab7, which are involved in the transport of clathrin-coated vesicles, revealed that entry of HCVpp requires delivery to early but not to late endosomes (40). N-linked glycosylation processing events in the ER are important for the secretion of several enveloped viruses. ER α-glucosidase I and II are involved in the trimming of terminal glucose on the core oligosaccharides, and the resulting monoglucosylated glycoproteins are able to bind to the ER chaperones calnexin (CNX) and/or calreticulin (CRT). ER α-glucosidase inhibitors, DNJ or castanospermine, which block

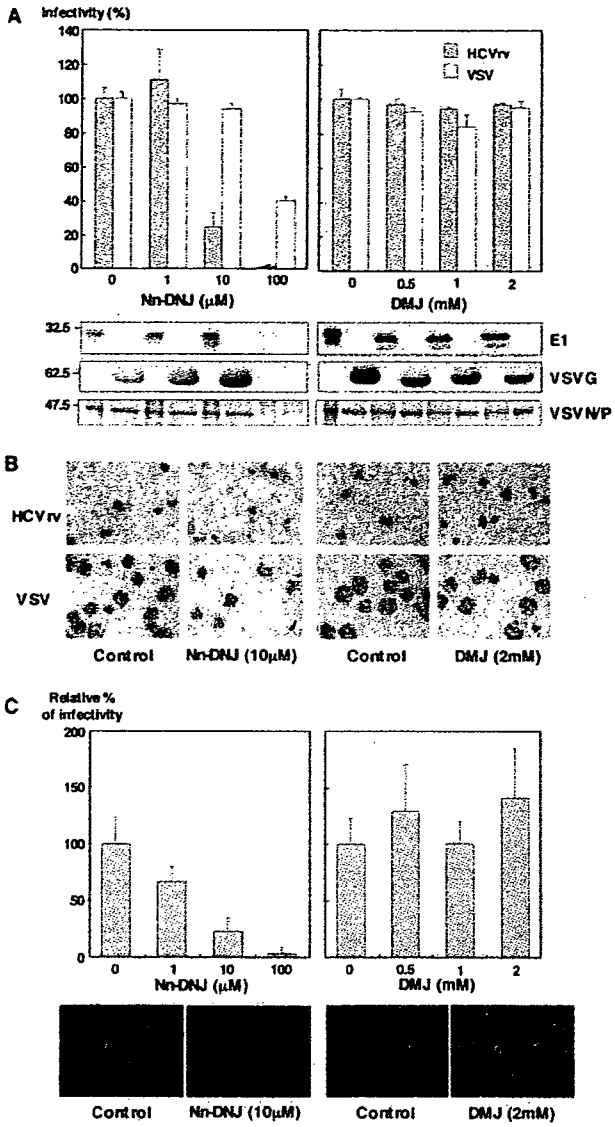

FIG. 8. Effects of ER α-glucosidase inhibitors on the infection with HCVrv and HCVcc. (A) (Top) Production of HCVrv and VSV in the presence of Nn-DNJ (left) or DMJ (right). Huh7 cells infected with HCVrv and VSV at MOIs of 0.1 and 0.01, respectively, were treated with various concentrations of Nn-DNJ or DMJ. Seventy-two hours (HCVrv) or 24 h (VSV) postinfection, culture supernatants were collected and titrated on Huh7 cells by a focus-forming assay. The results shown are from three independent assays, with the error bars representing the standard deviations. (Bottom) Purified viruses generated in Huh7 cells treated with Nn-DNJ or DMJ were analyzed by immunoblotting with anti-E1 (BDI198) and anti-VSVG (ab34774) or Coomassie staining. (B) Focus formation of HCVrv and VSV in the presence of Nn-DNJ or DMJ. Huh7 cells were infected with HCVrv or VSV, treated with Nn-DNJ (10 μM) or DMJ (2 mM) prior to an overlay of culture media containing 0.8% of methylcellulose, and stained with an anti-VSV N antibody after fixation at 72 h (HCVrv) and 24 h (VSV) postinfection. (C) (Top) Production of HCVcc in the presence of Nn-DNJ (left) or DMJ (right). Huh7.5.1 cells infected with HCVcc at an MOI of 0.01 were treated with various concentrations of Nn-DNJ or DMJ. Culture supernatants were collected and titrated by a quantitative core enzyme-linked immunosorbent assay at 96 h postinfection. (Bottom) Immunofluorescence assay of HCVcc infection in the presence of Nn-DNJ or DMJ. Huh7.5.1 cells were infected with HCVcc at an MOI of 0.01, treated with 10 μM of Nn-DNJ or 2 mM of DMJ prior to an overlay of culture media containing 0.8% of methylcellulose, and stained with an anti-NS5A antibody and Alexa 488-conjugated secondary antibody after fixation at 96 h postinfection. Cell nuclei were stained by Hoechst 33258. Pictures were taken using a fluorescence microscope by double exposure of the same fields with filters for Alexa 488 or Hoechst 33258.

the trimming step of N-linked glycosylation, have been shown to prevent the interaction of CNX and/or CRT with the folding glycoproteins, and the production of many enveloped viruses is inhibited by these inhibitors (41). In this study, we found that infection with both HCVrv and HCVcc was inhibited in a dose-dependent manner by treatment with Nn-DNJ, which is an N-alkylated derivative of DNJ exhibiting a stronger effect than DNJ. HCV E1 and E2 proteins were shown to interact with CNX and CRT, and these interactions were inhibited by the treatment with ER α-glucosidase inhibitors (12). One possible function of the HCV p7 protein, the formation of ion channels, has also been shown to be inhibited by the treatment with long-alkyl-chain iminosugar derivatives (50). Recently, it was reported that HCV-LPs produced in the presence of ER α-glucosidase inhibitors incorporated unprocessed, triglucosylated N-glycans and misfolded E1 and E2 proteins and lost their ability to bind hepatoma cell lines (11). Our results demonstrate that the modification of E1 and E2 proteins in the glycosylation steps in the ER is required to confer infectivity to HCVrv and HCVcc. The presence of E1 and E2 proteins on the surrogate viruses and HCVcc possessing high-mannose glycans indicate that these viruses are not released through the *trans*-Golgi network. In the case of West Nile virus, mature particles propagated in mammalian cells possess complex types of carbohydrates, in contrast to those generated in insect cells, which have high-mannose glycans (16). We still do not know the exact nature of modifications of the mature envelope proteins on authentic HCV particles. Further studies of the relationship between the modification of HCV envelope proteins and their infectivity are needed to clarify the life cycle of HCV. The neutralizing activity of antibodies against HCV have been assessed in the past using HCVpv (10, 43), HCVpp (3, 33, 42), and HCVcc (63, 65), as well as by the inhibition of binding of purified E2 protein to hCD81 (24, 53) and of HCV-LP to target cells (59). Sera from patients chronically infected with HCV and experimentally infected chimpanzees were shown to specifically neutralize HCVpp infection (3, 33, 42). In the present study, sera from patients infected with genotype 1b of HCV and anti-E2 monoclonal antibodies exhibited high levels of neutralization activity against infection with both HCVpv and HCVrv bearing HCV envelope proteins of genotypes 1a and 1b. One of the characteristics of HCV infection is the establishment of a persistent infection. Therefore, the high prevalence of neutralizing antibodies to the surrogate viruses and HCVcc suggests that HCV particles exhibiting similar phenotypes to surrogate viruses and HCVcc would be easily eliminated by neutralizing antibodies and thus not be able to participate in the establishment of a persistent infection. Recently, it was reported that HCV escapes from neutralizing antibody and T-cell responses by the continuous generation of escape

variants during chronic infection (51, 62). However, it was demonstrated that viral clearance in acute HCV infection was not correlated with the presence of neutralizing antibodies against HCVpp (33, 42), and 75% of HCVpp bearing HCV envelope proteins of various genotypes are not infectious (29). Therefore, it is reasonable to speculate that HCV particles exhibiting characteristics similar to those of the surrogate viruses are produced in large numbers and act as decoys in HCV patients, eliciting strong neutralizing antibodies against the viruses, and that a small portion of HCV particles exhibiting characteristics different from those of the surrogate viruses may participate in the establishment of persistent infection by escaping from the host immune surveillance system. The authenticity of the surrogate virus systems for the study of HCV infection remains controversial, and further studies are needed to clarify their profiles.

In conclusion, we generated replication-incompetent HCVpv and replication-competent HCVrv possessing HCV envelope proteins as novel surrogate models for the study of HCV. HCVpv and HCVrv were shown to have infection mechanisms similar to those of HCVpp and HCVcc. HCVrv has the following advantages compared to HCVcc: (i) infectious particles bearing HCV envelope proteins of various genotypes are capable of generating in various cell lines or primary cells, in contrast to the strict restriction of generating the infectious HCVcc in the Huh7-derived cell lines; (ii) isolation of escape mutants carrying mutations in the envelope proteins under various pressures may be easily obtained due to the higher replication efficiency than that of HCVcc; and (iii) in vivo investigation of the HCV envelope proteins for entry using humanized mice with human Hc and for immunogenicity for a future vaccine development are possible. Therefore, replication-competent HCVrv established in this study may provide valuable tools not only for understanding the entry mechanisms of HCV in a manner that is cell type and species dependent but also for developing novel therapeutics and vaccines.

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Oligomerization of Hepatitis C Virus Core Protein Is Crucial for Interaction with the Cytoplasmic Domain of E1 Envelope Protein[▽]

Kousuke Nakai,[1] Toru Okamoto,[1] Tomomi Kimura-Someya,[2] Koji Ishii,[2] Chang Kweng Lim,[1] Hideki Tani,[1] Eiko Matsuo,[1] Takayuki Abe,[1] Yoshio Mori,[1] Tetsuro Suzuki,[2] Tatsuo Miyamura,[2] Jack H. Nunberg,[3] Kohji Moriishi,[1] and Yoshiharu Matsuura[1]*

Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka,[1] Department of Virology II, National Institute of Infectious Diseases, Tokyo,[2] Japan, and Montana Biotechnology Center, The University of Montana, Missoula, Montana 59812[3]

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Hepatitis C virus (HCV) contains two membrane-associated envelope glycoproteins, E1 and E2, which assemble as a heterodimer in the endoplasmic reticulum (ER). In this study, predictive algorithms and genetic analyses of deletion mutants and glycosylation site variants of the E1 glycoprotein were used to suggest that the glycoprotein can adopt two topologies in the ER membrane: the conventional type I membrane topology and a polytopic topology in which the protein spans the ER membrane twice with an intervening cytoplasmic loop (amino acid residues 288 to 360). We also demonstrate that the E1 glycoprotein is able to associate with the HCV core protein, but only upon oligomerization of the core protein in the presence of tRNA to form capsid-like structures. Yeast two-hybrid and immunoprecipitation analyses reveal that oligomerization of the core protein is promoted by amino acid residues 72 to 91 in the core. Furthermore, the association between the E1 glycoprotein and the assembled core can be recapitulated using a fusion protein containing the putative cytoplasmic loop of the E1 glycoprotein. This fusion protein is also able to compete with the intact E1 glycoprotein for binding to the core. Mutagenesis of the cytoplasmic loop of E1 was used to define a region of four amino acids (residues 312 to 315) that is important for interaction with the assembled HCV core. Taken together, our studies suggest that interaction between the self-oligomerized HCV core and the E1 glycoprotein is mediated through the cytoplasmic loop present in a polytopic form of the E1 glycoprotein.

Hepatitis C virus (HCV) is the causative agent of chronic hepatitis C, leading to steatosis, cirrhosis, and hepatocellular carcinoma. It is estimated that over 170 million people are infected with HCV worldwide (5, 18, 37). HCV is an enveloped single-stranded plus-sense RNA virus in the *Hepacivirus* genus of the family *Flaviviridae*, which also includes the flaviviruses and pestiviruses (36). The genome of HCV encodes a polyprotein of approximately 3,000 amino acids which is cotranslationally and posttranslationally processed to generate at least 10 viral proteins (12). The structural proteins, the core and E1 and E2 envelope glycoproteins, are encoded in the N-terminal portion of the polyprotein, and the nonstructural proteins, thought to be required for replication of the viral genome, are encoded in the C-terminal region (11). The core protein, which interacts with viral RNA (47) to form the nucleocapsid, is liberated from the N terminus of the polyprotein by signal peptidase cleavage in the downstream E1 protein (at position 191), and the C-terminal transmembrane region of the core protein (residue 164 to 191) is further cleaved at residues 177 or 179 by the signal peptide peptidase (16, 43). The remaining hydrophobic region of the core protein (domain II; residues 119 to 174) has been shown to affect the efficiency of signal peptide peptidase cleavage and the intracellular localization of core protein (14, 44). Although the C-terminal transmembrane region of core protein and E1 were reported to interact with each other within the intramembrane space (25), the central hydrophobic region from residues 119 to 152 within domain II was also suggested to be responsible for the interaction between core and E1 (27).

Recently, in vitro replication of a JFH1 clone of HCV genotype 2a derived from a patient with fulminant hepatitis C was reported in a cell line that had been cured of its HCV replicon by treatment with interferon (23, 50, 51). However, this reverse genetics system is limited to the JFH-1 clone of genotype 2a and specific cell lines. Robust and reliable in vitro replication of other major genotypes of HCV such as genotypes 1a and 1b has yet to be developed. So far, biological functions of HCV envelope proteins have been characterized by using recombinant envelope proteins expressed in vitro, HCV-like particles produced in insect cells, and the pseudotyped virions based on vesicular stomatitis virus and retroviruses (8). The HCV polyprotein precursor must be specifically threaded through the membrane of the endoplasmic reticulum (ER) to undergo maturation to form the mature envelope glycoproteins (7). In the polyprotein, the C-terminal regions of E1 and E2 each contain a membrane-spanning domain as well as the hydrophobic signal peptide of the downstream viral protein (E2 and p7, respectively). These domains form hairpin structures that pass through the membrane twice, to allow processing by signal peptidase in the ER lumen. Upon signal peptidase cleavage, the C termini are thought to translocate into the cytoplasm to generate the type I membrane topology of the mature glycoproteins. The mature E1 and E2 glycoproteins

* Corresponding author. Mailing address: Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, 3-1, Yamadaoka, Suita, Osaka 565-0871, Japan. Phone: 81-6-6879-8340. Fax: 81-6-6879-8269. E-mail: matsuura@biken.osaka-u.ac.jp.

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remain noncovalently associated, interacting in part through their C-terminal transmembrane domains, which also mediate retention of the E1-E2 complex in the ER. Based on this model of membrane topology, the HCV envelope glycoproteins possess little or no cytoplasmic region. However, a physical association between E1 and the cytosolic core protein has been reported (25, 27), suggesting that the E1 glycoprotein is able to expose a cytoplasmic domain of sufficient length to interact with the core. In addition, the presence of the core protein has been shown to affect the folding of E1 (32).

We have previously suggested that the E1 glycoprotein may adopt a polytopic (double membrane-spanning) topology that coexists with the dominant type I form (35). In this study, we provide genetic evidence for a polytopic form of the E1 glycoprotein and for exposure of a centrally located cytoplasmic domain. Furthermore, we show that the cytoplasmic region of the polytopic form of E1 is required for interaction with amino acid residues 72 to 91 of the core protein.

MATERIALS AND METHODS

Cell culture. 293T cells were maintained in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) containing 2 mM L-glutamine, penicillin, and streptomycin and supplemented with 10% fetal bovine serum.

Plasmids. A cDNA of E1 glycoprotein was amplified from HCV type 1b strain J1 (1) by PCR using *Pfu* Turbo DNA polymerase (Stratagene, La Jolla, CA) and inserted between NheI and BamHI sites of pJW4303, which contains the signal sequence of tissue plasminogen activator and the bovine growth hormone polyadenylation signal (a kind gift from J. M. Mullins), to generate pJW383. For the deletion analysis, the plasmids pJW360 and pJW288 encoding residues 192 to 360 and 192 to 288, respectively, were amplified by PCR and cloned into pJW4303. The plasmids pJW383d1 and pJW383d2, containing deletions in residues 261 to 286 and 289 to 340, respectively, were generated from pJW383 by splicing of overlapping extensions (13, 15) as described previously (44). A cDNA fragment encoding core to E2 proteins of HCV strain J1 was amplified by PCR and cloned into pCAGGs-PUR (28), and glycosylation site mutations in the E1 protein were generated by the method of splicing by overlapping extension. For the yeast two-hybrid assay, pGBKT7HCVCore173 was used as bait, as described previously (38). The gene encoding amino acids 288 to 346 of HCV E1 protein was amplified from cDNA of strain J1 and introduced into NdeI and EcoRI sites of a pGADT7 vector (Clontech, Palo Alto, CA). In the same way, deletion mutants of core protein encoding residues 1 to 151, 1 to 25, 24 to 173, 38 to 173, 58 to 173, 72 to 173, and 92 to 173 were amplified by PCR and cloned into a pGBKT7 vector. The FLAG sequence was introduced between amino acids 195 and 196 of the cDNA encoding residues 1 to 383 of the HCV polyprotein and replaced Ala^{383} with Arg to avoid processing by signal peptidase and spacer amino acids (Gly-Gly-Gly-Ser), and influenza virus hemagglutinin (HA) sequence was added at the C terminus. The resulting cDNA fragment encoding core protein, FLAG tag, E1, and HA tag was cloned into a pcDNA3.1(+) vector and designated Flag-core-E1-HA (see Fig. 2D, below) and used for in vitro transcription and translation. Similarly, the FLAG sequence was introduced into the cDNA encoding residues 151 to 383 of the HCV polyprotein, and the HA sequence was added at the C terminus. The resulting cDNA fragment encoding the C-terminal hydrophobic/transmembrane region of the core protein, FLAG tag, E1, and HA tag was designated Flag-E1-HA (see Fig. 3A, below). The DNA fragments encoding residues 1 to 191 with amino acids 72 to 91 deleted were generated by splicing via overlapping extension and cloned into pCAGGS (CoreΔ72-91) (see Fig. 4A, below) (42). The DNA fragment encoding the cytoplasmic domain of the E1 protein with a C-terminal HA tag was amplified by PCR and introduced at HindIII and SacII sites of pEGFP-C3. pCAGGS plasmids encoding core to p7 replacing residues 304 to 307, 308 to 311, 312 to 315, 316 to 319, 320 to 323, 324 to 327, or 328 to 331 with Ala were generated by using splicing with overlapping extension (see Fig. 6A, below).

Antibodies. Mouse monoclonal antibody to HA tag (HA11) and anti-FLAG antibody (M2) were purchased from Covance (Richmond, CA) and Sigma, respectively. Mouse monoclonal antibodies to core protein (clones 11-7, 11-10, and 11-14) were gifts from S. Yagi (2). Anti-E1 mouse monoclonal antibody (clone 0726) was prepared by immunization using the membrane fraction of the CHO L10 cell line, which constitutively expresses HCV envelope proteins (30). Anti-E2 monoclonal antibody (clone 187) was a generous gift from M. Kohara.

Yeast two-hybrid assay. A yeast two-hybrid assay was carried out by using Matchmaker system 3 (Clontech) according to the manufacturer's protocol. The bait vector pGBKT7HCVcore 173 (38) or empty plasmid was transfected into *Saccharomyces cerevisiae* strain AH109 together with the prey vectors, pGADT7-based constructs (see Table 1, below). The yeast cells possessing pGBKT7/p-53 and pGADT7/large T antigen were used as positive controls, while yeast cells possessing pGBKT7 and pGADT7 were the negative controls. These transfected yeast colonies were cultivated on dropout plates lacking Trp, Leu, His, and Ade (test plates) or plates lacking Trp and Leu (control plates) and then incubated at 30°C for 1 week.

Transfection, immunoblotting, and immunoprecipitation. Liposome-mediated DNA transfection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was described previously (38). Transfected cells were cultured at 2×10^5 cells/well in a six-well plate, harvested 30 to 48 h posttransfection, washed twice with phosphate-buffered saline (PBS), and incubated at 4°C for 30 min in 0.25 ml of lysis buffer (20 mM Tris-HCl [pH 7.4], 135 mM NaCl, 1% Triton X-100, and 10% glycerol supplemented with 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, and 5 mM Na_3VO_4). After freezing and thawing, lysed cells were centrifuged at 20,000 × *g* for 5 min. The resulting cleared lysate was stored at −80°C prior to use for immunoprecipitation and blotting. Immunoprecipitation was carried out according to the method described previously (44). Briefly, lysates were preincubated at 4°C for 5 h in the lysis buffer with or without 1 mM $MgCl_2$ and 0.1 mg/ml of yeast tRNA (Sigma) prior to immunoprecipitation. The resulting lysates (0.2 ml) were gently rotated with 1.0 μg of anti-FLAG, anti-HA, or mixed mouse monoclonal anti-HCV core antibodies or mouse monoclonal antibody to the E1 protein at 4°C for 3 h with or without 1 mM $MgCl_2$ and 0.1 mg/ml of yeast tRNA. The immunocomplex was gently rotated at 4°C for 3 h with 10 μl of 50% (vol/vol) protein G-Sepharose 4 Fast Flow beads (Amersham Pharmacia Biotech, Franklin Lakes, NJ) with or without 1 mM $MgCl_2$ and 0.1 mg/ml of yeast tRNA and then centrifuged at 20,000 × *g* for 30 s. The precipitated beads were washed five times with 0.5 ml of lysis buffer containing or lacking 1 mM $MgCl_2$ and 0.1 mg/ml of yeast tRNA and then boiled in 50 μl of the loading buffer. The boiled samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins in gels were transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore, Bedford, MA) and then blotted with primary antibody and secondary horseradish peroxidase-conjugated antibody. The immunocomplexes on membranes were visualized with Super Signal West Femto substrate (Pierce, Rockford, IL) and detected by using an image analyzer LAS-3000 (Fujifilm, Tokyo, Japan).

Protease protection assay of HCV proteins synthesized by in vitro transcription/translation. A plasmid encoding a FLAG-core-E1-HA protein was transcribed under the control of a T7 promoter by using the RiboMax large-scale RNA production system with Ribo m^7G cap analog (Promega, Madison, WI). In vitro translation was carried out in the presence of [^{35}S]methionine-cysteine (Amersham, Piscataway, NJ) by using rabbit reticulocyte lysate and canine pancreatic microsomal membrane (Promega). Translated sample was diluted sevenfold with PBS and then mixed with tosylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin (Sigma) at a final concentration of 2 μg/ml. The mixture was incubated at 30°C for 60 min with or without 0.5% Nonidet P-40, and then soybean trypsin inhibitor (Sigma) was added at a final concentration of 20 μg/ml. Digestion products were immunoprecipitated with anti-FLAG antibody.

Indirect immunofluorescence analysis. 293T cells were washed with PBS at 40 h after transfection and fixed with 3% paraformaldehyde in PBS for 20 min at room temperature. The fixed cells were permeabilized with 0.2% Triton X-100 for 3 min at room temperature and blocked with nonfat milk solution. Cells were incubated with the anti-E1 antibody for 60 min at 37°C and then with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (IgG; TAGO, Burlingame, CA). HCV E1 protein was visualized by fluorescence microscopy (TE300; Nikon, Tokyo, Japan).

Velocity sedimentation with sucrose gradients. Transfected 293T cell were suspended in MNT buffer (20 mM 2-morpholinoethanesulfonic acid, 100 mM NaCl, 30 mM Tris-HCl [pH 8.6], and 0.1% Triton X-100) and then incubated at 4°C for 5 h with or without 0.1 mg/ml of yeast tRNA and 1 mM $MgCl_2$. Each sample was layered on top of 12 ml of sucrose with a 20 to 60% gradient and then centrifuged in a Beckman SW 41Ti rotor (Beckman Coulter, Tokyo, Japan) at 30,000 rpm for 3 h at 4°C. Centrifuged lysates were collected from the bottoms of the tubes and then concentrated with trichloroacetic acid. After washing with ethanol, concentrated proteins were subjected to SDS-PAGE and immunoblotting.

RESULTS

Prediction of the topology of the E1 protein in the membrane. Although a small fraction of the HCV envelope glycoproteins expressed in 293T cells is translocated onto the plasma membrane (3), the vast majority of E1 is retained in the ER membrane (6). Previously, we showed that both a central hydrophobic region of E1 (residues 260 to 288) and the C-terminal transmembrane domain (residues 360 to 383) are important for ER retention (29). As in the C-terminal hydrophobic region, the amino acid sequence of the central hydrophobic region is highly conserved among HCV isolates (4). To investigate the role of these two hydrophobic regions in the biogenesis of the E1 glycoprotein, we utilized the TMHMM algorithm (19), a computer program trained to identify potential transmembrane helical regions. The algorithm identified both hydrophobic regions as having a high probability of transmembrane helix (Fig. 1A). To examine the function of the hydrophobic regions as transmembrane domains, we constructed a series of deletion mutants in the E1 protein in which one or the other of the hydrophobic segments was absent (Fig. 1B). Mutant E1 glycoproteins were expressed in 293T cells, and the cellular localization of E1 proteins was determined by indirect immunofluorescence analysis (Fig. 1B). The full-length E1 (383) was detected only in permeabilized cells, consistent with its retention in the ER. The 383d2 mutant, which contains both hydrophobic regions but lacks the intervening hydrophilic region (residues 289 to 340), was also detected in the cytoplasm but not on the cell surface as the full-length E1. By contrast, deletion mutants lacking the central (383d1) or C-terminal (288 and 360) hydrophobic domains were detected on the cell surface in nonpermeabilized cells, suggesting that both the central and the C-terminal hydrophobic domains are required for retention of the E1 protein on the ER membrane. If the central hydrophobic domain traverses the ER membrane as predicted by the TMHMM program, the region between positions 288 and 360 would be expected to lie in the cytoplasmic space. Based on this model and on the results with E1 deletion mutants, we suggest that the E1 protein might be able to retain two membrane topologies: the conventional type I topology and a polytopic topology that spans the membrane twice with N and C termini in the ER lumen and an intervening cytoplasmic loop, as reported previously (35) (Fig. 1C). Recently, a similar polytopic form of the fusion glycoprotein of Newcastle disease virus was identified (31).

Mutational analysis of putative N-glycosylation sites of the E1 glycoprotein. To explore the membrane topologies of E1, we examined the utilization of potential glycosylation sites. The E1 protein of HCV strain J1 (1) contains seven N-glycosylation sequence motifs (Asn-X-Ser/Thr) at amino acid positions 196, 209, 233, 234, 250, 305, and 325 (Fig. 2A). The Asn residues at these possible N-glycosylation sites were individually replaced with Gln, and the mutant E1 glycoproteins were expressed as a core-, E1-, or E2-containing polyprotein in 293T cells. In all cases, the mutant polyproteins were expressed and properly processed by signal peptidase and signal peptide peptidase to generate the core, E1, and E2 proteins (Fig. 2B). The mutant E1 proteins displayed distinct glycoforms consistent with changes in glycosylation. The wild-type E1 glycoprotein exhibited a major band of 34 kDa and a minor band of 32 kDa.

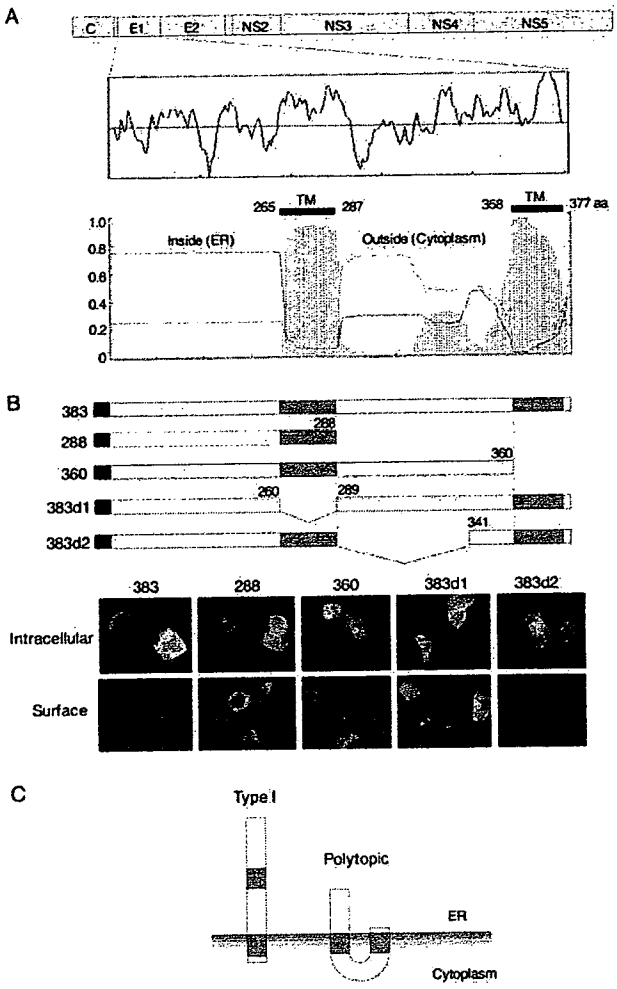

FIG. 1. Prediction of the membrane topology of the E1 protein. (A) Genome structure of HCV and a hydrophobic profile of the amino acid sequence of the E1 protein are shown at the top. The transmembrane helices in the E1 protein were predicted by the TMHMM program (19), and regions of high probability (amino acid residues 265 to 287 and 358 to 377) are indicated. (B) 293T cells transfected with the wild type (383) and deletion constructs were fixed with paraformaldehyde and permeabilized with Triton X-100 (intracellular) or not permeabilized (surface). E1 proteins were visualized with an anti-E1 monoclonal antibody and fluorescein isothiocyanate-conjugated anti-mouse IgG. (C) Possible topologies of the E1 protein on the ER. (Left) Type I topology model possessing a C-terminal transmembrane region; (right) a polytopic topology that spans the membrane twice, with both N and C termini in the ER lumen and with an intervening cytoplasmic loop.

The 325 mutant was unchanged from the wild-type E1, suggesting that the 325 position is not utilized, presumably due to an unfavorable NWSP motif in the genotype 1a protein (33). The 209, 233/234, and 250 mutants migrated faster than the authentic E1 protein and exhibited two bands of 32 and 30 kDa. The E1 of the 196 mutant was apparently not recognized by the monoclonal antibody directed to the N-terminal region of E1. In the 233 and 234 mutants, glycosylation occurred at the remaining Asn (234 or 233, respectively). These mutants comigrated with the wild-type E1 glycoforms, suggesting that

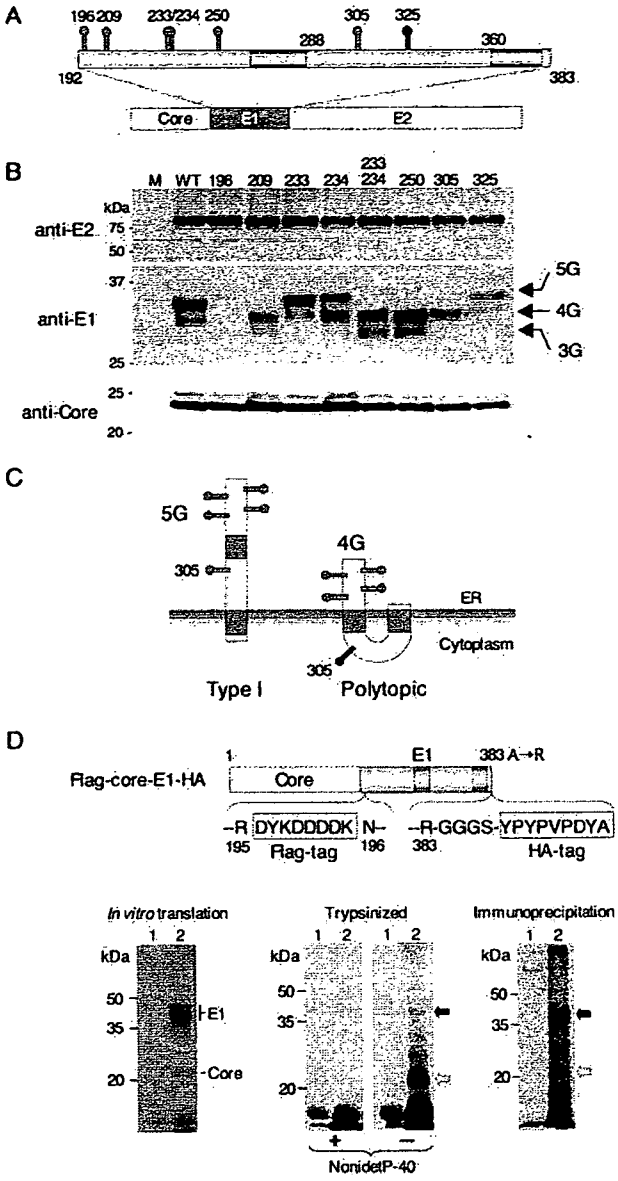

FIG. 2. Mutational analysis of N-glycosylation sites and protease protection assay of the E1 protein. (A) Positions of potential N-glycosylation sites (gray and black spikes) in the E1 protein are shown. (B) Asn residues in the possible N-glycosylation sites in the E1 protein were individually replaced with Gln. Mutant plasmids encoding the core, E1, and E2 polyproteins (A) were expressed in 293T cells, and processed core, E1, and E2 proteins were detected by immunoblotting. (C) Type I and polytopic topology models of E1 proteins bearing carbohydrates at positions of 196, 209, 234, 250, and 305 (5G) and 196, 209, 234, and 250 (4G), respectively. The 305 mutant would exhibit a single band of 4G irrespective of the topologic models. (D) Structure of the FLAG-core-E1-HA construct encoding the HCV core and E1 polyprotein carrying FLAG and HA tags in the N- and C-terminal regions of the E1 protein (top). (Bottom, left) In vitro translation of capped RNA transcribed from the FLAG-core-E1-HA (lane 2) and without RNA (lane 1) in the presence of [^{35}S]methionine-cysteine using rabbit reticulocyte lysate and canine pancreatic microsomal membrane. (Bottom, middle) Translated products of FLAG-core-E1-HA (lane 2) and without RNA (lane 1) were digested with trypsin in the presence (+) or absence (−) of 0.5% Nonidet P-40. (Bottom, right) Digestion products were immunoprecipitated with control (lane 1) and anti-FLAG (lane 2) antibody. Black and white arrows indicate protected and digested E1 protein, respectively.

only one or the other of the overlapping motifs can be utilized in the wild-type molecule. Glycosylation in this region was absent in the double mutant (233/234). The existence of two glycoforms of E1 may reflect incomplete and stochastic use of the available glycosylation sites or, alternatively, the presence of two discrete topological forms of E1 protein. For instance, the major band of 34 kDa in the wild-type glycoprotein might correspond to the type I topology form, with glycosylation at 196, 209, 234, 250, and 305 (5G), whereas the minor band of 32 kDa might correspond to the polytopic form of E1, bearing glycans at positions 196, 209, 234, and 250 (4G). In this regard, it is noteworthy that the 305 mutant of E1 exhibited only a single band of 32 kDa. The absence of a second glycoform is consistent with the putative cytoplasmic localization of Asn305 in a polytopic form of E1 (Fig. 2C). Taken together, this mutational analysis provides support to the model in which the HCV E1 glycoprotein is able to exist in either the type I or polytopic form. In the latter form, an extended cytoplasmic domain in E1 would be available to interact with the core protein in the virion.

Protease protection assay of the E1 protein. To confirm the presence of the cytoplasmic domain in the E1 protein, in vitro translation products of the HCV core and E1 polyprotein carrying FLAG and HA tags in the N- and C-terminal regions of the E1 protein, respectively, were digested with trypsin, and the protected portion of the E1 glycoprotein was immunoprecipitated by anti-FLAG antibody. As shown below in Fig. 4D, treatment of the translation products with trypsin in the presence of Nonidet P-40 resulted in complete digestion, and a 22-kDa band (major) and several <35-kDa faint bands were detected in the absence of the detergent. When in vitro-translated HCV core protein was treated similarly, no band was detected, irrespective of the presence of detergent (data not shown); therefore, the protected bands from trypsin digestion were derived from the E1 protein. Although the 22- to 35-kDa bands were specifically immunoprecipitated with anti-FLAG antibody but not with control antibody, the 35-kDa protein corresponding to the type I topology of the E1 protein resistant to trypsin digestion was dominant. This might be due to the difference in the reactivity of the anti-FLAG antibody, which recognizes the intact E1 protein more efficiently than digested ones. These results further support the presence of the polytopic form of HCV E1 glycoprotein, which has a cytoplasmic region together with a type I topology in the ER.

HCV core protein binds to the E1 protein in the presence of tRNA. The HCV core protein undergoes extensive conformational changes upon binding to nucleic acid and self-assembling into nucleocapsid-like particles (20). To investigate the effects of nucleic acid on oligomerization of the core protein, lysates of 293T cells expressing HCV core protein were incubated in the presence or absence of yeast tRNA (20) and subjected to velocity sedimentation in a sucrose gradient. Oligomerized core protein was detected in fractions 1 to 4 in the presence of tRNA but not in those in the absence of tRNA (Fig. 3A). To specifically examine the interaction between HCV core and E1 proteins in the assembly of the nucleocapsid-like particles, we coexpressed the core protein with an E1 protein possessing a FLAG tag near its N terminus and an HA tag at the C terminus (Flag-E1-HA) (Fig. 3B, left). The transfected cells were lysed with Triton X-100, and the E1 protein

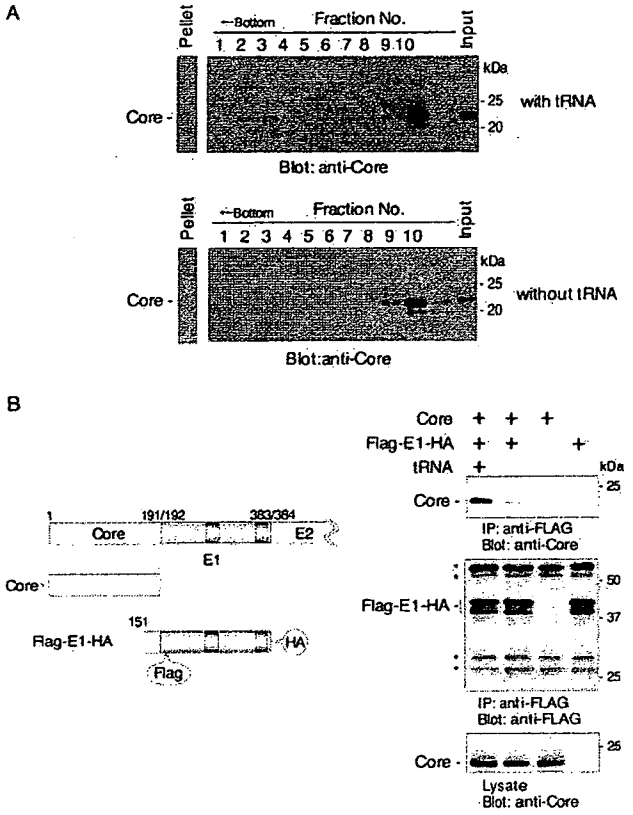

FIG. 3. HCV core protein binds to E1 protein in the presence of tRNA. (A) Cell lysates of 293T cells expressing HCV core protein were subjected to velocity sedimentation with a sucrose gradient in the presence or absence of tRNA. Oligomerized core protein was detected in fractions 1 to 4 in the presence of tRNA but not in those in the absence of tRNA. (B, left) cDNAs used for expression. FLAG-E1-HA encodes FLAG tag after the signal peptide and HA tag after the transmembrane region. (Right) Immunoprecipitation analyses. Cell lysates of 293T cells expressing core and FLAG-E1-HA proteins were immunoprecipitated by anti-FLAG antibody in the presence or absence of tRNA. The asterisks indicate nonspecific bands.

was immunoprecipitated by using an anti-FLAG antibody. Coprecipitation of core protein with E1 was assessed by Western blot analysis using a core-specific monoclonal antibody. Although HCV core protein was clearly coprecipitated with FLAG-E1-HA in the presence of tRNA, little association was seen in the absence of tRNA (Fig. 3B, right). Nonspecific precipitation of the core protein with tRNA was not observed (data not shown). Although a small amount of the intracellular core protein may already associate with viral RNA under the intracellular conditions, a large amount of RNA may be required for oligomerization that is detectable by the sedimentation assay. Together, our results suggest that tRNA facilitates oligomerization of the HCV core protein and potentiates the interaction between the core protein and E1.

The region spanning amino acid residues 72 to 91 in the HCV core protein is crucial for binding to the E1 protein in yeast. The interaction between the HCV core and E1 proteins likely occurs on the cytosolic side of the cell membrane and, thus, presumably involves the posited cytoplasmic loop region in the polytopic form of the E1 glycoprotein. To investigate the possibility for this specific interaction in cells, core protein lacking the transmembrane region (Core1-173) was examined for interaction with the putative E1 cytoplasmic loop region in a yeast two-hybrid system (Table 1). When Core1-173 was expressed with the E1 cytoplasmic region (residues 288 to 346), the yeast was able to grow on the dropout plate lacking Trp, Leu, His, and Ade, suggesting that the core protein associates with the cytoplasmic loop of the E1 protein in yeast. To determine the region of the HCV core protein responsible for the interaction with the cytoplasmic domain of E1, deletion mutants of the core were tested. Association in the yeast two-hybrid system was seen with Core24-173, Core38-173, Core58-173, Core72-173, and Core1-151 mutants, but not with Core92-173 and Core1-25. Nonspecific interaction of the GAL4 activation domain with these core mutants was not observed. These results suggest that the region spanning from amino acid residues 72 to 91 in the HCV core protein is important for interaction with the cytoplasmic domain of the E1 protein in yeast.

Amino acid residues 72 to 91 in the core protein are involved in oligomerization of the core protein and interaction with the E1 protein in mammalian cells. To examine the involvement of amino acid residues 72 to 91 of the HCV core protein in the interaction with the E1 protein in mammalian cells, FLAG-E1-HA was coexpressed with either a wild-type core or a deletion mutant lacking amino acid residues 72 to 91 (CoreΔ72-91) in 293T cells (Fig. 4A). Cell lysates were incubated with yeast tRNA, and FLAG-E1-HA was immunoprecipitated with anti-FLAG antibody. As shown in Fig. 4B (left), only the wild-type core protein, but not CoreΔ72-91, coprecipitated with E1. Self-oligomerization was also prevented by the deletion in CoreΔ72-91 (Fig. 4B, right). These results suggest that amino acid residues 72 to 91 in the HCV core protein play a crucial role in the interaction with the E1 protein and oligomerization of the core protein.

The E1 cytoplasmic domain interacts with the core protein in mammalian cells and inhibits the interaction with intact E1 protein in *trans*. To assess the involvement of the E1 cytoplasmic region in the interaction with core protein in mammalian

TABLE 1. Interaction between the core and the E1 cytoplasmic region in yeast

Bait	Growth with prey[a]			
	E1 cytoplasmic loop		No insert	
	Dropout	Control	Dropout	Control
Core1-173	+	+	−	+
Core24-173	+	+	−	+
Core38-173	+	+	−	+
Core58-173	+	+	−	+
Core72-173	+	+	−	+
Core92-173	−	+	−	+
Core1-151	+	+	−	+
Core1-25	−	+	−	+
No insert	−	+	−	+

[a] HCV core mutants were expressed as fusion proteins with the DNA binding region by using a bait plasmid. The HCV E1 cytoplasm region was expressed as a fusion protein with an activation domain by using a prey plasmid. Yeast growth was observed in dropout plates lacking Trp, Leu, Ade, and His (dropout) or plates lacking Trp and Leu (control). +, growth; −, no growth.

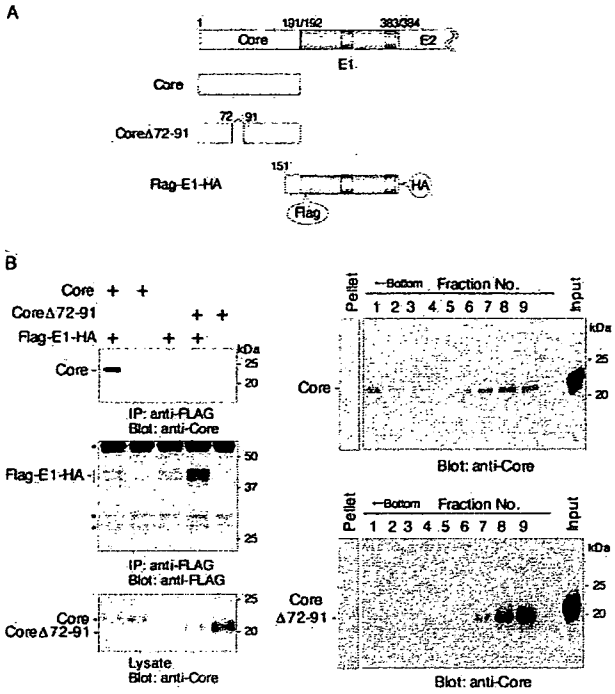

FIG. 4. Amino acid residues72 to 91 in the core protein are involved in oligomerization of the core protein and interaction with the E1 protein. (A) cDNAs used for expression. CoreΔ72-91 is an HCV core protein carrying a deletion of amino acid residues 72 to 91. (B, left) FLAG-E1-HA was coexpressed in 293T cells with either a wild-type core or CoreΔ72-91, and the interaction was analyzed by immunoprecipitation in the presence of tRNA. The asterisks indicate nonspecific bands. (Right) Oligomerization of a wild-type core or CoreΔ72-91 in the presence of tRNA. Wild-type core protein was self-oligomerized, but CoreΔ72-91 was not.

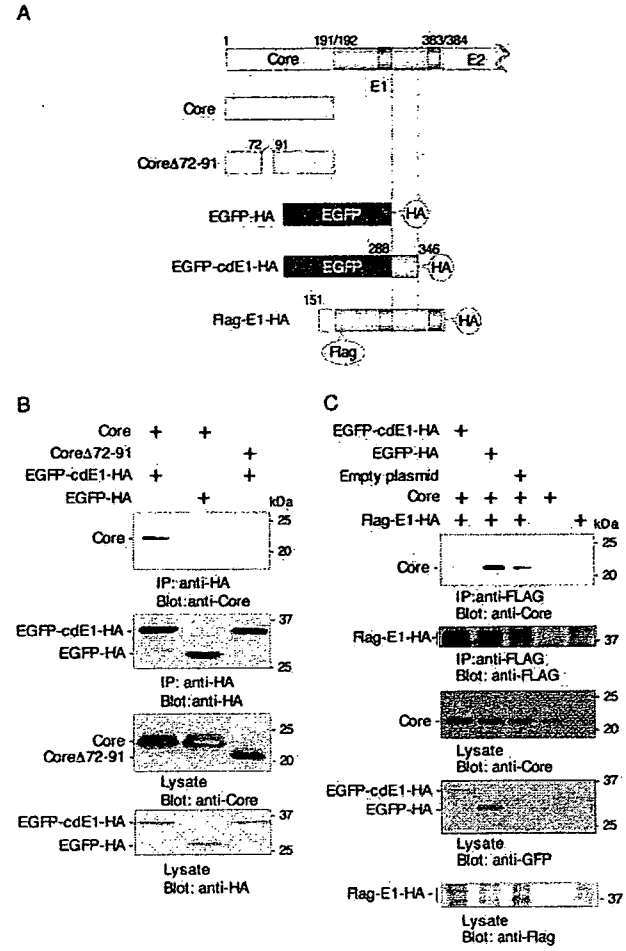

FIG. 5. Interaction of the E1 cytoplasmic loop with the core protein. (A) cDNAs used for expression. EGFP-cdE1-HA is an EGFP fusion protein carrying the E1 cytoplasmic region of amino acid residues 288 to 346 followed by an HA tag. (B) Wild-type core or CoreΔ72-91 was coexpressed with EGFP-cdE1-HA in 293T cells, and their interaction was analyzed by immunoprecipitation. EGFP-cdE1-HA coprecipitated with wild-type core protein, but not with CoreΔ72-91. (C) Inhibition of the interaction of the core protein with FLAG-E1-HA by expression of EGFP-cdE1-HA. Expression of EGFP-cdE1-HA but not of EGFP disrupted the interaction between core and E1 proteins.

cells, we constructed an enhanced green fluorescent protein (EGFP) fusion protein carrying the E1 cytoplasmic domain followed by an HA tag (EGFP-cdE1-HA) (Fig. 5A). Upon coexpression of EGFP-cdE1-HA with the wild-type core protein in 293T cells, the two proteins could be coprecipitated using anti-HA antibody (Fig. 5B). The mutant CoreΔ72-91 protein was unable to associate with EGFP-cdE1-HA (Fig. 5B). Together, these studies demonstrate that the cytoplasmic loop region of E1 is able to interact with the core protein and that core residues 72 to 91 are required for this association.

To further confirm the specificity of the interaction of the E1 cytoplasmic region with the core protein, we examined the ability of the EGFP-cdE1-HA protein to inhibit the association of the intact E1 protein (in Flag-E1-HA) with the wild-type core protein (Fig. 5C). Expression of EGFP-cdE1-HA but not EGFP-HA competed strongly with the interaction between core and the FLAG-tagged Flag-E1-HA protein. These results suggest that the cytoplasmic loop in the intact E1 glycoprotein can directly bind to HCV core protein. Interestingly, the EGFP-cdE1-HA protein was unable to inhibit this interaction in the context of the intact core and E1 and E2 polyproteins (data not shown), suggesting that expression of the core and E1 proteins in *cis* may prevent subsequent interaction with E1 expressed in *trans*.

Four amino acid residues, 312 to 315, in the cytoplasmic region of the E1 protein are important for interaction with the core protein. Alignment of the amino acid sequence of the E1 cytoplasmic region among different HCV genotypes revealed that the region from Gln^{302} to Pro^{328} is highly conserved (Fig. 6A). To determine residues in the E1 cytoplasmic region that are critical for interaction with the core protein, blocks of four residues each in the conserved region were replaced with Ala in the polyprotein (core, E1, E2, and p7) (Fig. 6A). These mutant polyproteins were expressed in 293T cells and immunoprecipitated with anti-core antibody; coprecipitated E1 protein was detected by immunoblotting using an anti-E1 monoclonal antibody (Fig. 6B). The replacement of four amino acid residues, 304 to 307, with Ala in the conserved region of the E1

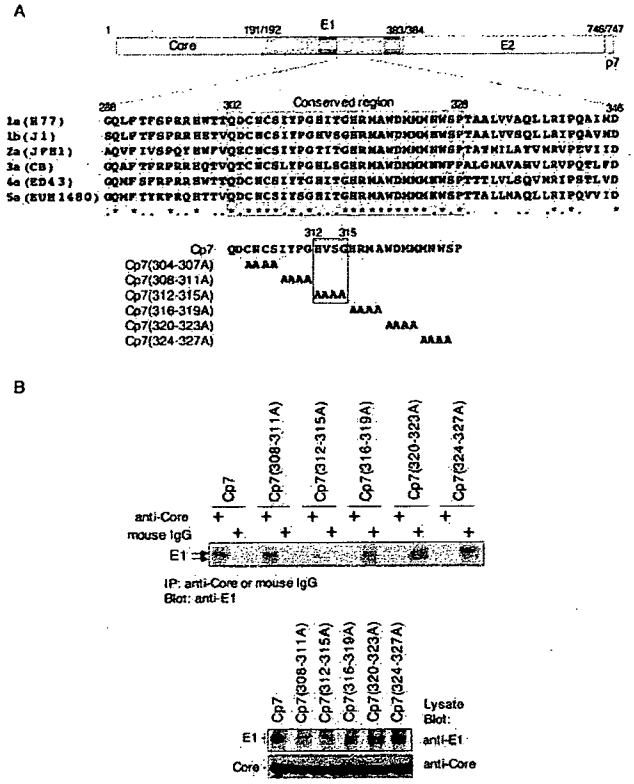

FIG. 6. Four amino acid residues, 312 to 315, in the cytoplasmic region of the E1 protein are important for interaction with the core protein. (A) Alignment of the amino acid sequence of the E1 cytoplasmic region among different HCV genotypes (1a, H77 [AF009606]; 1b, J1 [D89815]; 2a, JFH1 [AB047639]; 3a, CB [AF046866]; 4a, ED43 [Y11604]; 5a, EUH1480 [Y13184]). A conserved region from Gln^{302} to Pro^{328} is shown by gray shading. Mutant polyproteins consisting of the core, E1, E2, and p7 proteins with four residues each replaced by Ala in the conserved E1 region were constructed. Four amino acid residues, His^{312}, Val^{313}, Ser^{314}, and Gly^{315}, in the E1 cytoplasmic region of strain J1 and substitution of the amino acids with Ala in Cp7 (312-315A) are indicated by the box. (B) These mutant polyproteins were expressed in 293T cells and immunoprecipitated with anti-core antibody or nonspecific mouse IgG in the presence of $MgCl_2$ and tRNA. The E1 protein that coprecipitated with the core protein was detected by immunoblotting. The substitution of four amino acid residues, 304 to 307, with Ala in the conserved region of the E1 protein, Cp7 (304-307A), could not be examined due to the low level of expression.

protein could not be examined due to a low level of expression (data not shown). Among the mutant constructs examined, only the substitution at residues 312 to 315, Cp7 (312-315A), markedly diminished association with the core protein (Fig. 6B). These results suggest that this region in the E1 cytoplasmic domain of the J1 strain of HCV (His^{312}, Val^{313}, Ser^{314}, and Gly^{315}) is important for interaction with the core protein.

DISCUSSION

The biogenesis of the transmembrane glycoproteins involves a series of coordinated translation and membrane integration events that are directed by topogenic determinants within the nascent chains and that ultimately lead to the most favored topology for any given polypeptide (24). However, there is an increasing number of examples of glycoproteins that can assume multiple topological orientations. The large envelope protein of the hepatitis B virus, for instance, has been suggested to adopt distinct topologies that enable the protein to serve in virus assembly as a matrix-like protein and in virus entry as a receptor binding protein (22). An unglycosylated form of the HCV E2 protein has been identified and shown to interact with protein kinase R in the cytosol (45). In Newcastle disease virus, type I and polytopic forms of the fusion protein are present in the same cell, and the polytopic form is suggested to be involved in the membrane fusion event (31).

HCV glycoproteins E1 and E2 were shown to possess transmembrane domains and associate to form noncovalent heterodimers that are statically retained in the ER membrane upon recombinant expression (10, 29, 46). Previously, the E1 protein of genotype 1a was suggested to possess a single C-terminal transmembrane domain, based in part on its utilization of potential glycosylation sites (33) and on a model of the transmembrane domains of the E1 and E2 proteins, in which the C terminus reorients, upon signal peptidase cleavage, from the ER lumen to protrude slightly into the cytoplasm (7). In our study, we have suggested that the E1 protein can also adopt a polytopic topology in which the protein spans the ER membrane twice and includes an intervening cytoplasmic region. In this model, the membrane orientation of the C-terminal transmembrane region is inverted and translocation of the signal peptidase-cleaved C terminus is not required.

Our analysis revealed that the 305 mutant of the 1b genotype expressed by transfection exhibited a single band of 32 kDa, whereas that of genotype 1a expressed by recombinant vaccinia viruses has been reported to contain two bands (33). Although we do not know the reason for this discrepancy, it may relate to differences in the expression systems. HCV proteins expressed by vaccinia virus and Sindbis virus vectors formed disulfide-linked aggregates (9, 11, 34), and coexpression of a large amount of vaccinia viral proteins also may alter the proper processing of the expressed proteins, as suggested by Merola et al. (32). However, further work will be necessary to clarify the reasons for the differences in glycosylation patterns of E1 mutants obtained in the different expression systems.

Mottola et al. analyzed the determinants for ER localization of the E1 protein and showed that the juxtamembrane region of E1, between amino acid residues 290 and 333, was required for ER retention (41). This region lies within the ectodomain of the E1 protein in the type I topology and in the cytoplasmic region of the protein in the proposed polytopic form. ER localization determinants of transmembrane proteins have in general been located either in the cytosolic or in the transmembrane domain, not in the luminal ectodomain, except for the yeast Sec20 protein (41). Therefore, assignment of the ER localization signal to the cytoplasmic region of the E1 protein might further support the possibility of the polytopic topology model. Affinity purification and membrane reconstitution of the E1 protein carrying an affinity tag (S-peptide) in the putative cytoplasmic region are also consistent with this model (35). Together, these findings provide indirect support that the E1 glycoprotein can adopt a polytopic form.

As previously reported (20), oligomerization of the HCV core protein to form nucleocapsid-like particles requires the presence of stem-loop RNA structures, such as those in tRNA.

Here, we have demonstrated that self-assembly of the core protein occurs without envelope protein in the presence of tRNA and that tRNA is required for the association of E1 glycoprotein with the core protein, suggesting that oligomerization of the core protein may be a prerequisite for this interaction during virus assembly. Based on hydrophobicity and the clustering of basic amino acids, the HCV core protein is proposed to possess three domains: the N-terminal basic and hydrophilic region (domain 1; residues 1 to 118), a central basic and hydrophobic domain (domain 2; residues 119 to 174), and the hydrophobic signal sequence for E1 (domain 3; residues 175 to 191) (14). Biophysical characterization of the core protein indicated that the C-terminal residues 125 to 179 were critical for the folding and oligomerization of the core protein (21). Although our mutant HCV polyprotein containing Ala substitutions at residues 312 to 315 in the cytoplasmic region of the E1 protein exhibited a clear reduction in its interaction with the core protein, a substantial amount of residual binding was retained. These results suggest that regions other than the residues from 312 to 315 in the E1 protein are also involved in the interaction with the core protein.

In Semliki Forest virus, the cytoplasmic domain of the E2 glycoprotein, which corresponds to the E1 protein in HCV, has been shown to interact with the capsid protein (26, 49). Assembly of alphaviruses has also been found to require the specific interaction between the C-terminal cytoplasmic domain of the E2 protein and the capsid protein (17). Although the functional significance of the two forms of the HCV E1 protein is still unclear, the E1 cytoplasmic region among different HCV genotypes is well conserved and four amino acid residues, His^{312}, Val^{313}, Ser^{314}, and Gly^{315} of strain J1, were shown to be important for interaction with the core protein. Although the four amino acid sequences identified in strain J1 of genotype 1b are not strictly conserved among the different HCV genotypes (Fig. 6A), a pattern of polar-hydrophobic-polar-glycine residues can be discerned in all of them. The interaction of the cytoplasmic E1 protein with the core protein may indicate that the polytopic form is a mature E1 protein that is incorporated into virions.

In conclusion, the polytopic topology model of the HCV E1 protein and the interaction of oligomerized core protein with the cytoplasmic region of the E1 protein may provide clues to aid in understanding the biosynthesis and assembly of the HCV structural proteins. HCV core protein is also involved in the development of liver steatosis, type II diabetes mellitus, and hepatocellular carcinoma in transgenic mice (39, 40, 48). A detailed knowledge of the assembly of HCV particles will provide the basis for the development of effective therapeutics for chronic hepatitis C.

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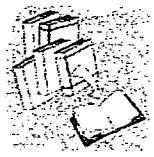

Host factors involved in the replication of hepatitis C virus

Kohji Moriishi and Yoshiharu Matsuura*

Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan

SUMMARY

Hepatitis C virus (HCV) is the major causative agent of blood-borne hepatitis. The majority of HCV-infected individuals develop chronic hepatitis, which eventually progresses to liver cirrhosis, and hepatocellular carcinoma. Although the precise mechanisms of entry, replication, assembly, egress and pathogenesis of HCV are largely unknown, information about viral receptor candidates has accumulated by the development of pseudotype viruses and an *in vitro* replication system of the HCV JFH1 strain. Furthermore, the autonomous RNA replication system based on the artificial viral genome revealed that HCV replicates in the intracellular replication complex composed of viral and host proteins. Recently, an immunosuppressant, cyclosporin A and inhibitors for sphingolipid synthesis and chaperon were reported to inhibit the replication of HCV by counteracting the interplay between host and viral proteins. This review considers the current knowledge of the host proteins that participate in HCV replication and the possibility of developing novel therapeutics intervention for chronic hepatitis C. Copyright © 2007 John Wiley & Sons, Ltd.

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INTRODUCTION

Hepatitis C, which is caused by infection with hepatitis C virus (HCV), is a serious form of chronic hepatitis with steatosis and cirrhosis, and eventually leads to hepatocellular carcinoma [1]. HCV is classified into a member of genus *Hepacivirus* of the family *Flaviviridae* [1]. Epidemiological study reveals that 170 million individuals worldwide are infected with HCV, mostly through blood-borne infection [2]. Introduction of combination therapy with interferon alpha and ribavirin improved therapeutic efficacy, but had no effect on half of the individuals infected with a high viral load of HCV genotype 1 [3,4]. Therefore, effective therapeutic measures are required for the treatment of hepatitis C patients who are not responsive to chemotherapy. An HCV replicon system was established as a representative functional system composed of an antibiotic gene for selection and HCV genomic RNA for autonomous replication in the intracellular compartments around the endoplasmic reticulum (ER) [5]. Studies on HCV replication have used the replicon system, and small chemicals targeted to HCV proteins have been identified [6–10]. On the other hand, a pseudotype viral system based on the vesicular stomatitis virus and retrovirus has been developed to study the receptor determination and the entry mechanism [1]. Recently, an *in vitro* cell culture system for HCV of genotype 2a, which is highly sensitive to interferon therapy [11,12], has been developed [13–15]. However, a robust cell culture system for the HCV 1a and 1b genotypes, which are both the most prevalent genotypes in the world and resistant to interferon therapy, has not yet been successful.

HCV possesses a single positive strand RNA genome encoding a large polyprotein composed of approximately 3000 amino acid residues [1]. The polyprotein is cleaved by the viral proteases

*Corresponding author: Y. Matsuura, DVM, PhD, Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan.
E-mail: matsuura@biken.osaka-u.ac.jp

Abbreviations used
CaM, calmodulin binding domain; Dhh, Desert hedgehog; ER, endoplasmic reticulum; FBD, FK506-binding domain; FKBP, FK506-binding proteins; HCV, hepatitis C virus; Ihh, Indian hedgehog; MSP, major sperm protein; NS, nonstructural; ORPs, oxysterol-binding protein-related proteins; Ptc, Patched; Smo, Smoothened; Shh, Sonic hedgehog; VAMP, vesicle-associated membrane protein.