Morphological identification of hepatitis C virus E1 and E2 envelope glycoproteins on the virion surface using immunogold electron microscopy

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Abstract. It is known that hepatitis C virus (HCV) particles are spherical, 55-65 nm particles with fine surface projections of about 6 nm in length and with a 30-35 nm inner core. We have reported that free HCV particles labeled with gold particles specific to the HCV E1 glycoprotein are located in 1.14-1.16 g/ml fractions from plasma samples with high HCV RNA titers after sucrose density gradient centrifugation. However, the morphology of the HCV E2 glycoprotein on the virion has not yet been elucidated. To visualize HCV E2 localization on the virion, we used the same plasma samples where HCV particles were clearly shown. An indirect immunogold electron microscopic study was carried out using monoclonal and polyclonal anti-HCV E2 antibodies. HCV-like particles specifically reacted with the anti-HCV E2 antibodies. Moreover, to evaluate the localization of the HCV E1 and E2 glycoproteins on the virion surface, an immunogold electron microscopic study using double labeling with anti-HCV E1 antibodies and anti-HCV E2 antibodies was also performed. These particles also specifically reacted with both anti-E1 and E2 antibodies. This is the first report showing the presence of both HCV E1 and E2 glycoproteins on HCV virion surface in human plasma samples.

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Abbreviations: HCV, hepatitis C virus; ALT, alanine aminotransferase; PCR, polymerase chain reaction; EM, electron microscopy; RVV, recombinant vaccinia virus; ELISA, enzyme-linked immunosorbent assay; IFA, indirect immunofluorescence assay; WB, Western blot analysis; BSA, bovine serum albumin; Huh7, a human hepatoma cell line

Key words: hepatitis C virus, E1, E2, electron microscopy, virion

Introduction

Hepatitis C virus (HCV) is the main causative agent of non-A non-B hepatitis. It is estimated that 170 million individuals are infected with HCV worldwide (1). HCV is a hepatotropic, enveloped RNA virus that belongs to the genus *Hepacivirus* of the *Flaviviridae* family (2), and it is the leading cause of acute hepatitis, chronic hepatitis, liver cirrhosis and hepatocellular carcinoma in humans (1,3-6). The HCV genome is a positive-stranded RNA of 9.6 kb containing a single open reading frame and two untranslated regions (7-9). It encodes a polyprotein of 3010 amino acids, which is cleaved into single proteins by a host signal peptidase in the structural region and by HCV-encoded proteases in the nonstructural region. Structural components include the capsid protein and the envelope glycoproteins E1 and E2. The nonstructural components include NS2, NS3, NS4A, NS4B, NS5A and NS5B. The NS2, NS3, and NS 4A proteins function as proteases, the NS3 protein as helicase, and the NS5B protein as RNA-dependent RNA polymerase (1).

HCV E1 and E2 glycoproteins are possible virion envelope glycoproteins, and their molecular weights are 35 and 70 kDa, respectively (10,11). The comparison of HCV genome structure with flaviviruses suggests that HCV E1 (gp35) and E2 (gp70) glycoproteins interact forming heterodimer complexes as the basic subunit of the HCV virion envelope (11,12). However, this has not been confirmed morphologically. We previously demonstrated that HCV particles are spherical, 55-65 nm particles with fine surface projections of about 6 nm in length and with a 30-35 nm inner core by immunoelectron microscopic study using anti-HCV E1 antibodies (13-17). Free HCV particles were found in 1.14-1.16 g/ml fractions after sucrose density gradient

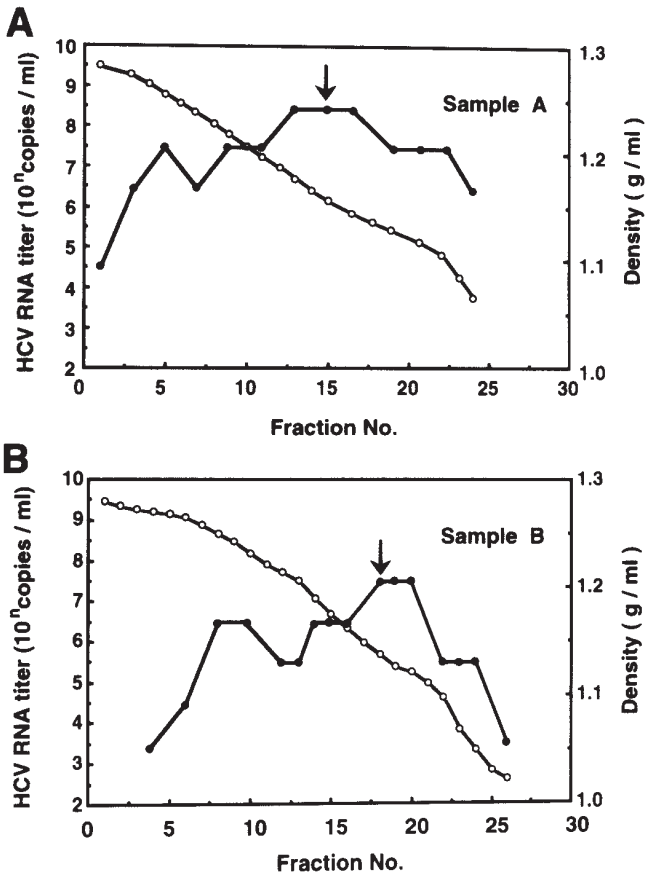

Figure 1. HCV-RNA titers in fractions from samples A and B obtained by sucrose density gradient centrifugation. HCV RNA titers (●) and buoyant densities (○) are shown: (A) sample A, (B) sample B. Arrows indicate the fractions (1.14-1.16 g/ml) in which HCV particles were successfully detected by immunogold EM using rabbit anti-HCV E1 polyclonal antibody (RR2).

centrifugation. However, the morphology of the HCV E2 glycoprotein on the virion has not as yet been elucidated.

In this study, we carried out indirect immunogold electron microscopy (EM) in order to evaluate the localization of the HCV E1 and E2 glycoproteins on the surface of HCV particles isolated from plasma samples with high HCV RNA titers.

Materials and methods

Virus samples. Plasma samples where HCV particles were clearly shown, were used to determine the HCV E2 localization on HCV particles. HCV particle isolation and indirect immunogold EM were performed as previously described (13). In brief, virus samples from HCV RNA-rich plasma sample A [alanine aminotransferase (ALT): 10^3 IU/l, HCV RNA: genotype 1b (18), $4x10^7$ copies/ml] and B (ALT: 10^9 IU/l, genotype 1b, $5x10^7$ copies/ml), and anti-HCV-negative plasma sample C (ALT: 121 IU/l) and D (ALT: 87 IU/l) were prepared as follows: 100 ml of plasma were centrifuged at 75000 g for 6 h at 4˚C and the suspension of the pellet was centrifuged again at 150000 g for 2.5 h at 4˚C. A 1000-fold concentrated suspension of the sample was layered on a 20-60% (w/w) continuous sucrose density gradient in TNE buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA), and centrifuged at 100000 g for 16 h at 4˚C. Sucrose fractions (500 μl) were collected from the tube bottom, and the sucrose densities were measured with an Abbé refractometer. The distribution of HCV RNA titers was determined using competitive polymerase chain reaction (PCR) (19,20). HCV RNA titers in fractions from samples A and B obtained by sucrose density gradient centrifugation were previously described (13,17). The density at which the highest HCV RNA titers (sample A, $5x10^8$ copies/ml; sample B, $5x10^7$ copies/ml) were found was 1.14-1.17 g/ml for sample A and 1.12-1.14 g/ml for sample B (Fig. 1). For preparing a 1000-fold concentrated virus sample, each sucrose fraction was diluted in 12 ml of PBS (pH 7.4), and spun down at 150000 g for 2.5 h at 4˚C. The pellets were then suspended in 100 μl of PBS. The fractions (1.14-1.16 g/ml) in which HCV particles were successfully detected by immunogold EM using rabbit anti-HCV E1 polyclonal antibody (13) were used for virus sampling.

Rabbit polyclonal and mouse monoclonal antibodies to HCV E2 glycoprotein. The rabbit polyclonal anti-HCV E2 antibody (RR6) was prepared and characterized as follows. The putative E2 gene of HCV genotype 1b (nucleotide position 1068-2430) (8,10) was cloned under the control of the ATL-P7.5 hybrid promoter of the vaccinia virus vector pSFB4 (21), and allowed to recombine with the Liter strain of vaccinia virus to give a vector recombinant vaccinia virus (RVV). Rabbits were infected intradermally with 10^8 p.f.u. of RVV and 2 months later were boosted twice with the purified putative E2 glycoprotein. Putative HCV E2 glycoprotein was expressed by RVV and purified by lentil lectin column chromatography and affinity chromatography using an anti-E2 monoclonal antibody. Mouse monoclonal antibodies (747, 843, 1518, 1671, and 1864) against the putative HCV [genotype 1b (17)] E2 glycoprotein were prepared by immunization of mice with purified recombinant E2 glycoprotein (gp70) expressed by RVV. The antibody RR6 and the monoclonal antibodies were screened by enzyme-linked immunosorbent assay (ELISA) using synthetic peptides and purified recombinant protein, indirect immunofluorescence assay (IFA) using RVV- and baculovirus-infected (22) rabbit kidney cells, and Western blot analysis (WB) using purified E2 protein region of HCV genotype 1b as antigens (8). The epitope of monoclonal antibodies was mapped using residues of 20 synthetic peptides whose adjacent peptides overlap by 10 amino acids corresponding to the amino acid sequence reported by Kato *et al* (7). The characteristics of anti-HCV E2 antibodies used as the primary antibody of the indirect immunogold reaction were determined (Table I). The antibody RR6 and the monoclonal antibodies reacted specifically with the putative HCV E2 glycoprotein, but it did not react with the putative HCV core, E1, or NS2 proteins. Specificity was determined by using primary antibodies from pre-immune normal rabbit serum, serum from a rabbit infected with the Lister strain of vaccinia virus and monoclonal antibody specific to human blood type A antigen as negative controls, or by omitting the use of the primary antibody.

Rabbit polyclonal and mouse monoclonal antibodies to HCV E1 glycoprotein. The rabbit polyclonal antibody (RR2) and

Table I. Characteristics of polyclonal and monoclonal antibodies to the putative HCV E2 glycoprotein.

Antibody	ELISA[a] titer	IFA[b] titer	WB[c] titer	Epitope[d] (amino acid position)[e]
Polyclonal				
RR6	10^6	$\geq 10^3$	$\geq 10^3$	
Monoclonal				
747	<0.1 μg/ml	<10 μg/ml	< 1 μg/ml	520-540
843	<0.1 μg/ml	<10 μg/m	<10 μg/ml	520-540
1518	<0.1 μg/ml	<10 μg/ml	<10 μg/ml	450-470
1671	<0.1 μg/ml	<10 μg/ml	<10 μg/ml	640-660
1864	<0.1 μg/ml	<0 μg/ml	<1 μg/ml	450-470

[a]Carried out using synthetic peptides and purified recombinant HCV E2 glycoproteins. [b]Performed using recombinant vaccinia virus- and baculovirus-infected cells. [c]Western blot analysis, using purified recombinant glycoproteins from the putative E2 glycoprotein region of HCV genotype 1b. [d]Mapped using 20-amino acid oligopeptides, each overlapping the adjacent oligopeptide by 10 amino acids. [e]Kato *et al* (7).

mouse monoclonal antibodies (159, 260, 305 and 1905) to the HCV E1 glycoprotein were described previously (13).

Electron microscopy and immunogold electron microscopy. For conventional EM, 3 μl of each virus sample was applied to formvar-coated and carbon-vaporized grids and then negatively stained with 2% phosphotungstic acid, pH 6.5. The grid was examined under a Hitachi H-800 electron microscope operated at 100 kV. Indirect immunogold EM was performed as previously described (13-17). In brief, 3 μl of each virus sample was adsorbed on the grid and then the semidried grid was floated for 5 min on a drop of TBS-BSA (100 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 2% bovine serum albumin) placed on parafilm in a moist chamber. The grid was then floated for 30 min on a drop of TBS containing 3% gelatin and then the excess gelatin from the drop of TBS-BSA was washed away. The grid was incubated for 60 min on a drop of primary antibody solution (diluted 1:100 in TBS-BSA) at room temperature, and then washed three times with TBS-BSA. After incubating the grid for 60 min in a drop of secondary antibody solution (diluted 1:20 in TBS-BSA), the grid was washed three times with TBS-BSA and once with TBS. The grid was negatively stained with 2% phosphotungstic acid for EM observation. Indirect immunogold EM was performed using rabbit anti-HCV E2 antibody RR6 at a dilution of 1:100 or a mixture of five monoclonal anti-HCV E2 antibodies (747, 843, 1518, 1671 and 1864), at a dilution of 1:10 as a primary antibody, and goat anti-rabbit IgG colloidal gold particles (10 nm in diameter; BioCell Research Laboratories, Cardiff, UK) or staphylococcal Protein A-conjugated colloidal gold particles (5 nm in diameter, BioCell Research Laboratories) as a secondary antibody. HCV particles (genotype 1b, 10^8 copies/ml of HCV RNA) in 1.14-1.16 g/ml sucrose fractions were incubated on carbon-coated copper grids with RR6 at a dilution of 1:100 and with a mixture of five clones of mouse anti-HCV E2 monoclonal antibodies at a dilution of 1:10. The HCV E1 and E2 glycoproteins on the virion were double labeled as follows. HCV particles were initially incubated with a mixture of four clones of mouse anti-HCV E1 monoclonal antibodies (159, 260, 305 and 1905) at a dilution of 1:10 and protein A-conjugated 5 nm-gold particles at a dilution of 1:20. Then, the sample was treated with rabbit anti-HCV E2 polyclonal antibody (RR6) at a dilution of 1:100 and with goat anti-rabbit IgG-conjugated 10 nm-gold particles at a dilution of 1:20. Finally, the samples were stained with 2% phosphotungstic acid for EM observation. In addition, HCV particles were initially incubated with a mixture of five clones of mouse anti-HCV E2 monoclonal antibodies (747, 843, 1518, 1671, and 1864) at a dilution of 1:10 and protein A-conjugated 5 nm-gold particles at a dilution of 1:20, and then reacted with the rabbit anti-HCV E1 polyclonal antibody (RR2) at a dilution of 1:100 and with goat anti-rabbit IgG-conjugated 10 nm-gold particles at a dilution of 1:20. Finally, the samples were stained with 2% phosphotungstic acid.

Results

Localization of the HCV E2 glycoprotein on the surface of the virion. HCV-like particles (55-65 nm) with fine 6 nm spikes were visualized in sucrose fractions at a density of 1.14-1.16 g/ml from samples A and B (13). The HCV-like particles of 55-65 nm in diameter showing specific gold labeling when treated with the rabbit polyclonal anti-HCV E2 antibody are shown in Fig. 2A and B. Antibody haloes and their specific binding to goat anti-rabbit IgG colloidal gold particles (10 nm) can be observed in samples A (Fig. 2A) and B (Fig. 2B) but not in sample C or D. Antibody haloes and specific gold labeling were not observed when the normal rabbit serum and the anti-vaccinia virus Lister strain serum was used as the primary antibody in samples A (Fig. 2C) and B. The mixture of five clones of anti-HCV E2 antibodies (159, 260, 305 and 1905) also produced specific gold labeling of an HCV-like particle (Fig. 2D and E). This type of specific reaction was confirmed in samples A (Fig. 2D) and B (Fig. 2E) but not in sample C or D. In Fig. 2D an HCV particle with a visible inner core structure of 35 nm in diameter was also labeled with staphylococcal Protein A conjugated gold

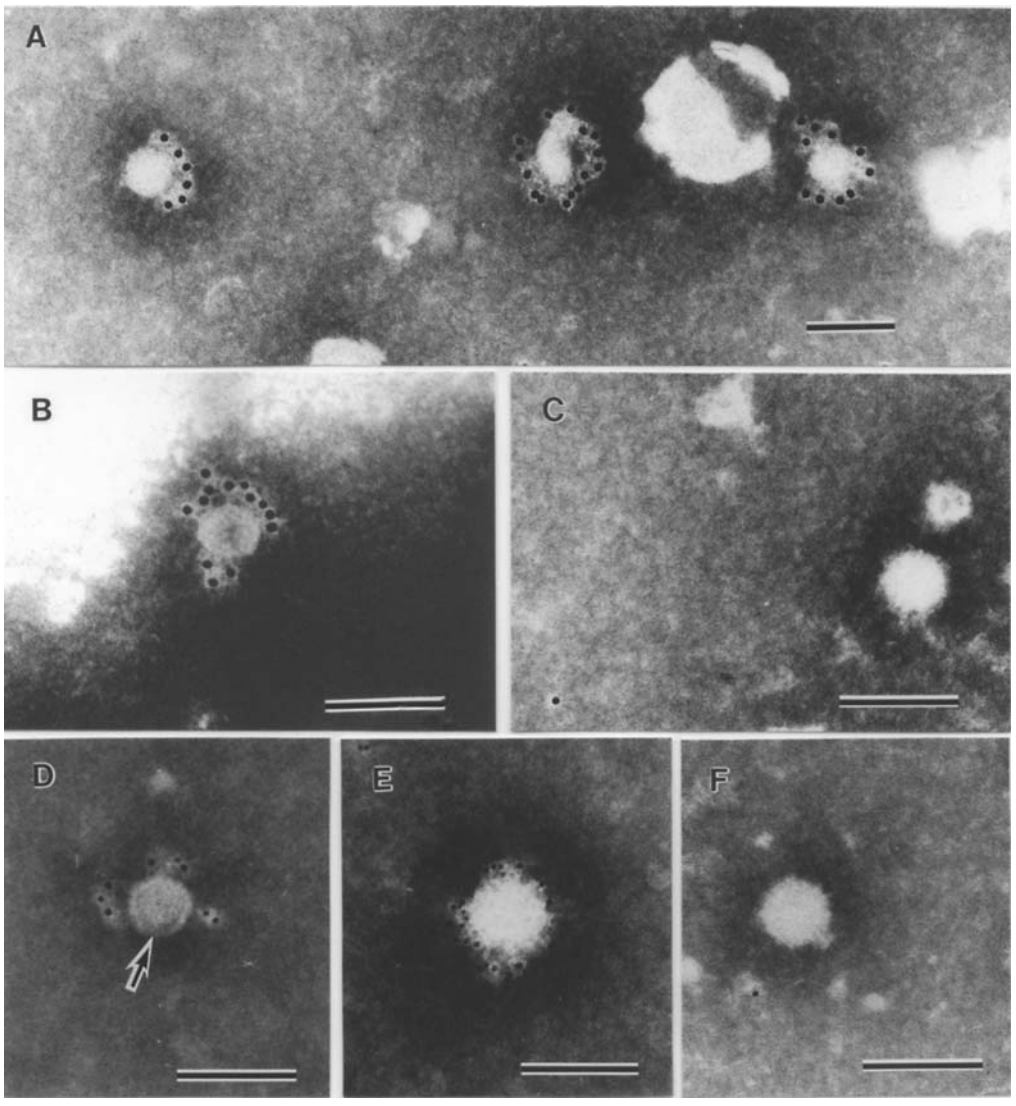

Figure 2. Immunogold electron micrographs of 55-65 nm HCV particles using rabbit polyclonal anti-HCV E2 antibody (RR6) and monoclonal anti-HCV E2 antibodies. HCV-like particles from sample A in (A), (C) and (E), and sample B in (B), (D) and (F) are shown. In (A) and (B), HCV-like particles were reacted with RR6 at a dilution of 1:100, and their antibody haloes were identified by binding to goat anti-rabbit IgG-conjugated colloidal gold particles (10 nm) at a dilution of 1:20. (C) Controls were prepared using rabbit polyclonal antibody to vaccinia virus Lister strain at a dilution of 1:100, following reaction with goat anti-rabbit IgG colloidal gold particles (10 nm) at a dilution of 1:20. In (D) and (E), HCV-like particles were reacted with a mixture of five monoclonal anti-HCV E2 antibodies, at a dilution of 1:10 and staphylococcal Protein A-conjugated colloidal gold particles (5 nm) at a dilution of 1:20. Specific labeling of gold particles on the surface of HCV-like particles can be noted. (D) An HCV-like particle with a visible inner core (indicated by an arrow) can be detected. (F) Controls were prepared using mouse monoclonal antibody to human blood type A antigen. The bars, 100 nm.

particles (5 nm). No specific gold labeling occurred in the presence of mouse monoclonal antibody to human blood type A antigen (Fig. 2F). The HCV-like particles specifically reacted with polyclonal and monoclonal antibodies to the HCV E2 glycoprotein.

Colocalization of the HCV E1 and E2 glycoproteins on the virion surface. When double labeling with different size colloidal gold was performed using monoclonal HCV E1 antibodies and the rabbit anti-HCV E2 polyclonal antibody (RR6), both staphylococcal Protein A-conjugated colloidal gold particles (5 nm) and goat anti-rabbit IgG colloidal gold particles (10 nm) were observed on the surface of the 55 nm HCV particle (Fig. 3A). Moreover, double labeling using the monoclonal HCV E2 antibodies and the rabbit anti-HCV E1 polyclonal antibody (RR2) also produced specific 5 nm and 10 nm gold labeling (Fig. 3B). Specificity of the immunogold labeling was confirmed using the mouse monoclonal antibody to the human blood type A antigen and protein A-conjugated colloidal gold particles (5 nm), and then using rabbit polyclonal antibody to vaccinia virus Lister strain and goat anti-rabbit IgG colloidal gold particles (10 nm) as negative controls, or by omitting the primary antibody. These negative control tests showed no specific gold labeling in 55-65 nm HCV-like particles with delicate surface spike-like projections (Fig. 3C). Both the HCV E1 and E2 glycoproteins colocalized on the surface of HCV virion.

Discussion

Recently, two *in vitro* HCV replication systems have been developed using an infectious HCV genotype 1b cDNA (23)

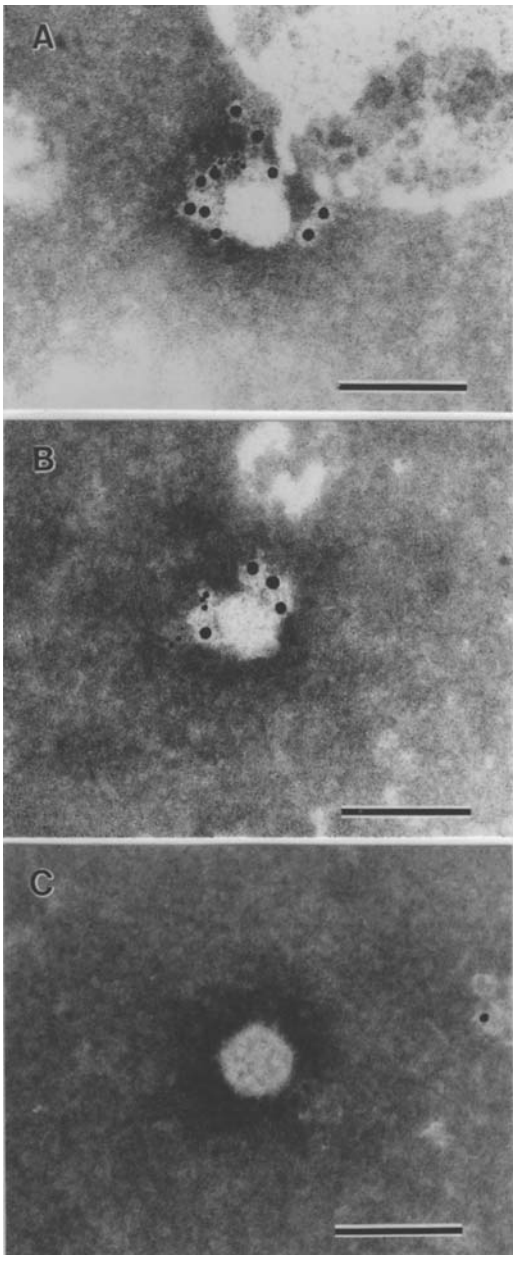

Figure 3. Immunogold electron micrographs of 55-65 nm HCV particles by double labeling with anti-HCV E1 and anti-HCV E2 antibodies. HCV-like particles from sample A are shown. (A) HCV-like particles were treated with a mixture of four monoclonal anti-HCV E1 antibodies at a dilution of 1:10 and staphylococcal Protein A-conjugated colloidal gold particles (5 nm) at a dilution of 1:20, and then with rabbit polyclonal anti-HCV E2 antibody (RR6); antibody haloes were identified by treating with goat anti-rabbit IgG-conjugated colloidal gold particles (10 nm) at a dilution of 1:20. (B) An HCV particle was detected using a mixture of five monoclonal anti-HCV E2 antibodies at a dilution of 1:10 and protein A-conjugated colloidal gold particles (5 nm) at a dilution of 1:20, and rabbit polyclonal anti-HCV E1 antibody (RR2) at a dilution of 1:100 and goat anti-rabbit IgG-conjugated colloidal gold particles (10 nm) at a dilution of 1:20. (C) Controls were prepared using mouse monoclonal antibody to human blood type A antigen at a dilution of 1:10, followed by treatment with Protein A-conjugated colloidal gold particles (5 nm) at a dilution of 1:20, treatment with rabbit polyclonal antibody to vaccinia virus Lister strain at a dilution of 1:100, and with goat anti-rabbit IgG colloidal gold particles (10 nm) at a dilution of 1:20. The bars, 100 nm.

and subgenomic replicons of the JFH1 genotype 2a strain (24). Heller *et al* (23) described an *in vitro* HCV replication system that is capable of producing viral particles in culture medium. A full-length HCV construct, CG1b of genotype 1b, known to be infectious (25), was placed between two ribozymes designed to generate the exact 5' and 3' ends of HCV after cleavage. After transfection into a human hepatoma cell line (Huh7), HCV-like particles, approximately 50 nm in diameter, increased in number and secreted into the culture medium. Sucrose density gradient centrifugation of the culture medium revealed colocalization of HCV RNA and structural proteins in a fraction at a density of 1.16 g/ml. HCV-like particles were observed in a fraction at a density of 1.16 g/ml. Wakita *et al* (24) developed subgenomic replicons of the HCV genotype 2a JFH1 strain cloned from an individual with fulminant hepatitis (26). After transfection into Huh7 cells, HCV particles, of approximately 55 nm in diameter, increased in number and secreted into the culture medium. These particles had a density of 1.15-1.17 g/ml in a 10-60% sucrose density gradient. HCV particles with an electron-dense inner core of 30-35 nm in diameter were identified by immunogold EM using monoclonal anti-HCV E2 antibody. Both studies showed that free HCV particles were about 55 nm spherical particles, had a density of about 1.16 g/ml and a 30-35 nm inner core. The morphology and the density of free HCV particles were quite consistent with our previous study (13). In Fig. 2, we demonstrate that the HCV E2 glycoprotein localized on the surface of 55-65 nm HCV particles containing a 30-35 nm inner core. The indirect immunogold electron microscopy detected immuno-gold-labeling in anti-HCV E2 antibody haloes surrounding the virions; haloes were produced by antigen-antibody reactions on the surface of virus-like particles, and they are good markers to discriminate HCV particles from other particles. In this way, the morphology of HCV virion can also be elucidated. Furthermore, our structural analysis of the HCV E1 and E2 envelope glycoproteins on HCV virions revealed that both HCV E1 and E2 envelope glycoproteins colocalize on the surface of virions in human plasma samples (Fig. 3).

The envelope glycoproteins have been shown to assemble into a noncovalent E1E2 heterodimer that is retained in the endoplasmic reticulum (27). This heterodimer is believed to be the prebudding form of an HCV glycoprotein complex (28). The E1E2 complex has been proposed as a functional subunit of HCV virions (11,12), and the E1E2 heterodimer is thought to be the functional unit of the HCV spike; low pH may induce dissociation leading to homooligomerization of the active form of the fusion protein (29,30). To date, it is known that both HCV E1 and E2 envelope glycoproteins are essential for receptor binding, host-cell entry, and membrane fusion (31,32). The HCV E2 glycoprotein also contains the binding site for CD81, a tetraspanin expressed on hepatocytes and B lymphocytes; CD81 is thought to function as a cellular receptor or coreceptor for the virus (33). Among the cellular factors mediating HCV entry into hepatocytes are tetraspanin CD81 (34-38), the human scavenger receptor SR-B1 (38,39), and probably the receptor for low density lipoproteins (40,41). However, the precise role of each receptor in HCV entry is still unclear and no vaccine is presently available. Further morphological studies on the binding site for CD81, the receptor SR-B1, and the receptor for low density lipoproteins of HCV virions from human plasma samples should be carried out.

In conclusion, both the HCV E1 and E2 glycoproteins are simultaneously present on the surface of the virion, providing definitive evidence that both the E1 and E2 envelope glycoproteins constitute the outer coat of fine spike-like projections of the HCV particle.

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