Apolipoprotein C1 Association with Hepatitis C Virus

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Received 2 May 2008/Accepted 22 July 2008

Accumulating evidence suggests that cellular lipoprotein components are involved in hepatitis C virus (HCV) morphogenesis, but the precise contribution of these components remains unclear. We investigated the involvement of apolipoprotein C1 (ApoC1) in HCV infection in the HCV pseudotyped particle system (HCVpp), in the recently developed cell culture infection model (HCVcc), and in authentic HCV isolated from viremic chimpanzees. Viral genomes associated with HCVcc or authentic HCV were efficiently immunoprecipitated by anti-ApoC1, demonstrating that ApoC1 was a normal component of HCV. The infectivities of HCVpp that had been mixed with ApoC1 and, more importantly, untreated HCVcc collected from lysates or media of infected HuH7.5 cells were directly neutralized by anti-ApoC1. Indeed, convalescent anti-HCV immunoglobulin G and anti-ApoC1 each neutralized over 75% of infectious HCVcc particles, indicating that many, if not all, infectious particles were recognized by both antibodies. Moreover, peptides corresponding to the C-terminal region of ApoC1 blocked infectivity of both HCVpp and HCVcc. Altogether, these results suggest that ApoC1 associates intracellularly via its C-terminal region with surface components of virions during viral morphogenesis and may play a major role in the replication cycle of HCV.

Hepatitis C virus (HCV) is a single-stranded, positive-sense RNA virus belonging to the Flaviviridae family. The HCV genome encodes a polyprotein that is co- and posttranslationally processed by host and viral proteases into at least 10 proteins, including 2 envelope glycoproteins, E1 and E2. The glycoproteins form heterodimers and are believed to be essential for HCV entry (32). Nevertheless, the mechanism by which HCV attaches to and enters the cells is not clear. For many viruses, entry into target cells is a multistep process that can involve the successive use of multiple attachment factors, receptors, and coreceptors (27). Several putative receptors have been proposed for HCV entry into cells: human tetraspanin CD81, tight junction component claudin 1, scavenger receptor class B type I, low-density lipoprotein (LDL) receptor, mannose binding lectins L-SIGN and DC-SIGN, asialoglycoprotein receptor, and glycosaminoglycans (GAGs) (3, 7, 10, 13).

Lipoproteins are synthesized mainly in the liver and intestines. HCV particles isolated from the plasma samples of HCV-infected patients and experimentally infected chimpanzees are associated with LDLs. LDL and HCV components are believed to form LDL-virus complexes, characterized by very-low-to-low buoyant density (1, 2, 23, 29, 30, 34). In addition, assays characterizing the recently developed consensus JFH-1 molecular HCV clone (HCVcc) (19, 33, 35) provided evidence that the infectious particles generated in vitro display sedimentation velocity and buoyant density profiles similar to those described for HCV particles isolated from the plasma samples of HCV-infected patients (15, 16). Moreover, it has been proposed that apolipoproteins E and B, components of lipoproteins, were associated with HCV particles and were closely involved in HCV morphogenesis (6, 9, 15, 16, 22).

HCV pseudotyped particles (HCVpp) contain the glycoproteins of HCV and are produced in 293T kidney cells that do not synthesize lipoproteins. We and others (20, 31) reported recently that high-density lipoprotein (HDL) was able to facilitate entry of HCVpp into cultured hepatoma cells. Several others have proposed that this enhancement of HCVpp infection involves a complex interplay between the hypervariable region of HCV E2 protein, scavenger receptor class B type I, and HDL (5, 31), even though a direct interaction between HCV envelope proteins and HDL could not be demonstrated (12, 31).

On the other hand, we showed that purified, exogenously supplied apolipoprotein C1 (ApoC1) alone enhanced HCVpp infection of HuH7 cells, and we proposed that ApoC1 plays a central role in the HDL-mediated enhancement of HCVpp infection (20). Dreux et al. (11) have since reported that ApoC1 is released from HDL by a triple interplay between the hypervariable region 1 of E2, HDL, and scavenger receptor B1 and that recruitment of the ApoC1 to the viral membrane promotes membrane fusion of HCV.

Apolipoprotein C1 is a water-soluble, 7.4-kDa protein associated mainly with HDL (>80%) but also found in LDL and very-low-density lipoprotein (VLDL). Several potential functions of this protein have been documented (18). ApoC1 can inhibit binding of VLDL to LDL receptor-related protein and is involved in the regulation of several lipases. ApoC1 accounts also for the ability of HDL to inhibit cholesterol ester transfer protein activity. In contrast, little is known about the regulation of ApoC1 synthesis, trafficking, secretion, and interaction with target cells. Here, we studied ApoC1 in the context of HCVpp, HCVcc, and circulating authentic HCV to discover whether

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† Published ahead of print on 30 July 2008.
ApoC1 is a biologically relevant component of hepatitis C virions.

MATERIALS AND METHODS

Abs used in this study. The anti-apoapolipoprotein C1 antibodies (Abs) were Biodesign International goat (K74110G) and rabbit (K74110R) polyclonal Abs and mouse (H11003M) monoclonal Ab, Abnova mouse (H0000341-M01) monoclonal Ab, and U.S. Biologicals mouse (A2299-5NB) monoclonal Ab. The anti-HCV core protein Ab was Anogen mouse (MO-I0015B) monoclonal Ab. The anti-HCV Ab consisted of immunoglobulin G (IgG) purified from chronic-phase serum of patient H infected with a genotype 1a strain of HCV.

Production of pseudotyped virus. Three million 293T cells were seeded in 100-mm flat culture dishes and allowed to adhere overnight. Cells were transfected with Lipofectamine Plus reagents (Invitrogen) per the supplied protocol. Briefly, a total of 4 μg of plasmid DNA, including 1.5 μg of a cytomegalovirus-Gag-Pol packaging construct, 1.5 μg of a murine leukemia virus-greengenesecent protein (GFP) plasmid, and 1 μg of an HCV E1E2 expression vector encoding one of six genotypes of HCV, was diluted in 250 μl of serum-free Dulbecco’s modified Eagle’s medium (DMEM) (4). Eight microliters of Plus reagent was added to the DNA solution and incubated at room temperature (RT) for 15 min. The mixture was then added dropwise to 250 μl of serum-free DMEM containing 12 μl of Lipofectamine reagent and incubated for 15 min. Cells were washed twice and covered with 2 ml of serum-free DMEM. The DNA-Lipofectamine mixture was added dropwise to the dishes, and cells were incubated at 37°C. Three hours later, cells were washed once and cultured in 7.5 ml of complete medium for 2 days. Transfection efficiency was consistently 80% or higher in baseline plate examination of GFP expression. Culture media were harvested and passed through a 0.45-mm filter to remove cells and debris. HCVpp infections were performed on the same day in order to avoid freezing-thawing of virus stocks. Focus-forming assays were performed in the absence of an agarose overlay since earlier tests established that the released virus remained localized so that at 2 to 3 days postinfection, the numbers of foci were the same with or without an overlay.

Pseudotyped virus infectivity assays. Huh7 cells were seeded at 4 × 10^4 cells/well in 24-well plates and cultured overnight. The next day, 100 μl of 293T cell supernatant containing HCVpp was added to 100 μl of 2× polybrene (Sigma) diluted in complete medium (final concentration, 4 μg/ml). Huh7 cell medium was reduced, and 200 μl of HCVpp plus polybrene was added to each well and incubated for 3 h to 6 h, with shaking every hour. The infection medium was then removed and replaced with 1 ml of complete medium. Three days later, medium was removed and cells were rinsed with 300 μl of phosphate-buffered saline (PBS; pH 7.4; Invitrogen), covered with 100 μl of trypsin solution (Bio-Whittaker), and incubated at 37°C for 30 min. Detached cells were transferred to a 1.5-ml Eppendorf tube, washed with 1 ml of PBS, resuspended in another 300 μl of PBS, and placed on ice. Twenty thousand cells were analyzed for GFP expression with a FACScan (BD).

Cell culture and HCV production. JFH1 viral stocks were produced as previously described (33, 34). Briefly, Huh7.5 human hepatoma cells (a generous gift from Charles Rice) were grown in DMEM (Celsior) supplemented with 10% fetal bovine serum. The plasmid pJFH1, containing the full-length cDNA of the JFH1 isolate (33), was linearized at the 3′ end of the HCV sequence by XbaI digestion, purified, and transcribed in vitro with the riboprobe system T7 (Promega) to generate HCV genomic RNA. Cells were transfected with the in vitro-transcribed RNA by using DMRIE-C transfection reagent (Invitrogen) as recommended by the manufacturer. Cel culture medium was collected 72 h after transfection, passed through a 0.45-mm filter, and used to infect naive Huh7.5 cells. Virus was adapted to grow in Huh7.5 cells by additional passages on naive Huh7.5 cells to produce a viral stock with a titer of 1 × 10^9 focus-forming units/ml.

Indirect immunofluorescence and cytofluorimetry. Huh7.5 cells seeded on eight-well chamber slides were washed in PBS and fixed in 100% acetone at RT. Cells were then washed two times with serum-free OptiMEM (Gibco), trypsinizing and pelleting the cells, and freezing the cell pellet on dry ice. Cells were lysed by adding 675 μl water, vortexing the mixture vigorously, and incubating at RT for 15 min, after which 67.5 μl 10X PBS was added, the mixture was centrifuged at 16,100 × g for 2 min, and the clarified supernatant was used as the source of virions.

Chimeric genotype 1a viruses in medium from infected Huh7.5 cells or plasma samples from infected chimpes were preadsorbed with GammaBind Plus Sepharose beads to remove IgG prior to the addition of Abs. The plasma was collected from the chimpanzees early in the acute phase of infection, before Abs to HCV were generated.

The indicated amounts of Abs were added to 100-μl plasma samples from experimentally infected chimpanzees or HCVcc particles in medium or lysate from infected Huh7.5 cells and incubated for 1 h at 37°C and then overnight at 4°C. A 60-μl 100-μl slurry of washed GammaBind Plus beads was added, and the mixture was incubated for 2 h at RT. The beads were washed three times in PBS, and the immunoprecipitated HCVcc RNA was extracted with Trizol and then detected by quantitative reverse transcription-PCR (RT-PCR; TaqMan).

Western blotting. After separation by SDS-polyacrylamide gel electrophoresis (PAGE), protein preparations were electrotherophoretically transferred to nitrocellulose membranes (Invitrogen) with an Xcel 2 blot apparatus (Invitrogen) and membranes were incubated with chimpemize anti-E2 monoclonal Ab followed by goat anti-human IgG conjugated to peroxidase (Pierce). E2 was detected by enhanced chemiluminescence (Pierce) as recommended by the manufacturer.

RESULTS

Enhancement of infection and neutralization of all six genotypes of HCVpp via ApoC1. Both HDL and human sera are able to enhance infectivity of HCVpp that contain the glycoproteins of HCV but not those that contain the glycoproteins of other viruses (5, 20, 31). We previously demonstrated that genotype 1a HCVpp infectivity is enhanced by ApoC1 (20). HCVpp are produced in 293T kidney cells which do not contain ApoC1. Since enhancement is specific for HCV glycoproteins and since the sequences of HCV glycoproteins vary widely among genotypes, we determined whether the ApoC1 effect is limited to selected genotypes of HCV or whether ApoC1 can broadly enhance the infectivity of HCVpp of all six genotypes.

Preincubation of HCVpp with ApoC1 resulted in a modest, but consistent, enhancement of infection for each of the six
genotypes (Fig. 1A). In contrast, infection by control pseudoparticles containing the RD114 feline endogenous virus glycoprotein was not enhanced by preincubation with ApoC1. It was reported recently that ApoC1 interacts directly with HCVpp (11). Therefore, it seemed possible that Abs to ApoC1 (anti-ApoC1) might neutralize the virus and perhaps would abrogate only the observed enhancing activity. Of note, HCVpp exposed to anti-ApoC1 in the absence of exogenous ApoC1 were not neutralized. In contrast, the addition of anti-ApoC1 to HCVpp of all six genotypes preincubated with ApoC1 not only prevented the enhancement but also neutralized the majority of virus present in each case (Fig. 1B). Moreover, a similar inhibition by anti-ApoC1 was observed when HCVpp were preincubated with HDL and, in addition, the enhancement by HDL was strongly suppressed when anti-ApoC1 and HDL were mixed prior to incubation with HCVpp (data not shown). Therefore, ApoC1 plays a critical role in the enhancing activity of HDL. As reported previously, infectivity of control RD114 pseudoparticles was not enhanced by preincubation with ApoC1; consistent with this result, these particles were detected by FACS analysis of GFP expression 3 days later. Percentage of enhancement or inhibition was calculated by comparison with results for HCVpp alone. Results are representative of two independent experiments.

Coimmunoprecipitation of HCV envelope glycoproteins and ApoC1. Coimmunoprecipitation assays were performed to confirm the association of ApoC1 with HCV envelope glycoproteins. 293T cells (lacking ApoC1) were transfected with the same E1E2 plasmid used for genotype 1a HCVpp generation and lysed after 2 days. The lysate was preincubated or not preincubated with ApoC1 and then immunoprecipitated with human monoclonal anti-E2 (Fig. 2A). Coimmunoprecipitation of E2 was observed and shown to depend on the presence of ApoC1 (Fig. 2A, lane 2). The same result was obtained in an independent assay (Fig. 2A, lane 3). Note that E2 detected in the cell lysate by the monoclonal Ab prior to immunoprecipitation (Fig. 2A, lane 7) displayed a diffuse migration pattern. This is likely due to detection of multiple glycoforms of E2, i.e., completely or partially glycosylated E2 proteins. Interestingly, following immunoprecipitation by anti-ApoC1 (Fig. 2A, lanes 2 and 3), the same monoclonal anti-E2 Ab detected only one glycoform corresponding to the fully glycosylated E2. It is interesting to note that in a similar ApoC1 coimmunoprecipitation experiment (Fig. 2B, lane 1) both HCV glycoproteins E1 and E2 were detected by immunoblotting. These results suggest that ApoC1 interacted selectively with HCV E1E2 glycoprotein complexes in which E2 was fully glycosylated. In order to
extend this result to HCV envelope glycoproteins associated with HCVpp, the experiment described above was repeated with concentrated HCVpp. The concentrated HCVpp was pre-incubated with ApoC1 and then immunoprecipitated with anti-ApoC1. The immunoprecipitates were separated by SDSPAGE, followed by immunoblot analysis with human monoclonal anti-E2 and mouse monoclonal anti-E1 (Fig. 2B, lane 2). We observed the coimmunoprecipitation of both E1 and E2 HCV glycoproteins. 293T cells do not produce ApoC1; thus, the coimmunoprecipitation of HCVpp could happen only through exogenously added ApoC1. Of note, the same experiment performed on control viral particles harboring the glycoproteins of RD114 (RD114pp) or VSV (VSV-Gpp) did not reveal any coimmunoprecipitation by anti-ApoC1 of these glycoproteins (data not shown). These data confirm that, directly or through an unknown partner, ApoC1 can specifically bind to HCV envelope glycoproteins. 293T cells do not produce ApoC1; thus, we extend this result to HCV envelope glycoproteins associated with HCVpp. The concentrated HCVpp was pre-incubated with ApoC1 and then immunoprecipitated with anti-ApoC1. The immunoprecipitates were separated by SDS-PAGE, followed by immunoblot analysis with human monoclonal anti-E2 and mouse monoclonal anti-E1 (Fig. 2B, lane 2). We observed the coimmunoprecipitation of both E1 and E2 HCV glycoproteins. 293T cells do not produce ApoC1; thus, the coimmunoprecipitation of HCVpp could happen only through exogenously added ApoC1. Of note, the same experiment performed on control viral particles harboring the glycoproteins of RD114 (RD114pp) or VSV (VSV-Gpp) did not reveal any coimmunoprecipitation by anti-ApoC1 of these glycoproteins (data not shown). These data confirm that, directly or through an unknown partner, ApoC1 can specifically bind to HCV envelope glycoproteins. 293T cells do not produce ApoC1; thus, we extend this result to HCV envelope glycoproteins associated with HCVpp. The concentrated HCVpp was pre-incubated with ApoC1 and then immunoprecipitated with anti-ApoC1. The immunoprecipitates were separated by SDS-PAGE, followed by immunoblot analysis with human monoclonal anti-E2 and mouse monoclonal anti-E1 (Fig. 2B, lane 2). We observed the coimmunoprecipitation of both E1 and E2 HCV glycoproteins. 293T cells do not produce ApoC1; thus, the coimmunoprecipitation of HCVpp could happen only through exogenously added ApoC1. Of note, the same experiment performed on control viral particles harboring the glycoproteins of RD114 (RD114pp) or VSV (VSV-Gpp) did not reveal any coimmunoprecipitation by anti-ApoC1 of these glycoproteins (data not shown). These data confirm that, directly or through an unknown partner, ApoC1 can specifically bind to HCV envelope glycoproteins. 293T cells do not produce ApoC1; thus, we extend this result to HCV envelope glycoproteins associated with HCVpp. The concentrated HCVpp was pre-incubated with ApoC1 and then immunoprecipitated with anti-ApoC1. The immunoprecipitates were separated by SDS-PAGE, followed by immunoblot analysis with human monoclonal anti-E2 and mouse monoclonal anti-E1 (Fig. 2B, lane 2). We observed the coimmunoprecipitation of both E1 and E2 HCV glycoproteins. 293T cells do not produce ApoC1; thus, the coimmunoprecipitation of HCVpp could happen only through exogenously added ApoC1. Of note, the same experiment performed on control viral particles harboring the glycoproteins of RD114 (RD114pp) or VSV (VSV-Gpp) did not reveal any coimmunoprecipitation by anti-ApoC1 of these glycoproteins (data not shown). These data confirm that, directly or through an unknown partner, ApoC1 can specifically bind to HCV envelope glycoproteins. 293T cells do not produce ApoC1; thus, we extend this result to HCV envelope glycoproteins associated with HCVpp. The concentrated HCVpp was pre-incubated with ApoC1 and then immunoprecipitated with anti-ApoC1. The immunoprecipitates were separated by SDS

FIG. 3. ApoC1 is associated with HCVcc. (A) Immunoprecipitation of HCV RNA in 100 µl of the cell lysate and 100 µl of medium from JFH1-infected Hu7.5 cells was performed in triplicate with 20 µg of goat anti-apoC1 Ab. Immunoprecipitated HCV RNA yield was then evaluated by quantitative RT-PCR (TaqMan). Irrelevant goat IgG (Zymed) was used as a negative control. (B) Intracellular HCVcc, extracellular HCVcc, and metapneumovirus particles were incubated with 5 µg of irrelevant mouse IgG (panels a, c, and e, respectively) or 5 µg of mouse anti-ApoC1 (panels b, d, and f, respectively) for 1 h at 37°C and then inoculated onto Hu7.5 cells. Three days later, infected cells were detected by immunofluorescence microscopy after staining with a mouse anti-HCV core protein Ab (Anogen) or with an anti-metapneumovirus rabbit polyclonal Ab. (C) Triplicate samples of extracellular HCVcc (solid bars) or intracellular HCV (open bars) were incubated with 5 µg or 10 µg of Biodesign polyclonal rabbit anti-ApoC1 before inoculation onto cells. Three days later, cells were stained with anti-HCV core protein Ab and viral foci were manually counted under code. The percentage of inhibition was calculated by comparison with levels for the IgG-treated controls.
both the extracellular and the intracellular viruses were intimately associated with ApoC1 (Fig. 3C). The reason for the difference in degree of neutralization of HCVcc particles from the two compartments is unknown and could be as trivial as a difference in degree of neutralization of HCVcc particles from a patient serum sample (serum H), or irrelevant IgG. The immune complexes were collected on beads, and the amount of infectious virus remaining in the supernatant was quantified by a focus-forming assay to determine the percentage of infectious virus precipitated.

**Both anti-ApoC1 and anti-HCV preferentially recognize infectious virions.** Immunoprecipitation studies were performed to compare the reactivity of anti-ApoC1 with that of anti-HCV from a hepatitis C patient. Since the patient was infected with the H77 genotype 1a strain, approximately 1.7% of available genomes, respectively (Fig. 4A). Irrelevant goat IgG was used as a negative control.

The immune complexes were collected on beads, and the amount of virus bound to the beads and the amount of unbound virus remaining in the supernatant were quantified by a TaqMan assay for viral genomes: unbound virus in the supernatant was tested also for infectivity. Over 10^5 HCV genomes were precipitated in a dose-dependent manner by goat anti-ApoC1. Comparison of the total number of genomes with that bound to beads indicated that, on average, 20 µg of goat anti-ApoC1 and 20 µg of anti-HCV IgG precipitated 21% and 8% of available genomes, respectively (Fig. 4A). Irrelevant IgG precipitated approximately 1% (data not shown). These results demonstrated that a significant proportion of HCVcc was tightly associated with ApoC1, but since fewer than 50% of the genomes were precipitated by either Ab, the results did not reveal whether anti-ApoC1 and anti-HCV recognized the same set of particles.

The amount of infectious, unprecipitated virus remaining in the supernatant was determined by focus-forming assays with Huh7.5 cells. The results for these assays were very interesting. Twenty micrograms of anti-ApoC1 decreased the amount of infectious virus by 77%, and patient anti-HCV IgG decreased it by 87% (Fig. 4B), compared to the level for the irrelevant control. Since the combined amount of virus precipitated from identical aliquots was 164% (77% plus 87%) and thus greater than the 100% present in the aliquots, at a minimum, the excess amount of precipitated virus (between 27% and 37% of the virus) had to be recognized by both Abs. Also, since the percentage of infectious virus precipitated was much higher than that for viral genomes precipitated, the data suggest that anti-ApoC1 and serum anti-HCV each preferentially precipitated virions that were infectious.

**Authentic HCV particles are associated with ApoC1 in vivo.** It was important to determine if the results from cell culture experiments were relevant in vivo. Therefore, immunoprecipitation tests were performed on early-acute-phase viremic plasma samples collected from chimpanzees that had been experimentally infected with a genotype 1a monoclonal HCV. Chimpanzee 1530 had been infected with the wild-type virus, and chimpanzee 96A008 had been infected with the same virus lacking hypervariable region 1 of the E2 glycoprotein; another plasma contained Abs to HCV. Controls consisting of irrelevant goat IgG were run in sets of four, and tests of anti-ApoC1 were run in sets of five. As shown by the error bars in Fig. 5, the levels of HCV RNA were repeatedly at least 20-fold greater in samples precipitated with anti-ApoC1 than in those precipitated with the irrelevant IgG. A lower number of genomes were precipitated in this assay than in the one represented in Fig. 4, reflecting the fact that there were 70 to 85 times more genomes in the cell culture samples than in the chimpanzee plasma samples (data not shown). Indeed, calculations revealed that 20 µg of anti-ApoC1 immunoprecipitated 14.7% and 20.4% of the HCV genomes in chimpanzee 1530 and chimpanzee 96A008 plasma samples, respectively, compared to the 0.6% precipitated from each plasma sample by the control IgG. Therefore, the percentages of authentic virions immunoprecipitated from chimpanzee plasma and of HCVcc
immunoprecipitated from cell culture medium were surpris-ingly similar (14 to 20% versus 27%, respectively). Unfortu-nately, the viruses in the chimpanzee plasma samples do not grow in cell culture, so precipitation of infectious virus could not be studied. However, the demonstration that a consider-able portion of HCV particles circulating in vivo are associated with ApoC1 suggests that ApoC1 is an important component of the normal HCV replication cycle.

**Apolipoprotein C1 interacts with Huh7 cells via HSPG.**

Since both HCVpp and HCVcc can form stable complexes with ApoC1 and Ab against ApoC1 can prevent these complexes from infecting Huh7 cells (Fig. 1 and 3), it is reasonable to postulate that ApoC1 binds to Huh7 cells and that this binding was impaired by anti-ApoC1. To date, it is not known if or how ApoC1 interacts with liver cells. On the other hand, interaction of apolipoprotein E (ApoE) with several types of cells has been investigated extensively (8, 14). ApoE initially interacts with heparan sulfate proteoglycans (HSPG) on the cell surface and is then transferred to the LDL receptor-related protein for internalization in a process known as the HSPG–LDL receptor-related protein pathway. Here, we sought to determine whether ApoC1 also interacts with hepatoma cells via HSPG. Intact Huh7 cells were first incubated with purified ApoC1 and then with an anti-ApoC1. A high level of binding of ApoC1 to the cell surface was detected by fluorescence-activated cell sorting (FACS) analysis (Fig. 6A). To test if ApoC1 binds to the cell surface via proteoglycans, we mixed heparin, a soluble GAG, with ApoC1 prior to incuba-tion with Huh7 cells. Coincubation of heparin and ApoC1 resulted in a marked inhibition of binding of ApoC1 to cells (Fig. 6B). To investigate whether binding of ApoC1 to the cell surface was mediated directly through GAGs, Huh7 cells were treated with a specific GAG lyase, heparinase I, which de-grades heparin and highly sulfated domains in heparan sulfate. Binding of ApoC1 to cells was clearly reduced following pre-treatment of Huh7 cells with heparinase I (Fig. 6C). To determine whether GAG sulfation is required for ApoC1 binding, we cultured Huh7 cells in the presence of sodium chloride, an inhibitor of sulfation. Incubation of Huh7 cells with sodium chloride for 24 h abolished ApoC1 binding (Fig. 6D). The specificity of this effect was confirmed by supplementing the sodium chloride-treated cells with an excess of sodium sulfate, which resulted in restoration of ApoC1 binding (data not shown). These results clearly demonstrated that ApoC1 inter-acts with proteoglycans of Huh7 cells via highly sulfated hepa-ran sulfate.

**The ApoC1 C-terminal region is responsible for the coimmunoprecipitation of envelope proteins and for the interaction with HSPG.**

To determine the specific regions of ApoC1 that interact with HSPG and envelope proteins (either directly or indirectly), overlapping peptides based on the ApoC1 se-quence (Fig. 7A) were synthesized and evaluated for their effects on HCVpp infection of HuH7 cells.

First, the region of ApoC1 that binds to cellular HSPG was de termined. Huh7 cells were exposed to 200 pmol of each ApoC1 peptide and then infected with HCVpp or with HCVpp preincubated with purified ApoC1 protein. FACS for detection of GFP demonstrated that infection by HCVpp alone was not significantly modified by any of the ApoC1 peptides tested (Fig. 7B, panel a). In contrast, peptides 7, 8, and 12 signifi-cantly inhibited infection with HCVpp that had been preincu-bated with purified ApoC1 protein (Fig. 7B, panel b), suggest-
ing that the C-terminal region of ApoC1 is responsible for the interaction with cellular HSPG. In an attempt to confirm this hypothesis, binding of ApoC1 peptides to cells was monitored by FACS. Five different monoclonal or polyclonal anti-ApoC1 Abs were tested for their abilities to detect the peptides bound to cells. Only one of these, rabbit polyclonal Ab (Biodesign), reacted: it detected the relatively efficient binding of peptide 7 to Huh7 cells (Fig. 7C, panel a). Cell surface binding of peptide 7 was completely abolished by pretreatment of the cells with heparinase, consistent with the results shown in Fig. 6C (data not shown). Peptide 8 appeared to bind less well (Fig. 7C, panel b), peptides 6 and 12 were not tested, and binding of peptides 4 and 5 to cells could not be detected with any of the five Abs (data not shown); these negative results with peptides 4 and 5 could reflect a failure of the Abs to react with these peptides, but given the results shown in Fig. 7B, it is more likely that these peptides did not bind to the cells. Certainly, all the data are consistent with the conclusion that the C-terminal
region of ApoC1, and more likely a region within peptide 7, binds to cellular HSPG.

A related strategy was used to determine the domain of ApoC1 that bound to the HCVpp. HCVpp were preincubated with individual ApoC1 peptides and then exposed or not exposed to rabbit polyclonal anti-ApoC1 (Biodesign) before infection of HuH7 cells. When HCVpp was incubated with peptides only, inhibitory activity was minimal (Fig. 7D, panel a). In contrast, after subsequent incubation with anti-ApoC1, HCVpp infectivity was strongly inhibited by exposure to peptide 12, and to a lesser extent by peptide 8, but, surprisingly, not by peptide 7 (Fig. 7D, panel b). Consistent with these results, preincubation of HCVpp with peptide 8 strongly inhibited the enhancement of infection by HDL also (data not shown). In toto, these results suggested that the C-terminal half of ApoC1 binds both to HSPG and to HCV envelope proteins and that peptide 7 contains the region for binding to HSPG whereas peptides 8 and 12 contain the region for binding HCV envelope proteins. Nevertheless, one cannot exclude that association of ApoC1 and envelope protein occurs via an unknown partner. Further studies are required to resolve this point.

C-terminal peptides of ApoC1 inhibit HCVcc infection of HuH7.5 cells. In order to determine the region of ApoC1 involved in inhibition of HCVcc infections (Fig. 3), we evaluated the effects of selected ApoC1 peptides on infection by HCVcc. HuH7.5 cells were exposed to individual ApoC1 peptides 4, 7, and 12 and then incubated with HCVcc that had not been preincubated with exogenous ApoC1. After 3 days of incubation, cells were stained with anti-HCV core protein Ab (Fig. 8A) and HCVcc foci were counted (Fig. 8B). Peptide 12 strongly inhibited HCVcc infection of HuH7.5 cells compared to what was found for HCVcc alone, while peptide 7 demonstrated only a moderate inhibitory effect. In contrast, HCVcc infection was apparently not appreciably influenced by peptide 4. These results confirmed those obtained with the HCVpp system and established that ApoC1, through its C-terminal half, played a key role in HCV infectivity.

DISCUSSION

Since the discovery that HCV circulating in blood was associated with lipoproteins, considerable effort has been expended to determine why. Immunoprecipitation and/or neutralization by anti-ApoC1 of all six genotypes of HCVpp, two genotypes of HCVcc, and two plasma isolates of HCV from viremic chimpanzees, as reported here, demonstrated that ApoC1 is already associated with circulating virions and suggest that this association occurs intracellularly.

We previously reported that HDL, through its association with ApoC1, was able to enhance infectivity of HCVpp. Recently, Dreux et al. (11) suggested that HDL is central to infectivity of HCV and that ApoC1 in HDL is transferred indirectly from HDL to HCVpp or HCVcc particles when they bind to cells: ApoC1 was shown to promote membrane fusion in a liposome system, leading to the proposal that infection of cells required a triple interplay between hypervariable region 1 of the E2 glycoprotein, HDL, and scavenger receptor Bl. This putative mechanism is at odds with our results demonstrating that ApoC1 associates with newly produced HCVcc prior to release from cells, that ApoC1 remains associated with extracellular HCVcc, and, more importantly, that in the chimpanzee model, ApoC1 is already associated with circulating virions prior to their attachment to cells. Although the HCVpp system has proven invaluable for many studies, it suffers from the fact that HCVpp are made in nonhepatic 293T kidney cells which do not synthesize lipoproteins. Moreover, HCVpp consist mainly of non-HCV components and are released from cells by an unconventional pathway. Production of HCVcc, in contrast, occurs in hepatic cells, the natural host for HCV, and the infectious particles are produced by a pathway that is thought to mimic the pathway used in vivo. For that reason, the HCVcc system may provide a more accurate picture of HCV interactions with host components.

The association of LDL and VLDL with HCV was discovered by analyzing virions collected from a patient (2, 22). Immunoprecipitation of these in vivo complexes indicated that they contained apolipoproteins B and E, but tests for C were apparently not performed. Studies with HCVcc strongly support the conclusion that VLDL is associated with infectious virions and suggest that this association occurs intracellularly.
during virus maturation and indeed is required for virus release (15, 16).

Huang et al. (17) demonstrated that HCV replication complexes in Hu7 cells contained proteins involved in lipid metabolism, including Apo E and Apo B, and that showed that inhibition of VLDL secretion by Apo B small interfering RNA or by an inhibition of microsomal triglyceride transfer protein inhibited the release of infectious HCV particles into the medium. Chang et al. (6) demonstrated that infectivity of HCVccc was positively correlated with level of Apo E and that Apo E small interfering RNA inhibited virus release. Finally, Gasta-

minza et al. (15, 16) reaffirmed that HCV coopts the VLDL pathway as its means for egress. It is important to note that ApoC1, like Apo B and Apo E, is a component of VLDL and represents about 5% of the total protein.

Secretion of HCV via the VLDL pathway is consistent with our detection of ApoC1-HCVccc complexes prior to virion release from cells. At present, it is not known if the ApoC1 associated with HCV within the cell has a specific function or whether the ApoC1 so acquired performs a function downstream. Our immunoprecipitation studies suggest that ApoC1 may have a role in infectivity of released virions. Quantitative comparisons of precipitated viral genomes versus infectious virions suggest that both anti-ApoC1 and polyclonal anti-HCV reacted preferentially with infectious virions (Fig. 4). Anti-HCV displayed a distinct preference for infectious virions since it precipitated only 8% of the total HCVccc present but that 8% included 87% of the infectious virus. In the same experiment, anti-ApoC1 precipitated 21% of the total HCV, which included 77% of the infectious particles. Given the high proportion of infectious virions precipitated by each Ab, it is reasonable to assume that the same particles were recognized by both Abs, consistent with a role for ApoC1 in infectivity. This conclusion was supported by the almost total neutralization of either intracellular or extracellular HCV by anti-ApoC1 (Fig. 4).

Binding studies demonstrated that purified ApoC1 binds to cells, specifically to HSPG via heparan sulfate (Fig. 6), a molecule previously implicated in the entry of HCVccc into Hu7 cells (21). This cell surface molecule serves as a docking site for a great number of pathogens (24, 28). Peptide binding studies determined that the C-terminal half of ApoC1 was the critical region. The C-terminal region was sufficient to inhibit infection by HCVpp (Fig. 7) or HCVccc (Fig. 8), presumably by competing for binding. Peptides from the C-terminal region were also able to bind to HCVpp and render them susceptible to neutralization by anti-ApoC1 (Fig. 7). Thus, the C-terminal domain of ApoC1 may interact with various partners, such as heparan sulfates or HCV envelope proteins. According to Rozek et al. (25, 26), ApoC1 forms two helices linked by an unstructured region. One can propose that, as shown for other apolipoproteins, distinct domains of the ApoC1 C-terminal amphipathic alpha-helix perform specific roles, such as interaction with lipids and binding to heparan sulfate or to HCV envelope proteins. Alternatively, interaction with ApoC1 ligands could be temporally regulated and may occur in a sequential manner. Further investigation will be needed to decipher ApoC1 structure-function relationships, particularly for its interaction with other proteins.

The ApoC1 protein associated with HCV could function solely as a facilitator of binding to cells, or it could perform other functions in addition. Although, as discussed above, it seems unlikely that HDL is the source of ApoC1 in HCV complexes, the demonstration by Drex et al. (11) of ApoC1-enhanced fusion of HCV with liposomes is intriguing and needs to be studied further. The involvement of the HVR1 as discussed previously (11, 12) may be relevant for the actual process of fusion, but it is clear from immunoprecipitation studies with the chimpanzee 96A008-derived HVR1 deletion mutant that ApoC1 associates stably with HCV even in the absence of HVR1.

The lack of anti-ApoC1 neutralization of RD114 (Fig. 1) and metapneumovirus (Fig. 3) suggests that it is the HCV glycoprotein(s) that drives the reaction with ApoC1. This is consistent with the demonstration that anti-ApoC1 coprecipitated E1 and E2 proteins from an E1E2-expressing cell lysate and from concentrated HCVpp (Fig. 2A and B) but not RD114 and VSV-G proteins from concentrated RD114pp and VSV-Gpp control particles. It is interesting to note that a single E2 glycoform was selected through coimmunoprecipitation with ApoC1 (Fig. 2A). If ApoC1 is involved in HCV morphogenesis, this mechanism of mature E2 subset selection may act as an additional quality control during formation of particles.

Since the ApoC family of proteins has a high affinity for lipid surfaces (for a review, see reference 18), perhaps the interaction of HCV glycoproteins with ApoC1 triggers association of HCV with cellular lipoproteins that aid in transporting virions out of cells: later in the virus replication cycle, the ApoC1 associated with the extracellular virions may aid the virus in entry into cells.

ACKNOWLEDGMENTS

This work was supported by the Intramural Research Program of the National Institutes of Health, National Institute of Allergy and Infectious Diseases.

We thank Takaji Wakita (National Institute of Infectious Diseases, Tokyo, Japan) for kindly providing the infectious molecular clone of JFH1, Stanley Lemon (University of Texas Medical Branch, Galveston, TX) for kindly providing the H77/JFH1 HCVcc chimera, and Harry Greenberg (Stanford University, Stanford, CA) for providing the A4 monoclonal Ab.

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