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Apolipoprotein(a) Inhibits Hepatitis C Virus Entry Through Interaction With Infectious Particles

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The development of different cell culture models has greatly contributed to increased understanding of the hepatitis C virus (HCV) life cycle. However, it is still challenging to grow HCV clinical isolates in cell culture. If overcome, this would open new perspectives to study HCV biology, including drug-resistant variants emerging with new antiviral therapies. In this study we hypothesized that this hurdle could be due to the presence of inhibitory factors in patient serum. Combining polyethylene glycol precipitation, iodixanol gradient, and size-exclusion chromatography, we obtained from HCV-seronegative sera a purified fraction enriched in inhibitory factors. Mass spectrometric analysis identified apolipoprotein(a) (apo[a]) as a potential inhibitor of HCV entry. Apo(a) consists of 10 kringle IV domains (KIVs), one kringle V domain, and an inactive protease domain. The 10 KIVs are present in a single copy with the exception of KIV type 2 (KIV_2), which is encoded in a variable number of tandemly repeated copies, giving rise to numerous apo(a) size isoforms. In addition, apo(a) covalently links to the apolipoprotein B component of a low-density lipoprotein through a disulfide bridge to form lipoprotein(a). Using a recombinant virus derived from the JFH1 strain, we confirmed that plasma-derived and recombinant lipoprotein(a) as well as purified recombinant apo(a) variants were able to specifically inhibit HCV by interacting with infectious particles. Our results also suggest that small isoforms are less inhibitory than the large ones. Finally, we observed that the lipoprotein moiety of HCV lipoviroparticles was essential for inhibition, whereas functional lysine-binding sites in KIV_7, KIV_8, and KIV_{10} were not required. *Conclusions*: Our results identify apo(a) as an additional component of the lipid metabolism modulating HCV infection. (HEPATOLOGY 2017;65:1851-1864)

The hepatitis C virus (HCV) is a small positive single-stranded RNA virus that belongs to the *Hepacivirus* genus in the Flaviviridae family and causes serious liver diseases in humans.[1] In the last two decades, the development of different cell culture models such as replicons, retroviral particles pseudotyped with HCV E1E2 envelope proteins (HCVpp), and recombinant viruses derived from JFH1 cell culture (HCVcc), has greatly contributed to increasing our knowledge about the HCV life cycle.[2,3] However, it is still challenging to amplify HCV clinical isolates across different genotypes in tissue culture. This would open new perspectives to investigate viral determinants responsible for the evolution and treatment outcome of

Abbreviations: apo, apolipoprotein; DMEM, Dulbecco's modified Eagle's medium; GFP, green fluorescent protein; HCV, hepatitis C virus; HCVcc, HCV produced in cell culture; HCVpp, HCV pseudoparticle; KIV, kringle IV-like domain; LBS, lysine-binding site; LDL, low-density lipoprotein; Lp(a), lipoprotein(a); MAb, monoclonal antibody; MLVpp, retroviral particle pseudotyped with the murine leukemia virus envelope protein; PBS, phosphate-buffered saline; PEG, polyethylene glycol; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SGIG, self-generated iodixanol gradient; VLDL, very low-density lipoprotein; VSVpp, retroviral particle pseudotyped with the vesicular stomatitis virus glycoprotein G; WGA, wheat germ agglutinin.

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hepatitis C. It could also be utilized in studies of resistance to new direct-acting antivirals and in the development of a vaccine.

A striking feature of HCV particles is their association with lipoproteins.[4-6] The nature of the association between HCV virions and lipoproteins remains undetermined. However, the characterization of HCVcc indicates that their lipid composition resembles that of very low-density lipoproteins (VLDLs) and low-density lipoproteins (LDLs),[7] and it has been suggested that HCV particles could be a hybrid structure composed of a virion moiety and a lipoprotein moiety, so-called lipoviroparticles.[4] Nevertheless, alternative models have also suggested that lipoproteins could associate with viral particles through the interaction between apolipoproteins and HCV envelope lipids or proteins.[5] In line with this association, several studies have shown that apolipoproteins such as apoE, apoB, apoA1, apoC1, apoC2, and apoC3 are associated with HCV particles and play a critical role in HCV entry (for review, see Douam et al.[6]).

Apo(a) is an apolipoprotein synthesized by the liver, which is found only in humans, nonhuman primates, and Old World monkeys.[8] In humans, apo(a) consists of 10 types of kringle IV-like domains (KIV), one kringle V-like domain, and an inactive protease-like domain, which are highly homologous to plasminogen domains.[8] Each of the 10 apo(a) KIV domains is present in a single copy with the exception of KIV type 2 (KIV_2), which is encoded in a variable number of tandemly repeated copies by the apo(a) gene, ranging from 3 to > 40 and gives rise to several apo(a) size isoforms with molecular masses between ~200 and ~800 kDa.[8] In human serum, apo(a) covalently links to the apoB component of LDL through a disulfide bridge to form lipoprotein(a) (Lp[a]).[9] An inverse correlation between the size of apo(a) isoforms and the serum levels of Lp(a) (ranging from < 1 to > 100 mg/dL) has been described,[10] and elevated plasma concentrations of Lp(a) have been identified as a risk factor for vascular diseases such as peripheral arterial disease, ischemic stroke, and coronary heart disease.[9] It has also been shown that Lp(a) is a preferential carrier of oxidized phospholipids in plasma.[11]

In this study, we demonstrated that human serum contains restriction factors that inhibit HCVcc infection, which could explain the difficulty in growing HCV clinical isolates in culture. Performing serum fractionation and mass spectrometric analyses, we identified apo(a) as a potential inhibitor of HCV infection. Using plasma-derived and recombinant Lp(a), we confirmed that Lp(a) inhibits HCVcc infection by interacting with infectious particles. We also used purified recombinant apo(a) variants to show that free apo(a) was sufficient for the inhibitory activity and to decipher the mechanism of inhibition.

Materials and Methods

CELL CULTURE

HuH-7-RFP-NLS-IPS cells have been described[12] and were grown at 37 °C, 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum.

HUMAN PLASMAS AND SERA, PURIFIED RECOMBINANT APO(a) PROTEINS, ANTIBODIES, AND LECTIN

Human plasmas and sera were obtained from Biobanque de Picardie. This certified Biological

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Resource Center (ISO 9001 and NF S 96–900) obtained authorization AC-2013-1827 from the French Ministry of Research and Higher Education to collect serum samples for scientific usage. All samples tested negative for HCV, hepatitis B virus, and human immunodeficiency virus. The different variants of recombinant apo(a) (6K, 12K, 17K, 17KΔLBS$_{10}$, 17KΔLBS$_{7-8}$, 23K, 27K, 33K, KIV$_{5-9}$, and KIV$_{10}$-P) were expressed and purified as described.[13] The proteins were assessed for purity by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie blue staining (Supporting Fig. S1). The in house "anti-1-4" anti-apo(a) mouse monoclonal antibody (MAb), which is specific to an N-terminal epitope of apo(a); a commercial anti-apo(a) rabbit MAb (EPR6474; Abcam); and the C4 anti-actin MAb (Sigma) were used for immunoprecipitation and western blot experiments. Wheat germ agglutinin (WGA) and WGA-coated beads were purchased from Vector Laboratories and Sigma, respectively.

HCVcc AND HCVpp PRODUCTION

The production of the JFH1-derived recombinant virus expressing a luciferase reporter gene (JFH1-CS-A4-RLuc-TM) has been described.[12] For immunoprecipitation experiments, we used highly infectious viral supernatants purified by iodixanol gradient. Five milliliters of virus were layered on the top of a 10%-40% continuous iodixanol gradient. Gradients were ultracentrifuged overnight at 130,000 *g* and 4 °C in a SW32.1 rotor. Fifteen fractions of 1 mL each were collected. The infectivity of each fraction was determined, and the seven most infectious fractions were pooled (fractions 5-11, with infectivity ranging between 10^6 and 10^7 relative light units). HCVpp were produced using a plasmid encoding genotype 2a HCV envelope glycoproteins (JFH1 strain) as described.[14] Retroviral particles pseudotyped with the murine leukemia virus envelope protein (MLVpp) or the vesicular stomatitis virus glycoprotein G (VSVpp) were used as control. Viruses were put in contact with HuH-7-RFP-NLS-IPS target cells at 37 °C in the presence or absence of the different samples. After 3 hours of contact, the medium was changed, and cells were further incubated for 48 hours (for HCVcc) or 72 hours (for HCVpp, MLVpp, and VSVpp) with complete medium before luciferase activities were measured as indicated by the manufacturer (Promega).

POLYETHYLENE GLYCOL PRECIPITATION

One volume of a polyethylene glycol (PEG)-6000 solution (35% in phosphate-buffered saline [PBS]) was mixed with 4 volumes of pooled sera. Mixtures were incubated overnight at 4 °C and centrifuged at 1500 *g*, 4 °C for 30 minutes. Pellets were resuspended in a volume of DMEM equivalent to the pooled sera starting volume. When we combined PEG precipitation with self-generated iodixanol gradient (SGIG) and size-exclusion chromatography, the pellets were resuspended in a volume of DMEM equivalent to one fourth of the pooled sera starting volume in order to concentrate the inhibitors.

SERUM FRACTIONATION USING SGIG

Serum/iodixanol mixtures (12% wt/vol) were obtained by mixing 18.4 mL of pooled sera with 4.6 mL of iodixanol solution (60% wt/vol; Optiprep; Sigma). Mixtures were transferred to Beckman Polycarbonate Aluminum Bottles, and 3 mL of PBS were carefully layered on top to fill the tube. Bottles were capped with Cap Assembly and centrifuged at 360,000 *g* for 5 hours at 4 °C using a 70Ti rotor and a Beckman OPTIMA L-100 K BioSafe ultracentrifuge, set at slow acceleration and deceleration. Fractions (1 mL) were collected from the top to the bottom. The lipoprotein content of some fractions was evaluated using Hydragel 7 Lipo + Lp(a) (Sebia), as recommended by the manufacturer.

SIZE-EXCLUSION CHROMATOGRAPHY

The experimental condition of the high-performance liquid chromatography method was performed according to the study of Tao et al.[15] Further details are available in the Supporting Information.

NANO–LIQUID CHROMATOGRAPHY–ELECTROSPRAY IONIZATION–TANDEM MASS SPECTROMETRIC ANALYSIS

Samples were loaded on 12% Mini-PROTEAN TGX Precast Protein Gels, and short electrophoresis was used to concentrate proteins in one band. Each

SDS-PAGE band was manually excised from the gels to be hydrolyzed according to Shevchenko et al.[16] All digested peptide mixtures were separated by online nano–liquid chromatography and analyzed by nano–electrospray ionization–tandem mass spectrometry. Experiments were performed on an Ultimate 3000 RSLC system coupled with an LTQ-Orbitrap XL mass spectrometer (ThermoFisher Scientific, Waltham, MA). Mass data acquisitions were performed using Xcalibur 2.3 software. Proteins were identified by automated database searching (Mascot 2.4; Matrix Science) against Swissprot from UniProtKB release 2015-09 nonindexed, for *Homo sapiens* taxonomy. Further details are available in Supporting Information.

PLASMA LP(a) ISOLATION

Lp(a) (density 1.06-1.11 g/mL) was isolated from normolipemic human plasmas by sequential ultracentrifugation (see Supporting Information for further details). Lp(a) concentration was assessed by apo(a) measurement by immunoturbidimetry on a chemistry analyzer (Dimension Vista 500; Siemens, Erlangen, Germany).

RECOMBINANT LP(a) PRODUCTION

To produce recombinant Lp(a), we used the plasmids pCMV-A14, pCMV-A18, and pCMV-A22 encoding apo(a) with 13 (13K), 17 (17K), or 21 (21K) KIV domains, which have been described[17] and were kindly provided by H. Dieplinger (Medical University of Innsbruck, Innsbruck, Austria). A plasmid encoding green fluorescent protein (GFP) was used as a negative control. Plasmids were transfected in HuH-7-RFP-NLS-IPS cells using GenJet In Vitro DNA Transfection Reagent for Huh-7 Cells (SignaGen Laboratories). Supernatants were recovered 72 hours posttransfection. Lp(a) concentration was assessed by apo(a) measurement by immunoturbidimetry on a chemistry analyzer (Dimension Vista 500).

WESTERN BLOTTING

After separation by SDS-PAGE in Mini-PROTEAN TGX Precast Gels (Bio-Rad), proteins were transferred overnight (30 V, 4 °C) to nitrocellulose membranes (Bio-Rad) using a Trans-Blot apparatus (Bio-Rad) and revealed with specific MAbs. Proteins of interest were revealed using Immobilon Western Chemiluminescent HRP Substrate (Millipore), as recommended by the manufacturer.

IMMUNOPRECIPITATION

Protein G Plus Agarose (Pierce) was incubated with the "anti-1-4" anti-apo(a) mouse MAb for 2 hours at 4 °C and then washed with PBS. Anti-apo(a) antibody–coated beads were then incubated for 4 hours with the supernatant of HuH-7 cells containing 200 μg/mL of recombinant 21K-Lp(a). Supernatant of nontransfected HuH-7 cells was used as a negative control. Alternatively, free 17K-apo(a) variant was also tested, using PBS as a negative control. After washing, the beads were finally incubated with purified HCVcc. Mixtures were incubated overnight at 4 °C. Beads were washed five times with PBS, and RNAs were extracted by using the QIAamp viral RNA kit (Qiagen, Valencia, CA). HCV genomes were quantified by quantitative RT-PCR as described.[18] Control tubes were used to confirm the presence of apo(a) on sepharose beads by western blot.

Results

HUMAN SERUM INHIBITS HCV INFECTION

Despite decades of research, it is still impossible to grow primary HCV isolates in cell culture. To test whether this hurdle could be due to the presence of inhibitory factors in patient serum, we sought to determine if HCV infection is inhibited by human serum. To this end, we inoculated HuH-7-RFP-NLS-IPS cells with a recombinant HCV expressing a luciferase reporter protein, in the presence of increasing concentrations of human serum derived from four HCV-seronegative individuals. Our results revealed that all the tested sera dose-dependently inhibited HCV infection, resulting in an 80%-90% decrease when human serum was added at a final concentration of 50% during inoculation (Fig. 1A). Interestingly, it was able to inhibit HCVpp to a similar extent (Fig. 1B), whereas MLVpp were only moderately inhibited (Fig. 1C). In addition, we checked and confirmed that the addition of such concentrations of human serum for only 3 hours did not have any effect on cell proliferation (Fig. 1D). Altogether, these results confirmed our hypothesis that human serum contains factors that specifically inhibit HCV.

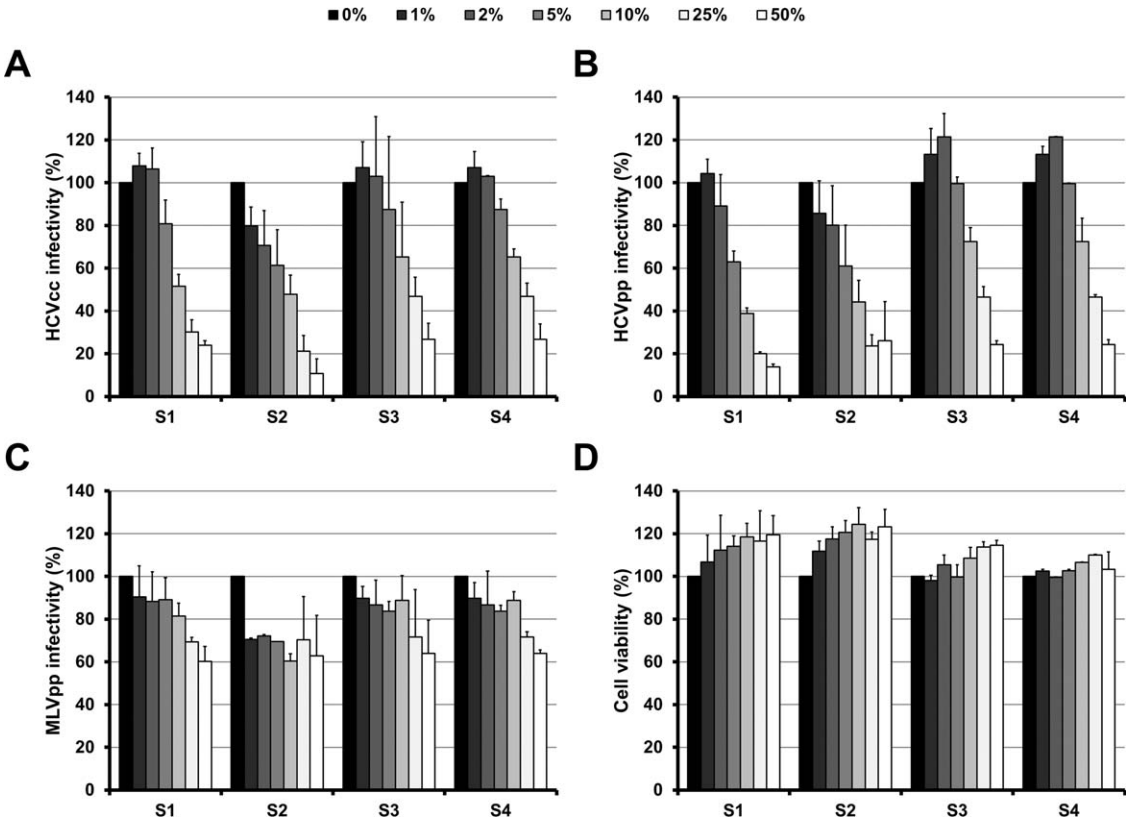

FIG. 1. Human serum inhibits HCV infection. HuH-7-RFP-NLS-IPS cells were inoculated with HCVcc (A), HCVpp (B), or MLVpp (C) for 3 hours in the presence of various concentrations of human serum (0%, 1%, 2%, 5%, 10%, 25%, or 50%) derived from four HCV-seronegative individuals (S1, S2, S3, and S4). Luciferase assays were performed on infected cells 48 hours (A) or 72 hours (B,C) postinfection. Results are expressed as percentages of infectivity compared to infection in the absence of human serum and are reported as the means ± standard deviation of three independent experiments. (D) Cell viability was evaluated 2 days after incubation with human serum in similar conditions. The results are expressed as percentages of viability compared to cells which had not been incubated with human serum and are reported as means ± standard deviation of two independent experiments.

HCV SERUM INHIBITORS ARE PELLETED BY PEG PRECIPITATION

PEG precipitation is commonly used for the recovery and concentration of viruses. In a search for a protocol which would allow the removal of inhibitory factors from HCV RNA-positive samples, we tested whether serum inhibitors are also precipitated by PEG. HCV-negative human serum was thus subjected to PEG precipitation, and the pellet obtained after centrifugation was resuspended in a volume equivalent to that of the supernatant. The inhibitory activity of various concentrations of the supernatant and the resuspended pellet was then evaluated and compared to that of the starting serum. We observed that the starting serum and the resuspended pellet dose-dependently inhibited HCV infection, whereas the supernatant obtained after centrifugation did not have any inhibitory activity (Fig. 2A). We therefore concluded that PEG precipitation could not be used to separate infectious virions and inhibitory factors from an HCV-positive sample. However, when we analyzed the protein content of each sample using Coomassie blue staining after SDS-PAGE, we observed that PEG precipitation allowed us to partially purify the inhibitory fraction compared to the starting serum and that several serum proteins, in particular albumin, were efficiently eliminated in the supernatant after precipitation (Fig. 2B).

HCV SERUM INHIBITORS ARE PRESENT IN LOW-DENSITY FRACTIONS AFTER SGIG

As mentioned, a striking feature of HCV particles is their association with lipoproteins. For this reason, we

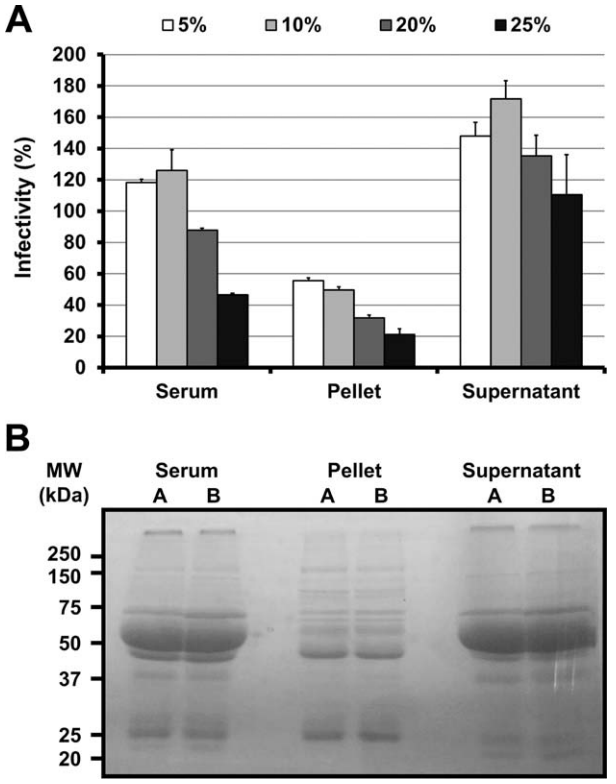

FIG. 2. Human serum inhibitors are precipitated with PEG. Human serum was subjected to PEG precipitation. After precipitation, the supernatant was recovered, and the pellet was resuspended in an equivalent volume of DMEM. (A) HuH-7-RFP-NLS-IPS cells were then inoculated with HCVcc for 3 hours in the presence of various concentrations (5%, 10%, 20%, or 25%) of the starting serum, the supernatant, or the resuspended pellet. Luciferase assays were performed on infected cells 48 hours postinfection. Results are expressed as percentages of infectivity compared to infection in the absence of sample and are reported as means ± standard deviation of a duplicate from a representative experiment. (B) The protein content of two sera (A and B) was evaluated by Coomassie blue staining after SDS-PAGE and compared to that of the supernatants and the resuspended pellets obtained after PEG precipitation. Abbreviation: MW, molecular weight.

also wondered whether density gradient would enable the recovery of an infectious fraction from an HCV-positive sample. To test this, we fractionated HCV-negative serum samples using SGIG and assessed the inhibitory activity of the different fractions obtained at the end of the experiment. This procedure enables the rapid separation of very low-density, low-density, and high-density fractions as confirmed by the presence of VLDLs in fractions 1-3 and LDLs in fractions 6-7, identified by the whitish and brownish colors, respectively (data not shown). We often observed an inhibitory activity of very low-density fractions (fractions 1-4) (Fig. 3A). The inhibitory activity of these fractions was highly variable from one serum to another and may correspond to the inhibition raised by VLDLs as demonstrated by Tao et al.[15] In contrast, in all the sera tested, we observed a higher level of inhibition with fractions 7-11 which present a density slightly higher than LDLs that peaked in fraction 6 (Fig. 3B). Fractions with higher density (fractions 12-22) did not show any effect on HCV infection. Because HCV is expected to be recovered in low-density and very low-density fractions after density gradient experiment, we concluded that SGIG could not be used to recover an infectious fraction from an HCV-positive sample. However, as with PEG precipitation, we observed that the SGIG procedure permits us to partially purify an inhibitory fraction and remove several serum proteins as observed after SDS-PAGE and Coomassie blue staining (Fig. 3C).

PURIFICATION AND IDENTIFICATION OF HCV INHIBITORS PRESENT IN HUMAN SERUM

In an attempt to purify and identify the inhibitory factors, we carried out serum PEG precipitation and fractionated the resuspended pellet by SGIG. We then analyzed the protein content of the most inhibitory fraction using mass spectrometry, and we identified around 100 proteins (data not shown). Consequently, we decided to concentrate 4-fold the inhibitory factors using serum PEG precipitation and to use SGIG as an intermediate purification step. In these conditions, we observed that low-density inhibitory factors were concentrated in fractions 6-13 with a maximal inhibition obtained using fractions 8 and 9 (88% and 90%, respectively; Fig. 4A). We then mixed fractions 8-9 (f8-9) and 10-11 (f10-11) and performed size-exclusion chromatography as a final purification step, as described by Tao et al.[15] For both samples, 80 fractions were obtained, and we observed that the inhibitory factors concentrated in fractions 45-55 (Fig. 4B,C). About 30 proteins were revealed by mass spectrometry in the inhibitory fractions obtained from f8-9, and 13 proteins were identified in fractions 47-67 obtained from f10-11 (see Supporting Table S1). Among them, we focused on apo(a) because this protein is known to covalently link to the apoB component of an LDL through a disulfide bridge to form Lp(a),[9] which concurs with an inhibitor that precipitates with PEG and that has a density slightly higher than that of LDL.

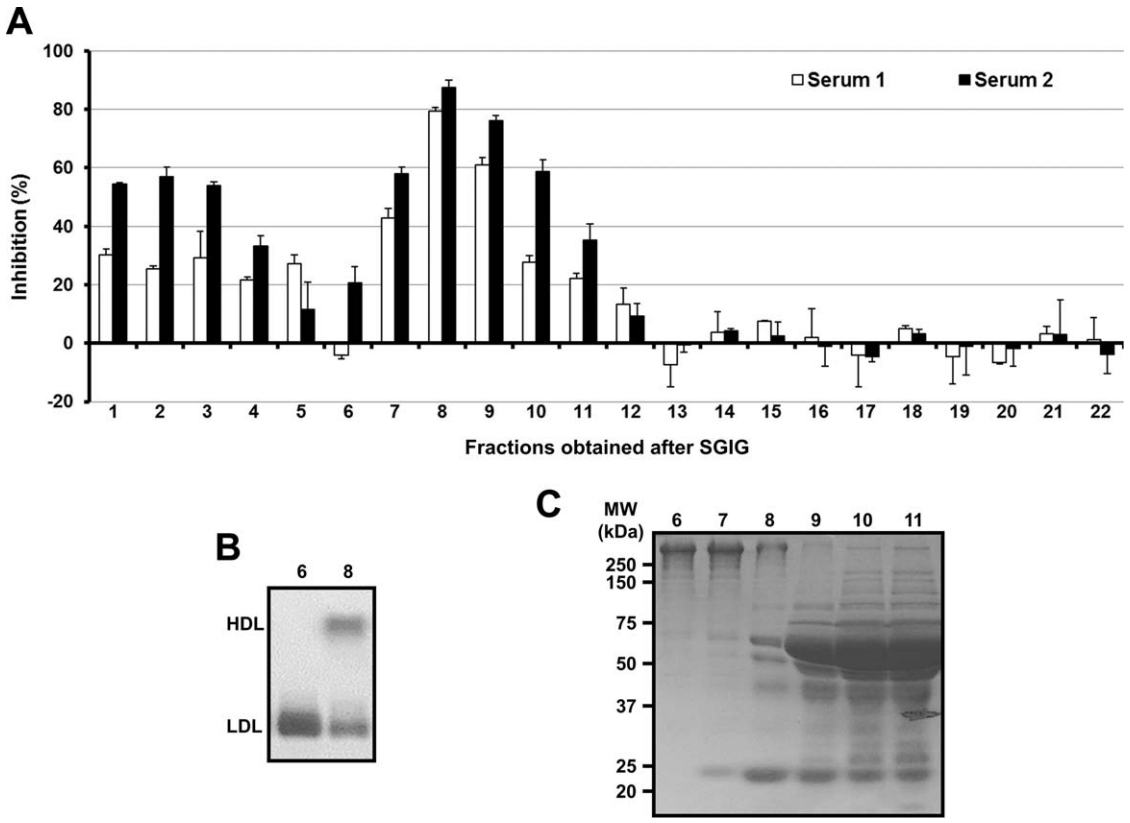

FIG. 3. Human serum inhibitors are concentrated in low-density fractions after SGIG. Human serum was subjected to SGIG. (A) After ultracentrifugation, 22 fractions were collected from the top to the bottom. The inhibitory activity of each fraction was evaluated by inoculating HuH-7-RFP-NLS-IPS cells with HCVcc for 3 hours in the presence of the fraction (concentration of 50%). Luciferase assays were performed on infected cells 48 hours postinfection. Results obtained with two different sera are shown. Results are expressed as percentages of infectivity compared to infection in the absence of sample and are reported as means ± standard deviation of a duplicate. (B) The lipoprotein content of fractions 6 and 8 obtained with "serum 1" was evaluated after electrophoresis on a buffered agarose gel and staining with a lipid-specific Sudan black stain (Hydragel 7 Lipo + Lp[a]). (C) The protein content of fractions 6-11 obtained with "serum 1" was evaluated by Coomassie blue staining after SDS-PAGE. Abbreviations: HDL, high-density lipoprotein; MW, molecular weight.

LP(a) INHIBITS HCV INFECTION

To investigate whether Lp(a) inhibits HCV, we first purified it from two different human plasmas using sequential ultracentrifugation. We observed that both samples dose-dependently inhibited HCVcc infection, even if the efficiency differed between samples (Fig. 5A). At 500 μg/mL, Lp(a) recovered from plasma 1 and 2 achieved 72% and 44% inhibition, respectively. Importantly, the presence of Lp(a) only during the 3-hour inoculation was sufficient to inhibit HCV, suggesting that the entry step was inhibited.

Because the apo(a) component of Lp(a) varies widely from one individual to another, we also tested the effect of recombinant Lp(a). HuH-7-RFP-NLS-IPS cells do not express apo(a) (see control cells transfected with a GFP plasmid) (Fig. 5B). In contrast, its expression was readily detected after transfection of three plasmids encoding apo(a) isoforms containing 13, 17, or 21 KIV domains (13K, 17K, or 21K, respectively; Fig. 5B). In addition, 72 hours posttransfection, the secreted Lp(a) concentration was readily measured by immunoturbidimetry in the supernatant of cells transfected with apo(a) plasmids, whereas it was undetectable in that of cells transfected with a GFP plasmid (data not shown). Interestingly, when we mixed HCVcc with recombinant Lp(a) containing supernatants during inoculation of naive HuH-7-RFP-NLS-IPS cells, we observed a dose-dependent inhibition of the infection (Fig. 5C). Importantly, the use of similar volumes of GFP-transfected cell supernatant did not have any effect on HCV infection (data not shown). In

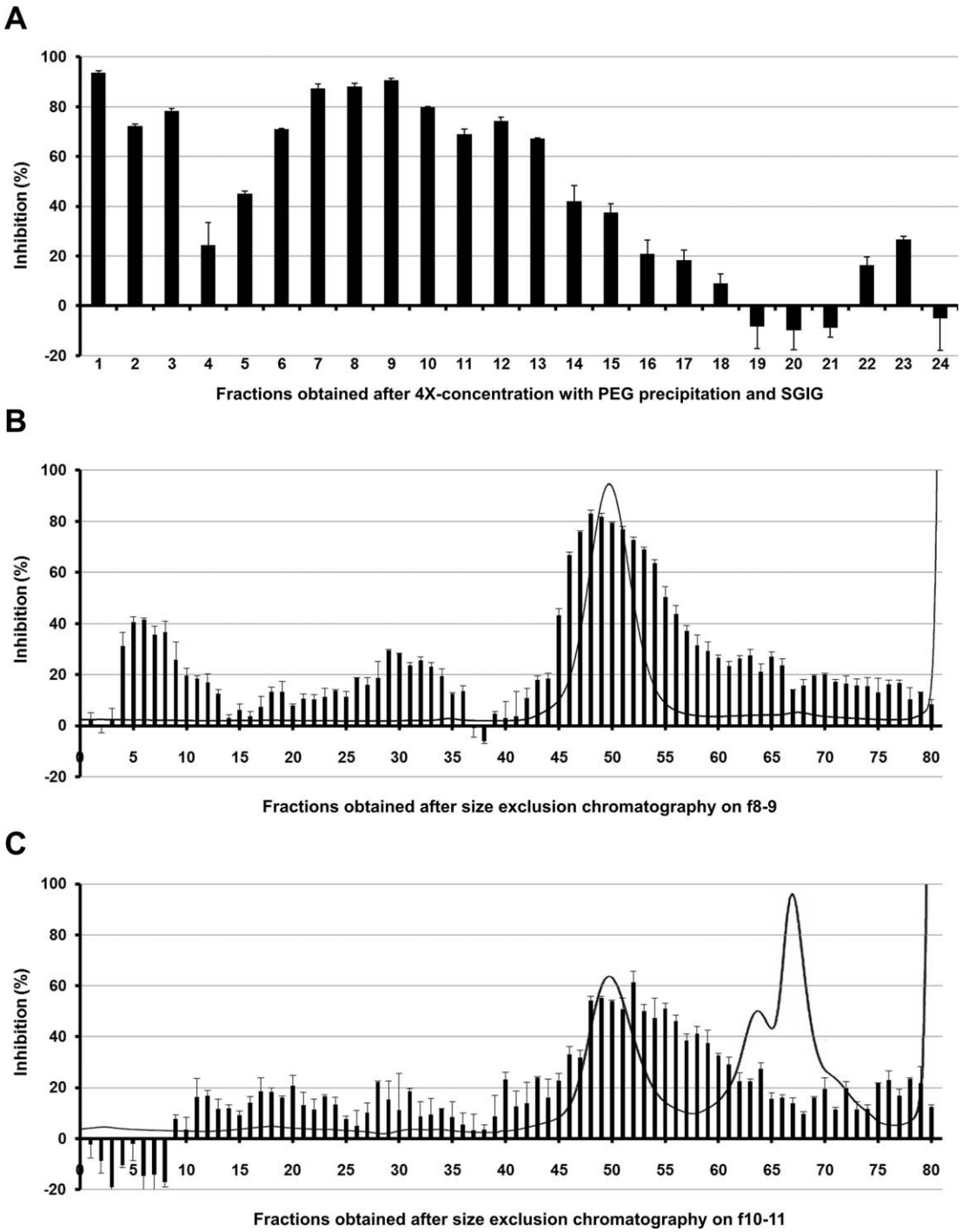

FIG. 4. Identification of inhibitory fractions obtained after PEG precipitation, SGIG, and size-exclusion chromatography. Serum inhibitory factors were concentrated 4-fold using PEG precipitation. The resuspended pellet was fractionated in 24 fractions using SGIG. Fractions 8-9 (f8-9) and 10-11 (f10-11) obtained after SGIG were subjected to size-exclusion chromatography as a final purification step. For both samples, 80 fractions were obtained. The inhibitory activity of the fractions obtained after SGIG (A) as well as size-exclusion chromatography on f8-9 (B) and f10-11 (C) was evaluated by inoculating HuH-7-RFP-NLS-IPS cells with HCVcc for 3 hours in the presence of each fraction (concentration of 50%). Luciferase assays were performed on infected cells 48 hours postinfection. Results are expressed as percentages of infectivity compared to infection in the absence of sample and are reported as means ± standard deviation of a duplicate. The relative protein content of the fractions obtained after size-exclusion chromatography is illustrated by the curves.

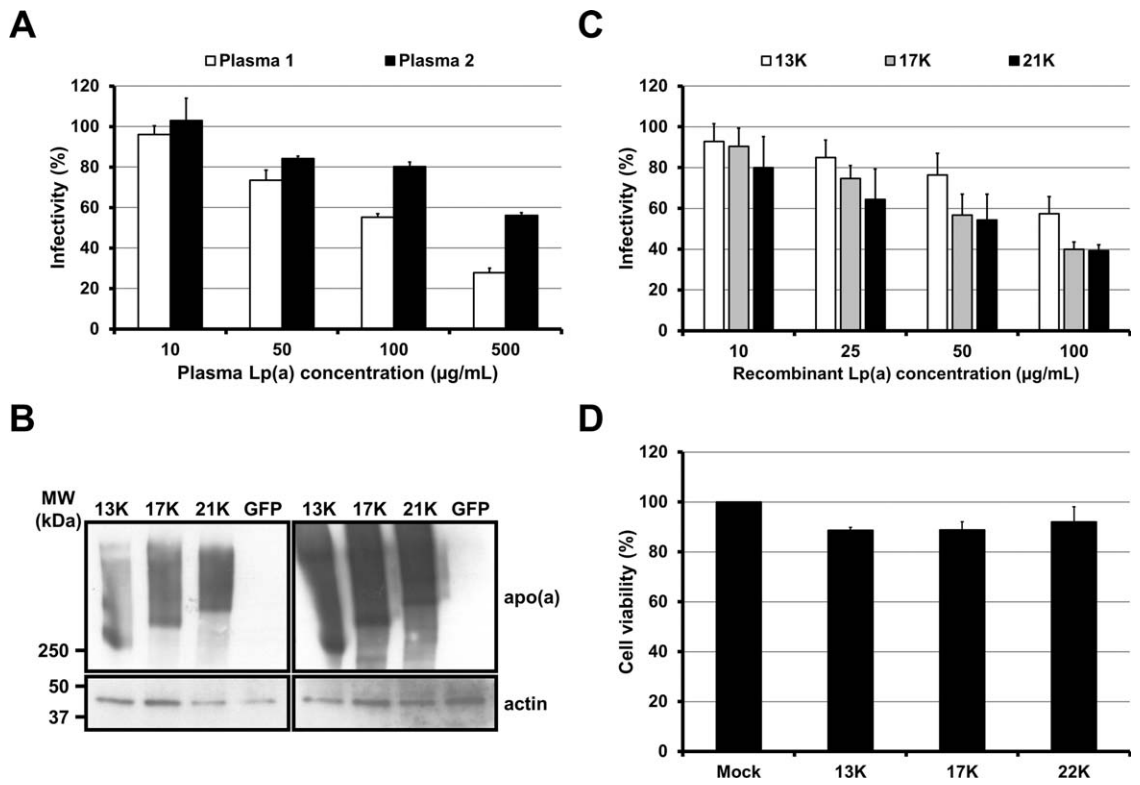

FIG. 5. Lp(a) inhibits HCV infection. (A) Inhibition assays were performed by inoculating HuH-7-RFP-NLS-IPS cells for 3 hours with HCVcc and various concentrations of Lp(a) purified from two different human plasmas. Luciferase assays were performed on infected cells 48 hours postinfection. Results are expressed as percentages of infectivity compared to infection in the absence of sample and are reported as means ± standard deviation of a duplicate from a representative experiment. (B) HuH-7-RFP-NLS-IPS cells were transfected with plasmids encoding apo(a) isoforms containing 13, 17, or 21 KIV domains (13K, 17K, or 21K, respectively). Transfection of GFP was used as a negative control. The transfection efficiency was evaluated after 48 hours by detection of apo(a) in cell lysates by western blotting using anti-apo(a) mouse MAb (anti-1-4, left panel) or rabbit MAb (EPR6474, right panel). Detection of actin was used as loading control. (C) Inhibition assays were performed by inoculating HuH-7-RFP-NLS-IPS cells for 3 hours with HCVcc and various concentrations of recombinant Lp(a) present in the supernatant of HuH-7-RFP-NLS-IPS cells, recovered 72 hours after transfection with plasmids expressing 13K, 17K, or 21K apo(a). Luciferase assays were performed on infected cells 48 hours postinfection. Results are expressed as percentages of infectivity compared to infection in the absence of sample and are reported as means ± standard deviation of three independent experiments. No effect was observed when using similar volumes of GFP-transfected cell supernatant as a negative control (data not shown). (D) HuH-7-RFP-NLS-IPS cells were incubated with recombinant Lp(a) (100 µg/mL) for 3 hours, and the cell viability was evaluated 48 hours later. The results are expressed as percentages of viability compared to cells which had not been incubated with recombinant Lp(a) and are reported as means ± standard deviation of two independent experiments. Abbreviation: MW, molecular weight.

addition, we did not observe any effect of Lp(a) containing supernatants on cell viability (Fig. 5D) or on HCV RNA replication (Supporting Fig. S2). Altogether, these results strongly suggest that Lp(a) inhibits HCV entry. Additionally, we noticed that the size of the apo(a) component correlates with the inhibition potency. For instance, at 25 µg/mL, 15%, 25%, and 46% inhibitions were observed with 13K, 17K, and 21K recombinant Lp(a), respectively, and 24%, 43%, and 46% inhibition were achieved at 50 µg/mL. This could be responsible for the variable inhibition observed with the apo(a) purified from plasma 1 and 2 in Fig. 5A. Interestingly, the Lp(a) concentrations in the four sera used in Fig. 1 were < 40, 885, 156, and 142 µg/mL for S1, S2, S3, and S4, respectively. The presence of apo(a) of different sizes could partly explain why all these sera showed a similar inhibitory effect on HCV infection. However, it must be kept in mind that the inhibition observed with nonfractionated sera may be due to the contribution of not only apo(a) but also other serum components. This was corroborated by experiments using an apo(a)-depleted serum obtained from the S2 serum thanks to beads coated with WGA, a lectin which was demonstrated to have high

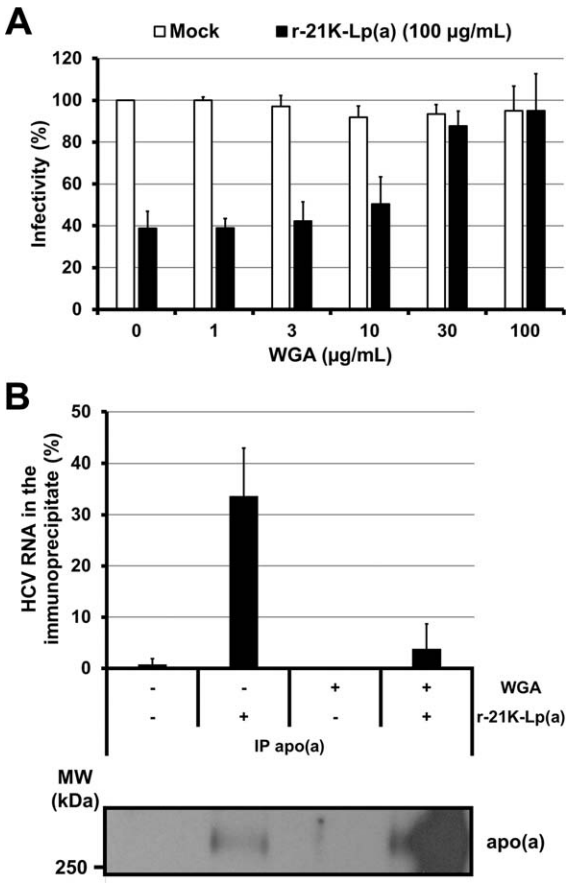

FIG. 6. Lp(a) interacts with HCV infectious particles. (A) RFP-NLS-IPS cells were inoculated for 3 hours with HCVcc in the presence or absence of 100 µg/mL of recombinant Lp(a) (r-21K-Lp[a], see Fig. 5C) and various concentrations of WGA. Luciferase assays were performed on infected cells 48 hours postinfection. Results are expressed as percentages of infectivity compared to infection in the absence of sample and are reported as means ± standard deviation of three independent experiments. (B) Recombinant 21K-Lp(a) contained in the supernatant of transfected HuH-7-RFP-NLS-IPS cells (200 µg/mL) was immunoprecipitated using anti-apo(a) antibody–coated beads. The supernatant of nontransfected HuH-7-RFP-NLS-IPS cells was used as a negative control. Mixtures were incubated overnight with purified HCVcc in the absence or presence of 100 µg/mL of WGA, and the amount of captured HCV RNAs was evaluated by quantitative RT-PCR. Results are expressed as the titer of HCV RNA in the immunoprecipitate (percentage of total HCV RNAs in the immunoprecipitate plus supernatant) and are reported as means ± standard deviation of three independent experiments. A picture of a western blot performed to confirm the presence of apo(a) on sepharose beads is shown at the bottom of the panel. Abbreviations: IP, immunoprecipitation; MW, molecular weight.

specificity for Lp(a). Indeed, when used at a concentration of 50%, only a 43% inhibition of HCV infection was observed with this apo(a)-depleted serum (data not shown).

LP(a) INTERACTS WITH INFECTIOUS HCV PARTICLES

Interestingly, we also observed that WGA restored HCVcc infectivity in a concentration-dependent manner when added during inoculation in the presence of recombinant Lp(a) (Fig. 6A). Thus, 100 µg/mL of WGA completely counteracted the inhibitory activity caused by 100 µg/mL of recombinant 21K-Lp(a). In the absence of Lp(a), WGA did not have any effect on HCVcc infectivity (Fig. 6A). Because WGA is known to specifically interact with apo(a), we sought to determine whether it could interfere with critical interactions between Lp(a) and infectious HCV particles. Recombinant 21K-Lp(a) contained in the supernatant of transfected HuH-7-RFP-NLS-IPS cells was immunoprecipitated using anti-apo(a) antibody–coated beads. Supernatants of nontransfected HuH-7-RFP-NLS-IPS cells were used as a negative control. The mixtures were then incubated overnight with purified HCVcc, and the amount of captured HCV RNAs was evaluated by quantitative RT-PCR. We observed that 33.6% of HCV RNAs were fixed to the beads in the presence of recombinant 21K-Lp(a), whereas almost all HCV RNAs (99.2%) were eliminated in the supernatant of immunoprecipitation in the absence of Lp(a) (Fig. 6B). In addition, we observed that WGA prevents Lp(a) from interacting with HCV particles as only 3.8% of HCV RNAs were coimmunoprecipitated in the presence of this compound. Altogether, these results suggest that Lp(a) decreases HCV infection by interacting with infectious viral particles.

APO(a) INHIBITS HCV INFECTION

In contrast to hepatoma cell lines, HEK293 cells do not synthesize apoB and produce free apo(a).[19] To investigate whether the apo(a) component alone was sufficient for the inhibitory activity, we thus tested the effect of several recombinant apo(a) variants purified from the conditioned medium of stably expressing HEK293 cells by affinity chromatography (Fig. 7A; Supporting Fig. S1). We observed that the different apo(a) isoform size variants were able to dose-dependently inhibit HCVcc infectivity (Fig. 7B). We also observed that small isoforms were less inhibitory than large ones, with a plateau for isoforms larger than 23K (Fig. 5C). Importantly, when replacing recombinant 21K-Lp(a) by free 17K-apo(a) variant in the immunoprecipitation assay described above, 39.9% of HCV RNAs were coimmunoprecipitated, demonstrating that apo(a) alone is sufficient to interact with infectious viral

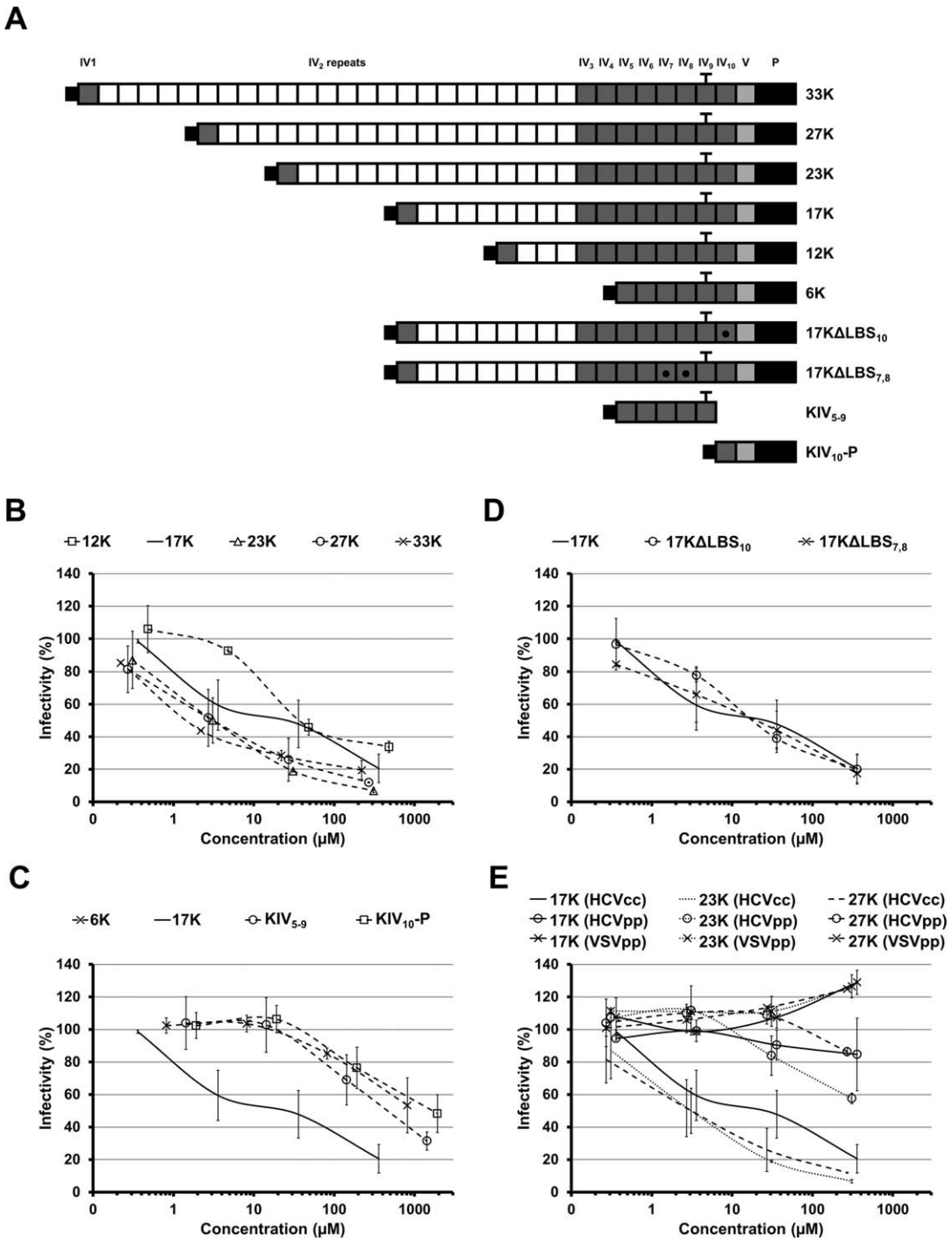

FIG. 7. HCV is inhibited by different recombinant apo(a) variants. (A) The schematic diagram shows the topology of the recombinant apo(a) variants that were purified from the conditioned medium of stably expressing HEK293 cells for this study (see Supporting Fig. S1). The reference 17K apo(a) variant corresponds to a physiological apo(a) isoform which includes all 10 types of KIV sequences present in apo(a) isoforms as well as the kringle V-like domain and protease-like (P) domains. Black dots denote the presence of amino acid substitutions that inactivate LBS. The bar above KIV_9 denotes the unpaired cysteine residue that mediates covalent attachment to apoB-100. (B-E) Inhibition assays were performed by inoculating HuH-7-RFP-NLS-IPS cells for 3 hours with HCVcc, HCVpp, or VSVpp and various concentrations (0.1, 1, 10, and 100 μg/mL) of the apo(a) variants. Luciferase assays were performed on infected cells 48 hours (for HCVcc) or 72 hours (for HCVpp and VSVpp) postinfection. Results are expressed as percentages of infectivity compared to infection in the absence of sample and are reported as means ± standard deviation of at least two independent experiments.

particles (data not shown). Interestingly, we also observed that the inhibition triggered by the 6K, KIV_{5-9}, and KIV_{10}-P truncated variants was very weak compared to that of the 17K isoform (Fig. 7C), suggesting that a substantial number of KIV_2 repeats is required for maximal inhibition. However, results obtained with these three variants must be interpreted with caution as they likely adopt a very different conformation compared to full-length apo(a) isoforms.[20] Several of the apo(a) kringle domains contain lysine-binding sites (LBSs) that play a key role in the pathogenic activity of Lp(a). For instance, weak LBSs in KIV_7 and KIV_8 mediate noncovalent interactions with apoB-100 prior to the disulfide bond formation, and a strong LBS in KIV_{10} enables the interaction of apo(a) with several biological substrates including fibrin. Remarkably, when comparing the inhibition potency of 17K, 17KΔ$LBS_{7,8}$, and 17KΔLBS_{10} apo(a) variants, we observed that point mutations which affect the functionality of the $LBS_{7,8}$ and LBS_{10} did not have any effect on the inhibitory activity (Fig. 7D). Furthermore, because they are produced from 293T cells, HCVpp are not associated with lipoproteins, which allowed us to investigate the importance of HCV association with lipoproteins for the apo(a)-induced inhibition. Using 17K, 23K, and 27K apo(a) variants, we showed that 100-fold higher concentrations were required to inhibit HCVpp compared to HCVcc (Fig. 7E). In contrast, HCVpp and HCVcc were similarly sensitive to inhibition by the JS-81 anti-CD81 MAb (Supporting Fig. S3). These results suggest that the lipoprotein moiety of HCV lipoviroparticles is essential for the inhibitory activity of apo(a). Importantly, we also demonstrated that recombinant apo(a) does not inhibit VSVpp, demonstrating that the inhibition is specific to HCV (Fig. 7E).

Discussion

In this study, we evaluated the presence of HCV restriction factors in human serum and observed that large serum particles (precipitated by PEG) with a density slightly higher than LDLs were able to strongly inhibit HCVcc infection. Mass spectrometric analysis identified apo(a) as a potential candidate responsible for this inhibition, and our results demonstrated that apo(a) decreases HCV entry, potentially through an interaction with infectious viral particles. A striking feature of HCV particles is their association with lipoproteins, and several studies have already demonstrated that apolipoproteins are associated with HCV particles and modulate HCV infection.[4-6] Indeed, it is now well established that apoE is required for HCV infectivity[21-23] and that apoC-I enhances HCV infectivity by promoting membrane fusion.[24,25] In addition, it has been suggested that apoC-III could increase HCV infectivity by reversing lipoprotein lipase–mediated inhibition,[26] whereas apoH could inhibit HCV,[27] potentially through interaction with the viral particles.[28]

The concentration of apo(a) in human serum is highly variable from one patient to another (ranging from < 1 to > 100 mg/dL), and an inverse correlation between the size of apo(a) isoforms and the serum levels of Lp(a) has been described. Importantly, we observed that this range of apo(a) concentrations is able to inhibit HCVcc in our infection assay. Thus, it is very likely that Lp(a) contributes to a significant part of the inhibition by human serum, as observed in Fig. 1. Results obtained using isoform size variants also suggest that the larger the apo(a), the stronger the inhibition is. A substantial number of KIV_2 repeats may thus be required for maximal inhibition. Whether these domains permit the interaction of apo(a) with HCV infectious particles will have to be investigated. On the other hand, this result may simply be explained by an increase of the steric hindrance. Little is known about the function of apo(a), but it is now known that Lp(a) is a preferential carrier of oxidized phospholipids in plasma.[9] However, mutation of the LBS_{10}, which is essential for the capture of oxidized phospholipids, did not have any effect on the inhibitory activity of apo(a). It is thus unlikely that oxidized phospholipids play a role in apo(a)-mediated HCV inhibition, and further studies will be needed to decipher the exact mechanism of HCV inhibition by apo(a).

Our results suggest that apo(a) inhibits HCV through an interaction with infectious particles. In addition, in contrast to HCVcc, we observed that HCVpp are only weakly sensitive to apo(a), suggesting that the lipoprotein moiety of HCV lipoviroparticles is essential for the inhibition. In human serum, apo(a) interact noncovalently with the apoB component of LDL through LBS_7 and LBS_8 before the formation of a disulfide bond, leading to the formation of Lp(a). However, our data suggest that not only apo(a) interacts with HCV infectious particles but also Lp(a). Additionally, we observed that mutation of LBS_7 and LBS_8 did not have any effect on HCVcc inhibition by apo(a). Thus, the interaction of apo(a) with HCV does not seem to involve apoB but potentially another component of the lipoprotein moiety of HCV lipoviroparticles. It is now well established that large amounts of apoE are associated with infectious HCVcc and that the

level of apoE correlates very well with infectivity.[21] In addition, Boyer et al. recently demonstrated that apoE and HCV glycoproteins form a protein complex associated with HCVcc and suggested that a small amount of apoE is associated with E1E2 on HCVpp.[29] Thus, it would be interesting to test whether apoE is a requisite for apo(a) inhibition, which could explain the relative resistance of HCVpp to inhibition compared to HCVcc. Alternatively, the involvement of other lipoprotein components cannot be excluded.

The development of a culture system enabling the amplification of primary HCV isolates would open new perspectives for the investigation of viral determinants responsible for the evolution and treatment outcome of hepatitis C as well as for the development of a vaccine. Saeed et al. recently demonstrated that SEC14L2 expression in hepatoma cell lines promotes HCV RNA replication following inoculation with patient sera, through enhancement of vitamin E–mediated protection against lipid peroxidation.[30] In addition, Harak et al. elegantly showed that inhibition of phosphatidylinositol 4-kinase alpha in HuH-7 cells allows efficient replication of patient-derived viruses.[31] In 2013, Steenbergen et al. demonstrated that the production of HCVcc was improved when using HuH-7 cells that had been differentiated by supplementing tissue culture medium with human serum for 7 days.[32] In these conditions, cells undergo growth arrest, have a hepatocyte-like morphology, and increase the expression of hepatocyte differentiation markers, which enables the production of large amounts of virus that more closely resemble HCV present in the serum of infected patients. However, to develop a culture system for the amplification of primary HCV isolates, it is also important to study the effect of human serum on the viral entry step. It has been shown that 2% human serum could facilitate the entry of HCV in a serum-dependent and HCV strain–dependent manner.[24,33,34] However, it is tempting to inoculate cells with higher concentrations of serum to increase the infection with clinical isolates, and we demonstrated in this study that high concentrations of human serum inhibit HCV infection. In addition, we previously showed that highly infectious sera displayed a specific profile with low levels of several cytokines/growth factors that could impact hepatocyte biology and infection efficiency.[35] Finally, in addition to innate and adaptive humoral immunity components,[36,37] several factors in human serum, such as serum amyloid A,[38] lipoprotein lipase,[39] VLDLs,[15] and oxidized LDLs,[40,41] have been shown to inhibit HCV infection. In this study, we identified apo(a) as an additional inhibitory factor present in human serum. Preliminary results suggest that depletion of apo(a) decreases the inhibitory potency of human serum. However, because we also demonstrated that apo(a) is associated with HCV viral particles, it seems complicated to remove this component from HCV-seropositive sera to increase the efficiency of the infection and facilitate the amplification of primary HCV isolates.

In conclusion, we identified apo(a) as an additional component of the lipid metabolism modulating HCV infection through interaction with lipoviroparticles. Our results bring insight into the tight link between HCV and lipoproteins. Our study could also open new perspectives for the study of apo(a) function.

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