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Characterization of Hepatitis C Virus Intra- and Intergenotypic Chimeras Reveals a Role of the Glycoproteins in Virus Envelopment

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Hepatitis C virus (HCV) is highly variable and associated with chronic liver disease. Viral isolates are grouped into seven genotypes (GTs). Accumulating evidence indicates that viral determinants in the core to NS2 proteins modulate the efficiency of virus production. However, the role of the glycoproteins E1 and E2 in this process is currently poorly defined. Therefore, we constructed chimeric viral genomes to explore the role of E1 and E2 in HCV assembly. Comparison of the kinetics and efficiency of particle production by intragenotypic chimeras highlighted core and p7 as crucial determinants for efficient virion release. Glycoprotein sequences, however, had only a minimal impact on this process. In contrast, in the context of intergenotypic HCV chimeras, HCV assembly was profoundly influenced by glycoprotein genes. On the one hand, insertion of GT1a-derived (H77) E1-E2 sequences into a chimeric GT2a virus (Jc1) strongly suppressed virus production. On the other hand, replacement of H77 glycoproteins within the GT1a-GT2a chimeric genome H77/C3 by GT2a-derived (Jc1) E1-E2 increased infectious particle production. Thus, within intergenotypic chimeras, glycoprotein features strongly modulate virus production. Replacement of Jc1 glycoprotein genes by H77-derived E1-E2 did not grossly affect subcellular localization of core, E2, and NS2. However, it caused an accumulation of nonenveloped core protein and increased abundance of nonenveloped core protein structures with slow sedimentation. These findings reveal an important role for the HCV glycoproteins E1 and E2 in membrane envelopment, which likely depends on a genotype-specific interplay with additional viral factors.

Globally, an estimated 160 million people are chronically infected with hepatitis C virus (HCV) (1) and are therefore at a high risk for developing severe liver damage, including hepatic steatosis, fibrosis, cirrhosis, and hepatocellular carcinoma (2). Due to error-prone RNA replication, HCV is a highly variable virus, and based on phylogenetic analyses, viral isolates are grouped into seven genotypes (GTs), which are associated with specific global regions and modes of transmission (3). Importantly, different HCV GTs display differential treatment response rates, and the clinical course of infection also differs, indicating that, in addition to host factors, viral factors play a role in the outcome of infection/treatment. Specifically, infections by HCV GTs 1 and 4 are more difficult to treat with interferons than infections by HCV GTs 2 and 3, and GT3 is associated with liver steatosis (4). The current standard therapy, a combination of pegylated alpha interferon and ribavirin combined with one of two available protease inhibitors, is associated with serious side effects and does not achieve an effective response in every patient (5).

HCV is a hepatotropic RNA virus that establishes a chronic infection in the majority of cases. The HCV genome, 9.6 kb in size, encodes a single polyprotein that is cleaved by cellular and viral proteases into at least 10 different proteins: the structural proteins core, E1, and E2; the ion channel p7; and the nonstructural proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B (6). The genetic variability exhibited by HCV facilitates immune evasion and contributes to viral persistence. The development of in vitro cell culture systems, including subgenomic replicons (7), HCV pseudoparticles (8, 9), and, ultimately, the JFH1-based infection system (10–12), have allowed detailed investigation of multiple aspects of the viral life cycle (13). With respect to assembly and release of infectious HCV particles, essential roles of viral and host factors have been defined. On the one hand, cellular factors involved in the secretion of lipoproteins, including the microsomal triglyceride transfer protein (MTP), apolipoprotein B (ApoB), and ApoE, have been implicated in HCV particle production (14–16). On the other hand, it was demonstrated that besides the canonical structural proteins core, E1, and E2, other viral factors, including p7, NS2, NS3, NS4B, NS5A, and NS5B, also play crucial roles in the production of infectious viral progeny (17–24).

The two viral glycoproteins E1 and E2, which show the highest variability among the HCV proteins (3), are embedded in the virus envelope, mediate uptake of HCV into liver cells, and are the main target for neutralizing antibodies. However, their role in the production of infectious particles is incompletely defined. Both are type I membrane proteins with a C-terminal transmembrane domain (TMD) and a large N-terminal extracellular domain. Their biogenesis is an intricate process involving various endoplasmic reticulum (ER)-resident chaperones, including calnexin, calreticulin, and BiP (25–27). Localization of the glycoproteins in the ER and signals for heterodimerization are determined by the TMDs...
(28–30). E2 folding is independent of other viral proteins, whereas the maturation of E1 requires the coexpression of E2 (31). The two envelope proteins are heavily glycosylated, mostly at highly conserved sites (32), and several glycans are important for HCV assembly and/or infectivity (33).

Chimeric HCV GT2a genomes such as "F1-J6/JFH1" or "Jc1," consisting of J6CF and JFH1 segments, produce much higher virus titers than parental JFH1 or genotype 1 viral chimeras, indicating that viral determinants in the core to NS2 proteins modulate efficiency of virus production (10, 34). However, the role of the HCV glycoproteins E1 and E2 in this regard and in assembly and release in general is not well defined. Therefore, we used a genetic approach to investigate the function of different E1 and E2 variants in HCV assembly and release.

MATERIALS AND METHODS

Cell culture. Huh7.5 cells were grown in Dulbecco’s modified minimal essential medium (DMEM; Invitrogen, Karlsruhe, Germany) supplemented with 2 mM l-glutamine, nonessential amino acids, 100 μM of penicillin, 100 μg/ml of streptomycin, and 10% fetal calf serum (FCS) (DMEM complete).

Plasmid construction. The genomes and plasmids pPK-JFH1 (11), H77 (35), Con1 (7), J6CF (36), Jc1, JFH1/p7, JFH1/coreJ6, and JFH1/ H77/c3 were described previously (23, 34, 37). Further chimeric HCV constructs, JFH1/corep7, Jc1/E1E2JFH1, JFH1/E1E2J6, Jc1/E1E2H77, and JFH1/H77/C5/E1E2f6, were generated by a PCR-based strategy. The integrity of these constructs was verified by sequencing. Additional information, including the sequences of these constructs, is available upon request.

In vitro transcription, electroporation of HCV RNA, and quantification of viral core protein. In vitro transcriptions of the individual constructs were generated by linearizing by 5 to 10 μg of the respective plasmid by digestion for 1 h with MluI. Plasmid DNA was extracted with phenol and chloroform, after precipitation with ethanol, dissolved in RNase-free water.

In vitro transcription reaction mixtures contained 80 mM HEPES (pH 7.5), 12 mM MgCl2, 2 mM spermidine, 40 mM dithiothreitol (DTT), 3.125 mM each ribonucleoside triphosphate, 1 μl of RNasin (Promega, Mannheim, Germany) per μl, 0.1 μg plasmid DNA/μl, and 0.6 μl of T7 RNA polymerase (Promega) per μl. After incubation for 2 h at 37°C, an additional 0.3 μl of T7 RNA polymerase/μl reaction mixture was added, followed by another 2 h at 37°C. Transcription was terminated by the addition of 1.2 μl of RNase-free DNase (Promega) per μg of plasmid DNA and 30 min of incubation at 37°C. The RNA was extracted with acidic phenol and chloroform, precipitated with isopropanol, and dissolved in RNase-free water. The concentration was determined as described previously (41). In brief, Huh7.5 cells were seeded into 96-well plates at a density of 1 × 104 cells per well 24 h prior to inoculation with dilutions of filtered cell culture supernatant (at least six wells were used per dilution). Two to 3 days later, cells were washed with PBS, fixed for 20 min with ice-cold methanol at −20°C, washed three times with PBS, and then permeabilized and blocked for 1 h with PBS containing 0.5% saponin, 1% bovine serum albumin, 0.2% dried skim milk, and 0.02% sodium azide. Endogenous peroxidases were blocked by incubating cells for 5 min with PBS containing 0.3% hydrogen peroxide. After washing three times with PBS and once with PBS containing 0.5% saponin (PBS-saponin), NASS was detected with a 1:1,000 dilution of hybridoma supernatant 9E10 (10) in PBS-saponin for 1 h at RT or overnight at 4°C. Cells were washed as described above, and bound 9E10 was detected by incubation with peroxidase-conjugated antibodies specific to murine IgG (Sigma-Aldrich, Steinheim, Germany) diluted 1:200 in PBS-saponin. After 1 h of incubation at RT, cells were washed as specified above. Finally, peroxidase activity was detected by using the Vector NovaRED substrate kit (Linaris Biologische Produkte GmbH, Wertheim, Germany).

Immunohistochemical staining and virus titration. Virus titers were determined as described previously (41). In brief, Huh7.5 cells were seeded into 96-well plates at a density of 1 × 104 cells per well 24 h prior to inoculation with dilutions of filtered cell culture supernatant (at least six wells were used per dilution). Two to 3 days later, cells were washed with PBS, fixed for 20 min with ice-cold methanol at −20°C, washed three times with PBS, and then permeabilized and blocked for 1 h with PBS containing 0.5% saponin, 1% bovine serum albumin, 0.2% dried skim milk, and 0.02% sodium azide. Endogenous peroxidases were blocked by incubating cells for 5 min with PBS containing 0.3% hydrogen peroxide. After washing three times with PBS and once with PBS containing 0.5% saponin (PBS-saponin), NASS was detected with a 1:1,000 dilution of hybridoma supernatant 9E10 (10) in PBS-saponin for 1 h at RT or overnight at 4°C. Cells were washed as described above, and bound 9E10 was detected by incubation with peroxidase-conjugated antibodies specific to murine IgG (Sigma-Aldrich, Steinheim, Germany) diluted 1:200 in PBS-saponin. After 1 h of incubation at RT, cells were washed as specified above. Finally, peroxidase activity was detected by using the Vector NovaRED substrate kit (Linaris Biologische Produkte GmbH, Wertheim, Germany).

Metabolic radiolabeling of proteins, immunoprecipitation, and Western blotting. Twenty-four hours after electroporation, Huh7.5 cells were washed with PBS, starved in methionine-cysteine-free medium for 1 h, and incubated for 6 h in methionine-cysteine-free DMEM supplemented with 100 μCi/ml of Express Protein labeling mix (PerkinElmer, Rodgau-Jügesheim, Germany). Cell lysates were prepared by using 1 ml of ice-cold NPB per well of a 6-well plate (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS), supplemented with a complete protease inhibitor cocktail, as recommended by the manufacturer (Roche). Lysates were cleared by centrifugation at 13,800 × g for 15 min at 4°C. The cleared lysates were used for immunoprecipitation using the E2-specific antibody AP33 (42). Immune complexes were resolved by denaturing SDS-PAGE and detected by autoradiography. For immunoprecipitation without radiolabeling, the cells were harvested at 48 h posttransfection by trypsination, and cell pellets were lysed in 230 μl lysis buffer (PBS supplemented with 1% Triton X-100 and a protease inhibitor cocktail [Complete, Roche]). Out of these, 200 μl was used for immunoprecipitation with AP33, and 20 μl was kept for the inputs (one-fifth of the input). For each immunoprecipitation, 25 μl of protein G-agarose beads (Roche) was washed 3 times in PBS. Subsequently, washed beads were incubated with 3.3 μg antibody diluted in PBS for 2 h at 4°C under rotation. In parallel, cell lysates were preclarified on protein G-agarose in the absence of antibody for 2 h at 4°C. Afterwards, preclarified cell lysates were added onto the washed antibody–bound beads and incubated overnight at 4°C, under constant rotation, in PBS–1% TRI-
ton X-100. Beads were then washed 5 times in PBS–1% Triton X-100 (three times quickly and twice with a 5-min incubation time under rotation) and finally washed once quickly in water before protein elution in Laemmli buffer and SDS-PAGE analysis.

Freeze-and-thaw lysates of HCV-transfected cells. Huh7.5 cells were transfected with different HCV genomes, cell culture supernatants were harvested at 48 h posttransfection, and virus titers were determined by a 50% tissue culture infective dose (TCID_{50}) assay. Cell-associated infectivity was determined essentially as described previously (23). Briefly, cells were extensively washed with PBS, scraped, and centrifuged for 5 min at 400 × g. Cell pellets were resuspended in 1 ml of DMEM containing 5% FCS and subjected to three freeze-thaw cycles using liquid nitrogen and a heating block set to 37°C. Samples were then centrifuged at 10,000 × g for 10 min at 4°C to remove cell debris, and cell-associated infectivity was determined by a TCID_{50} assay.

Rate-zonal centrifugation. HCV-transfected cells seeded into 10-cm dishes were scraped in 250 μl TNE buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 2 mM EDTA) at 48 h posttransfection and subjected to five cycles of freezing and thawing. Postnuclear supernatants collected after centrifugation of the crude lysate for 5 min at 4°C at 1,700 × g were layered on top of preformed continuous 0 to 30% sucrose-TNE gradients and spun at 270,000 × g for 1 h at 4°C in a Sorvall TH-641 ultracentrifuge rotor. Subsequently, 10 fractions (1 ml each) were harvested from the top, and the respective refractive index was measured by refractometry.

Statistical analysis. Statistical analysis was done with R (http://www.r-project.org/). Due to small sample sizes, a two-sample Mann-Whitney U test was used to assess the difference between sample distributions. P values were calculated, and differences are reported as significant if the P value was ≤0.05. Differences were considered not significant if P values of >0.05.

RESULTS

Features of HCV core and p7 determine efficiency of particle production by intragenotypic HCV chimeras. It has been described that the intragenotypic chimeric genome Jc1, composed of the region encoding core up to the first putative transmembrane segment of NS2 of the J6CF isolate and the remainder of the genome of the JFH1 isolate, replicates at levels similar to those of JFH1 but supports much higher levels of production of infectious HCV particles (34). We assumed that the higher titers achieved and faster kinetics of virus production must be due to determinants residing in the J6CF sequence from core to p7. Thus, we constructed chimeric HCV genomes by transfecting J6CF-derived core (JFH1/core6), p7 (JFH1/p76), and a combination of both (JFH1/corep76) into JFH1 (Fig. 1A). These chimeras were transfected into highly permissive Huh7.5 cells, in parallel with Jc1 and JFH1 as reference genomes. The kinetics of release of infectivity from transfected cells were determined with a limiting-dilution assay (23). As previously described, JFH1 titers were comparatively low at 24 h posttransfection and increased to 10^{4} TCID_{50}/ml at later time points. In contrast, peak Jc1 titers were 100-fold higher with 10^{6} TCID_{50}/ml (34) (Fig. 1B). Interchange of J6CF p7 into JFH1 increased viral titers 10-fold over the titers of the parental genome, and core transfer yielded even higher titers, 50-fold over JFH1 titers. Insertion of both J6CF-derived proteins core and p7 enhanced viral titers 100-fold, reaching a level comparable to that of Jc1. To rule out that the differences observed were not caused by changes in RNA replication, translation, or specific infectivity of viral particles, extra- and intracellular core protein levels were determined by an ELISA (Fig. 1C). Intracellular core amounts showed only slight differences for all genomes tested, and the level of released core protein grossly correlated with supernatant infectivity. Thus, efficiency of virus assembly/release was affected by core and p7 exchanges. These results indicate that core and p7 are crucial determinants for viral particle production in the context of JFH1-J6-intergenotypic chimeras.

Glycoprotein genes modulate efficiency of virus production within intragenotypic HCV chimeras. To investigate the relevance of E1 and E2 genes for the efficiency of virus production by
intrageneric GT2a chimeras, we constructed a JFH1 genome with the glycoproteins of J6CF (JFH1/E1E2J6) and a chimeric Jc1 genome with the glycoproteins of JFH1 (Jc1/E1E2JFH1) (Fig. 2A). After transfection of these genomes into Huh7.5 cells, release of infectivity into the cell culture supernatant over time was determined. As depicted in Fig. 2B, the transfer of JFH1-derived E1 and E2 into Jc1 caused a modest decrease in virus titers compared with the titers of the parental Jc1 genome. Exchange of J6CF-derived glycoproteins into JFH1 yielded titers of about 10^4 TCID_{50}/ml at 72 h posttransfection, which is comparable to the titers of the parental JFH1 genome (Fig. 2B). Levels of intracellular core protein accumulating in transfected cells showed only slight differences between the chimeric genomes, arguing for nearly equivalent levels of RNA replication (Fig. 2C). The differences in virus production between Jc1- and JFH1-based genomes were also comparable, with the differences of the amounts of core protein released from transfected cells indicating that the specific infectivity conferred by the glycoprotein transfer was not affected (Fig. 2C). In summary, these data suggest that within intrageneric GT2a-GT2a chimeras, glycoprotein genes can be exchanged without causing gross changes in virus production efficiency.

These findings raised the hope that non-GT2a-derived envelope protein genes could also be transferred to the highly efficient GT2a chimera, permitting efficient virus production of particles carrying these glycoproteins. To test this, we next generated a chimeric Jc1 genome encoding the glycoproteins of GT1a isolate H77 (Jc1/E1E2H77). In parallel, we inserted the J6CF-derived envelope proteins into the GT1a-GT2a chimera JFH1/H77/C3 (34), to create JFH1/H77/C3/E1E2J6 (Fig. 3A). As noted previously (34), Jc1 and JFH1/H77/C3 differ with respect to the extent and kinetics of accumulation of infectious virions, with 10^6 TCID_{50}/ml for Jc1 and only 10^2 TCID_{50}/ml for JFH1/H77/C3 (Fig. 3B). The replacement of J6CF glycoproteins by H77 envelope proteins suppressed release of infectious particles more than 1,000-fold. Remarkably, exchange of J6CF-derived glycoproteins into JFH1/H77/C3 enhanced production of infectious particles 50-fold (Fig. 3B). To rule out that these effects were due merely to isolate-specific differences in assembly competence of J6CF- and H77-derived glycoproteins, we performed similar experiments with intergeneric glycoprotein chimeras between J6CF and Con1 (GT1b) and observed similar results (Fig. 3D and E). The replacement of J6CF glycoproteins by Con1 envelope proteins suppressed infectious particle production dramatically, and at 48 h, viral titers were 50-fold enhanced for JFH1/Con1/C3/E1E2J6 in comparison to those for JFH1/Con1/C3 (Fig. 3D). Thus, both GT1-derived E1-E2 genes strongly reduced virus production in the context of the GT2a chimeric genome, whereas J6CF-derived genes facilitated release of infectious progeny from the GT1a-JFH1 and the GT1b-JFH1 chimeric genomes (Fig. 3). Intracellular core amounts were similar for all tested genomes, demonstrating comparable replication efficiencies, and the levels of extracellular infectivity correlated with the extracellular core levels, indicating that virus assembly/release was affected by the glycoprotein exchanges (Fig. 3C and E).

These data suggested that genotype 1a glycoproteins in the Jc1 backbone decrease the number of secreted infectious particles. This may be attributable to defective virion assembly or impaired release of virions. To distinguish between these two possibilities, we compared the quantities of intracellular infectious viruses prepared by repetitive freeze-and-thaw cycles and extracellular infectious virions produced upon transfection (Fig. 4). In the case of Jc1/E1E2H77, total infectivity was reduced by several orders of magnitude (Fig. 4A), and the parallel decrease of intra- and extracellular infectivity indicates a specific impairment of HCV assembly rather than release (Fig. 4B). The titer-enhancing effect of JFH1/H77/C3/E1E2J6 is also reflected in the amounts of intracellular infectious particles and core protein levels, consistent with
more efficient assembly of this glycoprotein chimera than of the parental construct JFH1/H77/C3 (Fig. 4B and C). In conclusion, transfer of GT1a-derived glycoprotein into Jc1 abrogated efficient HCV assembly, indicating genetic incompatibilities between the glycoproteins and other viral factors. On the other hand, insertion of J6CF-derived E1-E2 genes into the JFH1/H77/C3 or JFH1/Con1/C3 chimeras increased virus assembly of these chimeras. This finding suggests that glycoprotein compatibility with viral factors other than core, p7, and the N-terminal portion of NS2, which are derived from JFH1 (GT2a) in these chimeras, could influence virus production.

Heterologous glycoproteins within intergenotypic chimeras do not cause gross changes in polyprotein processing, protein interactions, and subcellular localization of viral proteins. To explore the molecular mechanism underlying the assembly defect apparent in intergenotypic glycoprotein chimeras, we first compared the subcellular localizations of core, E2, and NS2 between the different chimeric genomes. As shown in Fig. 5A, E2 was localized at the ER compartment, and no obvious differences between the parental constructs Jc1 and JFH1/H77/C3 and the glycoprotein chimeras Jc1/E1E2H77 and JFH1/H77/C3/E1E2J6 were observed (Fig. 5A). Comparable staining patterns of the different glycoprotein chimeras were also observed for the core protein and NS2, indicating no major influence of heterologous glycoprotein insertion on viral protein subcellular localization.
To assess whether the intergenotypic glycoprotein chimeras possessed a defect in glycoprotein processing, we analyzed the processing of E2, p7, and NS2 by pulse labeling of Huh7.5 cells transfected with the parental Jc1 and JFH1/H77/C3 genomes and the corresponding glycoprotein chimeras. Immunoprecipitations using an E2-specific antibody showed that E2, E1, and NS2 were precipitated in the case of Jc1, as previously described for JFH1 (Fig. 5B) (23). No major differences in precipitation patterns of the different mutants could be observed; larger amounts of viral proteins were detected only for Jc1/E1E2H77, which might be attributed to differences in the affinity of the antibody for E2 proteins of different genotypes (Fig. 5B). Furthermore, these results
show that processing at the E2-p7 and p7-NS2 junctions occurred and yielded comparable quantities of E1-E2 complexes. To confirm these findings, we investigated the physical interaction by immunoprecipitation of E2 followed by Western blotting against NS2 (Fig. 5C). Huh7.5 cells were transfected with the indicated constructs, and E2-containing immunocomplexes were captured from lysates that were prepared at 48 h posttransfection. As observed by radioimmunoprecipitation, similar quantities of NS2 could be pulled down for the different mutants (Fig. 5C). As noted above, we were able to detect a truncated form of NS2, especially in the input. The nature and function of this product still remain elusive (43). In summary, swapping of genotype 1 glycoproteins into Jc1 had no major influence on subcellular localization, processing, and interactions of viral proteins.

**HCV glycoproteins are important for core protein envelopment in a genotype-specific fashion.** To further dissect the defect in morphogenesis caused by insertion of the GT1a-derived heterologous glycoproteins into Jc1, we assessed the resistance of intracellular core to proteolytic digestion by proteinase K. In these assays, HCV-transfected cells are ruptured by repetitive cycles of freezing and thawing. Subsequently, proteinase K is added, and the amount of core protein resistant to this treatment, presumably due to envelopment into a lipid membrane, is quantified by Western blotting and ELISA (44, 45). A fraction of these lysates is treated with Triton X-100, which dissolves all membranes and proves that the concentration of proteinase K used was sufficient to cleave core protein. As depicted in Fig. 6A, for the parental genomes Jc1 and JFH1/H77/C3, about 30% and 15% of intracellular core was resistant to protease digestion, respectively, and had already acquired an envelope (Fig. 6A). The transfer of genotype 1a-derived glycoproteins into Jc1 reduced the amount of protected core, indicating that the amount of core protein accessible to proteolytic digestion was increased. The insertion of E1/E2 from J6CF into JFH1/H77/C3 had no detectable influence on core protection, which may be due to the modest dynamic range of this assay (Fig. 6A). To further corroborate that insertion of H77-derived E1-E2 genes into Jc1 decreases core protein envelopment, we analyzed the sedimentation velocity of core protein structures derived from this chimera and the parental Jc1 genome. Previously, we had shown that deletion of glycoproteins or disruption of p7 function caused a defect in capsid envelopment, which resulted in an increase of core protein structures with a slow sedimentation behavior in these rate-zonal gradients (44). Similar to our previous observations (44), core proteins derived from the parental Jc1 genome sediments rapidly, displaying a single peak in fraction 7 of our gradient (Fig. 6B). In contrast, the bulk of core protein structures originating after transfection of the glycoprotein chimera Jc1/E1E2H77 sediments slowly, with a peak in fraction 3 representing nonenveloped capsids, as shown recently (Fig. 6B) (44). As observed for the protease digestion assay, no major differences were observed for the parental JFH1/H77/C3 genome and JFH1/H77/C3/E1E2j6 (Fig. 6B, bottom). Notably, however, both constructs yielded clearly lower levels of rapidly sedimenting core protein species than did Jc1, suggesting that large numbers of these complexes are a hallmark of viral genomes with highly efficient virus production. In conclusion, intergenotypic glycoprotein chimeras accumulated as nonenveloped capsid-like structures, revealing an important role for the glycoproteins in membrane envelopment.

**DISCUSSION**

HCV envelope glycoproteins play important roles in different steps of the viral life cycle. During viral entry, they interact with host cell receptors and are involved in fusion of the viral and host cell membranes. In the context of virus assembly, glycoproteins are involved in the formation of viral particles. The glycoproteins are synthesized as a single polyprotein that is cleaved by viral and cellular proteases to yield functional glycoproteins. The glycoproteins are important for the morphogenesis of viral particles, including the formation of viral envelopes. The glycoproteins are also important for viral entry and fusion with the host cell membrane.

**FIG 6** Proteinase K digestion and rate-zonal separation of HCV glycoprotein chimeras. (A) HCV genome-transfected detergent-free cell lysates were subjected to a proteolytic digestion protection assay as follows. Lysates were separated into three parts: (i) untreated, (ii) treated with 50 μg/ml proteinase K (PK) for 1 h on ice, or (iii) lysed in 5% Triton X-100 prior to proteinase K treatment. The amount of protease-resistant core was determined by a core-specific ELISA. Mean values and standard errors of data from 5 independent experiments are shown. (B) Postnuclear supernatants of cell lysates obtained by repetitive cycles of freezing and thawing at 48 h posttransfection were layered on top of a preformed continuous 0 to 30% sucrose density gradient and subjected to rate-zonal centrifugation for 1 h at 270,000 × g. Core content was measured along the gradient by ELISA and normalized to the total core amount in the lysate. Representative values of 3 independent experiments are plotted.
cellular membranes. In the late steps of the viral life cycle, E1 and E2 are crucial for viral assembly, as deletion of the glycoprotein abrogates viral particle production (11, 44). However, the precise role of the glycoproteins in HCV morphogenesis is poorly understood. To further dissect the role of the E1 and E2 glycoproteins in virion morphogenesis, we generated chimeric viral genomes, replacing the glycoprotein regions with corresponding regions from different isolates and genotypes. Intragenotypic chimeric HCV genomes revealed that core and p7 are crucial determinants of viral particle production, whereas glycoprotein chimeras between J6CF and JFH1 were fully functional and produced particles with comparable efficiencies. These findings raise the hope that at least within GT2a, different GT2a-derived glycoprotein genes could be shuffled into the Jc1 backbone in order to permit assessment of their functional properties. In contrast, HCV assembly of intergenotypic glycoprotein constructs was highly attenuated, although no major effect on subcellular localization and interaction of viral proteins was observed. However, swapping of GT1 glycoproteins into a GT2a genome led to the accumulation in nonenveloped capsid-like structures, revealing an important, genotype-dependent role of the glycoproteins in viral envelopment.

Chimeric HCV genotype genomes like Jc1, consisting of J6 and JFH1 segments, produce much higher virus titers than the parental JFH1 or genotype 1 viral chimeras (34). To identify determinants in the core to NS2 proteins that modulate the efficiency of virus production, we generated chimeric viruses between J6CF and JFH1. Combination of both J6CF-derived proteins core and p7 enhanced viral titers 100-fold, which are comparable to those of the parental JFH1 or genotype 1 viral chimeras (34). To identify determinants of optimal HCV entry (48). In the present study, E1 and E2 were swapped together into the backbone of another genotype, and all isolates used have been shown to be entry competent (49). Interestingly, the transfer of genotype 1 glycoproteins into Jc1 had no major influence on subcellular localization of the viral proteins E2, core, and NS2, at least as far as they are detectable by the resolution of our assay (Fig. 5A). In addition, polyprotein processing at the E2-p7 and p7-NS2 junctions was not influenced by the heterologous glycoproteins (Fig. 5B). The HCV NS2 protein has been implicated as a key regulator of HCV assembly and interacts with several proteins, including E2, during HCV assembly (21, 22, 50–52). We therefore tested the interaction of E2 with NS2 by immunoprecipitation experiments and observed no defects in interaction profiles between the parental genomes and the glycoprotein chimeras. A genetic incompatibility with p7 might be possible, but we were recently unable to show a direct interaction between p7 and E2 (38). However, ratezonal centrifugation of detergent-free cell lysates demonstrated an accumulation of slow-sedimenting, core complexes in the case of the genotype 1a glycoprotein chimera (Fig. 6). These core protein structures resemble the ones accumulating upon transfection of Jc1 with deleted glycoproteins or inactive p7, which lacked a lipid envelope (44). In line with this, core protein derived after transfection of the glycoprotein chimera Jc1/E1E2H77 was less resistant to protease K digestion. These findings corroborate the notion that insertion of the heterologous GT1a-derived envelope proteins had decreased membrane envelopment of core protein, which in turn limits assembly and release of infectious progeny. Notably, JFH1/H77/C3 and JFH1/H77/C3/E1E2J6 produced modest infectious titers and intermediate levels of rapidly sedimenting core protein structures compared with the highly efficient Jc1 (Fig. 6B). Thus, efficient membrane envelopment of core, which is estimated by the proteinase K assay and the ratezonal gradient, albeit with modest dynamic ranges of these assays, may be a hallmark of those viral genomes with highly efficient assembly growing to high infectious titers. Future work should address which viral determinants and host factors specifically determine this critical step in virus morphogenesis.

Notably, we observed that insertion of J6CF-derived E1-E2 into JFH1/H77/C3 and JFH1/Con1/C3 chimeras increased virus production by these chimeras. Presently, we cannot strictly rule out that this is due to a higher permissiveness of J6CF-derived E1-E2 for HCV assembly than H77- or Con1-derived E1-E2. However, our observation that J6CF-derived E1-E2 did not increase virus production in the context of JFH1 makes this explanation less likely. Alternatively, it is possible that insertion of the J6CF-derived E1-E2 proteins increases virus production of the intergenotypic GT1-GT2a chimeras JFH1/H77/C3 and JFH1/Con1/C3 by facilitating the interplay of the envelope proteins with other viral proteins derived from the GT2a portion of these chimeras. Thus, these findings suggest that the HCV envelope proteins may cooperate with viral determinants resident downstream of the N-terminal domain of NS2 in a genotype-specific fashion. In turn, this interplay may be important to coordinate core protein envelopment during assembly. Clearly, more work is needed to fully understand the network of envelope protein interactions during HCV assembly.

In summary, we characterized intra- and intergenotypic glyco-
protein chimeras in the HCV assembly process. First, we demonstrated that the concerted action of core and p7 facilitates efficient virion production in the context of GT2a chimeric genomes. While exchange of glycoproteins from the same genotype was fully functional, genotype 1-derived transfer of E1-E2 drastically reduced virus particle production. Capsid protection and envelopment assays unveiled a novel role of the HCV glycoproteins in viral membrane envelopment.

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