Regulation of core expression during the hepatitis C virus life cycle

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Core plays a critical role during hepatitis C virus (HCV) assembly, not only as a structural component of the virion, but also as a regulator of the formation of assembly sites. In this study, we observed that core is expressed later than other HCV proteins in a single viral cycle assay, resulting in a relative increase of core expression during a late step of the viral life cycle. This delayed core expression results from an increase of core half-life, indicating that core is initially degraded and is stabilized at a late step of the HCV life cycle. Stabilization-mediated delayed kinetics of core expression were also observed using heterologous expression systems. Core stabilization did not depend on its interaction with non-structural proteins or lipid droplets but was correlated with its expression levels and its oligomerization status. Therefore in the course of a HCV infection, core stabilization is likely to occur when the prior amplification of the viral genome during an initial replication step allows core to be synthesized at higher levels as a stable protein, during the assembly step of the viral life cycle.

INTRODUCTION

Hepatitis C virus (HCV) is a small, enveloped, single-stranded positive RNA virus, belonging to the Flaviviridae family. With about 160 million persons infected globally, HCV is a major public health problem (Lavanchy, 2011). For many years, studies of the molecular and cellular aspects of its infection have been hampered by the lack of a cell culture system. However, with the recent development of a HCV cell culture (HCVcc) system, regulatory mechanisms of the HCV infection can now be investigated in the context of a complete life cycle.

HCV genome contains a single ORF. Its translation leads to the production of a polyprotein precursor, which is cleaved into ten polypeptides by cellular and viral endopeptidases. The N-terminal third of the polyprotein contains the structural proteins core, the capsid protein, and E1E2, a heterodimeric envelope glycoprotein. They are followed by the non-structural proteins p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B (Lindenbach et al., 2007). Non-structural proteins NS3-4A, NS4B, NS5A and NS5B are necessary and sufficient for replication of the genomic RNA (Lohmann et al., 1999). On the other hand, the assembly and budding of new infectious particles is likely to involve all the structural and non-structural proteins (Bartenschlager et al., 2011; Miyanari et al., 2007). Mechanisms regulating the transition from the translation/replication step to the assembly step of the HCV life cycle have remained elusive.

Among the viral proteins, core appears to be a major organizer of the assembly step. Core recruits active replication complexes to the assembly site by interacting with NS5A (Masaki et al., 2008; Miyanari et al., 2007). NS2 and p7 also regulate the assembly step, by interacting with each other and with envelope glycoproteins and non-structural protein NS3 (Jirasko et al., 2010; Popescu et al., 2011; Stapleford & Lindenbach, 2011).

To study mechanisms potentially regulating the transition from replication to assembly, we investigated if proteins specifically involved in assembly are expressed with the same kinetics as those that are involved in both replication and assembly. It is generally assumed that all HCV proteins are expressed with similar kinetics, since all HCV proteins...
are initially synthesized from a single precursor. However, the expression of a protein actually results from the balance between its synthesis and degradation. By measuring the kinetics of expression of HCV proteins in a single cycle assay, we confirmed that most HCV proteins are expressed with very similar kinetics. However, we also unexpectedly observed a time lag for core expression.

RESULTS

Core is expressed later than other HCV proteins during the HCV life cycle

To determine the kinetics of expression of HCV proteins, we measured their relative expression levels at different time points in a single cycle assay using Huh-7w7, CD81-deficient Huh-7-derived cells (Rocha-Perugini et al., 2009). We focused our study on proteins only involved in assembly (core, E1, E2 and NS2), and compared their expression with that of two markers of replication complexes (NS3 and NS5A), which are involved in both replication and assembly. As shown in Fig. 1(a), all proteins were undetectable at 12 h post electroporation. Expression levels rapidly rose between 12 and 36 h, to reach a maximal value at 48 h. Then, at 72 h post electroporation, we observed an apparent decrease of expression for all HCV proteins. This apparent decrease can be attributed to the fact that infected cells (24 ± 1 % in these experiments) probably grew more slowly than uninfected cells. Accordingly, the total amounts of HCV proteins per well did not decrease at 72 h post electroporation, when values were not normalized to the protein content of the lysates (not shown). These results indicate that HCV protein expression levels reached equilibrium after approximately 36–48 h of expression.

Core expression levels appeared to increase less rapidly than those of the other HCV proteins tested (Fig. 1a). Most HCV proteins were found to accumulate with a half-maximal value of 25–27 h (Fig. 1b). In contrast, the half-maximal accumulation of core was reached at approximately 33 h, indicating a 6–8 h lag. To rule out that this delay in core expression could in fact result from different sensitivities of the antibodies used for quantification (Fig. S1, available with the online Supplementary Material), we repeated the quantification with a series of antibodies to core, E1 and E2. Although some variations were observed for a protein probed with different antibodies, the results confirmed the delay of core expression (Fig. 1c). When core and NS3 were quantified by ELISA, core reached higher expression levels than NS3 at 48 h, and then decreased at 72 h, whereas NS3 levels did not (Fig. 1d). Compared with their expression at 48 h, the relative expression at 24 h was lower for core (13 %) than for NS3 (29 %), consistent with results obtained by immunoblot quantification. The percentage of secreted core protein increased over time (Fig. 1e), and correlated with infectious titres (Fig. 1f). However, core release was less than 1 %, indicating that the lower levels of core expression at 24 h could not be explained by an excess of secretion.

To confirm the delay of core expression in an unbiased manner, we introduced an HA epitope tag in core or in NS5A, in order to compare core and NS5A expression with the same antibody. The insertion of an HA tag in core or in NS5A did not alter replication efficiency, as assessed by immunoblot analysis of E1 expression (Fig. 2a). Again, slower kinetics of core expression were observed using an anti-HA antibody (Fig. 2a). The delay in core expression was very similar to that observed with the untagged virus (Fig. 2b).

To further confirm the kinetics of core expression by an independent method, we analysed core expression by immunofluorescence. Huh-7 cells were infected with HCVcc, and double-labelled with antibodies to core and NS5A, at 32 or 72 h post-infection (p.i.). These two time points were chosen to have well-detectable signals for the early time point (32 h) and to have a major part of the population of infected cells at a late step of the HCV life cycle for the late time point (72 h). The experiment was also performed in electroporated Huh-7w7 cells. All core-expressing cells also expressed NS5A, while a number of cells expressing NS5A did not express any detectable core, whichever time the cells were fixed (Fig. 3a). As shown in Fig. 3(b), there were more than two times more double-positive Huh-7 cells at 72 h p.i. than at 32 h p.i. Similar results were obtained with an HCVcc chimera expressing genotype 1a structural proteins (Fig. 3b). The number of double-positive cells reached about 95 % at 72 h in Huh-7w7 cells, and only about 50–55 % in Huh-7 cells (Fig. 3b). This difference probably resulted from the fact that infections are synchronized in Huh-7w7 cells and not in Huh-7 cells. Therefore, the Huh-7 population contains infected cells at different stages of the viral life cycle, when most of the Huh-7w7 cells are at a late step. These results are consistent with core being expressed with slower kinetics than NS5A, regardless of the difference in sensitivity of the two antibodies, otherwise similar percentages of double-labelled cells would have been measured at both time points.

A delay in core expression was also visible in the labelling pattern of HCV foci. At 72 h p.i., isolated foci often displayed a few cells brightly stained with both anti-core and anti-NS5A antibodies, surrounded by cells with weaker labelling (Fig. 3c). This difference in labelling is likely to reflect the occurrence of at least two rounds of infections: the small group of brighter cells at the centre representing the primary infected cell later divided by one or two mitoses, and the cells at the periphery with weaker labelling probably resulting from a second round of infection. Considering this scenario of focus formation, we consistently observed that the difference of labelling intensities between primary and secondary infected cells was less important for NS5A than for core (Fig. 3d). We quantified the relative expression levels of both proteins in secondary infected cells as a percentage of the signal measured for the primary infected...
**Fig. 1.** Kinetics of expression of HCV proteins. (a) JFH1 genomic RNA was introduced by electroporation into CD81-deficient Huh-7w7 cells. The expression of indicated viral proteins was monitored by immunoblot at the indicated time post electroporation. (b) The signals were quantified and plotted against time. (c) Quantification with two anti-E1 antibodies (green), four anti-E2 antibodies (blue), and three anti-C antibodies (red). Expression levels were expressed as percentages of the 48 h value. (d) Quantification by ELISA of core and NS3 in cell lysates. (e) Quantification by ELISA of core release. (f) Infectious titres. Values are means ± SD of two experiments, each performed in duplicate.

**Fig. 2.** Kinetics of core and NS5A expression of HA-tagged viruses. (a) Genomic RNAs of HA-tagged viruses were electroporated into Huh7w7 cells and the expression of HA-core, NS5A-HA, E1 and tubulin was monitored. (b) Quantification of the signals. HA signals are means ± SD of two experiments, each performed in duplicates. E1 signals were quantified once.
cells of 10 well-isolated foci. We found a fourfold higher relative expression level of NS5A, as compared with core, in secondary infected cells (Fig. 3d). Again, this observation points to a delay in core expression, as compared with NS5A. Together with the results of the kinetics of expression, these data indicate that core is expressed with a time lag during the HCV life cycle.

Core is stabilized at a late stage of the HCV infection

All HCV proteins are co-translated as a single polyprotein, and thus synthesized in equimolar amounts. To assess whether the peculiar kinetics of core expression could result from a variation of its stability as the HCV life cycle progresses, we measured its half-life at two time points post-infection. We made use of a cycloheximide (CHX) chase assay to measure half-lives of core, and of E1 and NS5A as controls. When the analysis was performed at 32 h p.i., a sharp decrease in core expression levels was observed (Fig. 4a). In contrast, the expression levels of E1 (Fig. 4b) and NS5A (Fig. 4c) decreased more slowly during the chase. Immunoblot signals were quantified and plotted versus CHX chase times, in order to estimate HCV protein half-lives. Core half-life was found to be approximately 95 min, whereas E1 half-life was almost 9 h and NS5A half-life was longer than 4 h (Fig. 4d). Strikingly, when the analysis was repeated at a later time point (72 h p.i.), core appeared much more stable than at 32 h p.i. (Fig. 4a). There was a tenfold increase in core half-life, whereas E1 and NS5A half-lives were increased by less than two times (Fig. 4d). Taken together, these results indicate that core is initially unstable, and is much more stable later on. This result indicates that core undergoes a stabilization process during the HCV life cycle.

Core stabilization depends on expression levels and correlates with oligomerization

We next investigated if the stabilization of core is restricted to the context of an infection, or if it could also be observed in heterologous expression systems. Core was expressed in Huh-7 cells by transfection, together with E1E2 and GFP, and the kinetics of expression of the proteins were analysed by immunoblotting (Fig. 5a). Again, a lag was observed for core expression, as compared with E1, E2 and GFP (Fig. 5b), suggesting that the delayed core expression results from intrinsic properties of this protein.

To assess whether this delayed expression resulted from stabilization, core half-life was measured at 20 and at 40 h...
after transfection. These two time points were chosen because core signal was easily detectable at 20 h and was close to the maximal expression level at 40 h. As shown in Fig. 5(c), core half-life was approximately 90 min at the earlier time point, and increased about three times at the later time point (275 ± 15 min). In contrast, GFP half-life did not significantly vary over time. This result indicated that the stabilization process could indeed be observed in a heterologous expression system, although the extent of core stabilization was not as high as during an HCV infection.

**Fig. 4.** Change in core stability during the HCV life cycle. JFH1-infected Huh-7 cells were incubated for the indicated periods with cycloheximide (CHX, 100 μg ml⁻¹) to block protein translation, and the expression levels of core (a), E1 (b) and NS5A (c) were monitored by immunoblot at 32 and 72 h p.i. Expression levels at 32 h p.i. (black symbols) and at 72 h p.i. (white symbols) were plotted against CHX chase time. (d) Protein half-lives at 32 and 72 h p.i. from at least three independent experiments (mean ± SD).

**Fig. 5.** Core stabilization in heterologous expression systems. (a) Core, E1, E2 and GFP were co-expressed by transfection in Huh-7 cells, and their expression was monitored by immunoblot at the indicated time post-transfection. (b) The signals were quantified and plotted against time. For each protein, the expression levels were expressed as percentages of the 60 h value, and are means ± SD of three experiments each performed in duplicate. (c, d) Core and GFP were expressed in Huh-7 cells by transfection (c), or by recombinant adenovirus transduction at different m.o.i. (d), and their half-lives were measured using the CHX chase assay at 20 or 40 h post-transduction (values are mean ± SD, from at least three independent experiments). (e) Transfected Huh-7 cells were treated with lactacystin for 8 h at 20 h post-transfection and the expression of core and tubulin was monitored by immunoblotting.
Then, to test the impact of core expression levels on core half-life, we made use of an adenoviral vector. Huh-7 cells were transduced with recombinant defective adenoviruses expressing HCV core, or GFP as a control, at different multiplicities of infection, in order to express core and GFP at different levels, and their half-lives were measured 20 h or 40 h later. The half-life of core did not significantly vary in 20 h samples (from 85 ± 7 to 101 ± 10 min). In contrast, we observed a dramatic increase of core half-life (from 171 ± 16 to 2108 ± 103 min) in 40 h samples (Fig. 5d), corresponding to 2- to 21-fold increases of core half-life, depending on adenovirus m.o.i. GFP half-life was only slightly longer in 40 h samples compared with 20 h samples (1.2-fold increase), but did not vary with the expression levels of the protein. These results indicate that core is stabilized in a manner dependent on its time and level of expression.

Core is degraded by the proteasome (Shirakura et al., 2007; Suzuki et al., 2001). To test if low amounts observed at 20 h resulted from proteosomal degradation, we incubated core-expressing cells with lactacystin, a potent proteasome inhibitor, for 8 h, and quantified core by immunoblot (Fig. 5e). Similar levels of core were observed in control and treated cells, suggesting that core is not degraded by the proteasome at early time points of expression.

The time-dependent stabilization of core suggests that it undergoes a conformational change over time that makes it more stable. The fact that core stabilization also depends on the level of expression of the protein suggests that a concentration-dependent process, such as oligomerization, could drive the mechanism of stabilization. To test this hypothesis, we determined the kinetics of core oligomerization. Core monomers and oligomers from transfected cells were separated by ultracentrifugation (Fig. 6a). A major part of core protein (92 ± 11 %) was detected in supernatants at 20 h post-transfection. In contrast, at later time points, core partitioned mainly into pellets (65 ± 20 and 87 ± 6 % at 40 and 60 h, respectively) (Fig. 6b). More than 96 % of the non-oligomeric protein GFP remained in supernatants up to 60 h post-transfection. This indicates that core is initially expressed as non-pelletable forms, such as monomers, dimers, or small oligomers, and is later included into larger, pelletable oligomeric forms. The kinetics of core oligomerization and stabilization were very similar, suggesting a role of core oligomerization in the mechanism of stabilization.

**Effect of non-structural proteins and lipid droplets on core stabilization**

Core interacts with NS5A, and this interaction is thought to be crucial for HCV particle assembly (Masaki et al., 2008; Miyanari et al., 2007). To test the impact of NS5A, and more generally of non-structural proteins, on core stabilization, core was expressed in Huh-7 cells containing a subgenomic replicon. To verify that NS5A actually interacts with core in replicon-containing cells, we first checked whether core was able to recruit replication complexes to the surface of lipid droplets (LDs) in core-transfected, replicon-containing cells, as it does in JFH1-infected cells (Miyanari et al., 2007). As reported previously for a genotype 1b replicon (Shi et al., 2003), NS5A was not associated to LDs in cells harbouring a JFH1 subgenomic replicon in the absence of core expression (Fig. 7a). In contrast, when core was expressed, NS5A labelling was almost completely redistributed to the vicinity of LDs, where core was also localized (Fig. 7a). This core-mediated recruitment strongly suggests that interactions between core and NS5A occur in replicon-containing cells expressing core. Therefore, this experimental setting was relevant for investigating the impact of non-structural proteins on core stability. Similar values of core half-life were obtained in naïve and replicon-containing cells, both at 20 h (90 ± 12 min in Huh-7 cells; 109 ± 18 min in replicon cells), and at 40 h post-transfection (269 ± 18 min in Huh-7 cells; 304 ± 27 min in replicon cells) (Fig. 7b). These data indicate that the presence of non-structural proteins and the active replication of viral RNA have no impact on core half-life, or on the mechanisms that lead to core stabilization.

Core also interacts with LDs, and this interaction is important for HCV assembly (Boulant et al., 2007). To test if LDs have any influence on core stability, we treated...
core-expressing cells with oleic acid (OA). This treatment resulted in an increase of both the number and the size of LDs (Fig. 7c). Image analysis revealed that mean LD size increased 2.6-fold and their number per cell about 1.5-fold (data not shown), resulting in an increase of total LD surface per cell of approximately four times. In the presence of OA, core half-life was about two times longer, both at 20 h (92 ± 2 min without OA; 174 ± 19 min with OA) and at 40 h (252 ± 18 min without OA; 484 ± 29 min with OA), suggesting that LDs have a positive influence on core stability (Fig. 7d). It is worth noting that the treatment with OA, although positively affecting core half-life at both time points, had no effect on the extent of core stabilization (about 2.8-fold, in these experiments). These data suggest that LDs have a positive effect on core half-life, but no major contribution to the mechanism leading to core stabilization.

DISCUSSION

For many viruses, progress through the life cycle is controlled by the successive expression of different sets of viral proteins over time. Many DNA viruses selectively express proteins during different steps of their life cycle, through transcriptional regulation of immediate early, early and late genes. For some RNA viruses, the regulation of protein expression occurs through subgenomic RNA production or translational frameshifting. For Flaviviridae family members, these two types of regulatory processes have not been observed, except for frameshifting events in the core coding sequence of HCV (Walewski et al., 2001; Xu et al., 2001). However, the functional importance of this frameshifting event in the regulation of the life cycle of the virus has not been clearly established (McMullan et al., 2007; Vassilaki et al., 2008).

We now describe a new mode of selective protein expression for HCV core, through the stabilization of the protein. Since protein expression is the result of a balance between synthesis and degradation, and since all HCV proteins are initially synthesized in equimolar amounts, differences in expression kinetics likely result from differential stability. In our study, expression levels were normalized to their value at 48 h post-electroporation. Therefore, proteins with similar expression kinetics did not necessarily reach equivalent absolute expression levels. Nevertheless, all these proteins have equivalent relative expression kinetics, indicating that their degradation rates are constant, or at least do not significantly vary over time. Accordingly, we observed similar E1 and NS5A half-lives at both time points. This indicates that the expression profiles
of most HCV proteins are likely to reflect a steady accumulation in relation to the amplification of the genomic RNA in infected cells.

In contrast, the peculiar kinetics observed for core indicate that its expression involves different mechanisms. Such a difference in kinetics could in fact result from several potential mechanisms. Post-translational modifications of core at early time points could result in a lack of recognition by antibodies. However, this possibility is very unlikely, since the same delay was observed with viruses expressing tagged proteins. A differential usage during the HCV life cycle of an internal translation initiation site expressing tagged proteins. A differential usage during the course of the HCV life cycle, as the mechanism likely to be responsible for its delayed expression.

Core has been previously shown to be an unstable protein in heterologous expression systems (Shimoyama et al., 2006; Shirakura et al., 2007; Suzuki et al., 2001). Accordingly, we measured a half-life of about 90–100 min in this study, which indeed corresponds to a rather high turnover for a membrane protein. Interestingly, similar half-life values were obtained for core in heterologous expression systems and in HCV infected cells at early time points, indicating that the unstable nature of core protein is not restricted to heterologous expression systems. At later time points, core was found to be much more stable, and this stabilization was dependent on its levels of expression. We did not observe any impact of HCV non-structural proteins, again suggesting that this phenomenon mainly relies on intrinsic properties of the core protein, rather than on cues from other viral factors.

Core has been shown to be ubiquitylated by ubiquitin ligase E6AP and degraded by the proteasome (Shirakura et al., 2007; Suzuki et al., 2001). However, we did not observe any stabilization of core or any accumulation of ubiquitylated core in cells treated with lactacystin or MG132, two proteasome inhibitors, at early time points of core expression (Fig. 5e and data not shown). Indeed, this lack of action of proteasome inhibitors on core expression has already been reported by others (Hope & McLaughlan, 2000; McLaughlan et al., 2002). This suggests that this degradative pathway is not the one that operates at the early time points of core expression.

The time and concentration dependence of core stabilization suggests that core interferes with its own degradation. It is unlikely that core inhibits proteosomal activity, because long-term inhibition of the proteasome is toxic for the cells. Therefore it is more likely that the interference with its own degradation results from a post-translational modification that makes core less sensitive to degradation. As this post-translational modification appears to be concentration-dependent, an oligomerization-dependent process is the most likely. In support of this hypothesis, we observed a good correlation between core oligomerization and stabilization. Previous reports indicated that core is a protein prone to oligomerize in infected and transfected cells (Alsaleh et al., 2010; Klein et al., 2004; Kunkel et al., 2001; Majeau et al., 2004). Core is known to form dimers (Boulant et al., 2005), and is usually found in cell lysates and cell-free expression systems as a mixture of monomers/dimers and larger oligomers, which can be resolved by ultracentrifugation (Alsaleh et al., 2010; Klein et al., 2004). Although the dissociation constants relative to core oligomerization are unknown, it can be reasonably speculated that they are in a range that allows core to be in equilibrium between mono/dimeric and larger oligomeric forms inside the cell. Consequently, higher expression levels should drive more core protein towards oligomeric structures. Therefore, the stabilization of core can be interpreted as a transition from a mono/dimeric less stable form to an oligomeric, more stable form of the protein. In a cell-free expression system, core oligomers were previously shown to be more resistant to proteolysis than core monomers (Klein et al., 2004). By analogy, core oligomers would be the stable form of the protein in infected cells, whereas core monomers would be preferentially recognized and degraded by cellular quality control systems. Alternatively, we cannot exclude the possibility that core stabilization could actually result not directly from oligomer formation, but from another putative post-translational modification that would preferentially occur on core protein engaged in oligomers. We also cannot exclude that core, which is a membrane-associated protein, could partition over time in detergent-resistant microdomains of the ER membrane, and that its presence in pellets after ultracentrifugation reflects such an accumulation in insoluble membranes.

Core is obviously required during late stages of the HCV life cycle, when virion assembly occurs. In addition to its role as a structural component of the virion, core also orchestrates the formation of assembly sites at the ER/LD interface (Bartenschlager et al., 2011; Miyarari et al., 2007). An optimal expression of core is thus essential during late steps of the HCV life cycle, when assembly occurs. On the other hand, subgenomic replicon studies have indicated that core is not required for RNA replication (Lohmann et al., 1999), and it has been shown that core deletion and point mutations inhibiting HCV assembly have no impact on translation or replication of the viral genome (Alsaleh et al., 2010; Murray et al., 2007). All these data are in line with our finding that core is actually expressed with a lag of 6–8 h, and is thus expressed at low levels when replication begins. We propose that this oligomerization-mediated core stabilization is an adaptive mechanism that allows core to be expressed at higher levels during a late step of the HCV life cycle, when it is required for orchestrating HCV assembly.

METHODS

Chemicals. Dulbecco’s modified Eagle’s medium (DMEM), PBS, FCS, 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene
BODIPY 493/503) and DAPI were purchased from Life Technologies. Protease inhibitor mix (complete) was from Roche. Other chemicals were from Sigma.

Antibodies. Mouse anti-E1 mAb A4, anti E2 mAb A11 (Dubuisson et al., 1994) and rat anti-E2 mAb 3/11 (Flint et al., 1999) were produced in vitro by using a MiniPerm apparatus (Heraeus). Mouse anti-core ACAP-27 (Maillard et al., 2001) and anti-NS3 (48D39) mAbs were provided by J.-F. Delagneau (Bio-Rad). ACAP-27 epitope was mapped to residues 40–53 (Maillard et al., 2001). Rabbit polyclonal anti-E2 and mouse anti-core mAb 2H9 (IgG1) (Wakita et al., 2005) were provided by T. Wakita (National Institute of Infectious Diseases, Tokyo, Japan). Mouse anti-core mAb 2H9 (IgG1) (Wakita et al., 2005) obtained from Innogenics. Mouse anti-core mAb 6H6 (Dentzer et al., 2009) provided by C. M. Rice (the Rockefeller University, NY, USA). Rabbit anti-E1 mAb 1G4 was obtained from Invogenics. Mouse anti-β-tubulin mAb was from Sigma. Mouse anti-GFP and rat anti-HA (3F10) mAbs were from Roche.

Cell culture. Huh-7 (Nakabayashi et al., 1982) and Huh-7w7 (Rocha-Purigni et al., 2009) cells were grown in DMEM supplemented with glutamax-I and 10% FCS.

HCVcc. The virus JFH1-CSN6A4 used in this study contained cell culture adaptive mutations and a reconstituted A4 epitope in E1 (Goueslain et al., 2010).

A virus with a tagged core was constructed by inserting a tag (YPYDVPDYAI) containing an HA epitope (underlined sequence) between residues Ser2 and Thr3 of core. To insert an HA tag in NS5A, unique BglII and NrdI sites were first inserted by overlapping PCR between codons of Pro418 and Leu419. Synthetic oligonucleotides 5′-GATCTTATCCATACGATGTTCCAGATTACGCGATATCG-3′ and 5′-GCATACGCATTAGACCTGACGATCATTATCG-3′ were annealed and ligated into BglII and NrdI sites. The sequence inserted between NS5A residues 418 and 419 was RSPYDPVDYAISR. The constructs were verified by sequencing. The H77/JFH1 chimera, which incorporates the coding sequence of core to NS2 of H77 origin into JFH1, was as previously described (Maurin et al., 2011).

Viral genomic RNAs were produced and delivered into Huh-7 or Huh-7w7 cells by electroporation as described by Kato et al. (2003). For infection assays, subconfluent Huh-7 cells grown in a 6-well cluster were incubated with HCVcc in 200 μl of medium for 2 h, and the inoculum was replaced with fresh culture medium. For the single cycle assay, 4 × 105 Huh-7w7 cells were electroporated with 10 μg of in vitro transcribed viral RNA, and plated into 6-well clusters.

Subgenomic replicon. The plasmid pSGR-JFH1 (Kato et al., 2003) was processed as described for HCVcc. Electroporated cells were selected with 0.5 mg genetin ml−1.

Plasmids. To construct a core expression plasmid, a cDNA encoding residues 1–191 with a HA tag (YPYDVPDYAI) inserted between residues 1 and 2 was subcloned into pCIneo (Promega) between a Kozak consensus sequence and a stop codon. The construct was confirmed by sequencing. The plasmid used to express E1E2 glycoproteins (pC DNA-E1E2) contained the E1E2 coding sequence of the JFH1 strain. The GFP-expression plasmid (pEGFP-C1) was from Clonetech.

DNA transfections. Cells grown in a 24-well were transfected with 0.5 μg of plasmid DNA mixed with 2 μl Trans-IT L71 reagent (Mirus). Core and GFP-expressing plasmids were co-transfected in a 9:1 ratio.

Adenoviruses. The coding sequence of JFH1-CSN6A4 core residues 1–191 was introduced in a recombinant defective adenovirus vector (Ad:core) by homologous recombination in Escherichia coli, as described by Sérón et al. (2011). The recombinant adenovirus expressing EGP was described previously (Sérón et al., 2011). Viral stocks were titred in Huh-7 cells by the TCID50 method using immunofluorescence or GFP fluorescence to detect positive cells.

Immunofluorescence. Cells were rinsed three times with cold PBS, and lysed at 4 °C for 20–30 min in a lysis buffer containing 50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 1% (v/v) Triton-X, 0.1% (w/v) SDS, 1 mM PMSF, and a mix of protease inhibitors. The protein content was determined by the bicinchoninic acid method (Sigma). The signals were recorded using a LAS 3000 apparatus (Fujifilm). Quantification of unsaturated signals was carried out using the gel quantification function of ImageJ.

Cycloheximide (CHX) chase assay. Cells were incubated with 100 μg CHX ml−1 for up to 8 h. Proteins were quantified by immunoblotting. Proteins expression levels were plotted against time, and the half-life was calculated from the decay constant of the fitted exponential curve.

Core oligomerization. Cells were lysed at 4 °C for about 20 min in lysis buffer without SDS. Cell lysates were centrifuged at 600 g, and post-nuclear supernatants were centrifuged at 100000 g and 4 °C for 30 min. High-speed supernatants and pellets were analysed by immunoblotting.

Immunofluorescence. Indirect immunofluorescence labelling was performed as previously described (Rouillé et al., 2006). LDs were stained with BODIPY 493/503 (0.5 μg ml−1; Invitrogen).

ELISA. Core was quantified by a fully automated chemiluminescent microparticle immunoassay (Architect HCVAg, Abbott; Descamps et al., 2012). NS3 was quantified with a kit from BioFront Technologies.

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