Hepatitis C virus p7 protein is localized in the endoplasmic reticulum when it is encoded by a replication-competent genome

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INTRODUCTION

Hepatitis C virus (HCV) is a major aetiological agent of chronic hepatitis, which can lead to liver cirrhosis, liver failure and hepatocellular carcinoma, and is also the leading single indicator for liver transplantation in the developed world. The current treatment for HCV is a combination of pegylated α-interferon and ribavirin, which is only effective in about 50 % of patients. Moreover, the current therapy is expensive, has adverse side effects and can lead to the emergence of resistant virus strains (Fargion et al., 2004).

HCV belongs to the family Flaviviridae, genus Hepacivirus, and is a small enveloped virus with a positive-sense single-stranded RNA genome of about 9.5 kb, which encodes a polyprotein of about 3000 aa (Houghton, 1996). The HCV polyprotein is co- and post-translationally cleaved into at least 10 individual viral proteins. A small protein, p7, is located at the junction of the structural and non-structural (NS) proteins (Lin et al., 1994), but it is not known if it is a structural or an NS protein. In an in vivo study, p7 was shown to be essential for HCV synthesis, as viral RNA with the p7 region deleted was non-infectious in chimpanzees (Sakai et al., 2003).

The p7 protein is a small, hydrophobic integral membrane protein of 63 aa and comprises two hydrophobic α-helices, TM1 and TM2, and a basic loop located in the cytoplasm (Carrere-Kremer et al., 2002). Studies using recombinant expression plasmids have shown p7 to be mainly localized in the endoplasmic reticulum (ER) (Carrere-Kremer et al., 2002) and mitochondrial membranes (Griffin et al., 2004) while a small percentage of the overexpressed p7 protein was detected in the plasma membrane (Carrere-Kremer et al., 2002). However, a recent report, the localization of p7 in the mitochondrial membrane was questioned and a mitochondrial-associated ER localization was suggested (Griffin et al., 2005).

It has recently been shown that the p7 protein forms ion channels in planar lipid bilayers (Griffin et al., 2003; Pavlovic et al., 2003; Premkumar et al., 2004). We have recently shown that the analogous p13 protein in GB virus B, the virus most closely related to HCV, also has ion channel activity (Premkumar et al., 2004) and, consequently, it is likely that ion channel proteins are important in the hepatacivirus replication cycle. p7 belongs to a family of...
proteins known as viroporins, which homo-oligomerize to form ion channels in cellular membranes (Gonzalez & Carrasco, 2003). The p7 protein appears to have an essential role in the HCV life cycle, as demonstrated for other viral ion-channel-forming proteins, like the influenza virus M2 protein (Pinto et al., 1992; Takeda et al., 2002). However, p7 is dispensable for viral RNA replication, as replicons which lack the p7 gene replicate efficiently (Blight et al., 2000; Lohmann et al., 1999). Therefore, it has been concluded that p7 is not required for efficient HCV genome replication, but is essential for the production of infectious virions (Sakai et al., 2003).

The introduction of a robust cell culture system to grow and propagate the JFH1 strain of HCV has generated great opportunities to study HCV (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). In the present study, we engineered two constructs of the JFH1 genome, namely eJFH1 and hJFH1, in which the p7 protein was tagged with either eGFP or the HA epitope, respectively. We used these recombinant genomes to investigate the localization of the p7 protein when it is expressed from the HCV polyprotein precursor in an authentic virus replication system.

**METHODS**

**Cells and plasmids.** The HL1 cell line, a cured replicon cell line established in our laboratory (D. Li, unpublished data) and HuH7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco-BRL) supplemented with 10% fetal bovine serum (PA Biologicals), 100 IU penicillin ml⁻¹ and 100 µg streptomycin ml⁻¹. The wild-type and replication-defective (GDD to GND in NS5B) full-length JFH1 cDNA constructs were kindly provided by Dr T. Wakita (Wakita et al., 2005). E. coli strain JM109 (Promega) was used in all transformation experiments.

**Construction of the recombinant cDNA.** The nucleotide sequence of the JFH1 strain of HCV (Kato et al., 2001) was used to design the primers. To facilitate cloning of the recombinant fragments, the wild-type and GND mutant cDNA clones of JFH1 were digested with XbaI and NotI to remove the HCV sequences downstream of NS2, then blunt-ended and recircularized using standard ligation protocols (Sambrook et al., 1989). The recombinant plasmids (containing HCV IRES, core, E1, E2, p7 and partial NS2) were termed wtJFH-N and GJFH-N, respectively. The recombinant HA-p7 constructs were then synthesized by the use of HCV-specific primers tailed with the nucleotide sequence of the HA tag (YPYDVPDYA) at their 5’ end. Using the JFH1 wild-type construct as templates in PCR and fusion PCR, we generated the fused genes encoding the HA tag downstream of the nucleotide sequences for amino acids 1–4 (ALEK) followed by the full-length p7 (Fig. 1). The primers used in this experiment are shown in supplementary Table S1 (available in JGV Online). We also used fusion PCR to construct a chimeric eGFP-p7 protein (the eGFP gene was a gift from Dr J. Patrick Condreay; Condreay et al., 1999). The recombinant products were subsequently cloned into wtJFH-N at the SpeI site. Recombinant plasmids with the correct insert orientations were selected and the nucleotide sequence of the insert and flanking regions was confirmed by automated cycle sequencing. The recombinant plasmids were named wtJFH-N-eGFP and wtJFH-N-HA. These constructs were then digested with EcoRI and KpnI, and the released fragments containing IRES, core, E1, E2, eGFP/HA-p7 and partial NS2 were cloned into the wtJFH1 and mutant GNDJFH1 digested with the corresponding enzymes; the constructs were named eGFP-JFH1 (eJFH1), eGFP-GNDJFH1, HA-JFH1 (hJFH1) and HAGNDJFH1. The nucleotide sequence and orientation of the inserts were confirmed by using automated cycle sequencing with oligonucleotide primers FE2 and RNS2 (supplementary Table S1, available in JGV Online).

**In vitro RNA transcription.** RNA transcripts were synthesized as described previously (Huang et al., 2005). Briefly, the plasmids were linearized with XbaI, blunt-ended by Mung bean enzyme (New England Biolabs), purified by phenol/chloroform extraction and ethanol-precipitated. RNA was synthesized with the Megascript T7 transcription kit (Ambion). The RNA was either used immediately or stored at −80 °C before transfection.

**Transfection of cells with RNA.** To examine the replication competency of the recombinant genomes, 2–4 µg in vitro-generated RNA transcripts were transfected into an overnight culture of 5 × 10⁵ cells in six-well plates (Nunc) using DMRIE-C (Invitrogen), according to the manufacturer’s instructions. After 5 h, the transfection mixture was removed, the cells were washed twice with DMEM and complete medium was added. The cells were trypsinized and passaged every 3–5 days for seven passages as described by Zhong et al. (2005).

**Immunofluorescence (IF) and immunoblot analysis.** The cells were fixed with either acetone or 4% paraformaldehyde for IF analysis, then stained with goat anti-HCV core antibody (Biocytess) followed by Alexa Fluor 488-conjugated donkey anti-goat antibody (Molecular Probes). HA and eGFP tags were detected with a mouse monoclonal anti-HA antibody (Sigma Aldrich) and a rabbit anti-eGFP polyclonal antibody (Invitrogen), respectively. The HA and eGFP antibodies were detected with Alexa Fluor 488 goat anti-mouse antibody (Molecular Probes) and Alexa Fluor 488 goat anti-rabbit antibody (Molecular Probes), respectively. To stain the mitochondria, cells were incubated, 3 days post-transfection with 200 nM Mitotracker Red CMXRos (Molecular Probes) for 1 h, washed twice with PBS and permeabilized with 0.1% Triton-X-100 for 3–5 min, followed by IF. The ER compartments were labelled with either rabbit anti-calreticulin (Stressgen), mouse monoclonal anti-calnexin (Abcam), or Alexa Fluor 594 concanavalin A (Molecular Probes). The anti-calreticulin and the anti-calnexin antibodies were detected with AlexaFluor 594 donkey anti-rabbit antibody and Alexa Fluor

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**Fig. 1.** Schematic representation of JFH1 wild-type (wt) and mutant constructs which were generated in this study. eGFP was inserted between amino acids 4 and 5 of p7 by fusion PCR and the resulting construct was named eJFH1. The HA tag was inserted 4 aa (ALEK) downstream of the potential E2-p7 cleavage site followed by the full-length p7.
568 goat anti-mouse, respectively. In co-localization experiments, rabbit anti-calreticulin polyclonal antibody or Alexa 594-conjugated concanavalin A were used in conjunction with the anti-HA antibody, and anti-calnexin mAb was used in conjunction with the rabbit anti-eGFP antibody. The stained cells were examined with a Bio-Rad confocal microscope.

For immunoblot analysis, the cell pellets were resuspended in complete protease inhibitor cocktail (Roche) and lysed in EBC lysis buffer (50 mM Tris/HCl, pH 8.0, 140 mM NaCl, 100 mM NaF, 200 μM Na₃VO₄, 0.1 % SDS, 0.5 % NP-40; Griffin et al., 2005). A chemiluminescence kit (Amersham) was used to detect the immunocomplexes, according to the manufacturer’s instructions.

**Immunoelectron microscopy.** Cells were transfected with in vitro synthesized RNA transcripts of wild-type JFH1 and chimeric hJFH1. Two days post-transfection, the cells were fixed using 4 % paraformaldehyde with and without 0.1 % glutaraldehyde. The cells were examined by immunostaining as described previously (Mackenzie & Westaway, 2001).

**RESULTS**

**The recombinant genomes are replication-competent**

By using fusion PCR, the tag sequences were inserted at the N terminus of the p7 protein downstream of the E2 protein (Fig. 1). To confirm that genomes with an insert at the N terminus of the p7 protein were still replication-competent, we transfected either the Huh7 or HL1 cells with in vitro transcribed wild-type or recombinant JFH1 RNA. Since comparable results were observed when we used Huh7 cells and HL1 cells, only the data from the Huh7 cells are presented here. Two to three days post-transfection, the cells were fixed and examined by IF using anti-HA, anti-eGFP and anti-core antibodies. Cell lysates were also examined by immunoblot analysis with anti-core antibody. As shown in Fig. 2(a), HA- and eGFP-tagged p7 were readily detected by IF in approximately 50 % of the cells transfected with hJFH1 and eJFH1, respectively, while the GND mutant-transfected cells were negative (Fig. 2a top panel). The use of anti-eGFP or anti-HA resulted in a punctuate staining pattern (Fig. 2a lower panel), as reported previously (Griffin et al., 2004, 2005). In contrast to the above data, direct visualization of the cells showed no eGFP signal above the background (data not shown). Since the eGFP tag was not fluorescent when it was fused to p7, the remainder of the study focused on hJFH1 (encoding HA-p7), because this tag was smaller and consequently was considered to have less influence on p7 localization and function.

Using anti-core antibody, immunoblot analysis of the cells 3 days post-transfection with the wild-type and mutant RNA showed a band at the expected position of core protein (about 20 kDa) in the wild-type, hJFH1 and eJFH1 lanes, but not in GNDJFH1-transfected cells (Fig. 2b).

The cells transfected with the wild-type transcripts were passaged and remained core-antigen-positive by IF throughout passage 6 (data not shown). However, of the two chimeras, only the cells transfected with hJFH1 transcripts remained positive at passage 4 (Fig. 2c), while cells transfected with eJFH1 transcripts showed a dramatic reduction in the proportion of core-antigen-positive cells at passage 1 and were completely negative thereafter (data not shown). The supernatant of the cells transfected with the wild-type JFH1 genome was infectious on day 4 post-transfection, while no infectivity was detected even when 10-times concentrated hJFH1-derived supernatant was used to infect naïve Huh7 cells (data not shown). Consistent with these findings, ultrastructural analysis of cells transfected with wild-type JFH1 revealed a significant number of HCV particles in the cells, while in the cells transfected with hJFH1, no HCV particles were observed (Fig. 3). The particles were approximately 50 nm in diameter, as expected for HCV virions.

**The cleavage site at the E2/HA-p7 junction is functional**

Since the eGFP tag was not fluorescent when it was fused to p7 (see the above results), we only examined the HA-tagged p7 in this experiment. It has been shown that the insertion of an HA epitope at the N terminus of the p7 protein slightly improved the E2-p7 cleavage (Carrere-Kremer et al., 2004). The putative cleavage site at the E2/HA junction was predicted to be equally functional to that in the wild-type virus when the deduced amino acid sequences were examined by the Signal-P program (Nielsen et al., 1997; data not shown). To determine any changes in the cleavage process of the E2/HA-p7 precursor protein 2 days post-transfection, cells transfected with JFH1 and hJFH1 RNA were subjected to immunoblot analyses using the mAb AP33 (Owsianka et al., 2005) and an anti-HA mAb. As can be seen in Fig. 4(a), using the AP33 antibody, a major protein band with an approximate size of 60 kDa, as expected of the glycosylated form of E2, and a weak protein band with an approximate size of 70 kDa, as expected from the E2-p7 precursor protein, were observed with intensity comparable to the analogous wild-type proteins (Fig. 4). Using an antibody against the HA tag, a protein band at the approximate position of the HA-p7 protein (about 7 kDa) was observed in the cells transfected with hJFH1 recombinant genomes (Fig. 4). The HA-p7 band was not observed either in mock-transfected cells or in the cells transfected with the wild-type genome (Fig. 4). This experiment clearly showed that the predicted cleavage site at the E2-HA junction was functional and that the HA tag remained fused to the p7 protein. These data are in agreement with published data showing that insertion of an HA tag at the N terminus of p7 does not interfere with the cleavage process (Carrere-Kremer et al., 2004).

**Subcellular localization of the chimeric p7 protein**

As some controversy exists as to whether p7 localizes to mitochondria, we investigated the subcellular localization of p7 by IF analysis. To visualize cellular mitochondria we used...
Fig. 2. Detection of HCV-tagged p7 proteins in transfected cells. IF and immunoblot analysis of Huh7 cells transfected with transcripts of wild-type JFH1 and chimeras. (a) Huh7 cells were transfected with GND mutants (top panels), eJFH1 (left middle and bottom panels) or hJFH1 (right middle and bottom panels) and then stained with anti-eGFP and anti-HA antibody, respectively. (b) Detection of HCV core protein by Western blot analysis. G, GNDJFH1 transcript transfected cells; J, wild-type; H, hJFH1; E, eJFH1; M, MagicMark protein size marker (Invitrogen). (c) Huh7 cells were transfected with either GND mutant (upper left), wild-type (upper centre) or hJFH1 (lower panels) and passaged every 3–5 days as described in Methods. The cells at day 3 post-transfection (PT), passages 1 (P1) and 4 (P4) were stained with anti-core antibody. The nuclei of the cells were stained with propidium iodide (red).
the mitochondria-specific stain Mitotracker. After transfection of the cells with in vitro-synthesized RNA, co-staining of mitochondria and the different tagged p7 proteins did not reveal any co-localization (Fig. 5a). The tagged p7 protein appeared to localize primarily to the perinuclear region.

To examine if the tagged p7 protein was localized to the ER, anti-calreticulin polyclonal antibody or Alexa 594-conjugated concanavalin A (as used by Griffin et al., 2005) were used in additional co-localization experiments. Co-staining of HA-p7 and the ER (with the anti-calreticulin) demonstrated that the tagged p7 protein was localized in the ER (Fig. 5b). Similar results were generated using the Alexa 594 concanavalin-A conjugate (data not shown). We also did not observe any co-localization of tagged p7 in the Golgi apparatus (data not shown).

To further confirm the localization of HA-p7 in the ER, mock-transfected cells and the cells transfected with the wild-type and chimeric genomes were analysed by transmission immunoelectron microscopy using the anti-HA mAb. As shown in Fig. 3, the HA-tag was detected in the ER, but not in mitochondria of the cells transfected with the hJFH1 genome. No specific staining of the ER or other organelles was observed in the cells transfected with the wild-type genome. The presence of HCV particles was only observed in the cells transfected with the wild-type transcripts where they were observed within the lumen of the ER, within tubulo-vesicular clusters and in transit through the Golgi apparatus (arrows and arrowheads in Fig. 3).

The recombinant tagged genome reverted to wild-type

An initial aim of this study was to generate a recombinant JFH1 virus in which the p7 protein was tagged with a detectable stretch of amino acids, as antibodies to the p7 protein are not generally available. As described above, approximately 50% of the cells were positive for the HCV core protein 3 days post-transfection. However, when the cells transfected with hJFH1 and eJFH1 were passaged, the number of core-antigen-positive foci was reduced in passages 1–3, while there was no significant reduction in the number of core-antigen-positive cells after passage of
Huh7 cells transfected with the wild-type JFH1. The cells transfected with the wild-type JFH1 transcripts showed a cytopathic effect (CPE) at passage 1, as described by Zhong et al. (2005), whereas the cells transfected with hJFH1 only showed a CPE with approximately 80% cell death, resulting in cell detachment, at passage 3. Viral RNA was extracted from the supernatant of the cells transfected with either wild-type or hJFH1 at passage 4. A region encoding the C terminus of E2, p7 and the N terminus of NS2 was amplified by a one-step RT-PCR (Invitrogen) using genome-specific primers FE2 and RNS2 (supplementary Table S1, available in JGV Online) and products with the expected sizes of 742, 778 and 742 bp representing the wild-type, recombinant (hJFH1) and revertant (revJFH1) fragments were obtained (Fig. 6). The PCR products were then subjected to nucleotide sequencing. The sequences revealed no mutations either in the wild-type (data not shown) or in the viral RNA sequences of hJFH1, but the HA tag from the recombinant genome was found to be deleted precisely (data not shown). Consistent with this finding, the supernatant of passage 4 of the hJFH1 chimeric transfected cells was used to infect naïve Huh7 cells and was demonstrated to be infectious (data not shown), whereas in contrast, the supernatant of the cells transfected with the eJFH1 was never infectious (data not shown) and the hJFH1 supernatant fluids from passages 1–3 were not infectious, i.e. prior to reversion. To determine the HCV viral loads in the supernatants following RNA transfection, the Versant HCV RNA 3.0 bDNA assay (Bayer HealthCare) was used, according to the manufacturer’s instructions. As can be seen in Fig. 7(a), the viral RNA was undetectable in passage 3 of cells transfected with the GND replication-defective mutant or with eJFH1, but was detectable in hJFH1-transfected cells with a titre comparable to wild-type. The viral genomes were also detectable in passage 4 cells. We did not examine the time points beyond this because the genome reverted to wild-type as shown above. The RNA that was detected in the supernatants of the cells transfected with hJFH1 and eJFH1 at passages 0, 1 and 2 was probably due to residual RNA from transfection and nascent RNA as these supernatants were not infectious (see above).

To investigate further the point when the revertant emerged, the supernatants collected at passages 0–4 were subjected to RT-PCR analysis using oligonucleotide primers F2-revertant and R2-revertant (supplementary Table S1, available in JGV Online) to amplify the E2-p7 junction region. As shown in Fig. 7(b), at passages 0 and 1 only the chimeric product with an expected size of 148 bp was observed, while at passage 2, a wild-type product with an expected size of 112 bp was also observed. The chimeric product was subsequently lost and the wild-type product was dominant in passage 3 and the following passages (Fig. 7b). Thus the revertant emerged following two passages of the cells transfected with the chimeric hJFH1 genome. The PCR chimeric product might be a result of residual RNA from the transfection because the GND mutant RNA also was detectable in quantitative RT-PCR until passage 3 (Fig. 7a), although the IF data described above showed that core protein was only detected in cells transfected with the replication-competent (GDD) chimera.

**DISCUSSION**

In the present study we generated two replication-competent recombinant genomes of JFH1 in which the p7 protein was tagged with either an HA epitope or eGFP molecule. The recombinant genome with an HA tag reverted to wild-type after two passages, while the replication-competent eGFP-tagged genome did not survive as a recombinant genome or revertant. Nevertheless, transfection of cells with RNA encoding the recombinant genomes showed that the tagged p7 localized in the ER, but not the mitochondria or the Golgi apparatus.

Replication studies of HCV have been hampered by the lack of a robust cell culture system until recently, when several groups demonstrated that the JFH1 strain of HCV genotype 2a (Wakita et al., 2005; Zhong et al., 2005) and a chimeric form of this strain (Lindenbach et al., 2005) replicated efficiently in vitro. The nascent viral particles were also infectious in vivo (Lindenbach et al., 2006; Wakita et al., 2005). Very recently, it was also shown that a genotype 1a HCV strain can replicate in cell culture and also produces viral particles that are infectious in vitro (Yi et al., 2006).

The recent identification of the HCV p7 protein as a putative member of the viral ion channel family has established p7 as a potential novel antiviral target. Viral ion channel activity has been implicated in mediating virion entry, assembly, morphogenesis and secretion from host cells (Fischer & Sansom, 2002; Gonzalez & Carrasco, 2003). Although the
exact function of the HCV p7 protein is not known, the protein is essential for the production of infectious virions and a p7 deletion mutant or a virus with a mutation in the encoded cytosolic loop is non-viable (Sakai et al., 2003). Initially, we intended to generate chimeric viruses with a tagged p7 to facilitate tracking of the protein in the cells. However, neither of the recombinant genomes was able to generate nascent virus. Consequently, we examined the localization of the HA-p7 protein in subcellular compartments. The HCV p7 protein was first reported to localize in the ER (Carrere-Kremer et al., 2002), although no co-localization staining was performed in that report. More recently, an extensive co-staining study showed that when a N-terminal-tagged p7 was overexpressed in vitro from an expression vector, the protein was detected in mitochondria, but not in the ER, whereas if the protein was tagged at the C terminus it was detected in the ER (Griffin et al., 2005). However, none of these reports examined p7 localization when it was expressed in the context of the viral polyprotein during virus replication. In this study, in contrast to the results of previous studies, we detected the N-terminal-tagged p7 in the ER, but not in mitochondria.

**Fig. 5.** Localization of tagged p7 protein in subcellular compartments. (a) The transfected cells were stained with Mitotracker (red). eGFP-p7 and HA-p7 were stained with anti-eGFP (top panels) and anti-HA (lower panels), respectively. (b) The ER was stained with a rabbit anti-calreticulin (red) and HA-p7 was stained with a mouse anti-HA mAb.

**Fig. 6.** Genomic analysis of JFH1 and mutants. RT-PCR and PCR analysis of RNA derived from cell culture supernatants (passage 4) and plasmid DNA. Lanes: GND, replication-defective JFH1; JFH1, wild-type virus; hJFH1, plasmid-DNA-derived product; revJFH1, product from hJFH1 revertant. The markers on the left represent the pGEM DNA size marker (Promega).
and consequently, the accessibility of the tag inserted at the N terminus of the p7 protein in the context of polyprotein encoded by a replication competent genome to antibody appears to differ from that resulting from plasmid expression.

Collectively, and consistent with the published data (Griffin et al., 2005), we demonstrated that the HA-tagged p7 localized in the ER and the tag did not alter the localization of the protein in the cells. Our study confirmed the results of a recent publication in which it was demonstrated that the localization of different HCV structural proteins encoded by JFH1 replication-competent RNA also differed from that resulting from overexpression of the proteins from an expression plasmid (Rouille et al., 2006). We then examined the virus replication steps that were affected by the addition of an HA tag at the N terminus of p7 that prevented the production of virus from the chimeric genome. Recently, it has been documented that the level of expression from the luciferase gene encoded by recombinant HCV genomes is a valid indicator of the level of viral genome replication (Koutsoudakis et al., 2006). Although we did not have such an assay, we did not see any significant difference in the expression of the E2 proteins in the cells transfected with the recombinant genomes. Therefore, the HA tag at the N terminus of the p7 protein possibly abrogated the function of the protein or disrupted a critical interaction between this protein and another viral protein. Furthermore, because the cleavage kinetics of the mutants was not analysed in this study, we cannot exclude that mutations in p7 affect the kinetics of polyprotein cleavage.

In conclusion, this study demonstrated that the p7 protein encoded by a replication-competent full-length genome is localized in the ER. Moreover, the data presented here indicate a possible role for the p7 protein in virus assembly.

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