

Disrupting the association of hepatitis C virus core protein with lipid droplets correlates with a loss in production of infectious virus

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In infected cells, hepatitis C virus (HCV) core protein is targeted to lipid droplets, which serve as intracellular storage organelles. Using a tissue culture system to generate infectious HCV, we have shown that the coating of lipid droplets by the core protein occurs in a time-dependent manner and coincides with higher rates of virus production. At earlier times, the protein was located at punctate sites in close proximity to the edge of lipid droplets. Investigations by using Z-stack analysis have shown that many lipid droplets contained a single punctate site that could represent positions where core transfers from the endoplasmic reticulum membrane to droplets. The effects of lipid droplet association on virus production were studied by introducing mutations into the domain D2, the C-terminal region of the core protein necessary for droplet attachment. Alteration of a phenylalanine residue that was crucial for lipid droplet association generated an unstable form of the protein that could only be detected in the presence of a proteasome inhibitor. Moreover, converting two proline residues in D2 to alanines blocked coating of lipid droplets by core, although the protein was directed to punctate sites that were indistinguishable from those observed at early times for wild-type core protein. Neither of these virus mutants gave rise to virus progeny. By contrast, mutation at a cysteine residue positioned 2 aa upstream of the phenylalanine residue did not affect lipid droplet localization and produced wild-type levels of infectious progeny. Taken together, our findings indicate that lipid droplet association by core is connected to virus production.

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INTRODUCTION

Chronic infection by hepatitis C virus (HCV) affects about 170 million individuals worldwide and is a major cause of liver disease (Hoofnagle, 2002). HCV is an enveloped virus belonging to the genus *Hepacivirus* within the family *Flaviviridae* (Murphy *et al.*, 1995). The viral genome is a single-stranded, positive-sense RNA molecule approximately 9.6 kb in length that encodes a polyprotein of some 3000 aa. The polyprotein is cleaved both co- and post-translationally at the endoplasmic reticulum (ER) membrane by cellular and viral proteases to yield the mature viral proteins (Bartenschlager & Lohmann, 2000; Penin *et al.*, 2004). The structural proteins are located at the N-terminal end of the polyprotein and consist of the core protein, which forms the viral capsid, and two envelope glycoproteins, E1 and E2. The glycoproteins are released from the polyprotein by signal peptidase (SP) cleavage, whereas maturation of the core protein requires proteolysis by both SP and signal peptide peptidase (SPP) (Hussy *et al.*, 1996; McLauchlan *et al.*, 2002).

The mature form of core is a dimeric, α -helical protein that is separable into two domains, D1 and D2 (McLauchlan,

2000; Boulant *et al.*, 2005, 2006). D1 consists of the N-terminal 117 aa, while D2 begins at amino acid residue 118 and ends between amino acids 171 and 182. D2 is required for correct folding of D1 and is critical for the association of core with membranes (Boulant *et al.*, 2005, 2006). We have identified two amphipathic α -helices (HI and HII) separated by a short hydrophobic loop (HL) as the major structural features in D2 (Boulant *et al.*, 2006). HI and HII are positioned between amino acids 119 and 136, and 148 and 164, respectively. Folding of HI and HII requires a hydrophobic environment with both helices probably lying in-plane with membranous surfaces (Boulant *et al.*, 2006).

Following maturation by SP and SPP, core associates with lipid droplets (LDs) (Moradpour *et al.*, 1996; Barba *et al.*, 1997; McLauchlan *et al.*, 2002). LDs store intracellular neutral lipid (Murphy, 2001; Martin & Parton, 2005) and are composed of a hydrophobic core of triacylglycerol and cholesterol ester, which is encircled by a single leaflet of phospholipids that in turn is surrounded by a proteinaceous layer. D2 is able to target not only core but also heterologous proteins to LDs (Hope & McLauchlan, 2000; Boulant *et al.*, 2006); the critical residues required for LD association lie on the hydrophobic faces of HI and HII and within HL.

A supplementary figure is available with the online version of this paper.

Association of core with LDs occurs in HCV-infected hepatocytes in chimpanzees and in a wide variety of cell types, including hepatocyte-derived cell lines (Moradpour *et al.*, 1996; Barba *et al.*, 1997; McLauchlan *et al.*, 2002; Sato *et al.*, 2006). Until recently, propagation of HCV in tissue culture cell lines was not possible. However, production of infectious virus in human hepatoma cells has now been achieved with JFH1, a Japanese genotype 2a strain (Lindenbach *et al.*, 2005; Wakita *et al.*, 2005; Zhong *et al.*, 2005). In cells either producing or infected with JFH1, core is found attached to LDs (Rouille *et al.*, 2006). In this report, we have analysed the timing of LD association by HCV core produced by JFH1 and studied the impact of mutations introduced into its D2 domain. We have examined the effect of these changes on the attachment of core to LDs and release of infectious progeny to determine whether impairing the core–LD interaction reduces HCV production.

METHODS

Construction of plasmids. Plasmid pJFH1 was a gift from Takaji Wakita (National Institute of Infectious Diseases, Tokyo, Japan) (Wakita *et al.*, 2005). To generate mutants, pTopoCE1E2_{JFH1} was constructed by amplifying the sequence encoding structural proteins core to E2 (aa 1–750) from pJFH1 by PCR and cloning the PCR fragment into pcDNA3.3/V5-His Topo (Invitrogen). Mutants were generated from pTopoCE1E2_{JFH1} using the QuikChange site-directed mutagenesis kit (Stratagene). Oligonucleotides for site-directed mutagenesis were designed according to the manufacturer's guidelines. The sequence of each mutant was verified prior to cloning into pJFH1. To generate pJFH1 mutant plasmids, a 865 bp *FspAI*–*BsiWI* fragment from each pTopoCE1E2_{JFH1} mutant plasmid was ligated to pJFH1 cleaved with identical restriction enzymes.

Maintenance of tissue culture cells and treatment with MG132. HuH-7 cells were propagated in Dulbecco's modified Eagle's medium (DMEM) and treated with MG132 as described previously (McLauchlan *et al.*, 2002).

Antibodies. Antibodies used to detect HCV core (rabbit antisera R308), NS5A and human adipocyte differentiation-related protein (ADRP) have been described previously (Hope & McLauchlan, 2000; Macdonald *et al.*, 2003; Targett-Adams *et al.*, 2003). Rabbit antiserum against calnexin (Sigma) was used according to the manufacturer's instructions.

In vitro transcription and electroporation of JFH1 RNA. Wild-type (wt) and mutant pJFH1 constructs were linearized by *XbaI* digestion and treated with Mung Bean Nuclease (New England BioLabs). RNA was synthesized *in vitro* from linearized plasmids using the MEGAscript high yield transcription kit according to manufacturer's instructions (Ambion). Electroporation of RNA into HuH-7 cells was performed as described previously (Hope & McLauchlan, 2000; McLauchlan *et al.*, 2002).

Infection of cells with HCV. Unless otherwise stated, HuH-7 cells were electroporated with either wt or mutant JFH1 RNA for 3 days. Virus released into the growth medium at this time point was used to infect monolayers of naïve HuH-7 cells on coverslips. Prior to infection, medium containing supernatant virus was filtered using a 0.2 µm Minisart filter (Sartorius) and diluted with fresh DMEM. Infected cells were detected at 3 days post-inoculation by indirect

immunofluorescence using core and NS5A antibodies. Virus titres were determined by counting numbers of fluorescent foci on each coverslip (Wakita *et al.*, 2005).

Preparation of cell extracts, PAGE and Western blot analysis. Preparation of cell extracts, PAGE and Western blot analysis were performed as described in Boulant *et al.* (2006).

Separation of intracellular organelles on sucrose gradients. HuH-7 cells were electroporated with recombinant JFH1 RNA and used to seed 100 mm tissue culture dishes. After incubation for 3 days at 37 °C, cells were fractionated as follows at 4 °C. Cell monolayers were washed 3–4 times with PBS and once with homogenization buffer (HB; 20 mM HEPES-KOH, pH 7.4, 1 mM EDTA, 0.25 M sucrose, 1 mM PMSF). Cells were scraped into 600 µl HB and homogenized by passage through a 22-gauge needle. The homogenate was centrifuged at 500 g for 5 min and the supernatant was collected as a post-nuclear supernatant (PNS). For flotation gradient centrifugation, the PNS was adjusted to 2 ml with HB containing 1.2 M sucrose and applied above a 1.6 M sucrose cushion (2 ml) in a 14 × 89 mm centrifuge tube. The PNS was overlaid with 2 ml each of 1, 0.75, 0.5 and 0.25 M sucrose solutions prepared in HB. Gradients were centrifuged in a SW41Ti rotor at 100 000 g for 16 h at 4 °C. After centrifugation, fractions were collected, and proteins were precipitated with trichloroacetic acid [15% (v/v)] and acetone [35% (v/v)]. Pellets were resuspended in sample buffer.

Indirect immunofluorescence. Cells were fixed for 20 min in methanol at –20 °C. Methanol was the preferred fixative as both core and ADRP were difficult to detect following paraformaldehyde fixation (data not shown). After washing with PBS and blocking with PBS/CS (PBS containing 1% new-born calf serum), cells were incubated with primary antibody (diluted in PBS/CS) for 2 h at room temperature. Cells were washed extensively with PBS/CS and then incubated with secondary antibody (either anti-rabbit or anti-sheep IgG) conjugated to a fluorescent tag for 2 h at room temperature. After washing with PBS/CS and PBS, cells were rinsed with dH₂O before mounting on slides using Citifluor (Citifluor).

Microscopy. Cells were examined with an LSM510 META inverted confocal microscope. Images were recorded with a Plan-Apochromat × 63 lens (NA 1.4). For Z-stack analysis, 25 images were recorded at 0.14 µm intervals. For Fig. 5, analysis of the extent of colocalization was performed by LSM510 software. For the three-dimensional (3D) reconstructions in Fig. 6, Z-stack images were collected using optimum intervals, generating 20–25 slices per sample. Image stacks were deconvolved by 3D-blind deconvolution (20 iterations) using Autodeblur software (MediaCybernetics). 3D reconstructions were created with the five-dimensional-viewer extension in the iso-surface mode using a bin factor of 1.

RESULTS

HCV core protein colocalizes with LDs in JFH1-infected cells

From several reports, core protein, expressed either individually or as part of an HCV polyprotein, is directed to LDs in a wide range of cell lines (Moradpour *et al.*, 1996; Barba *et al.*, 1997; Hope & McLauchlan, 2000; McLauchlan *et al.*, 2002). More recently, the protein was also found associated with these structures in cells either transfected with infectious JFH1 RNA or infected with JFH1 virus (Rouille *et al.*, 2006). To confirm and extend this analysis,

HuH-7 cells were either electroporated with JFH1 RNA or infected with supernatant virus harvested from cells at 3 days after electroporation. Seventy-two hours following either electroporation or infection, cells were examined for the intracellular distribution of both core and NS5A by indirect immunofluorescence.

Core and NS5A displayed a spotty pattern in infected and electroporated cells, but there was little evidence of colocalization of the two proteins (Fig. 1a). To determine whether the distribution of core indicated association with LDs, cells were also probed with an antibody against ADRP, the most abundant cellular component on the LD surface in HuH-7 cells (Fujimoto *et al.*, 2004). This analysis revealed precise coincidence of core and ADRP, demonstrating that the viral protein attached to LDs in cells either electroporated with JFH1 RNA or infected with JFH1 virus

(Fig. 1b and data not shown). Further evidence for the LD association of core was obtained from biochemical fractionation of cells electroporated with JFH1 RNA (Fig. 1c). In sucrose gradients, a large proportion of the viral protein was found in fractions that contained ADRP (Fig. 1c, fractions 2–4). In addition, core co-migrated with calnexin (Fig. 1c, fractions 7–12), indicating that it was also present on the ER membrane, in agreement with previous biochemical fractionation studies (Schwer *et al.*, 2004; Suzuki *et al.*, 2005).

HCV core localizes to LDs in a time-dependent manner

It has been reported that core not only coats the surface of LDs but is also found in a small proportion of cells at punctate sites that are in close proximity to LDs (Rouille *et al.*, 2006). These data were obtained at later times post-transfection and infection (48 h and beyond). To determine whether there was evidence of any distinct localization at earlier times, the intracellular distribution of core was examined from 12 to 120 h following electroporation of cells with JFH1 RNA. From several experiments, three patterns of core localization were observed, depending on the duration of expression. At 12 and 24 h post-electroporation, core exhibited a punctate staining pattern that was positioned adjacent to ADRP in about 95 % of cells (Fig. 2a, b). By 38 h, the punctate pattern for core was the predominant phenotype but about 40 % of cells contained protein colocalized with ADRP on the surface of LDs. Beyond this time (48, 72 and 120 h), core colocalized with ADRP on the surface of droplets in about 80–95 % of electroporated cells and the punctate appearance of the protein could be found in the remaining cells. Cells infected with HCV revealed the same patterns for core that progressed from a punctate distribution adjacent to ADRP at early times of infection to complete colocalization with the cell protein at later times (data not shown). These results indicated a time-dependent loading of LDs by core. From Western blot analysis, the abundance of intracellular core also steadily rose during the period of increased loading of LDs (Fig. 2d). In parallel, the monitoring of infectious virus throughout the time-course showed that the highest rate of virus production was between 24 and 48 h, which coincided with a shift in the punctate distribution of core to complete colocalization with ADRP on the surface of LDs (Fig. 2c). These results suggest a possible link between progressive association of core with LDs and enhanced virus production for JFH1.

Mutations that block LD association also inhibit virus production

Recently, we demonstrated that the D2 domain of core from HCV strain Glasgow contained the sequences necessary for targeting the protein to LDs (Bouland *et al.*, 2006). Amino acids on the hydrophobic faces of HI and HII, and in HL were critical for LD association; in particular, a phenylalanine residue at position 130 (Phe-130) on the

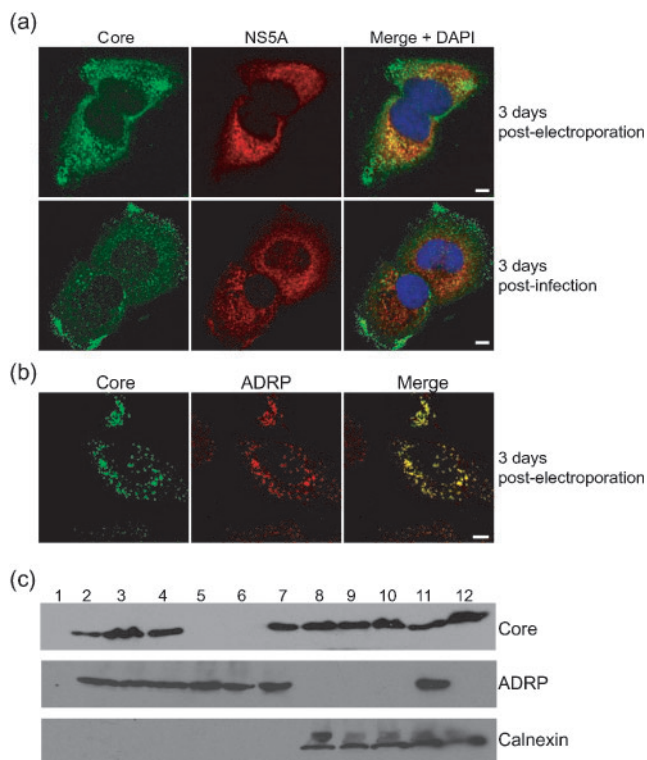


Fig. 1. Intracellular distribution of JFH1 core protein. (a and b) HuH-7 cells were either electroporated with JFH1 RNA or inoculated with medium from cells electroporated with JFH1 RNA. Cells were stained with the antisera indicated in (a) and (b). To show the location of nuclei, cells in (a) were counter-stained with DAPI. (c) Cofractionation of JFH1 core with cellular markers for LDs and the ER in sucrose density gradients. HuH-7 cells were electroporated with JFH1 RNA and lysed following incubation for 3 days at 37 °C. Extracts were subjected to centrifugation on sucrose gradients and fractions were removed from the top (1) to the bottom (12) of gradients. Samples were separated by SDS-PAGE and examined by Western blot analysis using antibodies against core, ADRP and calnexin. Bars, 5 µm.

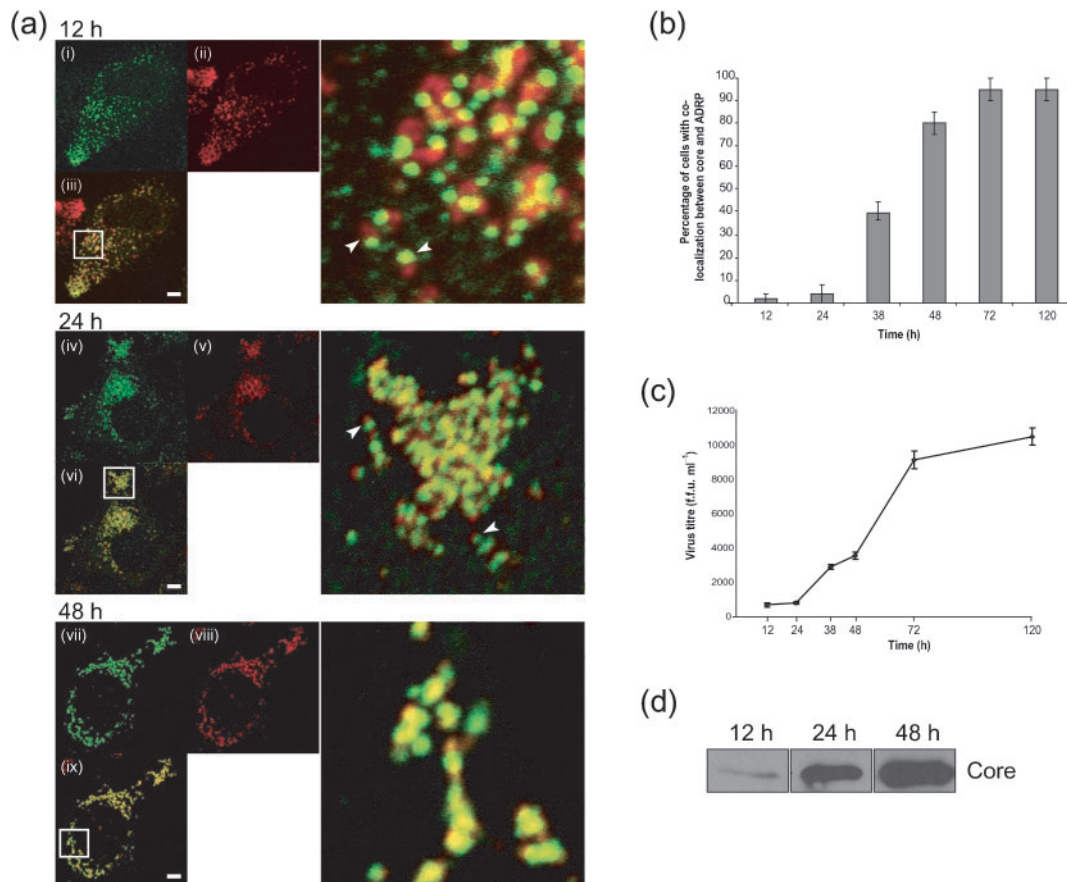


Fig. 2. Comparison between time-dependent loading of LDs with JFH1 core and production of infectious progeny. (a) HuH-7 cells were electroporated with JFH1 RNA and cells were examined by indirect immunofluorescence at 12, 24 and 48 h after electroporation. Cells were stained with core [panels (i), (iv) and (vii)] and ADRP antisera [panels (ii), (v) and (viii)]. Merged images are shown in panels (iii), (vi) and (ix). Zoomed areas of boxed regions in panels (iii), (vi) and (ix) are shown to the right. Arrowheads indicate sites where core protein does not colocalize with ADRP. (b and c) HuH-7 cells, electroporated with JFH1 RNA, were examined for the extent of colocalization between core and ADRP (b) and production of virus progeny (c) at the times indicated. Results were taken from three separate experiments and error bars indicate standard deviations. (d) HuH-7 cells were electroporated with JFH1 RNA and incubated at 37 °C. Cell extracts were prepared at the times indicated and examined by Western blot analysis using antiserum against core protein. Bars, 5 µm.

hydrophobic face of HI. Mutation of this residue to glutamic acid not only blocked targeting to LDs but also led to proteasomal degradation of core. By contrast, amino acids on the hydrophilic faces of the helices, including a cysteine residue (Cys-128) positioned only 2 aa upstream from the critical phenylalanine residue, did not affect either LD association or protein stability of the protein upon introduction of the same mutation. To determine whether core from strain JFH1 showed characteristics similar to those of strain Glasgow, codons for Phe-130 and Cys-128 were mutated to encode glutamic acid and introduced into pJFH1; the resultant mutants were pJFH1_{Phe-130} and pJFH1_{Cys-128}. Studies on cells electroporated with RNA from both mutants revealed that core could not be detected for JFH1_{Phe-130}, whereas the mutant form of the protein produced from JFH1_{Cys-128} was present at levels identical to those for wt JFH1 core (Fig. 3a, b). Moreover, core made

by JFH1_{Cys-128} retained the ability to associate with LDs (data not shown). The inability to detect core from JFH1_{Phe-130} did not arise from a defect in viral protein production, since NS5A was readily detected by both indirect immunofluorescence and Western blot analysis for this mutant (Fig. 3a, b). To confirm that the mutation at Phe-130 gave an unstable form of core protein, cells electroporated with JFH1_{Phe-130} RNA were treated with the proteasome inhibitor, MG132. In the presence of MG132, core produced by JFH1_{Phe-130} was readily detected at levels similar to those for the wt protein (Fig. 3c). The proteasome inhibitor did not affect the detected levels of wt core protein or the abundance of NS5A produced by either JFH1 or JFH1_{Phe-130} RNAs (Fig. 3c). No infectious progeny was detected from cells electroporated with JFH1_{Phe-130} but JFH1_{Cys-128} gave virus titres of 0.3–1.0 × 10⁴ per ml, which are in the same range as wt JFH1 (Fig. 2c). Attempts to

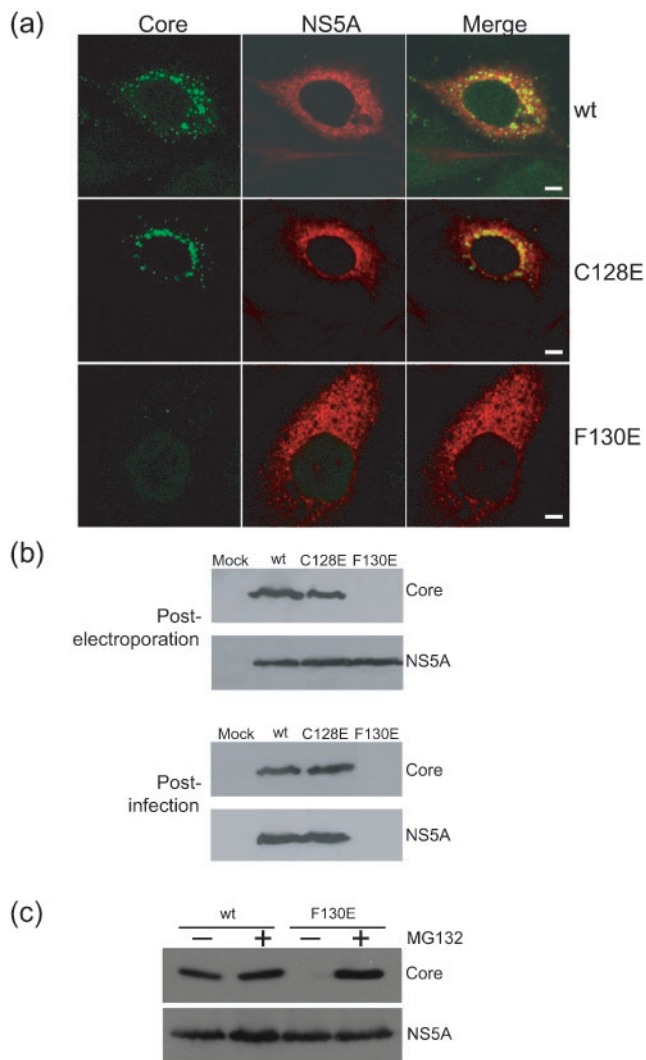


Fig. 3. Characteristics of mutants JFH1_{Cys-128} and JFH1_{Phe-130}. HuH-7 cells were either electroporated with RNA from wt and mutant JFH1 constructs [(a), (c) and upper panels in (b)] or inoculated with growth medium harvested from cells electroporated with RNA [lower panels in (b)]. Following incubation for 3 days at 37 °C, cells were either fixed with methanol for examination by indirect immunofluorescence (a) or extracts were prepared for Western blot analysis (b). In (c), cells were either treated or not treated with the proteasome inhibitor MG132 at a final concentration of 2.5 µg ml⁻¹ for 12 h prior to preparation of extracts for Western blot analysis. Cells and extracts were probed with antisera against core and NS5A. Bars, 5 µm.

detect virus released from cells treated with MG132 to stabilize Phe-130 mutant core were not successful since incubation of the cells in the presence of the compound for periods of greater than 12–16 h was highly cytotoxic. We concluded that the mutants in D2 of JFH1 core gave identical characteristics to the strain Glasgow protein expressed by the Semliki Forest virus (SFV) system (Boulant *et al.*, 2006). In addition, the inability to detect infectious progeny

with the JFH1_{Phe-130} mutant illustrated the obligate requirement for the core protein in virion production.

Since mutation at Phe-130 both abolished LD association and led to proteolytic breakdown of core, we wished to create a mutant that was defective for LD attachment but had greater constitutive stability. Such a mutant would determine whether association of core with LDs was linked to virion production. Previously, we described a mutant in which two proline residues in HL between HI and HII were converted to alanine residues (Hope *et al.*, 2002). These substitutions produced a stable form of core that did not associate with LDs although they did reduce the efficiency of its maturation by SPP. To examine these mutations in JFH1, a third mutant was generated termed JFH1_{DP} in which the codons for proline residues at positions 138 and 143 were substituted by alanine residues. In contrast to the JFH1_{Phe-130} mutant, core produced by JFH1_{DP} was detected at 72 h after electroporation in cells that expressed NS5A (Fig. 4a) but at slightly reduced levels compared with wt JFH1 as determined by Western blot analysis (Fig. 4b). Interestingly, the level of NS5A made by JFH1_{DP} was also slightly reduced compared with wt JFH1 (Fig. 4b). Similar to JFH1_{Phe-130}, infectious virus could not be detected in medium harvested from cells for up to 5 days after electroporation with JFH1_{DP} RNA. Moreover, neither core protein nor NS5A was present in cells inoculated with medium from JFH1_{DP} RNA-electroporated cells (Fig. 4b). Closer inspection of the intracellular location of core at 72 h after electroporation indicated that it gave a punctate appearance that was indistinguishable from that for wt core at the early stages after electroporation (compare Figs 2a and 4c). Staining for ADRP confirmed that the punctate spots for JFH1_{DP} core were located at sites in very close proximity to LDs and this distribution did not change at later time points when wt core coated the entire surface of LDs (Fig. 4c). These results provide further support for a link between the ability of core to associate with LDs and production of JFH1 virions. We noted also that LDs in cells electroporated with wt JFH1 RNA redistributed with time towards the periphery of the nucleus (Fig. 4c, upper panels). By contrast, LDs in cells electroporated with JFH1_{DP} RNA retained their diffuse cytoplasmic distribution at all time points (Fig. 4c, lower panels). The basis for this difference in behaviour by LDs is currently under investigation (S. Boulant and others, unpublished observations).

As stated above, the introduction of alanine at the two proline residues in HL of core protein from strain Glasgow impaired maturation of the protein by SPP (Hope *et al.*, 2002). These experiments utilized the SFV system for expression of core and examined extracts at 15 h after RNA electroporation; analysis of later time points was not possible due to apoptosis induced by SFV in tissue culture cells (Glasgow *et al.*, 1997, 1998). However, examination of JFH1_{DP} core gave no evidence for reduced SPP cleavage at 72 h (Fig. 4b). To establish whether maturation could be affected at earlier time points, core produced by JFH1_{DP} was analysed at different intervals after electroporation. At

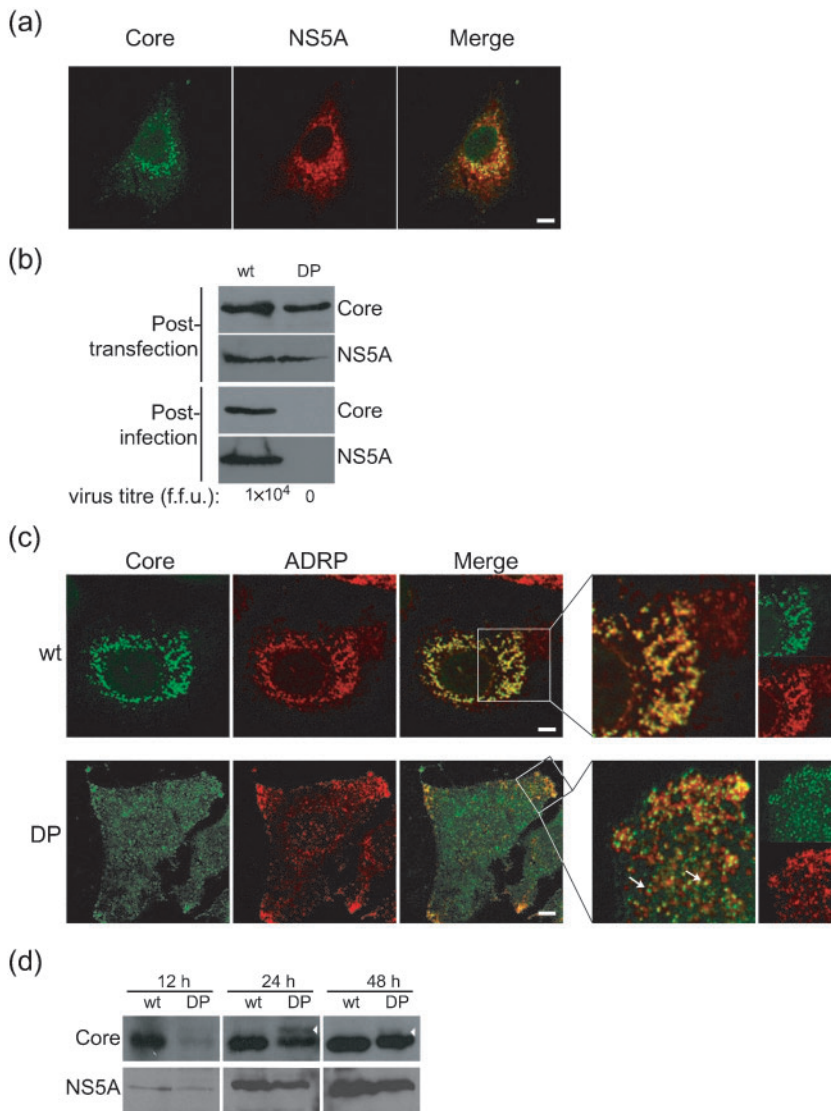


Fig. 4. Characteristics of mutant JFH1_{DP}. (a and c) HuH-7 cells were electroporated with wt (c) and JFH1_{DP} RNA (a and c) and fixed with methanol after incubation at 37 °C for 72 h. Cells were examined with the antisera indicated. A higher magnification of the boxed regions in the right-hand merged panels in (c) are shown. Punctate sites illustrating the juxtaposition of JFH1_{DP} core and ADRP are shown by arrows. (b and d) HuH-7 cells were electroporated with wt JFH1 and JFH1_{DP} RNAs and incubated at 37 °C for 72 h [upper panels in (b)] or the times indicated [panels in (d)]. For the lower panels in (b), cells were inoculated with growth medium harvested from cells electroporated with wt JFH1 and JFH1_{DP} RNAs and incubated at 37 °C for 72 h prior to harvesting. Cell extracts were examined by Western blot analysis using core and NS5A antisera. The core species generated by SP cleavage alone is indicated by arrowheads in (d). The panel showing levels of core protein at 12 h in (d) is overexposed to emphasize the low expression of JFH1_{DP} core at this time point. Bars, 5 µm.

12 h, the protein proved difficult to detect, but it was present as a doublet at 24 h (Fig. 4d). Based on comparison with wt protein, we predict that the JFH1_{DP} core species with reduced mobility corresponds to a product cleaved by SP and not SPP. However, only a single protein species was detected at 48 h that co-migrated with wt core, which was indicative of an SPP-cleaved product. Hence, we concluded that delayed cleavage by SPP was apparent only at early times of expression for JFH1_{DP} core.

Location of punctate sites on LDs

Observations from a large number of cells expressing wt core at 24 h and for JFH1_{DP} core, either treated or not treated with oleic acid (Supplementary Fig. S1 available in JGV Online), highlighted that the protein was apparently located only at a single punctate site on each LD (Fig. 4c, arrows). To determine the relative locations of core and ADRP more accurately we performed optical sectioning of

cells to generate Z-stacks in which sequential images could be analysed for coincidence of the two proteins in 3D. Examining images in x-y, x-z and y-z planes demonstrated precise colocalization between wt core and ADRP at 48 h (Fig. 5). By contrast, only partial overlap was evident between JFH1_{DP} core and ADRP in the x-y plane and almost no colocalization in either the x-z or y-z planes (Fig. 5). These data suggested that JFH1_{DP} core was located at a site that did not include ADRP but was in close proximity to the cellular protein. Identical results were obtained from analysis of wt core at 24 h after RNA electroporation (data not shown). To reconstruct the relative positions of ADRP and core located at punctate sites, Z-stacks were rendered for cells expressing wt and DP core at different times after electroporation to give a 3D representation of LDs (Fig. 6). This approach highlighted close juxtaposition of core to the surface of LDs and that the protein was present at a single site on the organelles at 24 h post-electroporation for wt core and at all time points

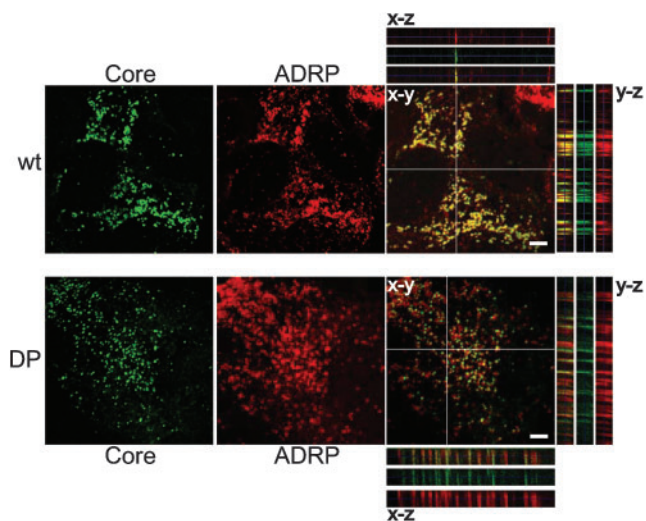


Fig. 5. Colocalization of JFH1 wt and DP proteins with ADRP. HuH-7 cells were electroporated with wt JFH1 and JFH1_{DP} RNAs and incubated at 37 °C for 48 h. After fixation with methanol, cells were stained for core and ADRP. Z-stack analysis of core and ADRP proteins was performed by confocal microscopy by recording 25 images from cells at 0.14 µm intervals. The locations of both proteins are shown separately and in x-y, x-z and y-z planes for merged images.

for DP core (Fig. 6a, b). The extent of coating of core on the surface of LDs was greater at 48 h and almost complete by 72 h post-electroporation (Fig. 6a).

DISCUSSION

Several reports have documented the intracellular localization of core and its association with LDs in either transiently or stably transfected cells (Moradpour *et al.*, 1996; Barba *et al.*, 1997; Hope & McLauchlan, 2000). Core also attaches to LDs in infected cells, suggesting that interaction with these organelles could be important in the HCV life cycle (Rouille *et al.*, 2006). In this study, we have extended analysis of the timing of attachment of core to LDs using the infectious strain JFH1 and examined the effects of introducing mutations into the domain required for LD association. Our data reveal that association of core with LDs occurs in a time-dependent manner and disrupting the ability of core to coat LDs coincides with a loss in production of infectious HCV.

Apart from LDs, core has been found located at the ER membrane, in mitochondria and in cell nuclei (Moradpour *et al.*, 1996; Yasui *et al.*, 1998; Okuda *et al.*, 2002; Schwer *et al.*, 2004). Such diverse reports pose questions regarding the relevance of systems that study the protein's properties in the absence of virus production. From a recent report using the JFH1 isolate, core was attached to LDs by 72 h in cells electroporated with infectious RNA and in

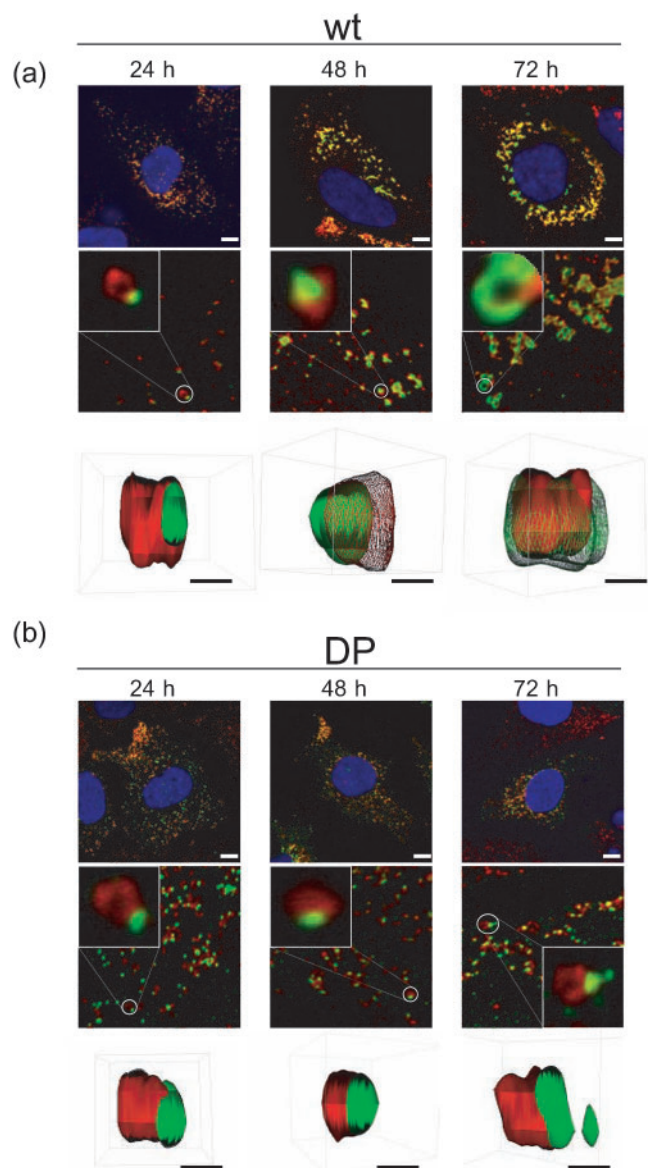


Fig. 6. 3D reconstruction of wt JFH1 and JFH1_{DP} core on LDs. HuH-7 cells were electroporated with wt JFH1 (a) and JFH1_{DP} (b) RNAs, and incubated at 37 °C for 24–72 h. After fixation with methanol, cells were stained for core and ADRP. Z-stack analysis of core and ADRP was performed by confocal microscopy by recording a series of 20–25 images depending on the cell thickness. 3D reconstructions of core (green) and ADRP (red) are shown for zoomed areas in the images. For wt JFH1 at 48 h, ADRP has been represented as a wire frame and not as a solid iso-surface due to extensive overlap with core protein. For wt JFH1 at 72 h, core has been represented as a wire frame. Bars, 5 µm for the confocal images and 0.5 µm for 3D reconstructions of LDs.

virus-infected cells (Rouille *et al.*, 2006). Core was also detected at sites proposed to be a membranous compartment associated with LDs. However, any time-dependent relationship between the two distributions for core was not studied. By examining cells from early to late stages after

electroporation and infection, we found that core was located initially at a single punctate site on each LD by 12 h and then it progressively surrounded the entire organelle from this time onwards. This time dependence for core to fully envelope LDs was also apparent upon induction of excess amounts of LDs (Supplementary Fig. S1 available in JGV Online). Hence, it is improbable that the punctate distribution of core results from limited availability of accessible sites on LDs. Rather, the coating of LDs by core is more likely to start from one position on LDs that is defined by a unique punctate site and then continues as a progressive process as the abundance of the protein increases. We consider that the two patterns of distribution described by Rouille *et al.* (2006) probably reflect cells expressing core at later times after infection or electroporation (when the protein is attached to LDs) and cells infected during the course of the experiment that have produced the protein for a shorter period (when core is located at punctate sites). Thus, our data are compatible with those of Rouille *et al.* (2006) and establish a time-dependent relationship between the two patterns of core distribution that these authors have described.

Mature core is generated at the ER membrane by SP and SPP and only fully processed protein is detected at the time core is located at punctate sites. Therefore, this distribution is apparently not a consequence of delayed protein maturation. We propose that the punctate sites may represent an intermediate location after processing where core is transferred between the ER membrane and LDs, and could indicate connections between the two organelles for loading of proteins onto LDs. Such a mechanism has been proposed for the transfer of ADRP from the ER membrane to LDs during their biogenesis (Robenek *et al.*, 2005, 2006). Moreover, recent evidence has demonstrated that Rab18, one of the Rab family of small GTPases, is found not only on the entire surface of droplets but also at punctate sites on LDs (Ozeki *et al.*, 2005; Martin *et al.*, 2005). Higher resolution analysis suggested that in cells overexpressing Rab18, LDs were juxtaposed to the ER membrane, which may indicate specific sites where LDs gain their proteins. Studies are in progress to determine whether any relationship exists between the punctate sites for Rab18 and those for HCV core.

Previously, we described the structural features contained within domain D2 for the core protein encoded by HCV strain Glasgow (Boulant *et al.*, 2006). Our studies identified two amphipathic helices (HI and HII) separated by an HL. It was considered likely that the hydrophobic amino acids on both helices and those in HL formed a contiguous stretch of residues, which interacted in-plane with the membranous surface of LDs. In the absence of any available infectious system at that time, the properties of the residues in the structural elements of D2 were analysed by mutagenesis and transient expression using an SFV vector. Among the mutants that were characterized, alteration of the phenylalanine residue at position 130 not only abolished LD association but also generated an unstable form of core. By contrast, mutation of a cysteine residue

at position 128 had no effect on either LD attachment or stability of the protein. Both Phe-130 and Cys-128 are highly conserved in all HCV strains, including JFH1. Since the introduction of these mutations into infectious JFH1 gave core proteins with characteristics identical to those studied with the SFV vector system, our current studies strongly support the hypothesis that hydrophobic residues in D2, which are critical for membrane association, also play a crucial role in maintaining the stability of the protein. Recently, it has been demonstrated that the abundance of core protein can be modulated through ubiquitinylation by the E3 ligase, E6AP (Shirakura *et al.*, 2007). These modulatory effects also influence levels of infectious virus progeny. Together with our findings on the instability of the Phe-130 mutant protein, we postulate that E6AP may have a role in degradation of core species, which are defective in either folding or membrane association, to prevent their incorporation into virus particles.

To examine directly whether there was a connection between LD association by core and virus production, it was necessary to examine a mutant that was more stable but did not associate with LDs. Hence a third mutant was created, termed JFH1_{DP}, in which two prolines were converted to alanines within HL. These residues have been suggested to correspond to the 'proline-knot motif', which is flanked by two α -helices in the LD-binding domain of the plant protein, oleosin (Lacey *et al.*, 1998; Alexander *et al.*, 2002; Hope *et al.*, 2002). The α -helices in oleosin could correspond to the amphipathic HI and HII helices in D2. In a previous study, these mutations in HCV core from strain Glasgow prevented LD association and reduced processing efficiency by SPP, but the mutant protein was relatively stable in contrast to the Phe-130 mutant (Hope *et al.*, 2002; Boulant *et al.*, 2006). At early times of expression, JFH1_{DP} core was detected as a doublet, which we presumed to correspond to SPP- and SP-cleaved forms of the protein. With longer periods of expression (48 h), only the SPP-cleaved form was detected in the JFH1 system. Maturation of core is a coordinated event in which SP cleaves initially and is then followed by SPP proteolysis (Lemberg & Martoglio, 2002). In our studies, there was no evidence for a core-E1 precursor, which would result from inefficient SP cleavage. Hence, we consider it likely that there was a delay only in SPP proteolysis for this mutant in JFH1. The mechanism for any delay in SPP cleavage is not known, but, conceivably the two prolines might introduce turns into the polypeptide backbone that facilitate correct folding or membrane integration for core. Perturbing these characteristics of the protein may be transmitted to the signal peptide between core and E1 and thereby reduce the ability of SPP to recognize the signal peptide as a substrate for cleavage. Other mutations in HL are also associated with inefficient SPP proteolysis but have no effect on SP cleavage (Okamoto *et al.*, 2004). Therefore, our results verify the contribution from other parts of core for optimal SPP proteolysis in addition to those located in the signal peptide region (McLauchlan *et al.*, 2002; Hope *et al.*, 2006).

Mutation of the prolines at positions 138 and 143 to alanine also reduced the abundance of core and NS5A, particularly at early times after RNA electroporation. We failed to recover increased amounts of the viral proteins by incubation of cells with the proteasome inhibitor MG132 (data not shown). Moreover, the number of cells expressing core and NS5A was about 10% lower for JFH1_{DP} as compared with wt JFH1 (50% for wt JFH1 compared with 40% for JFH1_{DP}; data not shown) at 24 h after electroporation, which only partially accounts for the lower abundance of the viral proteins synthesized by JFH1_{DP}. Therefore, the mechanism for reduced abundance of core and NS5A expressed by JFH1_{DP} at early time points is not apparent, although increased time for membrane integration due to protein misfolding could lower translational efficiency as well as SPP cleavage. Further studies are in progress to address this question.

Two pieces of evidence in our studies suggest a link between the association of core with LDs and virus production. Firstly, the change in distribution of wt core from sites juxtaposed to LDs at early times to complete envelopment of the organelles at later times coincided with a sharp rise in the production of infectious virus. Secondly, JFH1_{DP}, which did not give rise to virus progeny, expressed a core protein that was targeted to punctate sites indistinguishable from those identified for the wt protein at early times but JFH1_{DP} core did not proceed to coat LDs. We consider it likely that the punctate distribution for core triggered by these mutations corresponds to accumulation at the transfer site between the ER membrane and LDs. However, the protein is apparently blocked at such sites and not capable of completing the transfer to coat the LD surface, probably as a result of misfolding. Our data do not demonstrate formally that core present in virions was derived from a pool of protein attached to LDs and it is possible that the ability of core to associate with LDs is merely an indicator of the potential for the protein to promote virion assembly. Nevertheless, our results are consistent with the notion that core protein attached to LDs could be incorporated into virions. Such a pathway requires both association and disassociation of the protein from droplets to enable virus assembly, which is presumed to occur at the ER membrane. In the case of the JFH1_{DP} mutant, core may be trapped at the transfer site between the ER membrane and LDs, preventing its delivery to sites of virion assembly. Since wt core is able to transfer from the ER membrane to the surface of LDs, we propose that the reverse process can also occur and experiments are in progress to determine the mobility of the protein on both organelles. Such studies on the trafficking of core should provide greater insight into the relationship between LDs and HCV assembly.

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