Maturation of Hepatitis C Virus Core Protein by Signal Peptide Peptidase Is Required for Virus Production*

Paul Targett-Adams, Graham Hope, Steeve Boulant, and John McLauchlan

From the MRC Virology Unit, Institute of Virology, Glasgow G11 5JR, United Kingdom

Complete maturation of hepatitis C virus (HCV) core protein requires coordinate cleavage by signal peptidase and an intramembrane protease, signal peptide peptidase. We show that reducing the intracellular levels of signal peptide peptidase lowers the titer of infectious virus released from cells, indicating that it plays an important role in virus production. Proteolysis by the enzyme at a signal peptide between core and the E1 glycoprotein is needed to permit targeting of core to lipid droplets. From mutagenesis studies, introducing mutations into the core-E1 signal peptide delayed the appearance of signal peptide peptidase-processed core until between 48 and 72 h after the beginning of the infectious cycle. Accumulation of mature core at these times coincided with its localization to lipid droplets and a rise in titer of infectious HCV. Therefore, processing of core by signal peptide peptidase is a critical event in the virus life cycle. To study the stage in virus production that may be blocked by interfering with intramembrane cleavage of core, we examined the distribution of viral RNA in cells harboring the core-E1 signal peptide mutant. Results revealed that colocalization of core with HCV RNA required processing of the protein by signal peptide peptidase. Our findings provide new insights into the sequence requirements for proteolysis by signal peptide peptidase. Moreover, they offer compelling evidence for a function for an intramembrane protease to facilitate the association of core with viral genomes, thereby creating putative sites for assembly of nascent virus particles.

Recent estimates indicate that the global prevalence of hepatitis C virus (HCV) is about 2.2%, corresponding to ~130 million infected individuals (1). Chronic HCV infection accounts for a high proportion of cirrhosis and hepatocellular carcinoma and is therefore a leading cause of liver disease (2). The virus has a positive-sense, single-stranded RNA genome that encodes a polyprotein of some 3000 amino acids, which is cleaved both co- and post-translationally by viral and cellular proteases to produce the mature viral proteins (3, 4). The structural components of virus particles lie at the N terminus of the polyprotein and consist of core protein, which forms the virus capsid, and two envelope glycoproteins, E1 and E2. Maturation of the structural components is directed through cleavage by signal peptidase at signal peptides located between core-E1, E1-E2, and E2-p7 (5, 6). In the case of core protein, complete maturation requires further cleavage within the core-E1 signal peptide by signal peptide peptidase (SPP) (7, 8). This second proteolytic event is required to release core from the endoplasmic reticulum (ER) membrane, allowing its attachment to storage organelles termed lipid droplets (LDs) (8). Moreover, cleavage is required for generation of HCV virus-like particles in a system that may reflect the early stages of virion assembly (9). An identical process occurs for maturation of the core proteins encoded by GB virus-B (GBV-B) (10), the closest genetic relative to HCV (11, 12) and classical swine fever virus (CSFV) (13), a pestivirus that is more distantly related to HCV. In the case of GBV-B, preventing SPP cleavage by mutagenesis of the core-E1 signal peptide blocks both LD attachment of core in tissue culture cells and establishment of a productive infection in tamarins (10).

SPP is an intramembrane protease, which is related to presenilin, the protease within the γ-secretase complex that has a biological role in several key cellular functions including signaling and production of pro-amyloidogenic peptides (14, 15). Unlike presenilin, SPP apparently does not require additional cellular cofactors to promote cleavage (16, 17). It is considered that the primary function of SPP is to release signal peptide fragments from the translocon following signal peptide cleavage (15, 18). In addition, it may have a role in facilitating degradation of incorrectly folded proteins bound to the ER membrane (19). The range of substrates recognized by SPP has not been widely documented but includes the signal peptides of major histocompatibility (MHC) class I molecules that bind to HLA-E for presentation on the cell surface (20, 21). This process is critical for self-nonself recognition because surface expression of peptide-loaded HLA-E is needed to inhibit cell lysis by natural killer cells (22). In addition, interaction between SPP and the human cytomegalovirus (HCMV) immunoevasin US2 may promote removal and subsequent degradation of MHC heavy chain molecules attached to the ER (23).

Signal peptides are characterized by a tripartite structure; an N-terminal n-region of 7–13 amino acids that is often polar with a net positive charge, a central hydrophobic h-region of
6–15 amino acids, and a C-terminal c-region, containing polar residues (24). From in vitro translation studies, using microsomal membranes as a source of SPP, the sequence requirements for cleavage include the presence of small, helix-breaking amino acid residues in the h-region for optimal proteolysis although contributions from flanking sequences in the n- and c-regions also are believed to be important (18, 25). However, deriving a precise consensus motif has not been possible. Initial studies with the core-E1 signal peptide from strain Glasgow identified three amino acids, Ala-180, Ser-183, and Cys-184, within the h-region that were necessary for SPP cleavage (8); subsequent analysis revealed that the contribution of the latter two amino acids was far greater than Ala-180 for efficient proteolysis (18). The key role played by residues at positions 180, 183, and 184 in core maturation has been confirmed in a second strain (9). In other HCV strains, the role of these amino acids in SPP cleavage has been less clear (26) although technical differences likely contributes to the different results (27, 28). For the equivalent core-E1 signal peptide in GBV-B, a Ser residue within the h-region, in approximately the same position as Ser-183 in the HCV core-E1 signal peptide, is essential for SPP processing (10). Collectively, the available data indicate that the key residues required for SPP proteolysis lie in the h-region of signal peptides.

In this report, we examine the role of SPP proteolysis for production of HCV using the JFH1 strain that releases infectious progeny from tissue culture cells (29). The key residues involved in SPP maturation of HCV core encoded by this strain also have been identified. Finally, we utilize the properties of a mutant with delayed SPP cleavage to examine the impact of mature core protein on the location of replicated HCV RNA in virus-infected cells.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—Plasmids pSFV/CE1E2Glu and pSFV/CSpmtE1E2Glu have been described previously (8, 30). To generate pSFV/CE1E2FH11, a 2236-bp fragment encoding core, E1, and E2 from plasmid pJFH1 (29) (a gift from Takaji Wakita, National Institute of Infectious Diseases, Tokyo, Japan), which contains the full-length cDNA for strain JFH1 genomic RNA was produced from pTOPO/CE1E2FH11 (31) by BamHI and XbaI cleavage and ligated with 866- and 10172-bp fragments from pSFV-1 (generated by XbaI/Spel and BamHI/Spel digests, respectively). Mutants were generated using the QuikChange site-directed mutagenesis kit (Stratagene), and oligonucleotides for site-directed mutagenesis were designed according to the manufacturer’s guidelines. The sequence of each mutant was verified after cloning. Precise details for the construction of mutant plasmids can be obtained on request.

**Maintenance of Tissue Culture Cells**—HuH-7 cells were propagated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum.

**Antibodies**—Antibodies used to detect HCV core (rabbit antisera R308), E2 (monoclonal antibody ALP98), dsRNA (monoclonal antibody J2, English and Scientific Consulting Bt) and human ADRP have been described previously (30, 32–34). The antibody against SPP was supplied by Abcam.

**In Vitro Synthesis of RNA and Electroporation of Cells**—Wild-type (wt) and mutant pJFH1 constructs were linearized by XbaI digestion and treated with Mung Bean Nuclease (New England BioLabs). To produce SFV RNA encoding the HCV structural proteins, plasmids were digested with SpeI. RNA was synthesized in vitro from linearized plasmids using the MEGAscript high yield transcription kit according to the manufacturer’s instructions (Ambion). Electroporation of RNA into HuH-7 cells was performed as described previously (8, 30). To inhibit SPP cleavage, cells were incubated with 100 μM (Z-LL)_2 ketone immediately following electroporation until harvesting at 12–14 h after electroporation (10).

**Electroporation of siRNA into HuH-7 Cells**—HuH-7 cells were electroporated with in vitro transcribed JFH1 RNA and siRNA duplexes targeted against either human SPP (Ambion; GenBank™ accession number NM_003769) or a control siRNA composed of a scrambled sequence. Cells were electroporated as described previously (8), and the final concentration of siRNA was 1 nM.

**Infection of Cells and Determination of Virus Titers**—HuH-7 cells were electroporated with either wt or mutant JFH1 RNAs for 24–72 h, and virus released into the growth medium 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 Dulbecco’s modified Eagle’s medium. At 24–72 h postinoculation, the tissue culture 50% infective dose (TCID_{50}) was determined as described in Lindenbach et al. (35).

**Indirect Immunofluorescence and Three-dimensional Reconstructions**—Cells on 13-mm coverslips were fixed for 20 min in methanol at −20 °C. After washing and blocking with PBS, cells were incubated with primary antibody (diluted in PBS) for 2 h at room temperature. Cells were washed extensively with PBS and then incubated with secondary antibody (either anti-rabbit or anti-mouse IgG) conjugated to a fluorescent tag for 2 h at room temperature. After washing with PBS, coverslips were rinsed with H_2O before mounting on slides using Citifluor (Citifluor Ltd.).

Cells were examined with a LSM510 META inverted confocal microscope and images were recorded with a Plan-Apochromat x63 lens (NA 1.4). For three-dimensional reconstructions, Z-stack images were collected using optimum intervals, generating 60–80 slices per sample. Image stacks were deconvolved by three-dimensional blind deconvolution (10 iterations) using Autodeblur software (MediaCybernetics). Three-dimensional reconstructions were created with the 5D-viewer extension in the iso-surface mode using a bin factor of 1.

**Preparation of Cell Extracts and Western Blot Analysis**—To prepare extracts, cell monolayers were washed with PBS and solubilized in sample buffer (160 mM Tris–HCl, pH 6.7, 2% SDS, 700 mM β-mercaptoethanol, 10% glycerol, and 0.004% bromphenol blue) at a concentration of ~2 × 10^6 cells/ml sample buffer. Samples were heated at 100 °C for 5 min prior to electrophoresis through a 15% polyacrylamide gel (acrylamide/bis-acrylamide ratio of 37.5:1). For Western blot analysis, proteins were transferred to nitrocellulose membrane after separation on polyacrylamide gels. After blocking with PBSA containing 5% milk powder (Marvel), membranes were incubated with the
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Identification of Amino Acid Residues in the Core-E1 Signal Peptide of HCV Strain JFH1 Required for Efficient SPP Cleavage—Previously, we have shown that a triplet of mutations (A180V, S183L, C184V, hereafter referred to as ASC→VLV) in the core-E1 signal peptide of strains Glasgow and J1 impaired SPP cleavage, generating immature core, which was cleaved by signal peptidase and unable to associate with LDs (8, 27). Along with the different apparent molecular weights of signal peptidase- and SPP-cleaved core protein on polyacrylamide gels, the ability of core to attach to LDs has proven to be a convenient assay for determining the presence of mature SPP-cleaved protein (8, 9, 18, 37). The ASC→VLV mutant triplet was introduced into the core-E1 signal peptide sequence of JFH1 (JFH1mut1, in Fig. 2A) to test whether they had the same effect on SPP proteolysis in this strain. Initially, the effects of the mutations were examined in the Semliki Forest virus (SFV) vector, SFV1, as this strategy had been employed successfully for other HCV strains (8, 9, 27). HuH-7 cells were electroporated with in vitro transcribed RNAs from plasmids pSFV/CE1E2JFH1 and pSFV/Cmut1E1E2JFH1, which expressed the wt structural proteins from strain JFH1 and the ASC→VLV triplet mutation in the core-E1 signal peptide, respectively (Fig. 2A). For comparative purposes, we electroporated cells also with RNAs from pSFV/CE1E2Gla and pSFV/CspmtE1E2Gla, the corresponding wt and ASC→VLV mutant constructs for strain Glasgow (Fig. 2A). In agreement with our previous studies (8, 27), core protein from SFV/CE1E2Gla RNA had a slightly greater mobility compared with the protein produced by SFV/CspmtE1E2Gla RNA (Fig. 2B). This difference in migration arises from the cleavage events used to generate core from the wt (signal peptidase and SPP) and spmt (signal peptidase alone) constructs. The wt form of core from strain Glasgow colocalized with ADRP, a marker for LDs (Fig. 2C, panels vii ix). By contrast, the signal peptidase-cleaved core product made from SFV/CspmtE1E2Gla RNA had a reticular distribution corresponding to association with the ER membrane, and there was no evidence for attachment to LDs (Fig. 2C, panels x xi). For core made from SFV/CE1E2JFH1 and SFV/Cmut1E1E2JFH1 RNAs, we found that the protein produced by both constructs co-migrated by Western blot analysis (Fig. 2B). In addition, the wt and mutant core species colocalized with ADRP by indirect immunofluorescence (Fig. 2C, panels i vi), indicating their attachment to LDs. From this evidence, we concluded that the triplet of mutations, ASC→VLV, were not sufficient to block SPP proteolysis of the core-E1 signal peptide in HCV strain JFH1.

Given the different effects of the mutations in the core-E1 signal peptides of the two strains, we sought to determine whether other amino acids might influence the inability of the ASC→VLV triple mutation in strain JFH1 to impair SPP cleavage. Sequence comparison between the signal peptides of the two strains revealed two amino acid changes at the boundary of the h- and c-regions at positions 185 and 189 (Leu-185 and Ala-189 in strain Glasgow, Ile-185 and Val-189 in strain JFH1; underlined in Fig. 2A). To test whether these amino acids affected the behavior of core made by the ASC→VLV mutant of JFH1, amino acids at positions 185 and 189 were converted to Leu and Ala, respectively, creating a construct termed pSFV/

**RESULTS**

**Reduced Expression of SPP Lowers Production of Infectious HCV**—In a recent study of cellular factors that may play a role in HCV replication and virus production, siRNA targeted against SPP reduced production of infectious progeny by 6-fold (36). To validate further these data, HuH-7 cells were electroporated simultaneously with JFH1 RNA and siRNA duplexes as indicated. A, cells were harvested at 24-h intervals and examined by Western blot analysis with an antibody against SPP. The band corresponding to SPP has an arrow. B, supernatants were harvested at 24-h intervals, and virus titer was determined by measuring TCID_{50} values. Solid and dashed lines show the virus titers for cells electroporated with control and SPP siRNA duplexes, respectively.

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RNA was cleaved by signal peptidase and only about 20% of the protein was processed by SPP (Fig. 3B). By comparison, almost all of the detectable core generated from SFV/Cmut1E1E2JFH1 RNA was cleaved by SPP, in agreement with the data presented in Fig. 2B. Most of the cells (approx. 60%) expressing core made by SFV/Cmut2E1E2JFH1 RNA gave a reticular pattern for the protein (Fig. 3C, middle panel); in the remaining 40% of the cell population, core was located on LDs although there was also a background of reticular staining pattern in these cells (data not shown). Thus, the conversion of amino acids at positions 185 and 189 to Leu and Ala, respectively in the JFH1mut1 signal peptide impaired but did not block SPP cleavage completely. These results indicate that sequence differences between strains Glasgow and JFH1 in the core-E1 signal peptide contribute to the inability of the triple ASC→VLV mutation to prevent SPP processing in strain JFH1.

From our results with the corresponding signal peptide in GBV-B, SPP cleavage required a Ser residue within the h-region, and we suggested that the physico-chemical properties of this amino acid might contribute to generating a substrate recognition sequence for the protease (10). In addition, the signal peptide in CSFV that is cleaved by SPP contains a Thr residue in its sequence just beyond the proteolytic site (13). Given the close similarity in the properties of Ser and Thr, we focused attention on the Thr residue at position 186 in the core-E1 signal peptide of strain JFH1 because it could perhaps create a cryptic SPP cleavage site in the mut1 signal peptide that contains the ASC→VLV triple mutation. To explore whether Thr-186 could contribute to SPP proteolysis, we created two additional constructs, pSFV/Cmut3E1E2JFH1 and pSFV/Cmut4E1E2JFH1, in which this residue was mutated to Leu in the wt and JFH1mut1 sequences (JFH1mut3 and JFH1mut4 in Fig. 4A). Comparing the core species made by these constructs with those produced by SFV/CE1E2JFH1 and SFV/Cmut1E1E2JFH1 revealed that SFV/

Cmut2E1E2JFH1 (labeled as JFH1mut2 in Fig. 3A). In this series of experiments, we included also incubation of cells with (Z-LL)₂ ketone, an inhibitor of SPP (37, 38), to detect core species generated by signal peptidase cleavage. Results indicated that the predominant form of core produced from SFV/Cmut2E1E2JFH1 RNA was cleaved by signal peptidase and only about 20% of the protein was processed by SPP (Fig. 3B). By comparison, almost all of the detectable core generated from SFV/Cmut1E1E2JFH1 RNA was cleaved by SPP, in agreement with the data presented in Fig. 2B. Most of the cells (approx. 60%) expressing core made by SFV/Cmut2E1E2JFH1 RNA gave a reticular pattern for the protein (Fig. 3C, middle panel); in the remaining 40% of the cell population, core was located on LDs although there was also a background of reticular staining pattern in these cells (data not shown). Thus, the conversion of amino acids at positions 185 and 189 to Leu and Ala, respectively in the JFH1mut1 signal peptide impaired but did not block SPP cleavage completely. These results indicate that sequence differences between strains Glasgow and JFH1 in the core-E1 signal peptide contribute to the inability of the triple ASC→VLV mutation to prevent SPP processing in strain JFH1.

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A

JFH1wt
FFFSIFLLALLSCITVPVSA
JFH1mut1
FFFSIFLLVLLVLTVVPVSA
Glawt
CSFSIFLLALLSCITVPVSA
Glaspmt
CSFSIFLLVLLVLTVVPVSA

B

C

ADRP + DAPI
core
merge
i

JFH1wt

ii

iv

v

vi

vii

viii

ix

x

xi

xii

JFH1mut1

Glawt

Glaspmt

FIGURE 2. Effect of mutations in the core-E1 signal peptides of HCV strains Glasgow and JFH1 on SPP proteolysis. A, amino acid sequences of wt and mutant core-E1 signal peptides of HCV strains Glasgow and JFH1. Residues that differ at positions 185 and 189 between strains JFH1 and Glasgow are underlined. B and C, HuH-7 cells were electroporated with in vitro transcribed RNA from the indicated SFV constructs and incubated at 37 °C. In B, cells were harvested at 16 h after electroporation, and cell extracts were examined by Western blot analysis using R308 to detect core protein. Solid and open circles indicate core species cleaved by SPP and signal peptidase, respectively. In C, cells were fixed with methanol and probed with antibodies against ADRP and core protein (R308). To show the location of nuclei, cells were counterstained with DAPI. The scale bar in panel i represents 5 μm.
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The data obtained in the SFV system, JFH1mut1 and JFH1mut3 gave core protein that was processed by SPP, and there was no detectable species cleaved only by signal peptidase throughout the time course (Fig. 5A, lower panel). Consistently, we did note reduced abundance of core for JFH1mut1, particularly at 24 h, but did not explore further the basis of this lower amount of protein. For JFH1mut4, a single species of core corresponding to a signal peptidase-cleaved product was detected at 24 h, which also agrees with the behavior of this mutant in the SFV vector (Fig. 5A, upper panel). However, at 48-h post-electroporation, JFH1mut4 gave a low quantity of protein, which comigrated with mature, wt core produced by SPP. By 72 h, the abundance of this species increased such that there were approximately equal amounts of signal peptidase- and SPP-cleaved core.

\[ C_{mut3E1E2} \text{JFH1} \text{ gave only the SPP-cleaved form of core (Fig. 4B)}. \]

Thus, constructs containing the core-E1 signal peptide from JFH1 with a wt sequence, a triple ASC→VLV set of mutations or a T186L mutation all gave core proteins that were indistinguishable from species generated by SPP cleavage. In addition, core made by SFV/Cmut3E1E2JFH1 RNA colocalized with ADRP and therefore was targeted to LDs (Fig. 4C, panels i-iii). By contrast, SFV/Cmut3E1E2JFH1 generated a core species that had been cleaved by signal peptidase but not by SPP (Fig. 4B). Moreover, core made by this construct gave two intracellular distribution patterns. In one cell population, the protein had a reticular pattern corresponding to association with the ER membrane (Fig. 4C, panels iv-vi), whereas it gave a punctuate distribution in other cells (Fig. 4C, panels vii-ix). This punctuate distribution did not colocalize with ADRP and therefore did not correspond to association with LDs (Fig. 4C, panels x-xii). Hence, abrogating SPP cleavage at the core-E1 signal peptide in strain JFH1 required mutation at Thr-186 in addition to those made at amino acids Ala-180, Ser-183, and Cys-184.

Mutations that Block Efficient SPP Cleavage of HCV Core Also Impair Virus Production—To determine whether the core-E1 signal peptide mutants in strain JFH1 behaved in a similar manner in an infectious system, the amino acid changes in mut1, mut3, and mut4 were introduced into plasmid pJFH-1, the template for generating RNA that gives rise to supernatant from HuH-7 cells (29). Following electroporation of wt and mutant RNAs, the pattern of cleavage for core and titer of virus released from cells was monitored for 24–72 h. Apart from the advantage of determining the effects on virus production, this approach also extended the timescale for analyzing maturation of core compared with the SFV system, which is restricted to about 16–20 h after electroporation due to apoptotic effects on cells by the virus vector (40). In agreement with core. To address whether this lag in appearance of SPP-cleaved core protein resulted from reversion of the mutant sequences, we derived 19 clones from RT-PCR of JFH1mut4 RNA extracted from cells at 72 h post-electroporation. All of the clones contained nucleotide sequences identical to those in the signal peptide of JFH1mut4 (data not shown) indicating that reversion of the mutated residues did not account for our data. We conclude that the amino acid changes in JFH1mut1 lead to delayed SPP cleavage but do not completely abolish proteolysis.

From the TCID50 Values obtained, virus titers for JFH1mut1 and JFH1mut3 were 19- and 6-fold lower compared with wt JFH1 RNA at 24 h, but the differences were less marked at 48 and 72 h (Fig. 5B). We consider that the reduced amount of core protein made by JFH1mut1 at 24 h contributes to the greater reduction in virus titer at this time point. In the case of JFH1mut3, barely detectable levels of virus were found at 24 h (TCID50 of <10), and the relative level of virus production was 1000 times less compared with wt JFH1 (Fig. 5B). At both 48 and 72 h, the relative difference in titers compared with wt JFH1 was considerably reduced (to 64-fold). This pattern in the behavior of JFH1mut1 fits with the inability to detect SPP-cleaved core at 24 h whereas mature core is present at 48 and 72 h. These results provide formal evidence that maturation of core by SPP coincides with the assembly and release of infectious HCV.

From recent studies, the attachment of mature core to LDs correlates with virus production (31, 41). To determine whether the distribution of core produced by JFH1mut4 which did not associate with LDs in the SFV system, changed over longer time periods, we examined its intracellular localization for up to 72 h in the JFH1 system. In control experiments, wt core colocalized with ADRP from 24 h onwards, indicating association of the protein with LDs throughout the time course.

\[ \text{FIGURE 3. Effect on SPP cleavage by introducing amino acid residues encoded by strain Glasgow at positions 185 and 189 into the mut1 core-E1 signal peptide of strain JFH1. A, amino acid sequences of wt and mutant core-E1 signal peptides of HCV strain JFH1. B, HuH-7 cells were electroporated with } \text{in vitro} \text{ transcribed RNA from the indicated SFV constructs in the presence ( + ) and absence ( - ) of the SPP inhibitor, (Z-LL)}_2 \text{ ketone, and incubated at } 37^\circ \text{C. Cells were harvested at 16 h after electroporation, and cell extracts were examined by Western blot analysis using R308 to detect core protein. Solid and open circles indicate core species cleaved by SPP and signal peptidase, respectively. wt core produced by SFV/CE1E2JFH1 is shown also as a control for SPP-cleaved protein. C, cells were electroporated with } \text{in vitro} \text{ transcribed RNA from pSFV/CMut3E1E2JFH1 and fixed with methanol after incubation at } 37^\circ \text{C for } 16 \text{ h. Cells were probed with antibodies against ADRP and core protein (R308). To show the location of nuclei, cells were counterstained with DAPI.} \]
Core protein produced by JFH1mut4 had a punctate distribution at 24 h, but it did not colocalize with ADRP (Fig. 6, panels i and ii). This distribution corresponded to that observed with the SFV system in Fig. 4C (panels vii-xii). By 48 h, some punctate spots of core did localize with LDs but at relatively low frequency (Fig. 6B, panels iii and iv). However, core was readily found colocalized with ADRP at 72 h (Fig. 6B, panels v and vi). This change in the pattern of core distribution matched appearance of the SPP-cleaved form as detected by Western blot analysis (Fig. 5A) and in the rise in virus titer after 24 h (Fig. 5B). We conclude that these results confirm the need for SPP proteolysis to allow association of core with LDs. Moreover, they provide further supportive evidence for a role for attachment of core to LDs in the process of assembly and release of infectious HCV.

**SPP Cleavage of Core Is Required for Juxtaposition with Replicated HCV Double-stranded RNA**—Recently, it has been demonstrated that core recruits the HCV non-structural proteins and viral RNA to LD-associated membranes (33, 41). Using a monoclonal antibody that recognizes double-stranded (ds) RNA, we have established also that replicated HCV dsRNA can be found at the surface of core-coated LDs in infected cells at a higher frequency compared with cells harboring subgenomic replicons (33). The presence of HCV dsRNA on LDs potentially provides a site for interaction between virus genome and core to facilitate virus production. To examine whether maturation of core by SPP was important for colocalization with HCV RNA, the distribution of both core and viral dsRNA were analyzed for JFH1mut4 by indirect immunofluorescence.

We found very little overlap in the signals for core and dsRNA at 24 h after RNA electroporation, but, by 48 and 72 h, colocalization of the two signals was detected with increasing frequency (Fig. 7A). To reinforce the time-dependence for close association between core and HCV dsRNA for JFH1mut4, we performed Z-stack analysis on RNA-electroporated cells at 24-h intervals and generated three-dimensional reconstructions following deconvolution (Fig. 7B). In agreement with the immunofluorescence data presented in Fig. 7A, there were few contacts between core and dsRNA at 24 h. The frequency of their
close apposition increased at 48 h and was the most prominent phenotype at 72 h (Fig. 7B). Therefore, the increase in virus production for JFH1mut4 from 24 to 72 h coincides not only with the appearance of SPP-cleaved core but also the localization of HCV dsRNA to sites containing the viral protein.

DISCUSSION

In this report, we have identified amino acids within the core-E1 signal peptide of HCV strain JFH1, which are critical for efficient SPP cleavage. The most deleterious mutations gave delayed cleavage by SPP, enabling us to correlate production of mature core protein with release of infectious HCV. Moreover, SPP proteolysis was required for association of core with LDs and generation of sites of close juxtaposition between core and replicated HCV dsRNA.

Requirements for SPP Cleavage in the HCV Core-E1 Signal Peptide—A common feature in known SPP substrates is the presence of small, helix destabilizing residues in the h-region of signal peptides (18, 25). Analysis of the GBV-B core-E1 signal peptide in a cell-based system confirmed the need for a small amino acid within the h-region for SPP proteolysis, but maximal cleavage efficiency required a Ser residue at such positions (10). Hence, the hydroxyl group on Ser may participate in determining recognition and cleavage by SPP (10).

Both we (8, 27) and others (9) have demonstrated that mutation at 3 amino acids (Ala-180, Ser-183, and Cys-184) in the core-E1 signal peptide of HCV strains Glasgow and D) (both genotype 1a strains) blocked SPP cleavage. Within this triplet, Ser-183 and Cys-184 had a greater role in proteolysis (18). The same set of mutations in strain J1 (a genotype 1b strain) was shown to have either a slightly less deleterious effect (27) or no impact on core maturation by SPP (26); differences in the systems and contexts for expression of core used in the two studies are likely contributory factors underlying these contradictory conclusions (27). In the study presented here, mutations at these residues in strain JFH1 (a genotype 2a strain) did not reduce SPP cleavage efficiency using either the SFV system (in
which core was expressed as part of a polyprotein encoding the structural components) or full-length infectious JFH1 RNA that produced the entire viral polyprotein. A recent report on extensive mutagenesis across the core-coding region of strain JFH1 arrived at the same conclusion following conversion of amino acids 181–184 to Ala in the core-E1 signal peptide (42).

To try to clarify the disparate effects of mutating amino acids at positions Ala-180, Ser-183, and Cys-184, we performed additional mutagenesis in a JFH1 core-E1 signal peptide containing mutations at these residues (mut1). From our results, other amino acids within mut1 contribute to SPP cleavage. Firstly, converting amino acids at positions 185 and 189 from Ile and Val in JFH1 to Leu and Ala (creating mut2), which corresponds to the residues encoded at these positions in strain Glasgow, considerably impaired SPP cleavage. We are unable to explain the contribution that these amino acids may make to proteolysis. However, the results do illustrate the possible complexity for substrate recognition by SPP and the difficulties with predictions for optimal sequence requirements.

Secondly, mutating the Thr residue at position 186 in addition to the triplet ASC→VLV mutation (mut4) gave a delay in core maturation by SPP in the infectious JFH1 system. Thr has very similar properties to Ser and hence could substitute for Ser at position 183 in the triple ASC→VLV mutant to generate a cryptic SPP recognition site. This hypothesis would provide an explanation for the inability of an alanine-scanning mutant between amino acids 181 and 184 to influence virus production (42). The possibility for similar contributions by Ser and Thr in a SPP recognition motif is underlined by conversion of the HCMV gpUL40 signal peptide from an uncleaved to a cleaved substrate through replacement of two Arg residues with Thr at the junction of the h- and c-region (18). Moreover, a Thr residue is located 3 amino acids beyond the SPP cleavage site in the core-Erns signal peptide of CSFV (13).

Our results raise additional questions concerning SPP proteolysis in the core-E1 signal peptide. Firstly, in our previous studies and in the data presented in Fig. 1, there is a Thr residue at position 186 in the strain Glasgow sequence, which does not appear to facilitate SPP cleavage in Gla_spmnt that contains the triple ASC→VLV mutation. Other factors may prevent creation of a SPP-processing site by Thr-186 in this strain. For example, amino acids at positions 185 and 189 differ between strains Glasgow and JFH1 (Fig. 2A). As we have demonstrated, introduction of the residues encoded by strain Glasgow at these positions has a detrimental effect on proteolysis in the mut1 core-E1 signal peptide. Consequently, they may diminish the capability of Thr-186 to generate a motif for SPP recognition in strain Glasgow. Moreover, other sequences within the signal peptide and elsewhere

FIGURE 7. SPP Cleavage of core protein is needed for colocalization with HCV dsRNA. A and B, HuH-7 cells were electroporated with in vitro transcribed JFH1mut4 RNA and incubated at 37 °C. At 24-h intervals, cells were fixed with methanol and probed with antibodies against core protein (R308) and dsRNA (J2). In A, panels on the right-hand side show enlarged regions of interest (ROI) of merged images (indicated by a white rectangle). In B, three-dimensional reconstructions are shown for the areas marked by a white rectangle. Core protein that colocalizes with dsRNA (filled arrowheads) and is not associated with dsRNA (open arrowheads) is indicated. Scale bars in A and B represent 10 μm and 2 μm, respectively.
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...in core and E1 may influence SPP processing. In support of this view, mutation of Pro residues at positions 138 and 143 in the D2 domain of core, which lie about 30 amino acids upstream of the core-E1 signal peptide, impairs SPP cleavage (31, 43). A second aspect of our results is the delayed processing of JFH1mut4 that contains 4 mutations, which increase the hydrophobicity of the core-E1 signal peptide and remove amino acids expected to create a SPP recognition site. Therefore, even signal peptides that are apparently poor substrates may ultimately be recognized and cleaved by SPP. Such a notion fits with a proposed function for SPP to remove misfolded transmembrane domains as part of the ER quality control system (19).

From the above, several factors could contribute to SPP proteolysis in the HCV core-E1 signal peptide. These are likely to include structural elements determined by certain amino acids (for example in the D2 domain and core-E1 signal peptide) combined with more specific physico-chemical characteristics at key residues in the h-region of the signal peptide. We do anticipate that amino acids other than those identified in this study will contribute to the overall processing at the core-E1 signal peptide by SPP. More generally, our interpretation of the analysis described here may be relevant to understanding SPP cleavage of other signal peptides. The HCV core-E1 signal peptide could be an extremely efficient substrate for SPP, but there may be other signal peptides, which are cleaved with lower efficiency. Thus, different signal peptides may be processed at varying efficiencies, perhaps for regulatory purposes. To address this question, more studies are required to identify signal peptides, which are cleaved by SPP, and to examine functional roles for any cleaved products.

The Role of SPP Proteolysis in the Production of Infectious HCV—In our previous analysis, maturation of core by SPP was required for its association with LDs (8) but we were unable to examine whether this process was linked to virus production because release of infectious progeny from tissue culture cells was not available. By making use of a system that does allow virus production in hepatoma cells (29, 35, 39), the results in this report demonstrate that SPP cleavage of core is necessary for generating infectious HCV. The requirement for SPP proteolysis in the production of HCV fits with an identical role in GBV-B (10) and CSFV (13), two viruses that are relatives of HCV. Therefore, use of a cellular intramembrane protease has been adopted by three related viruses for maturation of their capsid proteins, a key event in the virion assembly pathway. It has been reported also that SPP interacts with the HCMV US2 protein, potentially to release MHC heavy chains from the ER membrane as part of an immune evasion mechanism for the virus (23). Hence, intramembrane proteases such as SPP could play diverse roles in the life cycles of viruses and this topic deserves further attention.

The delay in SPP maturation of core encoded by mutant JFH1mut4 is accompanied by time-dependent association of the protein with LDs. These events also coincide with a relative rise in the amount of virus produced by JFH1mut4. Our results strongly suggest that these events are connected and serve to underline the importance of LDs in the production of infectious HCV. It has been shown also that core is responsible for recruiting HCV RNA and the viral non-structural proteins to LD-associated sites (33, 41) but the involvement of core maturation has not been studied. From our results, core protein that has been processed only by signal peptidase, and which is not targeted to LDs, is not found juxtaposed to replicated HCV dsRNA. However, SPP cleavage allows not only attachment of core to LDs but also close positioning to HCV dsRNA. Therefore, our data suggest that complete maturation of core is required to create sites for close positioning of the protein with viral genomes, which could represent an early stage in the virus assembly pathway. Because mature core also is located on LDs, our studies lead us to conclude that targeting of core to LDs does not serve solely as a means of storing the viral protein but actively promotes and participates in virus assembly. The processes involved in the engagement of core, attached to LDs, with HCV RNA are currently under investigation.

To conclude, our studies have revealed the critical nature of intramembrane proteolysis by SPP in the assembly and release of HCV. Given the ongoing need for new approaches to treat chronic infection, this maturation event represents a potential step in the virus life cycle that could be targeted for antiviral therapy. SPP is related to presenilin, another intramembrane protease that is part of the γ-secretase complex, which is responsible for producing amyloid beta peptides in Alzheimer disease. Recent progress in clinical trials with compounds which target γ-secretase activity (44) offer the prospect that related compounds may be suitable as part of a regime in the treatment of chronic HCV infection.

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