Signal Peptide Peptidase-catalyzed Cleavage of Hepatitis C Virus Core Protein Is Dispensable for Virus Budding but Destabilizes the Viral Capsid*

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The capsid of hepatitis C virus (HCV) particles is considered to be composed of the mature form (p21) of core protein. Maturation to p21 involves cleavage of the transmembrane domain of the precursor form (p23) of core protein by signal peptide peptidase (SPP), a cellular protease embedded in the endoplasmic reticulum membrane. Here we have addressed whether SPP-catalyzed maturation to p21 is a prerequisite for HCV particle morphogenesis in the endoplasmic reticulum. HCV structural proteins were expressed using recombinant Semliki Forest virus replicon in mammalian cells or recombinant baculovirus in insect cells, because these systems have been shown to allow the visualization of HCV budding events and the isolation of HCV-like particles, respectively. Inhibition of SPP-catalyzed cleavage of core protein by either an SPP inhibitor or HCV core mutations not only did not prevent but instead tended to facilitate the observation of viral buds and the recovery of virus-like particles. Remarkably, although maturation to p21 was only partially inhibited by mutations in insect cells, p23 was the only form of core protein found in HCV-like particles. Finally, newly developed assays demonstrated that p23 capsids are more stable than p21 capsids. These results show that SPP-catalyzed cleavage of core protein is dispensable for HCV budding but decreases the stability of the viral capsid. We propose a model in which p23 is the form of HCV core protein committed to virus assembly, and cleavage by SPP occurs during and/or after virus budding to predispose the capsid to subsequent disassembly in a new cell.

Signal peptide peptidase (SPP)⁵ belongs to a novel family of polytopic membrane-associated aspartyl proteases that catalyze cleavage of peptide bonds within the hydrophobic environment of a lipid bilayer (for review see Ref. 1). SPP is highly conserved in higher eucaryotes from plants to humans, including insects (2, 3). This membrane protein with nine proposed transmembrane domains exerts its action in the endoplasmic reticulum (ER), where it resides by virtue of a classical ER retrieval signal (3–5). The SPP substrates identified so far have in common a type II (N terminus facing the cytosol and C terminus facing the lumen) orientation but do not unequivocally point to a common role for SPP activity. In humans, SPP carries out the intramembrane cleavage of signal peptides of polymorphic major histocompatibility complex class I molecules after they have been cleaved off by signal peptidase; the short N-terminal signal peptide remnants then serve as epitopes presented at the cell surface by nonpolymorphic human lymphocyte antigen-E molecules for immune surveillance (6). Thus, SPP activity may promote the liberation from the ER lipid bilayer of some bioactive signal peptide fragments. Another role of SPP activity may be to protect cells from the toxicity of some signal peptides, e.g. the signal peptide of eosinophil cationic protein (7). Finally, the substrate spectrum of SPP may not be restricted to signal peptides but extend to transmembrane domains of polytopic proteins (8). The recent finding that a misassembled transmembrane domain of a polytopic protein interacted with SPP has suggested a role for SPP in the ER quality control of membrane proteins (9). Beside cellular substrates, an internal signal sequence of the polypeptide of hepatitis C virus (HCV) was shown to interact with and be cleaved by SPP (10, 11). This makes SPP a potential target for therapeutic intervention, but a clear understanding of the role of SPP in the HCV life cycle is needed before this approach can be envisaged.

HCV was discovered by molecular cloning, but its life cycle has remained speculative due to difficulties encountered in reproducing infection in tissue culture cells (12). Entry of this
enveloped virus into target cells most likely involves virion internalization by endocytosis followed by envelope-mediated fusion upon endosomal acidification (13). The viral capsid must then be disassembled to begin translation of the positive strand RNA genome, but no data are available regarding the uncoating process. Flanked by 5' and 3' noncoding regions, the single long open reading frame of the HCV genome encodes a polyprotein of about 3,000 amino acids. During and after translation, this polyprotein undergoes a series of proteolytic cleavages by host and viral proteases, which generate the structural and nonstructural proteins, respectively. The nonstructural proteins are sufficient for replication of the viral genome but are not believed to be contained in virus particles, whereas the structural components (including core protein and two envelope glycoproteins, E1 and E2) assemble with the viral genome to form progeny virions. That HCV morphogenesis most certainly proceeds through budding of nascent particles into the ER lumen is supported by several arguments as follows: (i) the analogy with other viruses of the Flaviviridae family (12, 14); (ii) the rare available EM images of liver tissue from HCV-infected patients or chimpanzees showing putative particles in ER-related cisternae of hepatocytes (15, 16); (iii) the orientation of HCV core and envelope proteins with respect to the ER membrane; and (iv) the accumulation of E1 and E2 envelope glycoproteins in the ER due to ER retention signals (17).

HCV core protein is the most N-terminal component of the viral polyprotein and terminates with a signal peptide (Fig. 1A) (18). This signal peptide directs the nascent polypeptide chain to the ER membrane and induces translocation of the downstream E1 region into the ER lumen, while leaving the core protein region on the cytosolic side. Cleavage by signal peptidase at the luminal side of the ER separates E1 from the so-called immature form of core protein, containing 191 residues, which migrates in SDS-PAGE with an apparent molecular mass of 23 kDa (p23). This complete form of HCV core protein is anchored in the ER lipid bilayer by the C-terminal signal peptide (10). Intramembrane cleavage catalyzed by SPP generates the so-called mature form of core protein, which migrates in SDS-PAGE with an apparent molecular mass of 21 kDa (p21). The precise position of the SPP cleavage site has been debated (10, 19–21), but a direct mass spectral analysis of HCV core protein processed in insect cells has recently suggested that p21 ends with residue 177 (22). Because cleavage by SPP removes the C-terminal anchor of core protein, it loosens its attachment to the ER membrane and allows it to travel to other organelles within the cell. Indeed, a characteristic feature of the intracellular distribution of HCV core protein is its association with the surface of lipid droplets (10, 23–27). This was demonstrated not only in a variety of tissue culture cells, but also in liver biopsies from HCV-infected patients and chimpanzees. A fraction of HCV core protein was also shown to localize to the outer membrane of mitochondria in several cell lines (28, 29). When SPP-catalyzed cleavage to generate p21 was inhibited, however, none of these subcellular locations was reached, and the unprocessed p23 was retained in the ER membrane (10, 29).

Because core protein in sera from HCV-infected patients has an identical molecular mass to p21, this form is considered to constitute the HCV virion capsid (18, 21). It might be inferred from this observation that cleavage of core protein by SPP is a prerequisite for HCV particle morphogenesis. As a capsid protein is expected to reside at the virus budding site, however, the propensity of the processed p21 to move to other sites seems paradoxical and may be related to at least some of the many other reported functions of the HCV core protein (18). By contrast, p23 is not free for trafficking from the ER to other organelles within the cell (10, 29) and may therefore be prone to virus particle budding. This would imply that HCV morphogenesis can occur when SPP-catalyzed cleavage to generate the p21 form of HCV core protein is abolished. The aim of this study was to decide between these two opposite hypotheses regarding the role of SPP in the HCV life cycle. Two independent approaches, one pharmacological and the other genetic, were used to inhibit SPP-catalyzed cleavage of core protein in two complementary experimental systems that allow the study of HCV morphogenesis in a cellular context. The first uses a recombinant Semliki forest virus (SFV) replicon to express HCV structural proteins in mammalian BHK-21 cells (30, 31). Because HCV budding is abortive in this system, it does not allow the isolation of virus-like particles but instead appears as a choice model for EM visualization of budding events in situ in the ER. In the second system, expression of HCV structural proteins is achieved in insect Sf9 cells by means of a recombinant baculovirus vector, which leads to the recovery of enveloped virus-like particles (32–35). These have been shown to resemble putative virions isolated from HCV-infected patients on the basis of biophysical, morphological, and antigenic properties. The results of our study not only show that SPP activity is dispensable for HCV particle budding but further point to a role for SPP in destabilizing the viral capsid. These findings are consistent with a model in which p23 is the form of HCV core protein that is committed to virus particle morphogenesis, and cleavage by SPP occurs during and/or after virus budding in the ER to predispose the capsid to subsequent disassembly during virus uncoating in a new target cell.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—The pFastBac-HCV1a construct was generated by inserting into the multiple cloning site of the vector pFastBac [Invitrogen] the BamHI-BgIII fragment of the pGmAc-5'-NTRm-NS2A plasmid (35), a generous gift from M. Duonor-Cécutti and G. Devauchelle (CNRS UMR 5160, Saint-Christol-lès-Alès, France). This fragment contains the portion of the cDNA of the HCV-H strain (genotype 1a) coding for all HCV structural proteins and the first 5 amino acids of NS2 protein, downstream of the complete 5'-noncoding region modified by the mutation of its two AUG codons (35). For expression in BHK-21 cells, this fragment was also inserted into the unique BamHI site of the vector pSFV1 (36), yielding the pSFV-HCV1a construct. The plasmid pFastBac-HCV1b (originally designated as pFastBacHCV.S in Ref. 32), which contains the portion of the cDNA of the HCV-J strain (genotype 1b) coding for all HCV structural proteins and the first 21 amino acids of NS2 protein downstream of the 71 last nucleotides of the 5'-noncoding region, was a kind gift from T. J. Liang (NIDDK, National Institutes of Health, Bethesda). For expression in BHK-21 cells, the 2432-bp NruI-NdeI fragment of this...
plasmid was subcloned into the pSFV1 vector modified by the insertion of restriction sites for NruI and NdeI immediately downstream of the BamHI site, yielding the pSFV-HCV1b construct.

**Site-directed Mutagenesis**—Oligonucleotide-directed mutagenesis by the Kunkel method (37) was used to introduce mutations into the sequence encoding the signal peptide at the core-E1 junction. To do this, the 2534-bp HindIII-BglII fragment of pGM-Ac-5’NTRm-NS2A and the 1702-bp EcoRI-NotI fragment of pFastBac-HCV1b, which contained the sequences to be mutated, were subcloned into the vectors pGEM-3Zf(+) and pGEM-11Zf(+) (Promega), respectively. Mutagenesis was designed both to introduce an Spel restriction site and to replace residues Ala<sup>180</sup>, Ser<sup>183</sup>, and Cys<sup>184</sup> of HCV polyprotein by Val, Leu, and Val, respectively (Fig. 1A). The mutated fragments were then inserted in place of the homologous wild-type (WT) fragments into the pFastBac-HCV1a and pFastBac-HCV1b plasmids. For expression in mammalian cells, they were also inserted in place of the homologous WT fragments into the pSFV-HCV1a and pSFV-HCV1b plasmids. The presence of the desired mutations was checked both by restriction map analysis and DNA sequencing.

**Mammalian Cell Culture and Transfection**—BHK-21 cells were grown in Glasgow minimal essential medium (Invitrogen) supplemented with 10 mM HEPES (pH 7.3), 10% tryptose phosphate broth (Sigma), and 5% fetal calf serum (PAA Laboratories) at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere. For transfection, the WT and mutated forms of pSFV-HCV1a or pSFV-HCV1b plasmids were linearized at the Spel and Spfl site, respectively, and served as templates for <i>in vitro</i> transcription by the Sp6 RNA polymerase. Typical reactions were performed for 2 h at 37 °C in a mixture containing 40 mM Tris-HCl (pH 7.9), 10 mM NaCl, 6 mM MgCl<sub>2</sub>, 2 mM spermidine, 10 mM dithiothreitol, 1 mM each of ATP, CTP, and UTP, 0.5 mM GTP, 1 mM of the cap analog m<sup>7</sup>G<sup>(5’)</sup>ppp<sup>(5’)</sup>G (Invitrogen), 1.6 units/µl recombinant RNasin<sup>®</sup> ribonuclease inhibitor (Promega), and 0.6 units/µl Sp6 RNA polymerase (Promega). For the negative control, recombinant SFV RNA coding for β-galactosidase (SFV-lacZ) was synthesized from the Spel-linearized pSFV<sub>3</sub>-lacZ plasmid (36). BHK-21 cells in mid-log phase were detached with trypsin, suspended in phosphate-buffered saline (PBS), mixed with the capped transcripts in an electroporation cuvette (0.4-cm gap), and pulsed twice at 0.83 kV, 25 microfarads, in a Gene Pulser<sup>®</sup> II electroporator (Bio-Rad). Immediately after electroporation, cells were plated and cultured for 20 h in growth medium, containing 20 µM (Z-LL)<sub>2</sub>-ketone (Calbiochem) where indicated.

**Insect Cell Culture and Recombinant Baculovirus Generation**—<i>Spodoptera frugiperda</i> Sf9 cells were grown in monolayer cultures at 28 °C in SF-900 II SFM medium (Invitrogen) supplemented with 5% fetal calf serum (PAA Laboratories). The WT and mutated forms of pFastBac-HCV1a and pFastBac-HCV1b were used to generate recombinant baculoviruses by using the Bac-to-Bac<sup>®</sup> Baculovirus Expression System (Invitrogen) according to the manufacturer’s protocols. For the negative control, the plasmid pFastBac<sup>TM1</sup>-Gus (Invitrogen), which contains the cDNA coding for β-glucuronidase, was used to generate the recombinant baculovirus BV-gus. Recombinant baculoviruses harvested from the supernatant of transfected Sf9 cells were amplified by subsequent rounds of Sf9 cell infection until a high titer was achieved. For all expression experiments, Sf9 cells in mid-log phase were infected with the appropriate recombinant baculovirus at a multiplicity of infection of 5 and cultured for 3–4 days. Where indicated, (Z-LL)<sub>2</sub>-ketone was added every 12 h to the growth medium at a final concentration of 20 µM.

**Isolation of HCV-like Particles and Capsids**—Recombinant baculovirus-infected Sf9 cells were washed three times in PBS and collected by centrifugation. Cells were ruptured by three freeze-thaw cycles, using dry ice plus ethanol for freezing and a 37 °C water bath for thawing, and suspended in TNE buffer (50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 0.5 mM EDTA) containing 0.2 mg/ml of the protease inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride (ICN Biomedicals) plus RNasin<sup>®</sup> (complete TNE buffer). Homogenization further proceeded with 10 passes through a 23-gauge needle. After low speed centrifugation, the supernatant was pelleted over a 2-ml cushion of 20% (w/w) sucrose in complete TNE, at 77,000 x g for 90 min at 4 °C. The pellet was allowed to dissolve in complete TNE buffer for 2 h at 4 °C, before loading on a sucrose gradient made in complete TNE. To partially purify enveloped HCV-like particles, this consisted of an 8-ml continuous 30–65% (w/w) sucrose gradient overlaid with a 1.5-ml layer of 15% (w/w) sucrose, itself covered with a 0.5-ml layer of 10% (w/w) sucrose. Isopycnic ultracentrifugation was performed at 150,000 x g for 20 h at 4 °C, followed by fractionation from the bottom of the tube into 18 fractions of 0.6 ml. The density of each fraction was determined by measuring the refractive index of a 10-µl aliquot with an Abbe refractometer (Atago) at a constant temperature of 20 °C. To isolate capsids of HCV-like particles, a protocol was adapted from the “spin-thru” method described for the purification of mature human immunodeficiency virus (HIV) capsids (38). Briefly, isopycnic ultracentrifugation was performed as described above except that the 15% sucrose layer contained 60 mM octyl β-D-glucopyranoside (nOG) (Sigma). In this method, the 15% sucrose layer serves as a barrier to minimize mixing of the virus and detergent interfaces prior to centrifugation, and the enveloped particles pass through the detergent interface at the quickest possible speed, thus minimizing the time that particles are incubated in detergent.

**Western Blotting**—For analysis of HCV structural protein expression, recombinant SFV RNA-transported BHK-21 cells or recombinant baculovirus-infected Sf9 cells were lysed in reducing sample buffer. Cell lysates or fractions collected from sucrose gradients were electrophoresed in SDS-15% polyacrylamide gels under reducing conditions, and subsequently transferred onto nitrocellulose membranes (PROTRAN<sup>®</sup>, Schleicher & Schuell). Membranes were blocked with PBS containing 0.05% Tween 20 and 5% skimmed milk and probed overnight at 4 °C with monoclonal antibodies against HCV core protein (1856, Virostat), E1 glycoprotein (A4 (39), kindly provided by J. Dubuisson, CNRS UPR 2511, Institut Pasteur de Lille, Lille, France), or E2 glycoprotein (AP33 (34), kindly provided by A. H. Patel, MRC Virology Unit, Institute of Virology, Glasgow, UK), diluted 1:10,000, 1:750, and 1:500 in blocking buffer, respectively. Excess antibody was removed with four
Role of SPP in HCV Particle Morphogenesis

washes, and the second-step antibody (horseradish peroxidase-linked mouse-specific sheep antibody; Amersham Biosciences) diluted 1:2,000 in blocking buffer was allowed to bind for 1 h. After several washes, protein bands were visualized using membrane incubation in SuperSignal® West Pico Chemiluminescence Substrate (Pierce) and exposure to BioMax Light films (Eastman Kodak). Blots used for multiple hybridizations were stripped of antibodies by washing for 30 min at 55 °C in stripping buffer (125 mM Tris-HCl (pH 6.7), 2% SDS, 100 mM β-mercaptoethanol), and reprobed as described above.

Capsid Stability Assays—Freshly prepared capsids were tested for stability under different conditions. After isopycnic ultracentrifugation using the spin-thru method described above, the fractions with densities ranging from 1.23 to 1.26 g/ml were pooled and diluted 1:10 in TNE buffer, in high salt TNE buffer (TNE containing 1 M instead of 100 mM NaCl), or in low pH TNE buffer (TNE adjusted to pH 5.5). Following incubation at 37 °C for various times, samples were split in half. The total proteins contained in one-half were precipitated by adding trichloroacetic acid to a final concentration of 15% in the presence of 0.1 mg/ml bovine serum albumin; the precipitate was spun down at 13,000 × g in a microcentrifuge, washed with acetone to remove the trichloroacetic acid, and resuspended in reducing sample buffer for Western blotting detection of total HCV core protein. The other half was subjected to ultracentrifugation at 77,000 × g for 90 min at 4 °C, and the resulting pellet was resuspended in reducing sample buffer for Western blotting detection of pelletable HCV core protein. The NIH ImageJ version 1.33 software was used for quantitative evaluation of the relative intensities of the bands. For each incubation time, the capsid stability index was calculated as follows: 100 × (intensity of the band of total core protein at the given time/intensity of the band of pelletable core protein at time 0)/(intensity of the band of total core protein at the given time/intensity of the band of total core protein at time 0).

EM Analyses—For in situ visualization of HCV-like particle budding, recombinant SFV RNA-transfected BHK-21 cells were fixed for 1 h with 3% glutaraldehyde in phosphate buffer (0.1 M NaH2PO4, 0.1M Na2HPO4, pH 7.2), and post-fixed for 2 h with 1% OsO4 in 0.1 M cacodylate buffer. Fixed cells were washed in water for 5 min, then in 0.1 M cacodylate buffer for 15 min, and finally in 0.2 M cacodylate buffer for 30 min. They were transferred to 30% methanol for 10 min, stained for 1 h with 2% uranyl acetate in 0.1 M cacodylate buffer containing 30% methanol, followed by washing in 30% methanol. Cells were then dehydrated in a graded ethanol series to propylene oxide, and embedded in Epon 812. For observation, ultra-thin sections were contrasted with 4% uranyl acetate and lead citrate and examined under a Zeiss 902 electron microscope at 80 kV. Areas of convoluted ER with HCV-like particles budding toward the dilated lumen were measured using the NIH ImageJ version 1.63 software, and the number of budding particles arrested at early or late stages of the budding process was counted.

For visualization of enveloped HCV-like particles or capsids, immunoreactive fractions collected after isopycnic ultracentrifugation were pooled, diluted in complete TNE buffer, and centrifuged at 77,000 × g for 90 min at 4 °C. The pellet was then allowed to dissolve slowly in complete TNE. For direct examination, preparations were loaded onto collodion carbon self-coated copper grids (Sigma) for 10 min and stained with 1% ammonium molybdate (pH 7.4) for 1 min. For immunogold labeling, preparations were loaded onto collodion-carbon self-coated nickel grids (Sigma) for 10 min. Grids were blocked with PBS containing 5% goat serum (Sigma) and probed for 3 h with monoclonal antibody against HCV E2 glycoprotein (AP33, diluted 1:10 in blocking buffer) or core protein (1856, diluted 1:50 in blocking buffer) for examination of enveloped particles and capsids, respectively. Excess antibody was removed with four washes, and the second-step antibody (gold particle-conjugated mouse-specific goat antibody; British BioCell) diluted 1:30 in blocking buffer was allowed to bind for 1 h. After several washes, negative staining was carried out as described above. Samples were examined under a Phillips CM-10 electron microscope at 80 kV.

RESULTS

Visualization of Budding Events—To examine the role of SPP in the initiation of HCV budding, we made use of the only experimental system that, to date, has allowed the visualization of budding events in cultured mammalian cells (30, 31). This relies on expression of the portion of the HCV genome encoding the structural proteins in BHK-21 cells by means of a recombinant SFV replicon. We used two distinct HCV sequences, one of genotype 1a and the other of genotype 1b, to generate the recombinant RNAs SFV-HCV1a and SFV-HCV1b, respectively. As shown in Fig. 1B, transfection of BHK-21 cells with either of these constructs led to high level expression of HCV structural proteins. Western blot analysis of the cell lysates with a monoclonal antibody specific to HCV core protein revealed one major band with an apparent molecular mass of 21 kDa, corresponding to p21, and one faint band with an apparent molecular mass of 23 kDa, corresponding to p23 (Fig. 1B, bottom, WT1a and WT1b). Thus, p21 is the predominant form of HCV core protein at steady state in these cells, as is the case with other tissue culture cells expressing at least a partial HCV polypeptide encompassing the virus structural proteins (18). Reprobing the blots with monoclonal antibodies specific to E1 or E2 showed that the envelope glycoproteins were also correctly processed from the partial HCV polyprotein (Fig. 1B, middle and top, WT1a and WT1b), as described previously (30, 31). Major ER morphological differences were observed by EM between most of the cells transfected with recombinant SFV-HCV1a or SFV-HCV1b RNAs and cells transfected with the control recombinant SFV-lacZ RNA (Fig. 2). Indeed, whereas the ER was homogeneously distributed throughout the cytoplasm of the control cells (Fig. 2, Mock), areas of convoluted membranes with a dilated lumen were present in cells expressing the HCV structural proteins (Fig. 2, WT). The self-assembly of proteins at these convoluted membranes was observed as electron-dense patches, which in some cases appeared as hemispherical structures, 50 nm in diameter, budding toward the dilated ER lumen (Fig. 2, WT). Budding of HCV-like particles was abortive, however, because very few particles, if any, seemed to be fully released from the ER membrane. All of these
Role of SPP in HCV Particle Morphogenesis

ultrastructural changes have been reported previously using another HCV sequence of genotype 1a (30, 31).

To determine whether the p23 form of HCV core protein is capable of initiating virus budding, we treated transfected BHK-21 cells with a well characterized SPP inhibitor, the substrate mimic (Z-LL)$_2$-ketone (40). When lysates of cells transfected with recombinant SFV-HCV1a or SFV-HCV1b RNAs and cultured in the presence of the SPP inhibitor were subjected to Western blot analysis with monoclonal antibody to core protein, one major band of 23 kDa and one faint band of 21 kDa were detected (Fig. 1B, bottom, WT1a+inh and WT1b+inh). Thus, an almost complete inhibition of SPP-catalyzed cleavage of HCV core protein was achieved in this experimental system, as reported by others (40). The pattern of HCV envelope glycoprotein expression was similar in cells treated with (Z-LL)$_2$-ketone and in untreated cells (Fig. 1B, middle and top, compare WT1a+inh and WT1b+inh with WT1a and WT1b, respectively), confirming that the compound did not affect signal peptidase-catalyzed processing of the polyprotein. EM examination of treated cells revealed numerous areas of convoluted ER with a dilated lumen. A typical area is shown in Fig. 2 (WT+inh), with a large number of HCV-like particles budding from the convoluted membranes toward the dilated lumen. Importantly, various stages of the budding process were visualized, including particles that seemed to have moved forward to a late stage of budding and were only tethered to the ER membrane by a stalk (Fig. 2, WT+inh, see the high magnification images). In these (Z-LL)$_2$-ketone-treated cells as in untreated cells, very few particles seemed to be fully released from the ER membrane. Strikingly, however, areas of convoluted ER displayed a higher density of budding particles in cells treated with the SPP inhibitor as compared with untreated cells, and the majority of the viral buds was seen at a later stage of the budding process (Table 1). These phenomena were seen in repeated experiments with both genotypes.

As a second approach to inhibiting SPP-catalyzed cleavage of HCV core protein, we replaced residues Ala$^{180}$, Ser$^{183}$, and Cys$^{184}$ of HCV polyprotein by Val, Leu, and Val, respectively (Fig. 1A). This combination of amino acid substitutions, designed to remove a critical helix break required for SPP processing (41), was indeed shown to prevent maturation to p21 in the context of an HCV sequence of genotype 1a expressed in vitro or in BHK-21 cells (10), although discordant results were obtained in other experimental systems (11). We introduced the corresponding mutations into each of the HCV sequences under study (Fig. 1A), and hence generated the recombinant SFV-HCV1a/Sppmt and SFV-HCV1b/Sppmt RNAs. All three HCV structural proteins were expressed in BHK-21 cells transfected with either of these mutated constructs, albeit at a lower level than in cells transfected with the corresponding WT constructs (Fig. 1B, Sppmt1a and (Z-LL)$_2$-ketone where indicated (+inh). C, SF9 cells were infected by the recombinant baculoviruses BV-qus (Mock), BV-HCV1a (WT1a), BV-HCV1a/Sppmt (Sppmt1a), BV-HCV1b (WT1b), or BV-HCV1b/Sppmt (Sppmt1b) and cultured in the presence of 

FIGURE 1. Inhibition of SPP-catalyzed cleavage of core protein. A, schematic representation of the portion of HCV polyprotein encompassing the virus structural proteins. Signal peptides are indicated by hatched boxes. The arrows indicate the sites of the cleavages catalyzed by signal peptidase (SP); the arrowhead indicates the SPP cleavage site. The sequence at the C terminus of core protein is presented for the WT HCV strains of genotypes 1a and 1b used in this study. Exchanged residues are shown for the mutated sequences. B and C, Western blot analysis of HCV structural protein expression. B, BHK-21 cells were transfected with the recombinant RNAs SFV-lacZ (Mock), SFV-HCV1a (WT1a), SFV-HCV1a/Sppmt (Sppmt1a), SFV-HCV1b (WT1b), or SFV-HCV1b/Sppmt (Sppmt1b), and cultured in the presence of 

VOLUME 281 • NUMBER 38 JOURNAL OF BIOLOGICAL CHEMISTRY 27683

SEPTMBER 22, 2006

173

191

A

B

C

anti-E2

anti-E1

anti-core

anti-E2

anti-E1

anti-core

S F S I F L A L L S C L T V P A S A

S F S I F L A L L S C L T I P A S A

S F S I F L A L L S C L T V P A S A

S F S I F L A L L S C L T I P A S A

1a WT Sppmt

1b WT Sppmt

Mock

WT1a

WT1a+inh

Sppmt1a

WT1b

WT1b+inh

Sppmt1b

83.0

62.0

47.5

32.5

25.0

16.5

83.0

62.0

47.5

32.5

25.0

16.5
The percentage of cells that tested positive for HCV proteins by immunofluorescence was also lower in the case of the mutants (data not shown). This was most certainly due to the lower level of transcripts generated from the SphI-linearized templates used for the mutated sequences as compared with the SpeI-linearized templates used for their WT counterparts (see “Experimental Procedures”). In cells transfected with recombinant SFV-HCV1a/Sppmt or SFV-HCV1b/Sppmt RNAs, HCV core protein was detected as a single species, which showed a higher molecular mass than p21 but migrated slightly faster than the WT p23 (Fig. 1B, bottom, Sppmt1a and Sppmt1b). That the exchanged amino acid residues increase the mobility of the mutated p23 in SDS-PAGE has been reported previously (42). Even upon longer exposure of the Western blots, no p21 could be detected, indicating that the mutations completely abolished SPP-catalyzed cleavage of HCV core protein in the context of both genotypes. Nevertheless, areas of convoluted ER membranes with HCV-like particles budding toward the dilated lumen were visualized in some of the cells transfected with SFV-HCV1a/Sppmt or SFV-HCV1b/Sppmt

### TABLE 1
Estimation of budding efficiency

| Surface analyzed | Density of budding particles per µm² | Early buds | Late buds | Total |
|-----------------|-------------------------------------|------------|-----------|-------|
| µm²             |                                     |            |           |       |
| WT              | 40.01                               | 1.82       | 0.27      | 2.09  |
| WT + (Z-LL)₂-ketone | 18.44                               | 2.98       | 12.09     | 15.07 |
| Sppmt           | 22.09                               | 2.80       | 16.93     | 19.73 |

* Areas of convoluted ER with HCV-like particles budding toward the dilated lumen were measured in at least five different cells.

Sppmt1b). The percentage of cells that tested positive for HCV proteins by immunofluorescence was also lower in the case of the mutants (data not shown). This was most certainly due to the lower level of transcripts generated from the Sphi-linearized templates used for the mutated sequences as compared with the SpeI-linearized templates used for their WT counterparts (see “Experimental Procedures”). In cells transfected with recombinant SFV-HCV1a/Sppmt or SFV-HCV1b/Sppmt RNAs, HCV core protein was detected as a single species, which showed a higher molecular mass than p21 but migrated slightly faster than the WT p23 (Fig. 1B, bottom, Sppmt1a and Sppmt1b). That the exchanged amino acid residues increase the mobility of the mutated p23 in SDS-PAGE has been reported previously (42). Even upon longer exposure of the Western blots, no p21 could be detected, indicating that the mutations completely abolished SPP-catalyzed cleavage of HCV core protein in the context of both genotypes. Nevertheless, areas of convoluted ER membranes with HCV-like particles budding toward the dilated lumen were visualized in some of the cells transfected with SFV-HCV1a/Sppmt or SFV-HCV1b/Sppmt.
FIGURE 3. Isolation of enveloped HCV-like particles. A–C, isopycnic centrifugation analysis. Sf9 cells were infected by the recombinant baculovirus BV-HCV1a and then cultured in the absence (A, WT) or presence of (Z-LL)2-ketone (B, WT+inh) or else infected by the recombinant baculovirus BV-HCV1a/Sppmt (C, Sppmt). Isopycnic centrifugation in sucrose gradients containing no detergent was used to isolate enveloped HCV-like particles. Eighteen fractions were collected from the gradients (numbered from bottom to top) and analyzed by Western blotting using monoclonal antibodies against HCV core protein (anti-core), E1 envelope glycoprotein (anti-E1), or E2 envelope glycoprotein (anti-E2). The density (d) of the immunoreactive fractions is given. Positions of molecular mass markers (in kDa) are indicated on the right. D, comparison of HCV core protein species expressed in cells and recovered in HCV-like particles. For each of the experiments presented above, fractions 9 of the gradient were subjected to Western blot analysis using monoclonal antibody against HCV core protein. Positions of molecular mass markers (in kDa) are indicated on the right.

ROLE OF SPP IN HCV PARTICLE MORPHOGENESIS

Because HCV budding is abortive in the mammalian BHK-21 cell model, virus-like particles cannot be isolated and hence characterized. We therefore decided to express the HCV sequences of genotype 1a and 1b in insect Sf9 cells by means of recombinant baculovirus vectors. Western blot analysis of lysates of cells infected by either BV-HCV1a or BV-HCV1b showed that the HCV structural proteins were efficiently expressed and correctly processed in this system (Fig. 2, Sppmt, see the high magnification images and see Table 1). We conclude that inhibition of SPP-catalyzed cleavage of HCV core protein by either of our inhibitory approaches not only did not prevent the initiation of budding, but instead tended to facilitate the viral morphogenesis process.

Recovery of HCV-like Particles—Because HCV budding is abortive in the mammalian BHK-21 cell model, virus-like particles cannot be isolated and hence characterized. We therefore decided to express the HCV sequences of genotype 1a and 1b in insect Sf9 cells by means of recombinant baculovirus vectors. Western blot analysis of lysates of cells infected by either BV-HCV1a or BV-HCV1b showed that the HCV structural proteins were efficiently expressed and correctly processed in this system (Fig. 2, Sppmt, see the high magnification images and see Table 1). We conclude that inhibition of SPP-catalyzed cleavage of HCV core protein by either of our inhibitory approaches not only did not prevent the initiation of budding, but instead tended to facilitate the viral morphogenesis process.

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with densities ranging from 1.23 to 1.26 g/ml (Fig. 5A), in good agreement with the density reported for the capsids of putative virions isolated from HCV-infected patients (44, 47).

To verify that p21 was assembled into capsids, these fractions were subjected to immunogold EM analysis using monoclonal antibody specific to HCV core protein. Gold-decorated capsids of the expected size of 30–40 nm in diameter (45, 48, 49) were indeed visualized, although most of the labeled material appeared as particulate structures of different shapes, likely resulting from the partial disintegration of capsids upon the isolation procedure (Fig. 6, A and B). The immunolabeling was highly specific, because the capsid structures were not labeled in samples incubated with irrelevant primary antibodies, and no labeling of any cellular or baculoviral structures was seen in similar preparations from cells infected by the irrelevant recombinant baculovirus BV-gus (not shown). Thus, p21 is

FIGURE 4. EM visualization of enveloped HCV-like particles. SF9 cells were infected by the recombinant baculoviruses BV-HCV1a (A and B) or BV-HCV1a/Sppmt (C and D), and isopycnic centrifugation in sucrose gradients containing no detergent was used to partially purify enveloped HCV-like particles. Fractions with densities ranging from 1.13 to 1.18 g/ml were collected for EM visualization after negative staining. A and C, representative electron micrographs showing the presence of enveloped HCV-like particles (white arrows). B and D, higher magnification images of enveloped HCV-like particles after immunogold labeling using monoclonal antibody against E2 envelope glycoprotein. Black arrowheads, baculovirus remnants. Bars, 100 (A and C) or 50 nm (B and D).

FIGURE 5. Isolation of capsids of HCV-like particles. A–C, isopycnic centrifugation analysis. SF9 cells were infected by the recombinant baculovirus BV-HCV1a and then cultured in the absence (A, WT) or presence of (Z-LL)_2-ketone (B, WT/inh) or else infected by the recombinant baculovirus BV-HCV1a/Sppmt (C, Sppmt). Enveloped HCV-like particles were partially purified using sucrose gradients containing no detergent (Without nOG), and capsids of HCV-like particles were isolated by the spin-thru method using gradients containing an nOG layer (With nOG). Eighteen fractions were collected from the gradients (numbered from bottom to top) and analyzed by Western blotting using monoclonal antibody against HCV core protein. The density (d) of the immunoreactive fractions is given. Positions of molecular mass markers (in kDa) are indicated on the right. D, comparison of HCV core protein species expressed in cells and recovered in capsids of HCV-like particles. For each of the experiments presented above, fraction 3 of the gradient containing an nOG layer (f3) and the crude lysate of cells from which HCV-like particles had been recovered (cl) were electrophoresed in parallel and then subjected to Western blot analysis using monoclonal antibody against HCV core protein. Positions of molecular mass markers (in kDa) are indicated on the right.
Role of SPP in HCV Particle Morphogenesis

conditions were tested to achieve a complete abolition of SPP-catalyzed cleavage of HCV core protein while minimizing the toxic effect of the compound in these cells (data not shown). However, the generation of p21 could be abolished only under conditions where HCV structural proteins were expressed at a much lower level than in untreated cells (Fig. 1C, compare WT1a+inh and WT1b+inh with WT1a and WT1b, respectively). Nevertheless, the p23 form of HCV core protein found in (Z-LL)2-ketone-treated cells behaved as p21 does in untreated cells. Indeed, p23 co-sedimented with E1 and E2 envelope glycoproteins at a peak density of 1.16 g/ml upon isopycnic centrifugation in a sucrose gradient containing no detergent (Fig. 3B). Furthermore, delipidation using the spin-thru method resulted in a shift in the sedimentation profile of p23 to a peak density of 1.25 g/ml (Fig. 5B). These results strongly suggested the assembly of p23 into capsids enclosed in enveloped HCV-like particles, although direct evidence by EM visualization could not be provided because of the toxic effect of (Z-LL)2-ketone on the level of protein expression.

The limitations encountered upon treatment of Sf9 cells with (Z-LL)2-ketone prompted us to test the Sppmt combination of amino acid substitutions as an alternative approach to inhibiting SPP-catalyzed cleavage of HCV core protein in these cells. Western blot analysis of lysates of cells infected by either BV-HCV1a/Sppmt or BV-HCV1b/Sppmt showed that HCV structural proteins were expressed at a level similar to that of WT (Fig. 1C, compare Sppmt1a and Sppmt1b with WT1a and WT1b, respectively). The HCV envelope glycoproteins were correctly processed from the polyprotein, as expected. With either of the genotypes tested, however, HCV core protein was detected as two species, the mutated p23 and p21 (Fig. 1C, bottom, Sppmt1a and Sppmt1b). Thus, the Sppmt combination of amino acid substitutions only partially inhibited SPP-catalyzed maturation to p21 in insect cells. Remarkably, however, when the cell lysates were subjected to isopycnic centrifugation to partially purify enveloped HCV-like particles, p23 was the only one of the two expressed forms of HCV core protein found to co-sediment with E1 and E2 at the expected density of 1.13–1.18 g/ml (Fig. 3, C and D). EM confirmed the presence at this density of enveloped HCV-like particles of around 50–60 nm in diameter, labeled specifically with monoclonal antibody to E2 (Fig. 4, C and D). Removal of the viral envelope by the spin-thru method resulted in a shift in the sedimentation profile of p23 to a density of 1.23–1.26 g/ml, consistent with p23 forming capsids in the HCV-like particles (Fig. 5, C and D). Indeed, capsids of 30–40 nm in diameter labeled specifically with monoclonal antibody to HCV core protein were visualized by immunogold EM (Fig. 6, C and D). These results not only show that p23 is able to assemble into capsids enclosed in enveloped HCV-like particles but further suggest that of the two forms of HCV core protein, p23 is that committed to virus particle assembly. Further reinforcing this idea, we consistently noticed in both Western blotting detection and EM visualization experiments that the amount of HCV-like particles recovered from cells infected by BV-HCV1a/Sppmt or BV-HCV1b/Sppmt was greater than that obtained from cells infected by their WT counterparts at identical multiplicities of infection. In addition, immunogold EM analysis of the material recovered by using the
Role of SPP in HCV Particle Morphogenesis

spin-thru method showed a higher proportion of capsids with structural integrity relative to the total amount of gold-labeled material in the p23-containing fractions obtained from cells infected by BV-HCV1a/Sppmt or BV-HCV1b/Sppmt compared with the p21-containing fractions obtained from cells infected by their WT counterparts. We conclude that inhibition of SPP-catalyzed cleavage of HCV core protein not only did not prevent but instead tended to facilitate the recovery of enveloped HCV-like particles enclosing p23 assembled into capsids.

Comparison of the Stability of p23 Versus p21 Capsids—Finally, we were intrigued by the above observation that capsids with structural integrity were more readily recovered when SPP-catalyzed cleavage of HCV core protein was inhibited. Although this could simply be explained by the higher yield of HCV-like particles obtained, we considered the additional hypothesis that capsids composed of p23 might be more stable and hence less damaged by the isolation procedure than capsids composed of p21. We therefore developed novel assays to compare the stability of p23 and p21 capsids in vitro. Capsids of HCV-like particles were isolated by the spin-thru method from Sf9 cells infected by BV-HCV1a or BV-HCV1a/Sppmt and then incubated at 37 °C for various times to trigger disassembly. Ultracentrifugation was performed to separate the pelletable capsid structures from solubilized core protein. As shown in the representative experiment presented in Fig. 7A, the amount of total HCV core protein did not decrease over the period of incubation, showing that core protein per se was stable in this in vitro assay, whatever the species (p21 or p23). The capsid structures made of HCV core protein were unstable, however, as demonstrated by the progressive decrease in the amount of peltable core protein over time. Although this was observed with both p21 and p23 capsids, the kinetics of capsid disassembly were slower for the latter than for the former (Fig. 7B). We also tested high NaCl concentration and low pH as additional triggers of capsid disassembly. Again in these assays, p23 capsids disassembled more slowly than p21 capsids (Fig. 7, C and D). Under all conditions tested, comparable results were obtained with the HCV sequence of genotype 1b (data not shown). Taken together, the data indicate that p23 capsids are more stable than p21 capsids.

DISCUSSION

The recent identification of SPP as the protease responsible for the generation of the mature form of HCV core protein has led to the suggestion that SPP activity might be a prerequisite for initiation of virus particle morphogenesis (10). The data we present here using two complementary cellular systems instead indicate that SPP-catalyzed cleavage to generate p21 is dispensable for this process. Indeed, inhibition of SPP activity or of recognition of p23 as a substrate for SPP not only did not prevent but instead tended to facilitate the visualization of HCV budding events in the ER of BHK-21 cells and the recovery of HCV-like particles from Sf9 cells. These results support and extend previous findings that p23 can assemble into capsids upon de novo synthesis in cell-free systems (50) and form virus-like particles when expressed in yeast cells (51). The capsids composed of p23 were further shown to be more stable than those composed of p21, suggesting a role for SPP in the virus particle disassembly process.

That (Z-LL)2-ketone abolished the generation of p21 in Sf9 cells corroborates previous studies suggesting that the signal peptide located at the core-E1 junction is a substrate for SPP in
insect cells as it is in mammalian cells (19, 22). This could be anticipated from the high degree of amino acid identity of SPP orthologs across species (2, 3). The SPP cleavage site in the HCV core protein sequence was recently mapped to residue 177 in insect cells (22). The conservation of this residue among HCV isolates and the observation that the mature form of HCV core protein expressed in insect and mammalian cells migrated with the same apparent molecular mass in SDS-PAGE (see Refs. 22, 32, 34, and this report) suggest that the same position is used whatever the species, although direct mass spectral analysis is needed to ascertain this. Nevertheless, a combination of amino acid substitutions (Sppmmt) that completely abolished the generation of p21 in BHK-21 cells caused only a partial inhibition of SPP-catalyzed cleavage in Sf9 cells. This could not be attributed to the HCV sequence itself, because the very same sequence context was used in the two cell types, and the discrepancy was observed with two distinct genotypes. Thus, subtle differences in the substrate requirements probably exist among enzymes from different species. The timing of the intramembrane cleavage catalyzed by SPP relative to the cleavage catalyzed by signal peptidase at the luminal side of the ER membrane may also differ among species. Using HCV polyprotein expressed in mammalian BHK-21 cells as a model substrate, Lemberg and Martoglio (41) established the rule that cleavage by signal peptidase is a prerequisite for cleavage by SPP. Using the insect Sf9 cell expression system, however, Hüssy et al. (19) showed that p21 could be detected under conditions where cleavage by signal peptidase at the core-E1 junction did not occur. This question should be re-addressed using the same sequence context to gain insight into the principles that govern SPP activity.

Not only do our data show that p23 is capable of forming capsids assembled into HCV-like particles, but several concordant observations further suggest that it may be the form of HCV core protein that is prone to virus morphogenesis. Indeed, budding of HCV-like particles was more readily visualized in BHK-21 cells when SPP-catalyzed maturation to p21 was inhibited by either of our two inhibitory approaches. The density of budding particles was consistently greater in repeated experiments, and even more convincing was the observation that the majority of particles appeared to have moved forward to a later stage of the budding process. In addition to these observations in situ, we noticed that a greater amount of HCV-like particles was recovered from Sf9 cells infected with BV-HCV1a/Sppmt or BV-HCV1b/Sppmt than from cells infected with their respective WT counterparts at identical multiplicities of infection. Most remarkably, p23 was the only form of HCV core protein found in the virus-like particles isolated from cells infected with BV-HCV1a/Sppmt or BV-HCV1b/Sppmt, even though these cells were also expressing p21. A number of nonmutually exclusive hypotheses may be put forward to account for the commitment of p23, rather than p21, to the virus morphogenesis process. Assembly of an enveloped virus requires colocalization of its structural components to the virus budding site. Although ER retention signals do indeed sequester E1 and E2 envelope glycoproteins at the HCV budding site (17), the propensity of the SPP-processed form of core protein to travel to other compartments is puzzling, and it would be more easily reconciled with HCV core protein functions other than that in virus morphogenesis (18). This paradox is strikingly exemplified by the observation that the majority of core protein localized primarily to the surface of lipid droplets and did not co-localize with E2 at the ER in cells harvesting a stably replicating full-length HCV genome (26). Unlike p21, p23 is not free for subcellular trafficking and remains confined to the ER (10, 29). Thus, the transmembrane domain of p23 may be essential to accumulate and concentrate HCV core protein at the virus budding site to initiate the virus particle assembly process. In addition, interactions could occur inside the budding membrane between the transmembrane domains of p23 and of envelope glycoproteins to guarantee the efficient incorporation of all the virus structural components into the assembling particle. Finally, the transmembrane domain of p23 may also directly contribute to the driving force of HCV budding. Consistent with this view, the HCV core protein alone was reported to be sufficient for initiating the budding of virus-like particles in BHK-21 cells provided that it was expressed in its full-length form of 191 amino acids (31).

As shown here and in a number of previous studies, p21 is the predominant form of HCV core protein detected at steady state in tissue culture cells expressing the complete HCV polyprotein or at least a partial polyprotein encompassing the virus structural proteins (18, 26). Core protein resolved from the liver or serum of HCV-infected patients was reported to have an apparent molecular mass comparable with that of p21 expressed in tissue culture cells, leading to the plausible suggestion that the HCV virion capsid is composed of the SPP-processed form of core protein (21, 52). Isolation and characterization of HCV virions have proven to be difficult, however, and a higher molecular mass was reported for core protein recovered from the circulating blood of HCV-infected patients in other studies (48, 53). Moreover, the efficiency of core protein processing by SPP has recently been suggested to differ among HCV genotypes (54). Obviously, only direct structural analysis could ascertain the biochemical composition of the HCV virion capsid.

If p21 were to be confirmed as being the only form of HCV core protein enclosed in the circulating virions of HCV-infected patients, then our work would imply that SPP is incorporated into the budding particle, as recently suggested in a model of yeast cells co-expressing human SPP and p23 (51). It might be argued that not all enveloped viruses can incorporate host cell proteins into their envelope and that not all membrane proteins can be incorporated in those viruses that do accommodate host cell proteins in their envelope (55). The prediction that SPP might be incorporated into the budding particles seems plausible, however, because of the following: (i) SPP is a resident protein of the ER membrane (3–5), which most certainly is the cellular membrane used to envelope HCV particles; (ii) SPP is devoid of bulky cytoplasmic or extracellular domains (5), which might be detrimental to incorporation into the budding particle; and (iii) most importantly, HCV core protein is a substrate for SPP (10, 11). Because the Sppmmt combination of amino acid substitutions was designed to inhibit recognition of p23 as a substrate for SPP (41), it might inhibit
the incorporation of SPP into the budding particle, thus accounting for the observed absence of p21 in the HCV-like particles recovered from SF9 cells infected with BV-HCV1a/Sppmt or BV-HCV1b/Sppmt.

The capsid of putative virions isolated from HCV-infected patients was suggested to be unstable and readily disintegrate upon delipidation (44–46). Using novel assays to assess the stability of capsids of HCV-like particles in vitro, we have now demonstrated that capsids composed of the mature p21 form of HCV core protein are very sensitive to disruption by various triggers. Interestingly, an intrinsic instability of the capsid after removal of the viral envelope was also suggested for other viruses, such as alphaviruses (56) or HIV (57, 58). Indeed, the spin-thru procedure that we used here to isolate capsids of HCV-like particles was adapted from a protocol developed to conserve the integrity of mature HIV capsids as much as possible (38). It should be remembered that a viral capsid protein must fulfill two opposite functions as follows: assembly required for morphogenesis of virus particles during the late steps of the viral cycle in the infected cell, followed by disassembly required for uncoating during the early steps of the viral cycle in a new target cell. Some viruses have been suggested to solve these paradoxical requirements by using proteolytic maturation as a switch from the assembly to the disassembly mode (55). In HIV for instance, a precursor protein is devoted to the assembly of an immature capsid shell, which is noninfectious, and proteolytic cleavage catalyzed by the viral protease during and after virus budding converts it into a relatively less stably packaged structure, which is infectious (57, 59). With these considerations in mind, our finding that the capsids composed of the SPP-processed p21 form of HCV core protein are less stable than those composed of the precursor protein p23 suggests that, in the life cycle of HCV, SPP may play a role similar to that of the viral protease in the life cycle of HIV, i.e. prime the viral capsid for disassembly during the subsequent round of infection.

The mechanism of HCV uncoating is unknown, but a growing body of evidence has suggested that entry mediated by the HCV envelope glycoproteins is a pH-dependent process (13, 60, 61). Interestingly, capsid disassembly in our in vitro assays was potentiated by exposure to low pH. This may simply be indicative of the nature of the intermolecular interactions within the capsid structure. Acidification was also found to potentiate the disassembly of HIV capsids in vitro, even though entry of this virus does not involve a pH-dependent step (58). Alternatively, our in vitro observation may have some biological relevance with regard to the trigger of HCV uncoating in vivo. In the case of influenza A virus, acidification of virion-containing endosomes triggers not only the fusogenic activation of the viral envelope glycoproteins but also the disassembly of the inner virion structure (62, 63). The latter effect is thought to result from a proton influx into the virion interior mediated by the virus M2 protein, which possesses an amantadine-sensitive ion channel activity. HCV also encodes a protein p7, which has the property of forming amantadine-sensitive ion channels (64–66), yet its precise role in the virus life cycle remains speculative. A key question is whether p7 is incorporated into HCV particles, as current evidence suggests that the homologous protein in Flaviviridae of the Pestivirus genus is not a major structural component of the virion (67).

In conclusion, the data are consistent with a model in which p23 is the form of HCV core protein committed to virus assembly, and cleavage by SPP occurs during and/or after virus budding in the ER to predispose the capsid to the necessary disassembly step upon infection of a new target cell. This model predicts that inhibition of SPP activity, although not inhibiting HCV budding, should nevertheless inhibit HCV infectivity. SPP was recently reported to be the protease responsible for the generation of the mature core protein of classical swine fever virus, also a member of the Flaviviridae (68). Inhibition of SPP activity reduces virus infectivity in this model, although it remains to be determined which step of the virus life cycle is affected. Long awaited systems for propagating HCV infection in tissue culture cells have now been made available (69–72). Although the virus particles released in the cell culture supernatant need to be further characterized, and morphogenesis in situ has not yet been described, this system should permit the determination of whether (Z-LL)2-ketone inhibits HCV infectivity. If this is indeed the case, then SPP would appear as a promising target for therapeutic intervention. It must be conceded that cellular proteins are less attractive than viral proteins for the development of antiviral strategies. The cellular substrates and functions of human SPP are probably far from being completely listed, and an added complication arises from the fact that the human genome encodes four additional homologs of SPP (1). On the other hand, SPP and SPP-like proteins are members of a family that also includes the presenilins, which are implicated in the pathogenesis of Alzheimer disease. Thus, the search for compounds targeting intramembrane-cleaving aspartyl proteases is urged on by a dual medical challenge.

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