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Characterization of the cleavage of signal peptide at the C-terminus of hepatitis C virus core protein by signal peptide peptidase

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Abstract

Production of hepatitis C virus (HCV) core protein requires the cleavages of polyprotein by signal peptidase and signal peptide peptidase (SPP). Cleavage of signal peptide at the C-terminus of HCV core protein by SPP was characterized in this study. The spko mutant (mutate a.a. 189–193 from ASAYQ to PPFPF) is more efficient than the A/F mutant (mutate a.a 189 and 191 from A to F) in blocking the cleavage of signal peptide by signal peptidase. The cleavage efficiency of SPP is inversely proportional to the length of C-terminal extension of the signal peptide: the longer the extension, the less efficiency the cleavage is. Thus, reducing the length of C-terminal extension of signal peptide by signal peptidase cleavage could facilitate further cleavage by SPP. The recombinant core protein fused with signal peptide from the C-terminus of p7 protein, but not those from the C-termini of E1 and E2, could be cleaved by SPP. Therefore, the sequence of the signal peptide is important but not the sole determinant for its cleavage by SPP. Replacement of the HCV core protein E.R.-associated domain (a.a. 120–150) with the E.R.-associated domain (a.a.1–50) of SARS-CoV membrane protein results in the failure of cleavage of this recombinant protein by SPP, though this protein still is E.R.-associated. This result suggests that not only E.R.-association but also specific protein sequence is important for the HCV core protein signal peptide cleavage by SPP. Thus, our results suggest that both sequences of the signal peptide and the E.R.-associated domain are important for the signal peptide cleavage of HCV core protein by SPP.

Introduction

Hepatitis C virus is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. HCV belongs to the genus *Hepacivirus* in the family *Flaviviridae*. HCV genome is a single, positive-stranded RNA with a nucleotide length of about 9.6 kb, encoding a large polyprotein precursor of approximately 3,000 amino acids. This polyprotein precursor, processed co- and post-translationally into at least 10 different viral proteins by host and viral proteases, is arranged in the order of NH2-C(21Kd)-E1(31Kd)-E2(70Kd)-p7-NS2(23Kd)-NS3(70Kd)-NS4A(8Kd)- NS4B(27Kd)-NS5A(58Kd)-NS5B(68Kd)-COOH. C, E1, and E2 are structural proteins while NS2-NS5B are non-structural proteins [1, 2]. The releases of C, E1, E2, p7 from the polyprotein are mediated by cellular signal peptidase located in the endoplasmic reticulum [2, 3]. The non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B) are released from the polyprotein after cleavage by HCV NS2-3 and NS3/4A proteases [2]. It has been reported that an

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additional HCV protein (ARFP/F protein) can be produced at the N-terminal region of C gene fragment [4].

HCV core protein is a multifunctional molecule. In addition to its structural property, HCV core protein has been demonstrated to regulate host cellular functions in cultured cells, e.g. gene transcription [5], signal transduction [6–8], and apoptosis [9, 10, 11]. Moreover, transgenic mice with HCV core protein developed liver steatosis and thereafter hepatocellular carcinoma [12, 13]. Thus, core protein is suggested to be involved in the pathogenesis of hepatitis C. HCV core protein is a 191-amino acid product released from the polyprotein by cleavage with cellular signal peptidase. This 191-amino acid core protein will be further processed by signal peptide peptidase (SPP) into the mature form of core protein with about 179 amino acids in size [14]. The cleavage by SPP promotes the release of core protein from ER membrane. Core protein is then free for subsequent trafficking to lipid droplets [15]. Furthermore, SPP was shown to promote the formation of HCV non-enveloped particles in yeast [16] and is required for HCV-like particle assembly in Semliki Forest virus replicon system [17]. Thus, SPP cleavage should play an important role in the HCV life cycle.

Signal peptide peptidase (SPP), a presenilin-related aspartic protease containing YD and LGLGD motifs, was identified recently [18]. SPP, an intramembrane-cleaving protease, is located in the ER membrane and promotes intramembrane proteolysis of signal peptides [19]. Signal peptide sequences generally have a tripartite structure, including an N-terminal region (n region) containing positively charged residues, a central hydrophobic region (h region) of 7–15 residues, and a short C- terminal region of 2–9 residues (c region) containing generally smaller and polar residues. This c region defines the cleavage site for signal peptidase. SPP recognizes the N- and C- terminal regions of the signal peptide and cleaves in the middle of the H region in the signal peptide [20]. However, it is still unknown why not all signal peptides are substrates for SPP.

It has been demonstrated that processing of signal peptide at the C-terminus of HCV core protein by SPP is not required for the cleavage by signal peptidase and the translocation of E1 protein into ER [21]. Moreover, Leu^{139}, Val^{140}, Leu^{144} of HCV core protein play crucial roles in the ER retention and SPP cleavage [21]. However, it is unknown whether the processing of signal peptide at the C-terminus of HCV core protein by signal peptidase is a prerequisite for the cleavage by SPP. The mechanisms of signal peptide cleavage by signal peptide peptidase are also unclear. In this study, the cleavage of signal peptide at the C-terminus of HCV core protein by SPP was characterized.

Materials and methods

Plasmid construction

Construction of various recombinant HCV structural proteins, DNA fragments were amplified by polymerase chain reaction (PCR) from p90/HCV FL-long pU (GI: 2316097) plasmid [22] containing full-length of HCV-H polyprotein type 1a. Various PCR primer pairs (HCV-1 and HCV-2, HCV-1 and HCV-3) were used to amply different DNA fragments encoding HCV proteins a.a. 1–200, a.a. 1–392 separately. After PCR reaction, DNA fragment was digested by restriction enzymes (*Eco*RI and *Xba*I) and cloned into pcDNA3 (Invitrogen, USA) expression vector (linearized by *Eco*RI/*Xba*I).

To construct A/F mutant (mutate a.a. 189 and 191 from alanine to phenylalanine), PCR primers (HCV-1 and A189/191F-AS) were used to amplify the 5′-end of the core gene fragment while PCR primers (A189/191F-S and HCV-3) were used to amplify the 3′-end fragment. These two DNA fragments were linked together by PCR using primers (HCV-1 and HCV-3). After PCR reaction, DNA fragment was digested by restriction enzymes (*Eco*RI and *Xba*I) and cloned into pcDNA3 (Invitrogen, USA) expression vector (linearized by *Eco*RI/*Xba*I). To construct spko mutant (mutate a.a. 189–193 from ASAYQ to PPFPF), a similar approach was performed except using SPKO-AS to replace A189/191F-AS, and SPKO-S to replace A189/191F-S.

The plasmid containing the spko mutant was used as a template, various PCR primer pairs (HCV-1 and HCV220-AS, HCV-1 and HCV240-AS, HCV-1 and HCV260-AS, HCV-1 and HCV280-AS) were used to amply different DNA fragments encoding HCV proteins a.a. 1–220, a.a.

1–240, a.a. 1–260, a.a. 1–280 separately with spko mutation.

To replace the signal peptide at the C-terminus of core protein with that at the C-terminus of p7 protein, PCR primers (HCV-1 and L172/791-AS) were used to amplify the 5′-end of the core gene fragment while PCR primers (L172/791-S and HCV-4) were used to amplify the DNA fragment encoding HCV a.a. 791–818. These two DNA fragments were linked together by PCR using primers (HCV-1 and HCV-4). After PCR reaction, DNA fragment was digested by restriction enzymes (*Eco*RI and *Xba*I) and cloned into pcDNA3 (Invitrogen, USA) expression vector (linearized by *Eco*RI/*Xba*I). To replace the signal peptide at the C-terminus of core protein with those at the C-termini of E1 and E2 proteins, a similar approach was performed except using L172/365-AS or L172/728-AS to replace L172/791-AS, L172/365-S or L172/728-S to replace L172/791-S, HCV-3 and E2S-AS to replace HCV-4. After construction, these plasmids can express HCV a.a. 1–172 plus a.a.791–818, a.a. 365–392, or a.a. 728–755.

Various PCR primer pairs (HCV707-S and HCV819-AS, HCV707-S and HCV839-AS) were used to amply different DNA fragments encoding HCV proteins a.a. 707–819, a.a. 707–839, separately. To construct spkoII mutant (mutate a.a. 807–811 from AYALD to PPFPF), PCR primers (HCV707-S and SPKOII-AS) were used to amplify the 5′-end of the gene fragment while PCR primers (SPKOII-S and HCV839-AS) were used to amplify the 3′-end fragment. These two DNA fragments were linked together by PCR using primers (HCV707-S and HCV839-AS). After PCR reaction, DNA fragment was digested by restriction enzymes (*Eco*RI and *Xba*I) and cloned into pcDNA3.1-V5-His A (Invitrogen, USA) expression vector (linearized by *Eco*RI/*Xba*I).

To construct expression plasmid encoding a peptide tag, DNA fragment of c-Myc tag (EQKLISEEDL) was amplified from pCMV-Tag 3B (Stratagene, USA), and subcloned into *Kpn*I and *Bam*HI sites of pcDNA3 (Invitrogen, USA) as pcDNA3-cMyc Tag. To construct the expressing plasmid encoding c-Myc tag and core protein a.a. 119–200 or a.a. 150–200, PCR primers (HCV119-S or HCV150-S and HCV2) were used to amplify the core gene fragment. After PCR reaction, DNA fragments were digested by restriction enzymes (*Bam*HI and *Xba*I) and cloned into pcDNA3-cMyc Tag (linearized by *Bam*HI and *Xba*I).

To construct the plasmid expressing the c-Myc tagged fusion protein containing first 50 amino acids of SARS-CoV M protein (with a.a. 4 changed from N to D [23] to block the N-glycosylation site) and a.a. 151–200 of HCV core protein, PCR primers (M-S3 and M50/C151-AS) were used to amplify the 5′-end of the SARS-CoV M gene fragment while PCR primers (M50/C151-S and HCV-2) were used to amplify the 3′-end fragment of HCV core gene. These two DNA fragments were linked together by PCR using primers (M-S3 and HCV-2). After PCR reaction, DNA fragment was digested by restriction enzymes (BamHI and XbaI) and cloned into pcDNA3-cMyc Tag (linearized by *Bam*HI and *Xba*I).

All the expression plasmids were verified by sequencing.

Protein expression in HuH7 cells

HuH7 cells were maintained in DMEM containing 10% fetal bovine serum, 1% Glutamine (200 mM, Gibco, USA), and 100 ug/ml penicillin/streptomycin (Gibco BRL, USA). $3-5 \times 10^6$ cells were seeded in 100 mm culture dish. After overnight incubation, cells were infected with recombinant vaccinia virus carrying T7 phage RNA polymerase [24]. Two hours after infection, cells were transfected with 2 ug plasmid by using Effectene trasfection kit (Qiagen, Germany). 21 h after transfection, recombinant proteins in the cells were analyzed. To inhibit the signal peptide cleavage by signal peptide peptidase [25], chemical $(Z\text{-}LL)_2$-keton (Calbiochem, USA) was used in the concentration of 20 uM.

Western blotting analysis

For Western blotting analysis, cells were dissolved in sample preparation buffer (67.5 mM Tris–HCl (pH 6.8), 5% 2-mercaptoethanol, 3% SDS, 0.1% bromophenol blue, 10% glycerol) after washing with PBS twice. The samples were treated at 100 °C in the sample buffer for 10 min before electrophoresis. SDS-PAGE was used to separate proteins with regular size while Tricine gel [26] was used to analyze protein with smaller size. SDS-PAGE gel after electrophoresis was transferred to PVDF paper (Pall Corporation, USA). All

procedures were carried out at room temperature. The PVDF paper was blocked in PBST (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4.7H_2O$, 1.4 mM KH_2PO_4, 0.1% Triton-X 100) with 5% milk for **1** h. After blocking, the PVDF paper was incubated with anti-c-Myc mouse monoclonal antibody (Oncogene, USA) or anti-core monoclonal antibody (HCM-071-5, AUSTRAL Biologicals, California, USA) diluted 1000-fold in PBST with 5% milk, for 3 h. The PVDF paper was then washed three times in PBST for 10 min. Afterwards, goat anti-mouse IgG conjugated with horseradish peroxidase (Amersham Biosciences Ltd, USA), which had been diluted 5000-fold in PBST with 5% milk, was added for another 1 h of incubation. After three more 10 min washes with PBST, the signal was developed by the "Western Lightning^{TM} Chemiluminescence Reagent Plus" kit (PerkinElmer Life Sciences, USA). If rabbit anti-HCV core polyclonal antibody [27] or anti-V5/AP antibody (Invitrogen, USA) was used as the primary antibody to carry out the assay, published procedures [27] were followed.

The proportion of different proteins (in Fig. 1 and 2) was quantified using software "Quantity one" (Biorad, USA).

Immunofluorescence and confocal analysis

About 2.5×10^5 cells were seeded in 35 mm culture dishes. After overnight incubation, cells were infected with recombinant vaccinia virus carrying T7 phage RNA polymerase [24]. Two hours after infection, cells were transfected with 0.4 ug plasmid by using Effectene trasfection kit (Qiagen, Germany). 21 h after transfection, recombinant proteins in the cells were analyzed. Cells were fixed by acetone/methanol (1:1), at 0 °C for 10 min. Fixed cells were washed with incubation buffer (0.05% NaN_3, 0.02% saponin, 1% skim milk in PBS) twice for 5 min each time, then incubated with rabbit anti-core antibody [27], or mouse anti-c-Myc monoclonal antibody (Oncogene, USA), which had been diluted 200 fold, at 37 °C for 30 min. Samples were washed with PBS three times (5 min each time at room temperature), then incubated with RITC–conjugated goat anti-rabbit (or anti-mouse) IgG antibody in 20× dilution at 37 °C for 30 min. Again, samples were washed with PBS three times (5 to 10 min each time at room temperature).

Cells were co-transfected with pEYFP-ER plasmid (BD, USA) when ER localization needs to be defined. DAPI (Merck, Germany) was used to stain DNA as the localization of nucleus.

Results

The spko mutant is more efficient than the A/F mutant in blocking the cleavage of signal peptide by signal peptidase

A previous study using A/F mutant (mutate a.a. 189 and 191 from alanine to phenylalanine) demonstrated that cleavage of signal peptide by SPP does not require previous processing at the signal peptidase cleavage site (a.a. 191/192) of HCV polyprotein [14]. However, another study

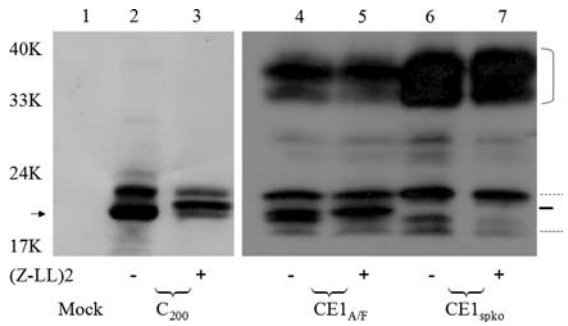

Figure 1. A/F mutation can't block the cleavage of signal peptidase completely. HuH7 cells were transfected with expression plasmids encoding HCV a.a. 1–200 in the absence (lane 2) or presence of SPP inhibitor ($Z(LL)_2$-ketone) (lane 3), HCV a.a. 1–392 with A/F mutation in the absence (lanes 4) or presence of SPP inhibitor (lane 5), or HCV a.a. 1–392 with spko mutation in the absence (lanes 6) or presence of SPP inhibitor (lane 7). After transfection, recombinant proteins were collected, subjected to SDS-PAGE analysis, followed by Western blotting assay using anti-core mAb. The arrow represents core protein with 179 a.a. in length, straight line represents core protein with 191 a.a. in length, and bracket marks the unprocessed C-E1 proteins with different levels of glycosylation. The protein bands marked by dotted line may represent degraded products from precursor C-E1 or core proteins.

using spko mutant (mutate a.a. 189–193 from ASAYQ to PPFPF) showed that the signal peptides have to be liberated from the precursor protein by signal peptidase cleavage in order to become the substrates for SPP [20]. The discrepancy between these two reports could be the different mutants used to block the cleavage by signal peptidase. To address this issue, the A/F and spko mutants of recombinant protein containing HCV core and E1 proteins were constructed separately and expressed in HuH7 cells (Fig.1). As a control, expressing plasmid encoding wild type HCV 1–200 a.a. was transfetcted into HuH7 cells (lanes 2 and 3). Core protein with 179 a.a. was the major protein detected when expressed in the absence of SPP inhibitor $(Z\text{-}LL)_2$-keton since the recombinant protein (a.a. 1–200) would be cleaved by signal peptidase and SPP (lane 2) while core protein with 191 a.a. was the major protein detected when expressed in the presence of SPP inhibitor since this recombinant protein would be cleaved by signal peptidase only (lane 3). Majority of the proteins detected in the A/F and spko mutants were unprocessed ones (marked by bracket, lanes 4–7). Expression level of different proteins in the A/F and spko mutants was quantified using software "Quantity one": Comparing with total intensity of the processed and unprocessed proteins (as 100%), the intensity of core protein with 179 a.a. in A/F mutant is 12.9% (lane 4) while in spko mutant is 7.5% (lane 6). Thus, consistent with the published results, core protein with 179 a.a. could be detected with greater amount from A/F mutant cell lysate than the spko mutant (lane 4 and 6 in Fig.1, the protein band marked by arrow). Moreover, core protein with 191 a.a. in length could be detected in the presence (lane 5, the protein band marked by straight line) or even absence of SPP inhibitor in the A/F mutant (lane 4) but not in the spko mutant in absence (lane 6) or even presence of SPP inhibitor (lane 7).

The cleavage efficiency of SPP is inversely proportional to the length of the C-terminal extension of the signal peptide

The above results that core protein with 179 a.a. could be detected with greater amount in A/F mutant cell than the spko mutant (lane 4 and lane 6 in Fig.1) may suggest that cleavage of signal peptide by signal peptidase could facilitate the further cleavage by SPP. The effect of signal peptidase on SPP could be due to the reduction in length of the C-terminal extension of signal peptide after cleavage. To address this viewpoint, recombinant core spko mutants with various length of C-terminal extension were constructed and expressed separately (Fig. 2). The expression level of different proteins was also quantified using software "Quantity one": Comparing with total intensity of processed and unprocessed proteins (as 100%), the intensity of core protein with 179 a.a. is 59.6% in HCV a.a. 1–200 with spko mutation construct (lane

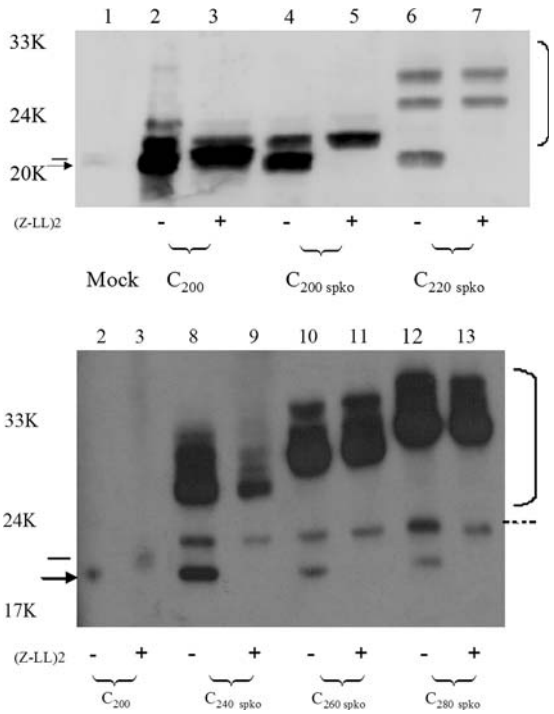

Figure 2. The length of C-terminal extension affects SPP cleavage. HuH7 cells were mock transfected (lane 1), transfected with expression plasmids encoding HCV a.a. 1–200 (lanes 2 and 3), HCV a.a. 1–200 with spko mutation (lanes 4 and 5), HCV a.a. 1–220 with spko mutation (lanes 6 and 7), HCV a.a. 1–240 with spko mutation (lanes 8 and 9), HCV a.a. 1–260 with spko mutation (lanes 10 and 11), HCV a.a. 1–280 with spko mutation (lanes 12 and 13), SPP inhibitor $(Z(LL)_2$-ketone) was treated in lanes 3, 5, 7, 9, 11, 13. After transfection, recombinant proteins were collected, subjected to SDS-PAGE analysis, followed by Western blotting assay using anti-core mAb. The arrow represents core protein with 179 a.a., straight represents core protein with 191 a.a., and bracket marks various unprocessed C-E1 proteins. The protein bands marked by dotted line may represent degraded products from precursor C-E1.

4), 43.4% in HCV a.a. 1–220 with spko mutation construct (lane 6), 21.2% in HCV a.a. 1–240 with spko mutation construct (lane 8), 12.4% in HCV a.a. 1–260 with spko mutation construct (lane 10), and 8.7% in HCV a.a. 1–280 with spko mutation construct (lane 12). Thus, the amount of core protein with 179 a.a. in length detected from these spko mutants is inversely proportional to the length of the C-terminal extension: the longer the C-terminal extension, the less SPP cleavage product was generated (Fig. 2). Again, core protein with 191 a.a. in length could not be detected after addition of SPP inhibitors in these spko mutant constructs (lanes 5, 7, 9, 11, 13).

The signal peptide sequence is not the sole determinant for its cleavage by SPP

Not all signal peptides are substrates for SPP. The substrate requirement for SPP is largely unknown. Up to now, only the signal peptide at the C-terminus of core protein but not those at the C-termini of E1, E2, and p7 has been demonstrated to be cleaved by SPP. To determine whether the signal peptide sequence is the determinant to be a substrate for SPP, recombinant core proteins with its original signal peptide replaced with signal peptides at the C-termini of E1, E2, or p7 were constructed and expressed separately (Fig. 3). The recombinant core protein with the signal peptide from the C-terminus of p7 protein could be cleaved by SPP (lanes 3 and 4 in Fig. 3, the protein band marked by arrow), but not those from the C-termini of E1 and E2 (lanes 5–8 in Fig. 3). Core proteins with 191 a.a. in length were detected in all of these recombinant protein chimeras (Fig. 3, the protein band marked by straight line) indicating signal peptidase cleavage. All of these recombinant core proteins with different signal peptides are ER-associated (data not shown) and can be cleaved by signal peptidase, indicating that the signal peptides of these chimeras are fully functional.

To test whether the signal peptide located at the C-terminus of p7 protein could be processed by SPP in its original structure, a recombinant protein containing p7 and truncated NS2 (a.a. 707–839) was constructed and expressed. No processed product generated by SPP was detected (lanes 4 and 5 in Fig. 4). These results were also verified by *in vitro* transcription/translation system using the

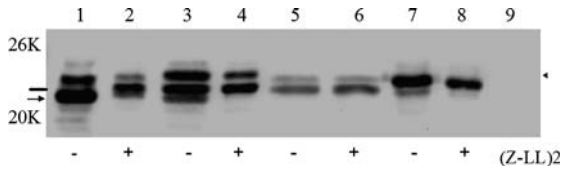

Figure 3. The recombinant core protein replacing its C-terminal signal peptide with the signal peptide located at the C-terminus of p7 protein, but not those located at the C-termini of E1 and E2, could be cleaved by signal peptide peptidase. HuH7 cells were mock transfected (lane 9), transfected with expression plasmids encoding HCV a.a. 1–200 (lanes 1 and 2), recombinant core protein with signal peptide derived from the C-terminus of p7 (lanes 3 and 4), recombinant core protein with signal peptide derived from the C-terminus of E1 protein (lanes 5 and 6), or recombinant core protein with signal peptide derived from the C-terminus of E2 protein (lanes 7 and 8). SPP inhibitor $Z(LL)_2$-ketone was treated in lanes 2, 4, 6, 8. After transfection, recombinant proteins were collected, subjected to SDS-PAGE analysis, followed by Western blotting assay using rabbit anti-core Ab. The arrow represents core protein with 179 a.a., straight line represents core protein with 191 a.a., arrowhead marks unprocessed core protein with 200 a.a..

same constructs (data not shown). Another recombinant protein containing p7 and truncated NS2 (a.a. 707–819) were constructed and expressed, and no processed product generated by SPP was detected either (data not shown).

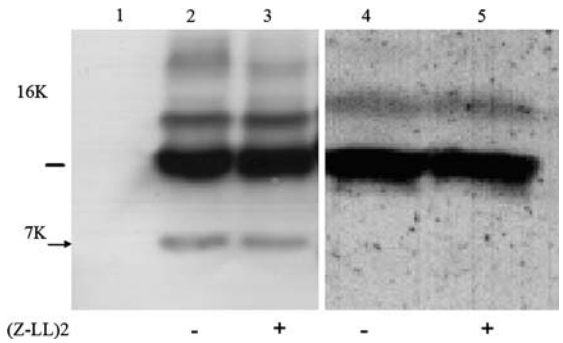

Figure 4. SPP does not cleave the signal peptide at the C-terminus of p7. HuH7 cells were mock transfected (lane 1), transfected with expression plasmids encoding HCV a.a. 707–839 plus V5-His tag fused at the C-terminus (lanes 2 and 3), or HCV a.a. 707–839 with spkoII mutation plus V5-His tag fused at the C-terminus (lanes 4 and 5). SPP inhibitor ($Z(LL)_2$-ketone) was treated in lanes 3, 5. After transfection, recombinant proteins were collected, subjected to SDS-PAGE analysis, followed by Western blotting assay using anti-V5 mAb. The arrow represents the polypeptide (HCV a.a. 810–839 plus V5-His tag) after the cleavage of this recombinant protein by signal peptidase between a.a. 809 and 810 while straight line marks the protein with a.a. 747–839 plus V5-His tag as there is another cleavage site for signal peptidase between E2 and p7 (a.a. 746–747).

E.R.-association is not sufficient for the signal peptide of HCV core protein to be cleaved by SPP

It has been demonstrated that replacing Leu^{139}, Val^{140}, and Leu^{144} of core protein with Ala inhibited its processing by SPP. Moreover, the mutated core protein was translocated into nucleus in spite of the presence of the unprocessed C-terminal signal-anchor sequence [21]. Why these hydrophobic residues are crucial for the processing of core protein by SPP is unknown. Previous studies have shown that core protein without the internal hydrophobic domain (a.a. 120- 150) is localized in nucleus rather than in ER [21, 27]. One possibility is that these hydrophobic residues (Leu^{139}, Val^{140}, and Leu^{144}) help core protein anchor on ER and facilitate its processing by SPP which is located in the ER membrane. To test this viewpoint, truncated core proteins with this hydrophobic domain (a.a. 119–200) and without this domain (a.a. 151–200) were constructed with N-terminal Myc-tag and expressed separately (Fig. 5A). Both of these truncated core proteins are ER-associated (center and bottom rows in Fig. 6) possibly due to lack of nuclear localization signal located at the N-terminus of core protein [28]. The signal peptide of truncated core protein without this hydrophobic domain was not processed by SPP (lanes 4 and 5 in Fig. 5A) because only two proteins, representing unprocessed fusion protein (marked by arrowhead) and the processed fusion protein cleaved by signal peptidase (marked by straight line), were detected both in the absence (lane 4) and presence of SPP inhibitor (lane 5) with equal amount. On the other hand, when truncated core protein containing this hydrophobic domain was expressed, only unprocessed fusion protein (marked by arrowhead in lane 2 of Fig. 5A) was detected in the absence of SPP inhibitor while two proteins representing unprocessed fusion protein (marked by arrowhead) and the protein cleaved by signal peptidase (marked by straight line) were detected in presence of SPP inhibitor (lane 3). The expected cleaved product of this truncated core protein by SPP (in lane 2) would be a little smaller than the protein cleaved by signal peptidase (marked by straight line in lane 3). The fact that it is not seen from the Western blotting (lane 2) suggesting that it is labile since the protein cleaved by signal peptidase (marked by straight line) could be detected in presence of SPP inhibitor (lane 3). The instability of this SPP-cleaved product should not be through proteasome-degrading pathway since proteasome inhibitor MG-132 could not stabilize this protein (data not shown). The results that the truncated core protein without the internal hydrophobic domain can not be cleaved by SPP, suggesting that the sequence of this region but not ER-retention contributes to the SPP processing at its C-terminal signal peptide. To strengthen this viewpoint, an ER-associated domain (a.a. 1–50)

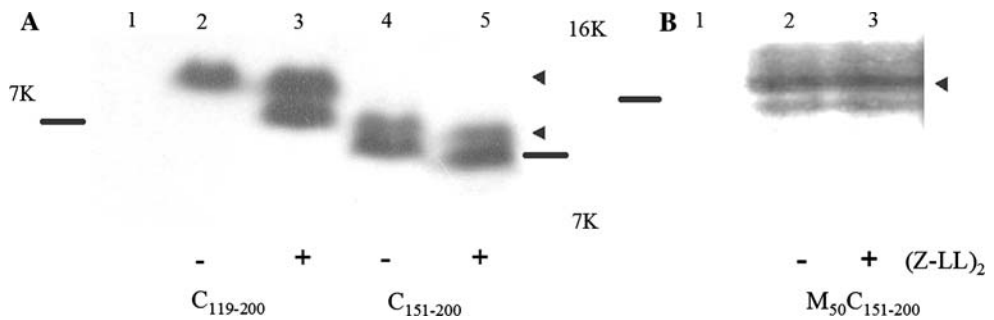

Figure 5. (**A**) The internal hydrophobic domain of core protein (a.a. 119–150) was important for its cleavage by SPP. HuH7 cells were mock transfected (lane 1), transfected with expression plasmids encoding HCV a.a. 119–200 plus c-Myc tag fused at the N-terminus (lanes 2 and 3), or HCV a.a. 150–200 plus c-Myc tag fused at the N-terminus (lanes 4 and 5). SPP inhibitor ($Z(LL)_2$-ketone) was treated in lanes 3, 5. After transfection, recombinant proteins were collected, subjected to SDS-PAGE analysis, followed by Western blotting assay using anti-Myc mAb. The straight line represents core protein after cleavage by signal peptidase while arrowhead marks unprocessed core proteins. (**B**) ER-association domain of SARS-CoV M protein did not facilitate SPP processing. HuH7 cells were mock transfected (lane 1), or transfected with the expression plasmid encoding c-Myc tag, first 50 a.a. of SARS-CoV M protein, plus HCV a.a. 151–200, in the absence (lane 2) or presence of SPP inhibitor ($Z(LL)_2$-ketone) (lane 3). After transfection, recombinant proteins were collected, subjected to SDS-PAGE analysis, followed by Western blotting assay using anti-Myc mAb. The straight line represents signal peptidase processed fusion protein while arrowhead marks signal peptidase unprocessed fusion protein.

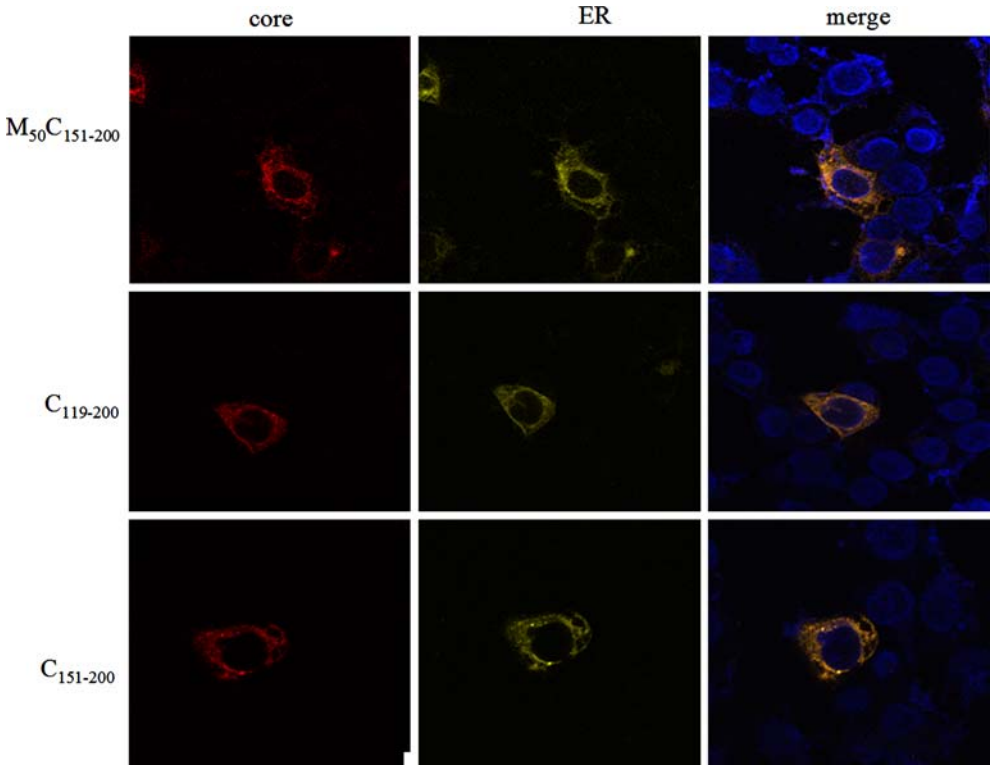

Figure 6. ER-association of various recombinant core proteins. HuH 7 cells were transfected with pEYFP-ER and different expressing plasmids encoding c-Myc tag plus HCV a.a. 151–200 (bottom row), c-Myc tag plus HCV a.a. 119–200 (center row), or c-Myc tag, first 50 a.a. of SARS-CoV M protein plus HCV a.a. 151–200 (top row). After transfection, cells were fixed and stained with anti-Myc mAb plus RITC-conjugated 2nd Ab to detect the recombinant proteins.

of SARS-CoV membrane protein (Hsieh and Lo, unpublished data) was constructed in front of the truncated core protein (a.a. 151–200) and expressed. Both unprocessed (marked by arrowhead) and signal peptidase processed (marked by straight line) fusion proteins were detected in equal amount, either in the absence (lane 2 in Fig. 5B) or presence of SPP inhibitor (lane 3 in Fig. 5B). Therefore, the signal peptide of this recombinant chimera core protein was not processed by SPP (Fig. 5B) though it is ER-associated (top row in Fig. 6).

Discussion

Majority of the proteins detected in the A/F and spko mutants were unprocessed ones (marked by bracket, lanes 4–7 in Fig. 1) indicating the inhibition of signal peptidase in these mutants. However, core protein of 191 a.a. in length could be detected in the presence or even absence of SPP inhibitor $(Z\text{-}LL)_2$-keton in A/F mutant (lanes 4 and 5 in Fig. 1) but not in spko mutant (lanes 6 and 7 in Fig. 1) of HCV C-E1 constructs. This result indicates that signal peptidase could still cleave the A/F mutant but not spko mutant to generate core with 191 a.a. in length. Therefore, the spko mutant is more efficient in blocking the cleavage of signal peptide by signal peptidase than the A/F mutant. The previous assumption that HCV core protein mutant with alanine 189 and alanine 191 replaced with phenylalanine should destroy the substrate requirements for signal peptidase [14, 29] is not absolutely correct.

Core protein of 179 a.a. in length could be detected with greater amount in spko mutants with shorter C-terminal extension (Fig. 2). Detectable amount can also be seen with longer C-terminal extension as found in the spko mutant of C-E1 construct (lane 12 in Fig. 2 and lane 6 in Fig. 1). Thus, the cleavage efficiency of SPP is inversely proportional to the length of the C-terminal

Table 1. PCR primers used to this study.

Name	Sequence
HCV-1	(5'-CGGAATTCAGGTCTCGTAGACCG-3')
HCV-2	(5'-GCTCTAGA**TTA**AAGCCCCGAGGAATT-3')
HCV-3	(5'-GCTCTAGA**TTA**GGCACTTCCCCCGGT-3')
HCV-4	(5'-GCTCTAGA**TTA**ACACGACGCGGCCAC-3')
E2S-AS	(5'-GCTCTAGA**TTA**ATTGAGTATTACGAG-3')
A189/191F-S	(5'-GTGCCCTTCTCATTCTACCAAGTGCGCAAT-3')
A189/191F-AS	(5'-GTAGAATGAGAAGGGCACGGTCAGGCA-3')
SPKO-S	(5'-CCCCCTCCTTTCCCTTTCGTGCGCAATTCCTCG-3')
SPKO-AS	(5'-GAAAGGGAAAGGAGGGGGCACGGTCAGGCA-3')
HCV220-AS	(5'-TGCTCTAGA**TTA**GATGGCATCGGCCGCCTC-3')
HCV240-AS	(5'-TGCTCTAGA**TTA**CACCCAACACCTCGAGGC-3')
HCV260-AS	(5'- TGCTCTAGA**TTA**ACGTCGAAGCTGCGTTGT-3')
HCV280-AS	(5'- TGCTCTAGA**TTA**CAGGTCCCCCACGTAGAG-3')
HCV707-S	(5'-CCGGAATTCATGTCAAGCATCGCGTCC-3')
HCV839-AS	(5'-GCTCTAGAGATGTAGCGCTTGTA-3')
HCV819-AS	(5'-GCTCTAGAGCCACACGACGCGGC-3')
SPKOII-S	(5'-CGGCCTCCTTTCCCTTTCACGGAGGTGGCCGCG-3')
SPKOII-AS	(5'-GAAAGGGAAAGGAGGCCGCTGAGGCAACGC-3')
L172/791-S	(5'-AACCTTCCTGGTTGCTACGGGATGTGGCCT-3')
L172/791-AS	(5'-AGGCCACATCCCGTAGCAACCAGGAAGGTT-3')
L172/365-S	(5'-AACCTTCCTGGTTGCGTGGGGAACTGGGCG-3')
L172/365-AS	(5'-CGCCCAGTTCCCCACGCAACCAGGAAGGTT-3')
L172/728-S	(5'-AACCTTCCTGGTTGCGACGCGCGCGTCTGC-3')
L172/728-AS	(5'-GCAGACGCGCGCGTCGCAACCAGGAAGGTT-3')
HCV119-S	(5'-CGGGATCCTTGGGTAAGGTCATC-3')
HCV150-S	(5'-CGGGATCCGCCCTGGCGCATGGC-3')
M-S3	(5'-CGCGGATCCATGGCAGACAACGGTACT-3')
M50/C151-S	(5'-TACATAATAAAGCTTCTGGCGCATGGCGTC-3')
M50/C151-AS	(5'-GACGCCATGCGCCAGAAGCTTTATTATGTA-3')

Note: *GAATTC, TCTAGA*, and *GGATCC* are the recognition sequences for EcoRI, XbaI, and BamHI respectively. ☐ means point mutation while bolded nucleotides represent stop codon.

extension of the signal peptide. Therefore, reducing the length of C-terminal extension of core protein signal peptide by signal peptidase cleavage should facilitate its further cleavage by SPP. The mechanism regarding the cleavage efficiency of SPP is inversely proportional to the length of the C-terminal extension of the signal peptide is unknown. It could be due to conformation change of protein substrate with longer C-terminal extension.

Proteases typically display a high degree of specificity which may be achieved by specific recognition of a distinct sequence. However, no such principal modes of control seem to be valid for SPP [19]. Not all signal peptides are substrates for SPP. The chimera core protein with signal peptide from the C-terminus of p7 protein, but not those from the C-termini of E1 and E2, could be cleaved by signal peptide peptidase (Fig. 3). Moreover, the signal peptide at the C-terminal of p7 in its native structure was not cleaved by SPP (Fig. 4). Thus, the core protein sequence may facilitate the cleavage of some foreign signal peptides. These results suggest that the sequence of the signal peptide itself is an important but not the sole determinant for its cleavage by SPP. It has been

demonstrated that helix-breaking residues within the transmembrane region of signal peptide are required for cleavage, and the flanking regions can affect the processing by SPP [20, 30]. A helix-breaking residue (Pro^{804}) in the signal peptide at the C-terminus of p7 may account for its cleavage by SPP when placed at the C-terminus of core protein.

Truncated core protein (a.a. 119–200) with the internal hydrophobic domain could be cleaved by SPP (Fig. 5A), indicating that the first 118 a.a. of core protein is dispensable for the cleavage by SPP.

A previous study showed that Leu^{139}, Val^{140}, and Leu^{144} of HCV core protein play crucial roles in the ER retention and SPP cleavage [21]. To determine whether ER retention or this protein sequence affects the cleavage of signal peptide by SPP, two truncated core proteins (a.a. 119–200 and a.a. 150–200) with or without this hydrophobic sequence were constructed and expressed. Only truncated core protein with this hydrophobic domain (a.a. 119–200) could be processed by SPP (Fig. 5A) though both proteins are ER-associated (Fig. 6). We also constructed a chimera core protein using the first 50 a.a. of SARS-CoV M protein to replace this hydrophobic domain. This chimera core protein could not be processed by SPP (Fig. 5B) though it is ER-associated (Fig. 6). Thus, these results suggest that not only E.R.-association but also the sequence of this hydrophobic domain is important for the HCV core protein signal peptide cleavage by SPP.

In addition to targeting downstream E1 protein into ER, signal peptide at the C-terminus of core protein may have other unknown functions. In human, the signal peptides of polymorphic major histocompatibility complex class I molecules contain a highly conserved sequence that is capable of binding to HLA-E [31]. This peptide-HLA-E complex when exposed on the cell surface, will specifically interact with an inhibitory receptor on natural killer (NK) cells, thereby monitoring indirectly the level of class I molecule expression [32, 33]. The free signal peptide of core protein (i.e. a.a. 180–191) after cleavage by signal peptidase and SPP may have a similar role like that of HLA-E. Alternatively, this short peptide (a.a. 180–191) may bind to Ca^{2+}/calmodulin and regulate the signal transduction pathway in the host cells, similar to the case of the hormone preprolactin (p-Prl) and the human immunodeficiency virus-1 gp160 [34].

In summary, our data indicate that the core spko mutant is more efficient than the A/F mutant in blocking the HCV core protein cleavage by signal peptidase. Prior cleavage of signal peptide by signal peptidase should facilitate its further cleavage by signal peptide peptidase. Moreover, both the sequences of the signal peptide and the ER retention domain (a.a. 120–150) but not the first 118 amino acids are important for the cleavage of HCV core protein by SPP.

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