

Short Communication

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Efficient cleavage by signal peptide peptidase requires residues within the signal peptide between the core and E1 proteins of hepatitis C virus strain J1

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Maturation of hepatitis C virus (HCV) core protein requires cleavage by signal peptidase (SP) and signal peptide peptidase (SPP) at a signal peptide between core and the E1 glycoprotein. For HCV strain Glasgow, amino acids Ala¹⁸⁰, Ser¹⁸³ and Cys¹⁸⁴ within the signal peptide have previously been shown to be essential for efficient SPP cleavage. By contrast, these residues apparently did not contribute to core maturation in HCV strain J1. In the present study, the source of this discrepancy has been analysed and it is concluded that interpretation of the strain J1 data was incorrect, due to the inability to separate wild-type and mutant forms of core on gels by using standard buffer systems.

Release of the hepatitis C virus (HCV) structural proteins (core, E1 and E2) from the viral polyprotein requires cleavage at signal peptides that separate the individual proteins (Fig. 1a). Proteolysis is directed by signal peptidase (SP), a cellular activity, thereby generating the N and C termini of glycoproteins E1 and E2. For core, a second cellular enzyme called signal peptide peptidase (SPP) cuts within the signal peptide between core and E1 (SigP_{core-E1}) to give the mature protein (Fig. 1a; Hüsey *et al.*, 1996; McLauchlan *et al.*, 2002). Mutations in SigP_{core-E1} from HCV strain Glasgow, a genotype 1a strain, revealed that amino acids Ala¹⁸⁰, Ser¹⁸³ and Cys¹⁸⁴ were necessary for efficient SPP processing (Lemberg & Martoglio, 2002; McLauchlan *et al.*, 2002). However, Okamoto *et al.* (2004) reported that these residues did not affect SPP cleavage and that the critical amino acids lay upstream of SigP_{core-E1} in strains J1 and H77, which are genotype 1b and 1a strains, respectively. Here, we examine the basis of these differences.

One possible source of the dissimilar conclusions was the use of different expression systems. In the study by Okamoto *et al.* (2004), the HCV proteins were expressed using vector pcDNA3.1, whereas we employed the Semliki Forest virus (SFV) vector system (McLauchlan *et al.*, 2002). To compare these systems, DNA fragments containing strain J1 sequences were amplified from the relevant pcDNA3.1 plasmids by using two primers (5'-GGGCAGATCTGCCGCCACCATG-AGCACAAATCCTAAACCCCAAAGA-3' and 5'-CAACA-GATGGCTGGCAACTAGAAAGGC-3'). The resultant PCR

products contained the coding region for aa 1–382 of strain J1, followed by 17 residues that incorporated an HA tag. After digestion with *BglIII/XbaI*, the PCR products were introduced into pSFV1 to generate two vectors, pSFV/C[wt]-E1-HA and pSFV/C[ASC/VLV]-E1-HA (Fig. 1a). pSFV/C[ASC/VLV]-E1-HA contained mutations in SigP_{core-E1} identical to those in pSFV/CspmtE1E2, the construct that expressed the structural proteins of HCV strain Glasgow (Fig. 1b; McLauchlan *et al.*, 2002); core made by these constructs will be referred to as core_{ASC/VLV} and core_{spmt} to discriminate between the strain J1 and Glasgow products, respectively. For completeness, we also included a construct, pSFV/C[IF/AL]-E1-HA, that contains mutations at Ile¹⁷⁶ and Phe¹⁷⁷ (Fig. 1b). Alteration of these residues was reported to impair SPP processing in strain J1 (Okamoto *et al.*, 2004).

The relative mobilities of wild-type (wt) and mutant core made from the pcDNA3.1 constructs in BHK cells were almost indistinguishable following electrophoresis on 12.5% polyacrylamide gels using a Tris/glycine buffering system (Laemmli, 1970), although core_{ASC/VLV} did migrate slightly above core_{wt} (Fig. 1c, lanes 2–4). Core made from the equivalent SFV constructs showed an identical pattern of electrophoretic mobilities in either BHK or HuH-7 cells (Fig. 1d, upper and lower panels, lanes 1–3). Core expressed by the pcDNA plasmids had reduced mobility compared with the species produced by the pSFV constructs, which resulted from a 22 aa extension at the N terminus of core (Okamoto *et al.*, 2004) that was removed in the pSFV constructs (Fig. 1c, compare lanes 1 and 2). With both expression systems, we found that Core-IF/AL-E1-HA and

A supplementary figure showing the stability of core_{wt} and core_{ASC/VLV} proteins that lack aa 125–144 is available in JGV Online.

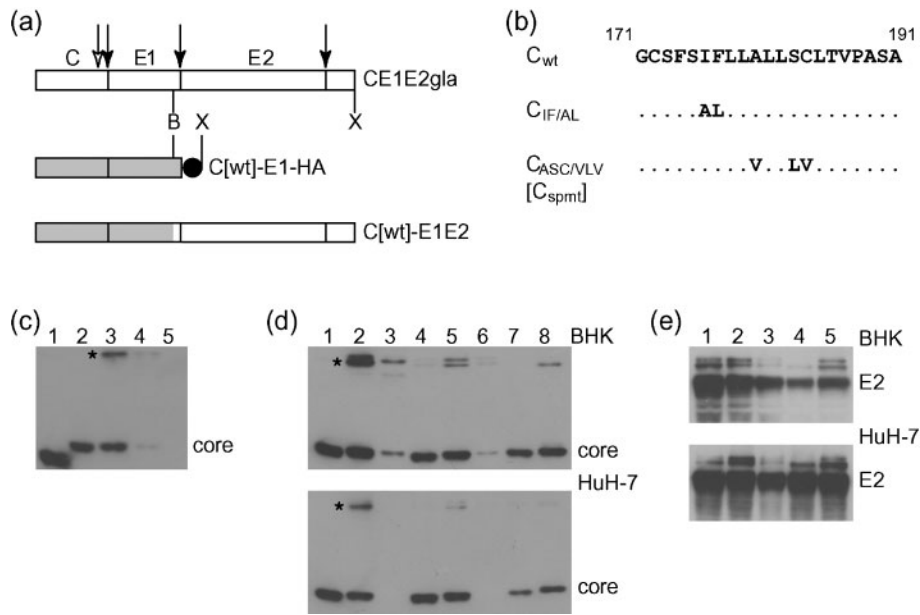


Fig. 1. Expression of wt and mutant forms of core from HCV strain J1. (a) Details of HCV proteins expressed by plasmids. The upper diagram shows the SP (filled arrowheads) and SPP (open arrowhead) cleavage sites that generate core (C), E1 and E2. Open and shaded boxes represent the coding sequences in plasmids for strains Glasgow and J1, respectively. B and X indicate positions of *Bam*HI and *Xba*I restriction-enzyme cleavage sites. ● marks the location of the HA tag. (b) Amino acid sequence of SigP_{core-E1} for wt and mutant proteins. The wt sequence is identical in strains Glasgow and J1. (c) Expression of wt and mutant core proteins from strain J1. BHK cells were transfected with pcDNA/Flag-Core-E1-HA (lane 2), pcDNA/Flag-Core-ASC/MLV-E1-HA (lane 3) or pcDNA/Flag-Core-IF/AL-E1-HA (lane 4) for 15 h at 37 °C. Western blot analysis was performed with R308, a core antiserum (Hope & McLauchlan, 2000). The positions of core and a higher-molecular-mass 46 kDa product (*) that represents core-E1 precursor protein are indicated. Lane 1 contains a sample from BHK cells electroporated with pSFV/C[wt]-E1E2 RNA and lane 5 contains a mock-transfected extract. (d, e) Expression of wt and mutant proteins from strains J1 and Glasgow. BHK (upper panels) and HuH-7 (lower panels) cells were electroporated with *in vitro*-synthesized RNA from pSFV constructs (McLauchlan *et al.*, 2002) and incubated at 37 °C for 15 h. Western blot analysis was performed with R308 antiserum (d) and ALP98 (e), an E2-specific antibody (Clayton *et al.*, 2002). The positions for core, putative core-E1 precursor proteins (*) and E2 are shown. Samples were: pSFV/C[wt]-E1-HA (d, lane 1); pSFV/C[ASC/MLV]-E1-HA (d, lane 2); pSFV/C[IF/AL]-E1-HA (d, lane 3); pSFV/C[wt]-E1E2 [(d), lane 4 and (e), lane 1]; pSFV/C[ASC/MLV]-E1E2 [(d), lane 5 and (e), lane 2]; pSFV/C[IF/AL]-E1E2 [(d), lane 6 and (e), lane 3]; pSFV/CE1E2gla [(d), lane 7 and (e), lane 4]; pSFV/C_{spmt}E1E2gla [(d), lane 8 and (e), lane 5].

Core-ASC/VLV-E1-HA constructs generated additional species, which have a similar apparent molecular mass to the predicted size of core-E1 precursor protein [marked by an asterisk in Fig. 1(c, d)]. The abundance of this precursor was reduced in HuH-7 cells (Fig. 1d, lower panel, lane 2). Moreover, both the pcDNA and pSFV constructs for Core-IF/AL-E1-HA consistently gave low levels of core in BHK cells (Fig. 1c, lane 4; Fig. 1d, upper panel, compare lanes 2 and 3), although the mutant protein detected did co-migrate with core_{ASC/MLV}. In HuH-7 cells, core protein from the SFV construct could not be detected (Fig. 1d, lower panel, lane 3).

For constructs that express a polyprotein, which consists of core and E1, but does not include E2, we have observed core-E1 precursor proteins under conditions where processing at SigP_{core-E1} is impaired by mutations in core (R. G.

Hope, S. Boulant & J. McLauchlan, unpublished data). This phenomenon is likely to arise from reduced efficiency in translocation at the endoplasmic reticulum (ER) membrane, which impairs cleavage at SigP_{core-E1} by cellular signalases. Any defect in translocation efficiency that gives rise to such precursors is reduced considerably by extending the length of the HCV polyprotein to include E2. To increase the polyprotein length made by the three pSFV constructs expressing strain J1 sequences, we inserted a *Bam*HI/*Xba*I fragment from pSFV/CE1E2gla to create pSFV/C[wt]-E1E2, pSFV/C[IF/AL]-E1E2 and pSFV/C[ASC/MLV]-E1E2. In each plasmid, amino acid codons 1–340 were derived from strain J1, whilst codons 341–829 were from strain Glasgow (Fig. 1a). This approach reduced the amounts of the core-E1 products, but did not improve separation of core_{ASC/MLV} from core_{wt} (Fig. 1d, upper and lower panels, lanes 4 and 5). In parallel, BHK and HuH-7 cells were electroporated with

RNA from strain Glasgow constructs pSFV/CE1E2gla and pSFV/CspmtE1E2. Again, the mutant protein (core_{smt}) migrated marginally above wt core (Fig. 1d, upper and lower panels, lanes 7 and 8). Only very low levels of core were made by pSFV/C[IF/AL]-E1E2, which correlates with the data obtained with construct pSFV/C[IF/AL]-E1-HA, but again, the detected protein co-migrated with core_{ASC/VLV} (Fig. 1d, upper panel, compare lanes 5 and 6). The basis for the low levels of core_{IF/AL} was not studied rigorously; however, pSFV/C[IF/AL]-E1E2 does produce quantities of E2 similar to those from the other pSFV constructs (Fig. 1e, upper and lower panels, compare lane 3 with lanes 1, 2, 4 and 5). Therefore, mutations at residues Ile¹⁷⁶ and Phe¹⁷⁷ not only impair SPP processing, but also decrease the stability of core. Due to differences in abundance of core_{IF/AL} compared with core_{wt} and core_{ASC/VLV}, particularly in HuH-7 cells, this mutant was not included in further experiments.

Based on the inability to distinguish core_{wt} from core_{ASC/VLV} on polyacrylamide gels, Okamoto *et al.* (2004) concluded that these residues were not important for SPP processing. For strain Glasgow, the difficulty with discriminating core_{wt} (which has been cleaved by SPP and ends putatively at about aa 179) from core_{smt} (which terminates at the SP site at aa 191) by gel electrophoresis using a Tris/glycine buffer system has been highlighted previously (McLauchlan *et al.*, 2002; Lemberg & Martoglio, 2003). Separation of the two forms was possible on multiphasic Tris/Bicine polyacrylamide gels. Paradoxically, core_{smt} had greater mobility than core_{wt} in this gel system, despite the longer length of the mutant protein (McLauchlan *et al.*, 2002; Lemberg & Martoglio, 2003). To examine in greater detail the core species made by pSFV/C[wt]-E1E2 and pSFV/C[ASC/VLV]-E1E2, RNA from these plasmids was electroporated into cells that were either treated or not treated with (Z-LL)₂ ketone, an inhibitor of SPP (Weihofen *et al.*, 2000, 2003), and extracts were analysed in both Tris/glycine and Tris/Bicine gel systems. In Tris/glycine gels, a single product corresponding to SPP-cleaved core was found for the wt form of the protein in untreated cells (Fig. 2a, lane 1); for cells treated with (Z-LL)₂ ketone, two species that represented SP- and SPP-cleaved core were detected (upper and lower bands respectively in Fig. 2a, lane 2). From quantification of the bands in lane 2 (Fig. 2a), SPP proteolysis was reduced by about 60 % by the inhibitor. For pSFV/C[ASC/VLV]-E1E2, only a single species was detected either in the presence or absence of (Z-LL)₂ ketone, which migrated more closely to the SP-cleaved wt core protein than to the SPP-cleaved form (Fig. 2a, compare lane 2 with lanes 3 and 4). The improved resolution of core species made by pSFV/C[wt]-E1E2 and pSFV/C[ASC/VLV]-E1E2 compared with the data presented in Fig. 1 was achieved by increasing the time taken to resolve the samples on gels. The inability to detect two species made by pSFV/C[ASC/VLV]-E1E2 in the presence of a specific SPP inhibitor indicated that the core protein produced from this plasmid is generated solely by SP. On Tris/Bicine gels, it was not possible to separate SPP- and SP-cleaved forms of core_{wt} (Fig. 2b, lane 2). However, the migration of core_{ASC/VLV} was greater than

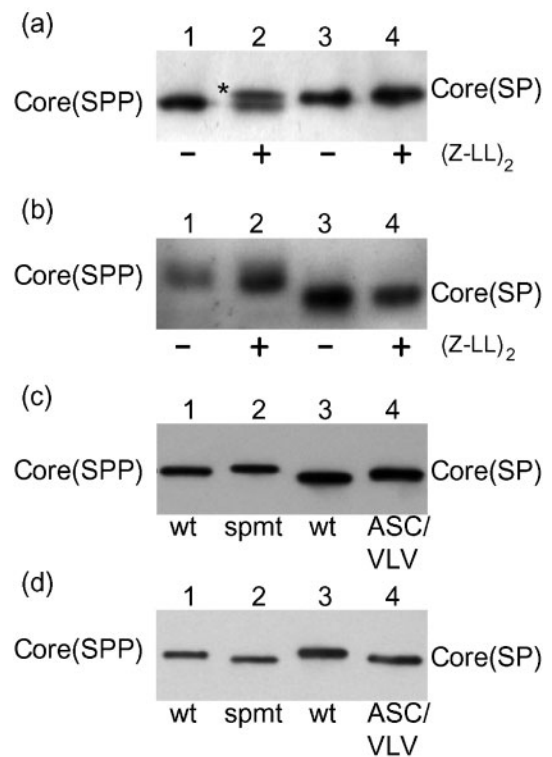


Fig. 2. Separation of core_{wt} and core_{ASC/VLV} on polyacrylamide gels by using Tris/glycine (a, c) and Tris/Bicine (b, d) buffer systems. (a, b) BHK cells were electroporated with RNA from pSFV/C[wt]-E1E2 (lanes 1 and 2) and pSFV/C[ASC/VLV]-E1E2 (lanes 3 and 4) for 11 h in the presence or absence of 100 μ M (Z-LL)₂ ketone as described by Weihofen *et al.* (2003). Bands corresponding to core_{wt} cleaved with SPP [core(SPP)] and core_{ASC/VLV} cleaved with SP [core(SP)] are indicated. The asterisk in (a), lane 2, denotes core_{wt} cleaved with SP. (c, d) BHK cells were electroporated with RNA from pSFV/CE1E2gla (lanes 1, marked wt), pSFV/CspmtE1E2 (lanes 2, marked spmt), pSFV/C[wt]-E1E2 (lanes 3, marked wt) and pSFV/C[ASC/VLV]-E1E2 (lanes 4, marked ASC/VLV) for 14 h. Bands corresponding to core cleaved with SPP [core(SPP)] and core cleaved with SP [core(SP)] are indicated.

that of core_{wt} (Fig. 2b, compare lanes 1 and 2 with lanes 3 and 4). This phenomenon was reproducible in three separate experiments and is identical to the migratory properties for proteins made from strain Glasgow, where the longer, SP-cleaved core_{smt} product migrates faster than the shorter, SPP-cleaved core_{wt} species (compare Figs 2c and d; McLauchlan *et al.*, 2002; Lemberg & Martoglio, 2003). Hence, the results indicate that core protein made from pSFV/C[ASC/VLV]-E1E2 is generated by SP and not by SPP. From the data obtained on the two gel systems, we conclude that mutation of Ala¹⁸⁰, Ser¹⁸³ and Cys¹⁸⁴ in SigP_{core-E1} in strain J1 does impair SPP processing, in agreement with our findings for the critical role that these amino acids play in SPP cleavage at SigP_{core-E1} from HCV strain Glasgow (McLauchlan *et al.*, 2002).

It has been established that inhibition of SPP processing at SigP_{core-E1} in strain Glasgow blocks attachment of core to lipid droplets (Lemberg & Martoglio, 2002; McLauchlan *et al.*, 2002; Weihofen *et al.*, 2003). Hence, the intracellular localizations of wt and mutant forms of core from strain J1 were examined. In BHK cells transfected with pcDNA/Flag-Core-E1-HA, core was present at the surface of lipid droplets (Fig. 3a–c). By contrast, core_{ASC/VLV} was distributed throughout the cytoplasm and was not directed to lipid droplets (Fig. 3d–f). In a small proportion of cells (approx. 5–10%), there was some staining for core_{ASC/VLV} around lipid droplets, which would be consistent with a minor fraction of mutant protein that is processed by SPP. To demonstrate further that core_{ASC/VLV} was not targeted to lipid droplets, HuH-7 cells were electroporated with RNA transcribed from the pSFV plasmids. Again, core_{wt} was localized to the surface of lipid droplets, whereas core_{ASC/VLV} was distributed throughout the cytoplasm in a reticular pattern, consistent with localization at the ER membrane (Fig. 3g–i). Identical patterns were found in BHK cells expressing core from the SFV plasmids (data not shown). In methanol-fixed cells, core_{ASC/VLV} co-localized

with E2, indicating that the mutant protein was present at the ER membrane (data not shown). These data for core_{ASC/VLV} agree with the results for core_{sprmt} produced by strain Glasgow (McLauchlan *et al.*, 2002) and demonstrate that the requirement for SPP cleavage to enable transfer of core from the ER membrane to lipid droplets is not a strain-specific characteristic.

In a final series of experiments to examine the similarity between the signal peptide mutants for strains Glasgow and J1, a region encoding aa 125–144 was removed from pSFV/C[wt]-E1E2 and pSFV/C[ASC/VLV]-E1E2. For strain Glasgow, removal of these amino acids generated an unstable form of core that was degraded upon cleavage by SPP (McLauchlan *et al.*, 2002). By contrast, introduction of mutations into SigP_{core-E1}, which impairs SPP cleavage, prevents degradation due to retention of core at the ER membrane. Analysis of the corresponding mutants in strain J1 gave results identical to those obtained for strain Glasgow (see Supplementary Figure, available in JGV Online), demonstrating further the similarity in the properties of core made by the two strains.

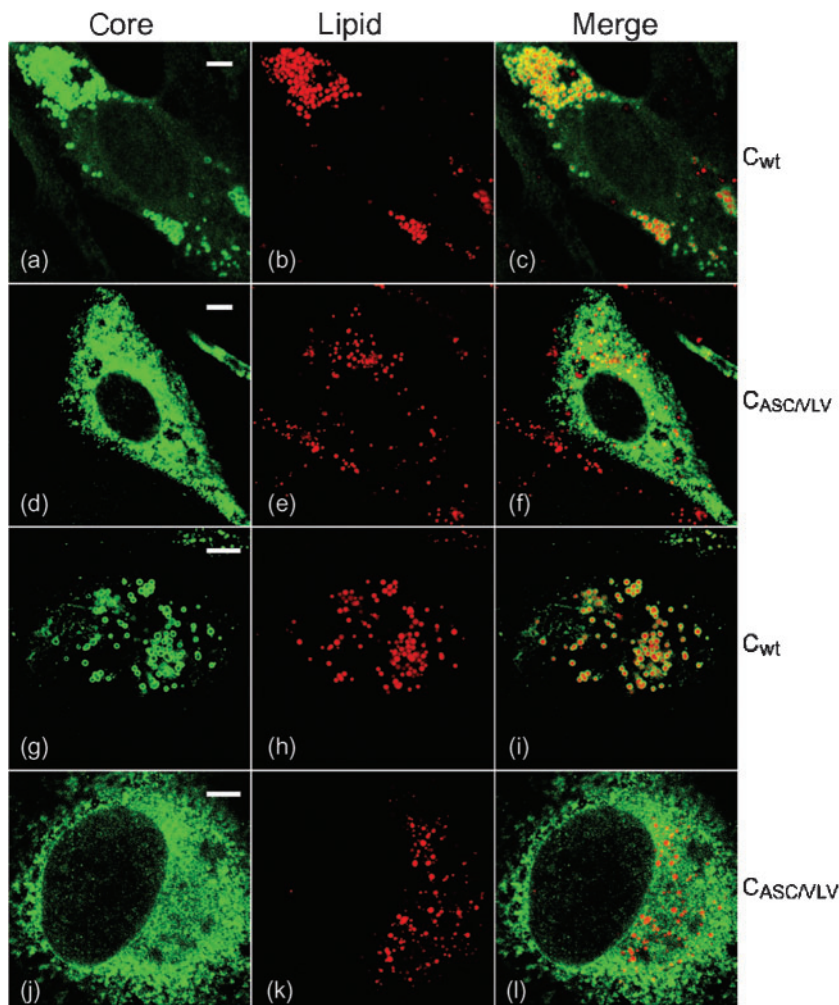


Fig. 3. Intracellular distribution of core_{wt} and core_{ASC/VLV}. BHK cells were transfected with pcDNA/Flag-Core-E1-HA (a–c) and pcDNA/Flag-Core-ASC/VLV-E1-HA (d–f). HuH-7 cells were electroporated with RNA from pSFV/C[wt]-E1E2 (g–i) and pSFV/C[ASC/VLV]-E1E2 (j–l). After incubation at 37 °C for 15 h, cells were fixed, probed with R308 antiserum (panels marked core) and stained with oil red O for lipid droplets (panels marked lipid; Hope *et al.*, 2002). Bars, 5 µm.

Contrary to the conclusions by Okamoto *et al.* (2004), our results demonstrate that amino acids Ala¹⁸⁰, Ser¹⁸³ and Cys¹⁸⁴ in SigP_{core-E1} for strain J1 are necessary for efficient processing by SPP. These data agree with the sequence requirements for SPP cleavage in strain Glasgow (McLauchlan *et al.*, 2002). We conclude that the disparity in the two published reports was not a consequence of the different expression systems employed or sequence differences elsewhere in the core protein, but the inability to separate core_{ASC/VLV} from core_{wt} reliably on polyacrylamide gels by using a Tris/glycine buffering system. These species could be resolved consistently on gels by using a Tris/Bicine buffering system, although the SP-cleaved form of core_{ASC/VLV} had greater mobility than wt core processed by SPP. It is likely that higher hydrophobicity of core_{ASC/VLV} compared with core_{wt}, which could enable increased binding of SDS to the mutant protein, accounts for its increased migration (Lemberg & Martoglio, 2003). In an analogous situation, two A β peptides (A β ₁₋₄₀ and A β ₁₋₄₂), which are major components in amyloid deposits, do not separate according to the normal relationship between mass and electrophoretic mobility in Tris/Bicine-buffered gels (Klafki *et al.*, 1996; Kawooya *et al.*, 2003).

Two other characteristics of core_{ASC/VLV} were identical to those of core_{sprmt} produced by strain Glasgow. Firstly, core_{ASC/VLV} failed to target lipid droplets and remained at the ER membrane. These mutations in SigP_{core-E1} do not apparently affect ER targeting of core in the context of a longer polyprotein, as processing of the glycoproteins in the ER lumen is not altered and core retains a reticular-staining pattern, consistent with association at the ER membrane. Secondly, removal of aa 125–144 led to degradation of SPP-cleaved core_{wt}, whereas core_{ASC/VLV} lacking this segment was stable. From these independent assays, we conclude that these amino acids, previously identified in SigP_{core-E1} for strain Glasgow, are also critical for efficient SPP processing of core from strain J1. Comparative studies revealed that SigP_{core-E1} is completely conserved in 85% of 450 HCV sequences deposited in public databases. The only changes identified were at either aa 178 (Leu to Ile in 2% of sequences) or 187 (Val to Ile in 13% of sequences). Therefore, amino acids at positions 180, 183 and 184 are invariant in the HCV polyprotein. Extrapolating from our results with two HCV strains from different genotypes, we propose that these amino acids in SigP_{core-E1} play a fundamental role in SPP cleavage for all strains of HCV.

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