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Running title: HCV core protein cleavage by SPP and viral assembly

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SUMMARY

Hepatitis C virus (HCV) core protein, expressed with a Semliki forest virus (SFV) replicon, self-assembles into HCV-like particles (HCV-LP) at the endoplasmic reticulum (ER) membrane, providing an opportunity to study HCV assembly and morphogenesis by electron microscopy. We used this model to investigate whether the processing of the HCV core protein by the signal peptide peptidase (SPP) is required for the HCV-LP assembly. We designed several mutants as there are conflicting reports concerning the cleavage of mutant proteins by SPP. Production of the only core mutant protein that escaped SPP processing led to the formation of multiple layers of electron-dense ER membrane, with no evidence of HCV-LP assembly. Our data shed light on the HCV core residues involved in SPP cleavage and suggest that this cleavage is essential for HCV assembly.

The hepatitis C virus (HCV) genome contains approximately 9,600 nucleotides. At both the 5' and 3' ends, untranslated regions flank a single open reading frame encoding a single polyprotein precursor of about 3,000 amino acids (aa). This precursor is co- and post-translationally processed by cellular and viral proteases, to yield three mature structural proteins [one core (C) and two envelope (E1 and E2) proteins] and six non-structural proteins involved in polyprotein processing and viral RNA replication (Grakoui *et al*., 1993). The hydrophobic sequence at the junction between the HCV core protein and the envelope glycoprotein E1 (amino acids 170 to 191 in the polyprotein) functions as a signal sequence and targets the nascent polyprotein to the ER membrane, inducing the translocation of E1 into the lumen of the ER (Lo *et al*., 1995). Cleavage by a signal peptidase in the ER lumen releases the N-terminal end of E1, leaving the 191-aa core protein anchored by the signal peptide (McLauchlan 2000). This 191-aa polypeptide (p23) is an immature form of the core protein, and is further processed by an intramembrane presenilin-type aspartic protease SPP (for signal peptide peptidase) (Weihofen *et al*., 2002). The site of the SPP cleavage is unclear but probably lies between residues 173 and 179, resulting in the elimination from the mature core protein of all or part of the N-terminal hydrophobic domain containing the signal peptide of E1 (McLauchlan *et al*., 2002). The resulting mature HCV core protein (p21) is thought to constitute the HCV capsid and therefore to be an integral component of the virion. Indeed, this mature form of the HCV core protein is the predominant form detected in virus particles purified from the sera of patients with chronic HCV infection (Yasui *et al*., 1998). However, processing of the HCV core protein by SPP has also been shown to release the core protein from the ER membrane leading to its trafficking to zones of the ER in which lipid droplets are produced (McLauchlan *et al*., 2002). During its trafficking, the proline-rich, central hydrophobic domain 2 of the HCV core protein (amino acids 119 to 173) is though to remain anchored in the cytoplasmic phospholipid monolayer of the ER, and is therefore thought to be present in the membrane surrounding the lipid droplets, which consists of a single phospholipid monolayer (Hope & McLauchlan, 2000).

Thus, retention of the HCV core protein in the ER membrane by its sequence signal may play an essential role in viral particle assembly by, at least transiently, preventing trafficking to the lipid droplets. It therefore remains unclear whether HCV core protein cleavage by SPP is an essential step for virion assembly and morphogenesis. Recent advances in the establishment of cell culture systems have made it possible to produce infectious HCV that can be efficiently propagated in cultured, naive hepatoma cells (Lindenbach *et al*., 2005; Wakita *et*

al., 2005; Zhong *et al*., 2005). However, it remains extremely difficult to observe virus assembly and morphogenesis in these cell cultures (P.R., personal observations). We have developed a model based on Semliki forest virus (SFV) replicon vectors, in which the production of the HCV core protein in mammalian cells leads to the assembly of this protein into HCV-like particles (HCV-LP) (Blanchard *et al*., 2003). This model displays abortive HCV-LP budding, but it remains a useful tool for studies of the early events of HCV assembly in eukaryotic cells (Roingeard *et al*., 2004). In this study, we use this HCV capsid assembly model to characterise the role of SPP cleavage in viral morphogenesis.

HCV core mutants. Previous studies have identified various residues in the transmembrane signal sequence domain of the HCV core protein that are involved in the intramembrane cleavage of this protein by SPP. It was initially demonstrated that the ASC180/3/4VLV HCV mutant core protein, which contains helix-favouring residues in place of the helix-bending and helix-breaking residues, was not processed by SPP (Lemberg & Martoglio, 2002; McLauchlan *et al*., 2002). However, a recent study of the same ASC180/3/4VLV mutant demonstrated efficient cleavage of this molecule by SPP (Okamoto *et al*., 2004). In the same study, it was shown that an IF176/7AL core mutant protein was not processed by SPP. These discrepancies were observed for the core proteins of both the 1a and 1b genotypes of HCV. They were not due to differences in the core protein sequence or in cell type, as both studies were conducted with BHK-21 cells. We therefore designed SFV replicon vectors encoding the ASC180/3/4VLV and IF176/7AL core mutants, to resolve these discrepancies and to address our own research questions. The SF173/4ML mutant core protein was found not to be processed by SPP in a third study (Yamanaka *et al*., 2002), and was therefore also investigated in our study. The C191 and C173 sequences were subcloned in SFV vectors from the HCV cDNA Dj 6.4 clone (genotype 1a) as previously described (Blanchard *et al.*, 2003). Site-directed mutagenesis within the C-terminal C191 signal sequence was performed with antisense primers leading to (i) generation of the three mutants ASC180/3/4VLV, IF176/7AL and SF173/4ML (Fig. 1A) and (ii) insertion of a stop codon at the 3' end of the core protein-coding region.

Cleavage of HCV wild-type and mutant core proteins by SPP. The BHK 21 cell line was cultured and electroporated as previously described (Blanchard *et al*., 2002). SDS-PAGE and western blotting analysis showed that the wild-type (WT) C191 core protein was fully cleaved by SPP in these cells, as previously reported (Blanchard *et al*., 2003) (Fig. 1B). In cells

transfected with the WT C191 construct and treated with various concentrations (60 μM, 100 μM or 150 μM) of $(Z\text{-}LL)_2$-ketone (Calbiochem), SPP cleavage was partially inhibited (Fig. 1B), as reported in previous studies using this commercially available SPP inhibitor (Weihofen *et al*., 2003). We used Image J gel analyser software to determine the amounts of p23 and p21 forms of HCV core protein in the scanned blots. Uncleaved and cleaved forms were detected in equal quantities in cells treated with 60 μM of $(Z\text{-}LL)_2$-ketone. Similar quantities of uncleaved (60%) and cleaved (40%) forms were detected at concentrations of 100 μM and 150 μM of $(Z\text{-}LL)_2$-ketone, indicating that this protease inhibitor was most efficient at a concentration of 100 μM (Fig. 1B). This is not surprising, as this compound is very efficient in cell-free translation/translocation assays, but less efficient in cellular assays because it does not readily cross the plasma membrane (Weihofen *et al*., 2003).

The ASC180/3/4VLV mutant core protein was the only mutant protein studied that remained uncleaved, resulting in detection of the p23 form of the core protein alone (Fig. 1B). This confirms the results obtained by Martoglio's group with this mutant (Lemberg & Martoglio, 2002; McLauchlan *et al*., 2002), but conflicts with the data reported by Okamoto *et al.* (2004). We found that the IF176/7AL mutant core protein was efficiently processed by SPP, leading to detection of the p21 core protein (Fig. 1B), despite a previous report of this protein being resistant to SPP cleavage (Okamoto *et al*., 2004). The SF173/4ML mutant core protein was also efficiently cleaved in our study (Fig. 1B). A total lack of processing (Yamanaka *et al*., 2002), or partial processing (Liu *et al*., 1997) had previously been reported for this protein. However, it should be pointed out that mutations in the HCV core protein have been shown to have a significant effect on the mobility of this protein in SDS-PAGE (McLauchlan *et al*., 2002). In addition, we cannot rule out the possibility that SPP processes the signal sequence of the core protein heterogeneously, at several sites, as observed for γ-secretase, an aspartic protease closely related to SPP that cleaves the transmembrane region of the amyloid-β precursor protein at several sites (Edbauer *et al*., 2003). These factors may account for difficulties in interpreting SDS-PAGE results for HCV mutant core proteins, and highlight the need for comparisons with relevant reference peptides. It should be also noted that the interaction of the HCV mutant core proteins with lipid droplets was not investigated in most of these previous studies. This led us to investigate the subcellular distribution of our HCV mutant core proteins.

Subcellular distribution of the HCV wild-type and mutant core proteins. This analysis was made with the WT and mutant constructs expressed in the human hepatoma FLC4 cell line (Aizaki *et al*., 2003) (Fig. 1C). Expression from SFV vectors was found to be less efficient in this cell line than in BHK-21 cells, allowing a more precise analysis of the subcellular core distribution and lipid droplet interaction. All the core proteins except the ASC180/3/4VLV mutant core protein were colocalized with lipid droplets (Fig. 1C). Nile red labeling in control cells transfected with β-Gal SFV RNA showed that lipids were evenly distributed throughout the cytoplasm. Transfection with the HCV WT C191 and C173 construct induced the clustering in the perinuclear area of large lipid droplets staining strongly for HCV core protein (Fig. 1C). The IF176/7AL and SF173/4MLmutant core proteins had a subcellular distribution similar to that of WT C191 (Fig. 1C). The ASC180/3/4VLV mutant core protein was weakly associated with some lipid droplets, but was mostly distributed in reticular patches throughout the cytoplasm (Fig. 1C). Thus, all the HCV core mutant proteins cleaved by SPP in SDS-PAGE analysis were colocalized with lipid droplets, like the WT core protein. This confirmed that the ASC180/3/4VLV mutant core protein was the only mutant core protein that escaped SPP processing.

Ultrastructural changes induced by the HCV wild-type and mutant core proteins. This study is the first to report the ultrastructural changes induced in cells by production of the HCV mutant core proteins ASC180/3/4VLV, IF176/7AL and SF173/4ML. BHK-21 cells transfected with the recombinant SFV RNAs encoding the two mutant HCV core proteins correctly cleaved by SPP (IF176/7AL and SF173/4ML) differed markedly in terms of ultrastucture (Fig. 2). As expected, the SF173/4ML mutant core protein gave a pattern similar to that of WT C191, with convoluted ER membranes displaying HCV-LP assembly (Fig. 2). Conversely, cells producing the IF176/7AL mutant core protein showed no sign of ER modification (Fig. 2), like cells transfected with β-Gal RNA or C173 RNA. This provides additional evidence that this mutant core protein is efficiently cleaved by SPP, and does not remain anchored in the ER membrane by its transmembrane sequence signal. This finding may also suggest that amino acids 176 and 177 (IF in the WT core protein) are located in the mature core protein after SPP cleavage and their replacement by other amino acids may hamper core protein multimerization.

Cells transfected with the recombinant SFV RNA encoding the ASC180/3/4VLV mutant HCV core protein, which is not processed by SPP, had a very unusual ultrastructure. Indeed,

convoluted electron-dense ER membranes were found to form multiple layers in these cells (Fig. 3). No HCV-LPs or marked clustering of lipid droplets was observed in cells producing this mutant core protein. These observations confirm that the ASC180/3/4VLV core protein remains anchored in the ER membrane by its transmembrane sequence signal. This unprocessed mutant protein therefore probably displays self-interaction, inducing the formation of multi-layer ER membranes, but not assembly to give HCV-LP. These data suggest that processing of the HCV core protein by SPP is required for assembly of the virus particle. We investigated the ultrastuctural changes induced in cells by production of the WT C191 core protein in the presence of $(Z\text{-}LL)_2$–ketone. Unfortunately, this commercially available SPP inhibitor is not very efficient in cellular assays, due to low plasma membrane permeability (Weihofen *et al*., 2003). We were able to achieve only 60% inhibition of SPP in cells transfected with the WT C191 core protein construct and treated with 100 μm concentration $(Z\text{-}LL)_2$–ketone. This treatment decreased the frequency of HCV-LP, which became much harder to detect, and led to the generation of multi-layer ER membranes similar to those found in cells producing the ASC180/3/4VLV mutant core protein (data not shown). Thus, cleavage of the transmembrane signal sequence of the HCV core protein by SPP appears to be an essential step in assembly of the viral particle. This conclusion is at odds with the recent results of analyses of WT C191 production in yeast cells (Majeau *et al*., 2005). The reasons for these discrepancies are unknown but may be due to differences in the HCV assembly mechanisms in yeast and mammalian cells. However, the intracellular HCV-LP assembly has not been well established by EM in yeast cell sections.

In conclusion, HCV core protein processing by SPP is required for the trafficking of the protein to the lipid droplets, but we suggest that it is also required for assembly into virus particles at the ER membrane. Our data provide new insight into the relevance of interactions between HCV and host cell factors, and suggest that SPP inhibitors are of potential value for the treatment of HCV infection.

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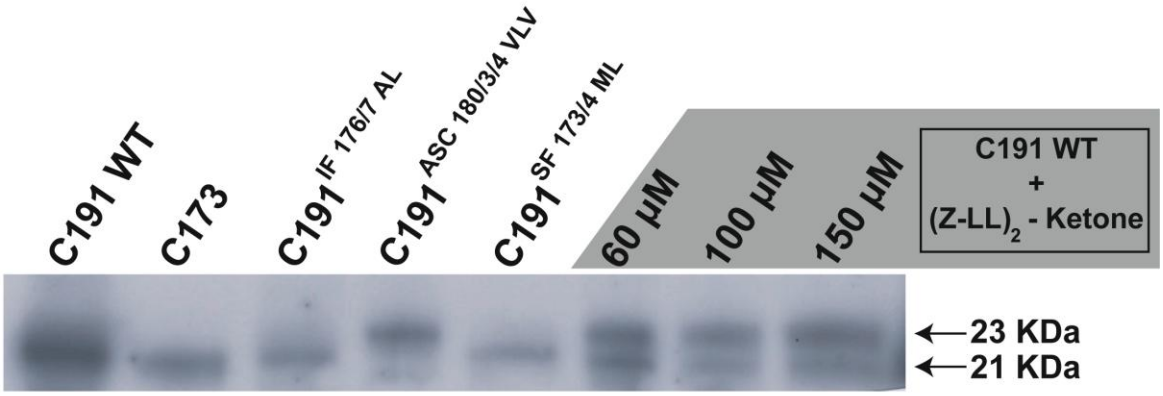
a

C191 WT	PGC**SF**S**IF**LL**A**LL**SC**LTVPASA
C191 IF 176/7 AL	. . . . . **AL** . . . . .
C191 ASC 180/3/4 VLV	. . . . . **V**. . **LV** . . . . .
C191 SF 173/4 ML	. . . **ML** . . . . .

b



c

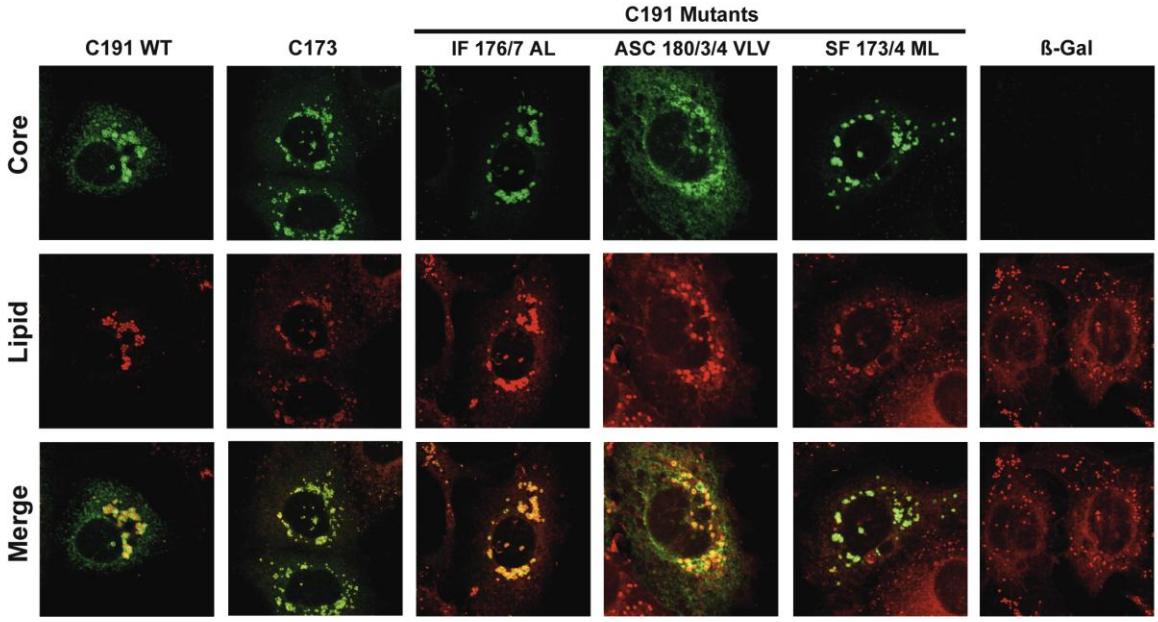


Fig. 1. Analysis of HCV core-E1 signal peptide processing. (a) Signal sequences of WT C191 HCV core protein and mutants (IF176/7AL, ASC180/3/4VLV and SF173/4ML). The transmembrane region of the signal sequence is underlined. (b): Production in BHK-21 cells of the WT and mutant C191 proteins and analysis of their processing by SDS-PAGE and western blotting, using the human monoclonal anti-HCV core antibody B12F8 (Esposito *et al.*, 1995). The mutants were compared with the WT C191 protein in the absence or presence of various concentrations of the SPP inhibitor $(Z\text{-}LL)_2$–ketone, and with the reference core protein C173. (c). Immunofluorescence of WT and mutant HCV core proteins combined with Nile Red staining of lipid droplets in FLC4 cells.

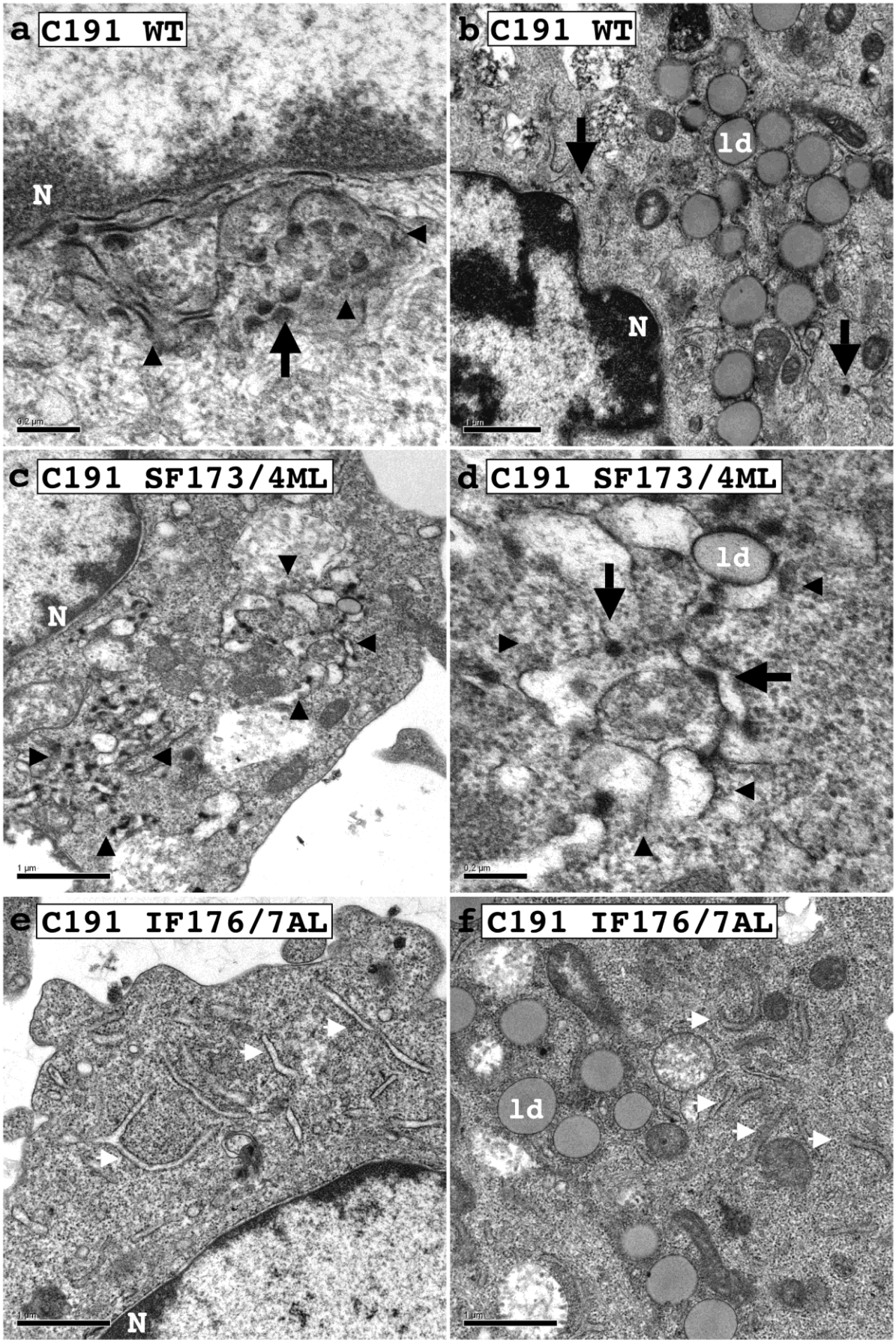

a C191 WT
N
0,2 µm
b C191 WT
ld
N
1 µm
c C191 SF173/4ML
N
1 µm
d C191 SF173/4ML
ld
0,2 µm
e C191 IF176/7AL
1 µm
N
f C191 IF176/7AL
ld
1 µm

Fig. 2. Electron micrographs of BHK-21 cells electroporated with WT C191 (a & b), C191 SF173/4ML (c & d), or C191 IF176/7AL (e & f) RNA. Micrograph in a shows a zone of convoluted ER membranes (delimited by arrowheads) in which HCV-LP assembly (arrows) was frequent. Micrograph in b shows that clusters of large lipid droplets were found in the perinuclear area next to ER membranes in which HCV-LP assembly (large black arrows) was detected. Cells transfected with C191 SF173/4ML RNA presented a pattern similar to that of the WT protein, with convoluted ER membranes (delimited by arrowheads in c) in which HCV-LP assembly (arrows in d) was detected. Cells transfected with C191 IF176/7AL RNA had normal ER structures, evenly distributed throughout the cytoplasm (white arrows), in which no HCV-LP was detected, as for cells transfected with β-Gal recombinant RNA (not shown). The only subtle change in ultrastructure of cells producing C191 IF176/7AL was the clustering of lipid droplets in the perinuclear area, next to normal ER membranes (micrograph in f). N, nucleus. ld: lipid droplet. ER lu: ER lumen. Bars in a and d, 0,2 μm. Bars in b, c, e and f, 1 μm.

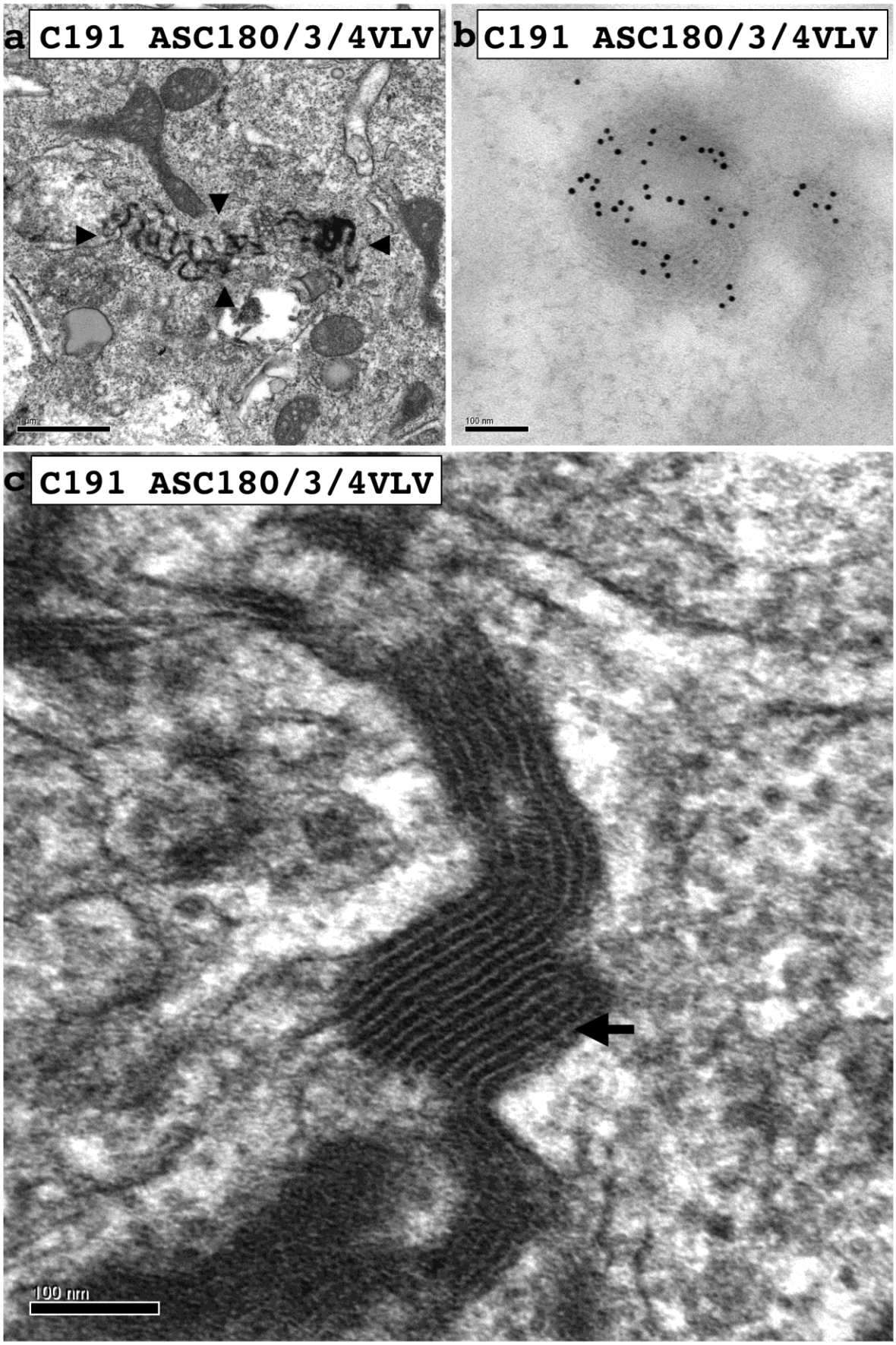

a C191 ASC180/3/4VLV
1 µm
b C191 ASC180/3/4VLV
100 nm
c C191 ASC180/3/4VLV
100 nm

Fig. 3. Electron micrographs of BHK-21 cells electroporated with the C191 ASC180/3/4VLV RNA, showing electron-dense ER membranes (delimited by arrowheads at low magnification in a). At high magnification (c), these structures were found to be formed by the interaction of multiple ER membranes (arrow). In b, an immunogold labeling with anti-core antibodies demonstrated the presence of high amount of core protein in these multi-layered ER membranes. Βαρσ ιν α , 0,2 μm. Bars in b and c, 100 nm.