Signal peptide peptidase promotes the formation of hepatitis C virus non-enveloped particles and is captured on the viral membrane during assembly

Nathalie Majeau, Valérie Gagné, Marilène Bolduc and Denis Leclerc

Infectious Disease Research Centre, Pav. CHUL, University of Laval, 2705 boulevard Laurier, Québec (Québec), Canada G1V 4G2

Correspondence
Denis Leclerc
Denis.Leclerc@crchul.ulaval.ca

Received 10 May 2005
Accepted 19 August 2005

The maturation of the core protein (C) of *Hepatitis C virus* (HCV) is controlled by the signal peptidase (sp) and signal peptide peptidase (spp) of the host. To date, it remains unknown whether spp cleavage influences viral infectivity and/or the assembly process. Here, evidence is provided that cleavage by spp is not required for assembly of nucleocapsid-like particles (NLPs) in yeast (*Pichia pastoris*). The immature NLPs (not processed by spp) show a density of 1·11 g ml^{-1} on sucrose gradients and a diameter of 50 nm. Co-expression of human spp (hspp) with C generates the 21 kDa mature form of the protein and promotes the accumulation of non-enveloped particles. The amount of non-enveloped particles accumulating in the cell was correlated directly with the expression level of hspp. Furthermore, immunocapture studies showed that hspp was embedded in the membranes of enveloped particles. These results suggest that maturation of the C protein can occur after formation of the enveloped particles and that the abundance of hspp influences the types of particle accumulating in the cells.

INTRODUCTION

Hepatitis C virus (HCV), a member of the genus *Hepacivirus* in the family *Flaviviridae*, is the major cause of chronic liver diseases such as cirrhosis and hepatocellular carcinoma (Lauer & Walker, 2001). The HCV genome is a single, positive-stranded RNA of 9600 nt, encoding a large polyprotein of 3010 aa (Choo *et al.*, 1989). The polyprotein is processed into 10 structural and non-structural components by cellular signal peptidase (sp) and signal peptide peptidase (spp) or by virally encoded proteases (Lin *et al.*, 1994; Moradpour *et al.*, 2002). These structural proteins include the core protein (C), located at the N terminus of the polyprotein, and the envelope glycoproteins E1 and E2. The primary function of C is the assembly and packaging of the RNA genome (Kunkel *et al.*, 2001; Majeau *et al.*, 2004). C is cleaved from the polyprotein by a host sp into a protein of 191 aa (p23) (Santolini *et al.*, 1994). The C-terminal region of the protein is hydrophobic, allowing the protein to anchor to the endoplasmic reticulum (ER). A second cleavage by human spp (hspp) separates the C terminus of C from the rest of the protein and generates the mature form of C, p21 (Hüssy *et al.*, 1996; Ogino *et al.*, 2004).

hspp is a presenilin-related aspartic protease that catalyses intramembrane proteolysis of signal peptides. This protein comprises seven to nine putative transmembrane regions embedded in the ER (Golde & Younkin, 2001) and exists as a functional homodimer of 95 kDa (Nyborg *et al.*, 2004). The cleavage of C by hspp is believed to be important, as only the hspp-matured form of C is found in HCV virions isolated from the blood of infected patients (Yasui *et al.*, 1998). This maturation is also linked to the migration of C to the nucleus, lipid droplets (Barba *et al.*, 1997; McLauchlan *et al.*, 2002) and mitochondrion-associated ER membrane (MAM) (Schwer *et al.*, 2004). The association of C with lipid structures appears to be crucial for the prevention of degradation of the protein by proteasomes (Suzuki *et al.*, 2001).

Circulating HCV particles in chronically infected patients can be divided into different populations: (i) lipoprotein-associated virions with densities of 1·00–1·06 g ml^{-1} on a sucrose gradient; (ii) free enveloped virions with a density of 1·08–1·11 g ml^{-1}; (iii) virus particles of 1·17–1·21 g ml^{-1}, associated with immunoglobulins; and (iv) non-enveloped particles with high densities of 1·22–1·25 g ml^{-1} (Miyamoto *et al.*, 1992; Hijikata *et al.*, 1993; Kanto *et al.*, 1994; Choo *et al.*, 1995; Prince *et al.*, 1996; Ishida *et al.*, 2001; André *et al.*, 2002). In chronic infections, the dominant population shifts from low-density to high-density particles with the progression of liver disease or inflammation (Kanto *et al.*, 1995; Watson *et al.*, 1996). A plausible explanation for the presence of non-enveloped particles in the blood is their release into the circulation by the lysis of infected hepatocytes that accompanies liver inflammation. Non-enveloped particles have also been detected as viral inclusions in the cytoplasm of liver cells of infected patients (Falcón *et al.*, 2003). However, the production of

non-enveloped particles and their secretion are not very well understood.

Yeast has been shown to be useful in the study of virus replication and assembly (Sakuragi *et al.*, 2002; Schwartz *et al.*, 2004). Expression of C in the yeast *Pichia pastoris* leads to the formation of nucleocapsid-like particles (NLPs) that are very similar in size and shape to the virus found in the blood of infected patients (Falcón *et al.*, 1999; Acosta-Rivero *et al.*, 2001). In this study, we investigated the effect of hspp cleavage on NLP assembly and formation in yeast. We observed the formation of NLPs in cells expressing C in the absence of maturation. Co-expression of hspp correlated with an increase in the number of non-enveloped particles. Interestingly, the hspp protein was present at the surface of the enveloped NLPs. It appears that the protein is captured together with the membranes during virion formation and is exposed at the surface of the virus. A model for HCV particle formation is discussed.

METHODS

Cloning of the HCV C, CE1E2 and hspp proteins and their expression in *P. pastoris*. The clone of *P. pastoris* expressing HCV C (1–191) has been described previously (Majeau *et al.*, 2004). Plasmid pCV-H77c, with a cDNA clone of strain H77 (genotype 1a) of HCV (generously provided by J. Bukh, NIH, Bethesda, MA, USA), was used to PCR-amplify the structural genes of HCV CE1E2, which contains the nucleocapsid protein and the two envelope proteins, E1 and E2. CE1E2 was amplified by PCR with primers 5′-AAAACGCAGAGATCTATTAATTAATAATAATGTCTACGAAT-CCTAAACCTCAAAGAAAAACC-3′ and 5′-AGCTGAATTCTTAC-GCCTCCGCTTGGGA-3′. DNA products were then digested with *Bgl*II and *Eco*RI and ligated into the yeast vector pPIC3.5K (Invitrogen) to generate the clone CE1E2 (aa 1–747). The vector containing the structural genes was linearized by digestion with *Pme*I. The DNA clone was introduced into *P. pastoris* GS115 by spheroblasting and transformants were selected on geneticin plates (G418) according to the protocol provided by Invitrogen.

C (1–179) was amplified by PCR using primers 5′-CCTCCCA-TGGTGAGCACGAATCCTAAACCTCAAGAAAAAACC-3′ and 5′-GACGGGATCCTCACAGAAGGAAGATAGAGAAAGAGCAACC-3′, and cloned as an *Nco*I–*Bam*HI fragment in vector pET-3d (Novagen). *Escherichia coli* strain BL21 Star (Invitrogen) was then used to express the protein as described previously (Majeau *et al.*, 2004).

Plasmid pDAW200 (generously provided by Professor Dr Ari Helenius and Dr Bruno Martoglio, ETH Zurich, Switzerland) was used to generate the hspp gene. hspp was amplified by PCR with primers 5′-CTTGTTGAATTCCGAGCTCACTAGTTCACC-3′ and 5′-AGAACT-CTAGAGCTTCCTGAGAGCTCGGC-3′. DNA products were then digested by *Eco*RI and *Xba*I and ligated into pPIC6 B (Invitrogen) to create the clone hspp. The vector containing the hspp gene was linearized by digestion with *Pme*I. The DNA clone was introduced by electroporation into *P. pastoris* that already contained the C or CE1E2 gene and transformants were selected on blasticidin plates (Invitrogen). Recombinant proteins were induced with 1 % methanol and extracted as described previously (Majeau *et al.*, 2004).

SDS-PAGE and Western blotting. After lysis, protein extracts were denatured by mixing with an equal amount of buffer-saturated phenol and vortexing for 2 min. The hydrophobic phase was recovered after centrifugation at 20 000 ***g*** for 5 min and mixed with 5 vols methanol/0·1 M ammonium acetate solution. Proteins were recovered after 1 h incubation at −20 °C and spun at 13 000 r.p.m. for 5 min. Pellets were washed with methanol and, after drying, mixed with SDS loading dye. Tris/glycine and Tris/Bicine SDS-PAGE were performed as described by Lemberg & Martoglio (2003). After migration, proteins were transferred to nitrocellulose membranes by using a Trans-Blot apparatus (Bio-Rad) and revealed with an anti-C antibody (C1–170; Majeau *et al.*, 2004) or with anti-hspp antibodies (generously provided by Dr Todd E. Golde, Mayo Clinic, Jacksonville, FL, USA).

Density-gradient centrifugation. After lysis, yeast extracts were spun at 20 000 ***g*** and the supernatant was passed through a 0·2 μm filter. Protein concentration was determined by BCA protein assays (Pierce) and equal loads of protein were layered onto a continuous (10–60 % w/w) sucrose gradient [50 mM sodium citrate, pH 6, and 300 mM NaCl (final concentration)] and centrifuged for 20 h at 4 °C at 120 000 ***g*** in an NVT65 rotor (Beckman). For some experiments, similar amounts of C as determined by ELISA were separated by ultracentrifugation. Fractions were collected, mixed with NP-40 (0·6 %) and analysed by ELISA using a polyclonal rabbit antibody against HCV C protein (C1–170) (Majeau *et al.*, 2004). When stated, yeast extracts were incubated with 0·6 % NP-40 for 30 min at 4 °C before loading onto the sucrose gradient. ER membranes were labelled by inducing yeast in medium supplemented with 0·1 μM ER-Tracker Green (glibenclamide BODIPY FL; Invitrogen). Fluorescence in the sucrose fraction was analysed on a Typhoon 9200 fluorescence imager (Amersham Biosciences). To measure intact mitochondria in the gradient fractions, 2·5 μM MitoTracker Red CM-H_2XRos (Invitrogen) was mixed with 100 μl sample diluted in 200 μl PBS and incubated at 25 °C for 1 h. Fluorescence was detected on a Typhoon 9200 imager (Amersham Biosciences).

Electron microscopy (EM). Samples were absorbed onto 400-mesh carbon–Formvar grids (Canemco) for 5 min. Grids were washed with TBS and stained for 5 min with filtered 2 % (w/v) uranyl acetate. Immunogold labelling was performed as described previously (Majeau *et al.*, 2004), using anti-hspp antibodies. Grids were examined under an electronic microscope with an acceleration voltage of 60 kV at a magnification of × 100 000.

Immunocapture. After sucrose-gradient centrifugation, protein samples (100 μl) were diluted in PBS (pH 7·2) and mixed with magnetic beads (Dynabead m-280) conjugated to a sheep anti-rabbit antibody that had previously been incubated for 30 min with rabbit anti-hspp antibody, and then washed carefully. After 1 h incubation at 4 °C, protein mixes were exposed to magnetic forces and washed three times with PBS. SDS loading dye was added to the tube and extracted proteins were analysed by Western blot using anti-C antibody as described above.

RESULTS

Expression of HCV C in *P. pastoris* and its maturation by hspp

Expression and maturation of the HCV C protein were analysed in the yeast *P. pastoris*. The HCV C protein (aa 1–191) and CE1E2 polyprotein (aa 1–747) were cloned and expressed under the control of a methanol-inducible promoter in *P. pastoris*. After 24 h induction, proteins were extracted, separated by SDS-PAGE and analysed by Western blot using an anti-C antibody (Fig. 1a). The 179 aa C recombinant protein expressed and purified from *E. coli* served as a control for the fully processed HCV C (Fig. 1a, lane 1). The full-length C expressed from the C construct

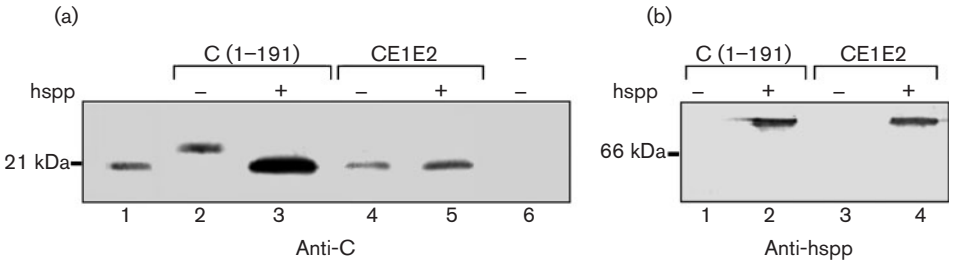

Fig. 1. Expression of HCV C protein and of hspp in *P. pastoris*. *P. pastoris* expressing HCV C (aa 1–191) or CE1E2 (aa 1–746), alone or with the hspp gene, was grown under inducing conditions for 24 h. (a) Yeast cells were lysed and equal loads of total protein were separated by SDS-PAGE and analysed by Western blotting using an anti-C antibody. As controls, extracts of *E. coli* expressing C (1–179) were loaded in the first lane and extracts of *P. pastoris* expressing the empty vector were loaded in the last lane. (b) Western blotting of C (1–191) and CE1E2; protein extracts were revealed by an anti-hspp antibody.

(1–191) appeared as a single band of 23 kDa (Fig. 1a, lane 2). This protein migrated more slowly than the control protein of 179 aa from *E. coli*, suggesting that processing of C (1–191) by spp to yield a protein of 177–179 aa (Hüssy *et al.*, 1996; McLauchlan *et al.*, 2002; Ogino *et al.*, 2004) is inefficient in *P. pastoris*. Expression of the polyprotein CE1E2 produced seven to ten times less C protein in the yeast than expression of the C (1–191) construct. Interestingly, the maturation of C from CE1E2 was complete after 24 h expression (Fig. 1a, lane 4). Acosta-Rivero *et al.* (2001) also observed the maturation of the C protein when the polyprotein CE1 (1–339) was used in *P. pastoris*. This result suggests that, in the context of the polyprotein, C adopts a different conformation in the C-terminal portion of the protein that allows a more efficient cleavage by the yeast spp-like enzymes.

To induce maturation of C (1–191) in *P. pastoris*, the hspp gene (Weihofen *et al.*, 2002) was cloned in a yeast vector and inserted into cells already transformed with C constructs. hspp in yeast was detected as a high-molecular-mass complex of approximately 100 kDa, as revealed by Western blotting using polyclonal anti-hspp antibodies (Fig. 1b). Consistent with our observation, hspp was shown to migrate in SDS-PAGE as a homodimer of 95 kDa that was resistant to SDS denaturation (Nyborg *et al.*, 2004). The co-expression of hspp with C (1–191) led to the production of a smaller form of C co-migrating with the recombinant control protein C (1–179) expressed in *E. coli*, suggesting a complete maturation of the C protein (Fig. 1a, lane 3). We also noticed that the C protein was more abundant when co-expressed with hspp (two to four times more protein). As the maturation of C in the context of the CE1E2 polyprotein was complete, co-expression with hspp did not produce any changes in the migration of the protein (Fig. 1a, lane 5); however, as for the C construct (1–191), the C protein content increased upon hspp expression.

hspp activity was revealed in a time-course experiment where C and hspp were expressed together with a methanol-inducible promoter (Fig. 2). A single band of 23 kDa was

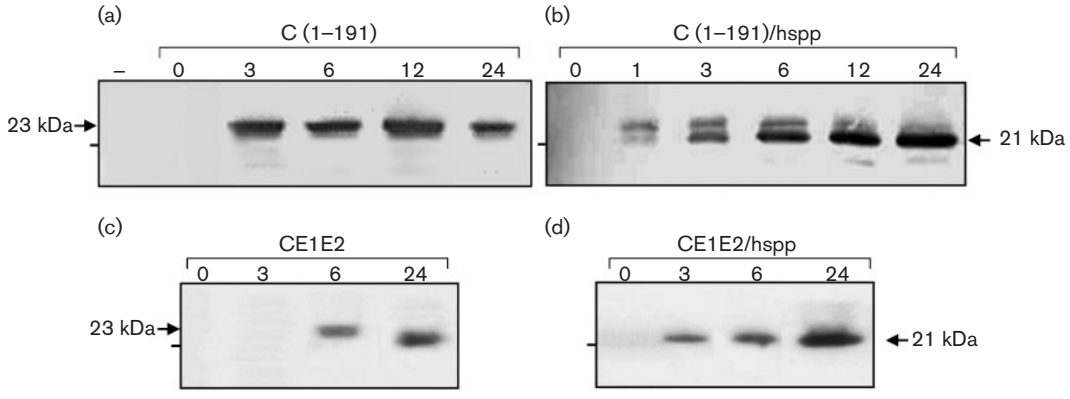

Fig. 2. Maturation of C protein in *P. pastoris* by hspp. *P. pastoris* expressing HCV C (1–191) or CE1E2, alone or with the hspp gene, was grown under inducing conditions for the induction times indicated (in h) at the top of each gel. Yeast cells were lysed and proteins [10 μg total protein for the C construct (1–191) and 25 μg for CE1E2] were separated by SDS-PAGE and analysed by Western blotting using an anti-C antibody. Extracts from mock-transfected cells are presented in the first lane of the gel (a). The mark on the left of each gel corresponds to a 20·5 kDa protein marker.

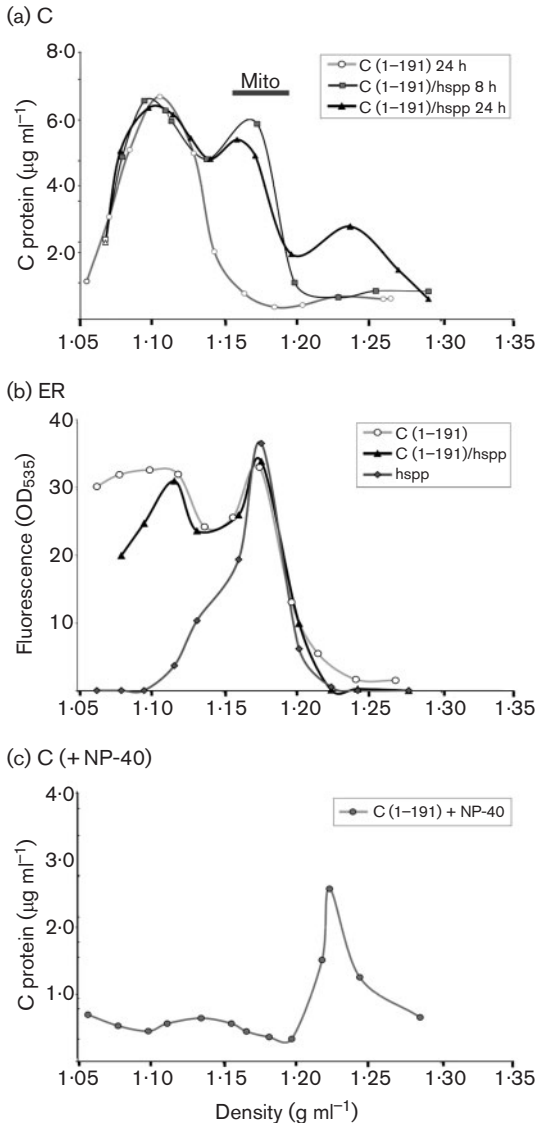

Fig. 3. Sucrose density-gradient fractionation of HCV particles produced in *P. pastoris*. (a) After 8 or 24 h induction, yeast cells expressing the C construct (1–191), with or without the hspp protein, were lysed and filtered with 0·2 μm membranes. Total protein (5 mg) was loaded onto a 10–60 % (w/w) continuous sucrose gradient and separated by ultracentrifugation. Collected fractions were treated with NP-40 (0·6 %) and C protein was analysed by ELISA with an anti-C antibody. Mitochondria were detected in the sucrose fractions with MitoTracker Red CM-H_2XRos. The bar shows the density where the MitoTracker was found. (b) Measurement of the fluorescence of 0·1 μM ER-Tracker Green (glibenclamide BODIPY FL) after separation on a sucrose gradient for yeast cells expressing C (1–191), C (1–191)/hspp or hspp alone, which were induced for 24 h in medium supplemented with 0·1 μM ER-Tracker Green (glibenclamide BODIPY FL) before sucrose-gradient separation. The fluorescence associated with ER membranes was detected by using a Typhoon 9200 fluorescence imager. (c) Sucrose density-gradient fractionation of protein extract of yeast expressing C (1–191) incubated with NP-40 (0·6 %) for 30 min at 4 °C before sedimentation on the gradient.

observed when the C construct (1–191) was expressed alone (Fig. 2a). The protein remained unprocessed even 72 h after induction (data not shown). However, co-expression of C (1–191) with hspp led to the appearance of a processed form (21 kDa) 1 h after induction (Fig. 2b). The 23 kDa precursor had been processed completely to 21 kDa, 12 h after induction of both proteins. In the context of the CE1E2 polyprotein, we observed that C was processed to the 21 kDa form by the yeast-encoded spp enzyme. However, co-expression of hspp accelerated the maturation of C; the maturation was completed after 3 h co-expression (Fig. 2d), instead of after 24 h when CE1E2 was expressed alone (Fig. 2c).

Effect of hspp on the accumulation of non-enveloped NLPs

Production in *P. pastoris* of NLPs similar in size and density to HCV virions has been shown previously (Acosta-Rivero *et al.*, 2001, 2003). To evaluate the importance of maturation on the formation of particles, C (1–191) clones, with or without hspp, were selected for sedimentation analysis. These clones showed similar levels of C mRNA on Northern blot (data not shown). Eight or 24 h after induction, proteins were extracted from yeast, clarified by centrifugation and applied to a sucrose-density gradient (10–60 % w/w) with an equal load of total protein. Fractions containing NLPs were analysed by ELISA. In the absence of hspp, the C protein was detected in fractions with a density of 1·08–1·12 g ml^{-1} (Fig. 3a). NLPs made of immature C protein showed a density similar to that of virions from the blood of infected patients (1·08–1·11 g ml^{-1}) (Miyamoto *et al.*, 1992; Kanto *et al.*, 1994), suggesting that these particles were enveloped with ER membranes. In this manuscript, we will call 'enveloped NLPs' those found at densities ranging from 1·08 to 1·11 g ml^{-1}. As described before for HCV virions (Miyamoto *et al.*, 1992), treatment of the samples with NP-40 before sedimentation was efficient at removing membranes associated with NLPs and shifted the C protein to densities of 1·22–1·25 g ml^{-1} (Fig. 3c), which corresponds to the density of non-enveloped particles. However, we observed fewer particles in samples treated with the detergent. As described by Thomssen & Bonk (2002), removing lipid membranes from the particles greatly affects the stability of the nucleocapsid.

Interestingly, co-expression of C with hspp generated a different pattern of sedimentation. Besides the peak at a density of 1·08–1·12 g ml^{-1}, the C protein was present at densities of 1·15–1·19 and 1·22–1·26 g ml^{-1} after 24 h induction (Fig. 3a). C was not detected in the dense fraction when induced for only 8 h. When samples were collected 3 h after induction, the two forms of C (21 and 23 kDa) (Fig. 2b, lane 3) were present in the fraction with a density of 1·11 g ml^{-1} (data not shown), suggesting that enveloped NLPs can be made of both the matured and the unprocessed forms of C.

It has previously been described that, after cleavage by spp, the C protein associates with the outer membranes of the mitochondria (Schwer *et al.*, 2004). In order to detect these

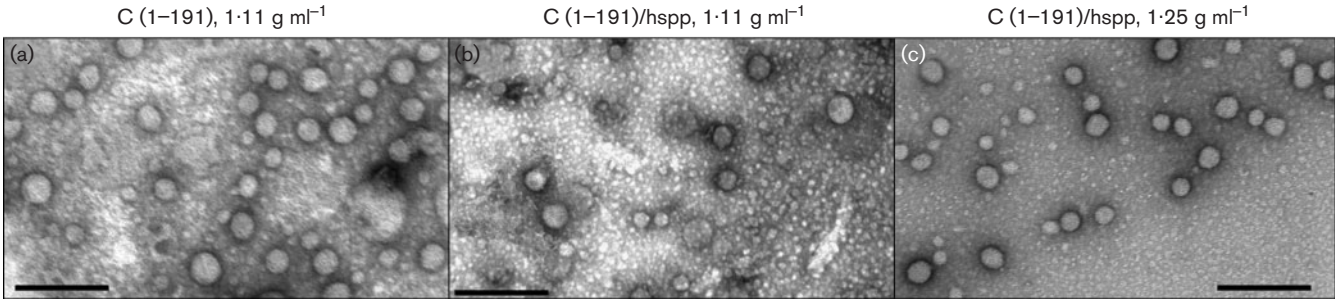

Fig. 4. Electron micrographs of HCV particles separated by sucrose-gradient centrifugation. Following centrifugation, the 1·11 g ml^{-1} fraction (a) from extracts of yeast expressing the C construct (1–191) and 1·11 g ml^{-1} (b) and 1·25 g ml^{-1} (c) fractions from yeast expressing it in combination with hspp were absorbed onto grids and washed before staining with 2 % uranyl acetate. Bars, 200 nm.

organelles, we mixed MitoTracker Red CM-H_2XRos, a reduced dye that becomes fluorescent when entering an actively respiring mitochondrion, with the sucrose-gradient fractions. We detected fluorescence in the fractions with densities of 1·16–1·20 g ml^{-1}, fractions previously described to contain mitochondria (Lee *et al.*, 1969; Walworth *et al.*, 1989) and corresponding to the second peak of mature C protein. As for ER membranes, they were detected in the sucrose fraction by using glibenclamide conjugated to a fluorochrome (ER-Tracker Green dye). Glibenclamide binds to the sulphonylurea receptors of ATP-sensitive K^+ channels, which are prominent on the ER. This dye is highly selective for the ER and rarely stains mitochondria. Yeast expressing the C construct (1–191), hspp or both was induced in medium containing 0·1 μM ER-Tracker Green dye for 24 h. Extracts were separated on a sucrose gradient and fluorescence in the samples was evaluated on a phosphorimager. As shown in Fig. 3(b), in the hspp extract, ER membranes were present at densities of 1·16–1·20 g ml^{-1}, as for the mitochondria. However, the presence of C shifted some of the ER membranes to less-dense fractions. ER membranes are expected to be associated with the NLPs with a density of 1·11 g ml^{-1}.

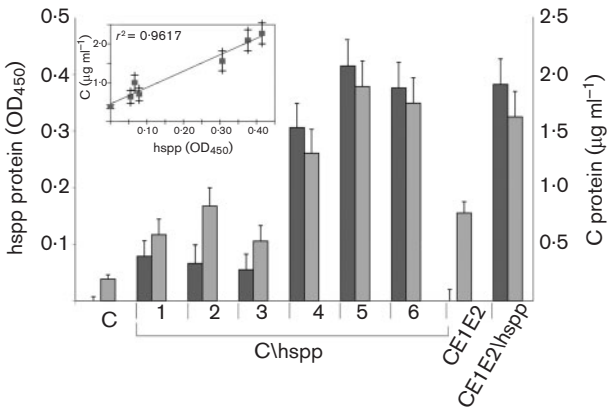

Fig. 5. Effect of co-expression of C and hspp on the accumulation of non-enveloped particles. *P. pastoris* cells expressing the C construct (1–191) were co-transformed with the hspp gene to generate several transformants expressing different levels of hspp (numbered 1–6). C concentration was determined by ELISA and 60 μg C from the different extracts was separated on a 10–60 % (w/w) sucrose gradient. Fractions at a density of 1·25 g ml^{-1} were analysed by ELISA with an anti-C antibody and the results were compared with the hspp content in total extracts, determined by ELISA using an anti-hspp antibody. Dark-shaded bars, hspp protein; light-shaded bars, non-enveloped NLPs. The small graph shows the direct correlation between the level of hspp and the accumulation of non-enveloped particles; $r^2 = 0{\cdot}9617$.

Particles of different densities in the fractions were visualized by EM (Fig. 4). In fractions with a density of 1·11 g ml^{-1} from C (1–191)-expressing cells, particles of 35–60 nm in diameter were observed (Fig. 4a), suggesting that the immature protein of 23 kDa can assemble into NLPs. These particles were similar to C (1–191)/hspp NLPs present at the same density (Fig. 4b) and resembled enveloped virus particles isolated from infected patients (Kaito *et al.*, 1994). Non-enveloped NLPs from C (1–191)/hspp-expressing cells, detected at a density of 1·25 g ml^{-1}, showed a diameter of 28–45 nm (Fig. 4c), i.e. within the size range of non-enveloped particles characterized previously (Maillard *et al.*, 2001). In the 1·17 g ml^{-1} density fraction, we observed membranes, aggregated material and mitochondria, but NLPs were not found.

To correlate the accumulation of non-enveloped NLPs with the amount of hspp, we selected clones of yeast expressing different concentrations of the hspp protein. For each isolate, the same amount of C was separated by sucrose-gradient centrifugation. We observed a correlation between the variations of C protein in the 1·25 g ml^{-1} fraction and the expression level of hspp in the cells ($r^2 = 0{\cdot}96$) (Fig. 5). We showed earlier that the rate of C maturation in the

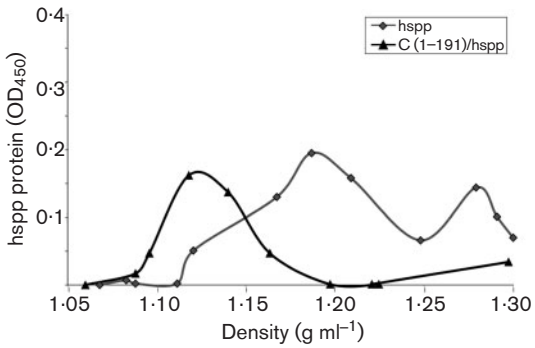

Fig. 6. Analysis of hspp sedimentation on sucrose gradients. Yeast extracts were filtered and 5 mg protein was separated on a 10–60 % (w/w) continuous sucrose gradient. Protein samples were analysed by ELISA with an anti-hspp antibody.

context of the CE1E2 polyprotein increased upon expression of hspp. We also subjected these samples to the sedimentation experiment. As for C isolates, increasing hspp activity resulted in a higher accumulation of non-enveloped particles (Fig. 5). These results suggest that the amount of hspp in the cell has a direct impact on the accumulation of non-enveloped NLPs.

Association of hspp with enveloped NLPs

The hspp protein has been shown to be associated with the ER membrane (Martoglio & Golde, 2003). As ER membranes are a component of HCV virions (Dubuisson *et al.*, 2002; Roingeard *et al.*, 2004), we hypothesized that hspp could become sequestered during the budding process. To explore its association with the different forms of NLPs, we searched for its presence in the different fractions of the sucrose-density gradient. As expected, hspp, in the absence of C, was recovered with fractions of a density between 1·18 and 1·20 g ml^{-1}, with rough ER membranes (Walworth *et al.*, 1989) (Fig. 6). However, co-expression of the C protein triggered a change in sedimentation of the protein. We observed a shift of all the hspp toward the 1·11 g ml^{-1} fraction, containing enveloped particles. In cells with high expression of hspp, the protein could also be detected at a density of 1·20 g ml^{-1}, probably due to saturation of the NLPs (data not shown).

To confirm the presence of hspp at the surface of the NLPs, we captured the particles by using an anti-hspp antibody. A Western blot was performed to detect the presence of C within the isolated complex (Fig. 7a). Antibodies against hspp were able to pull down the C protein from the enveloped NLP fraction of the C (1–191)/hspp extract

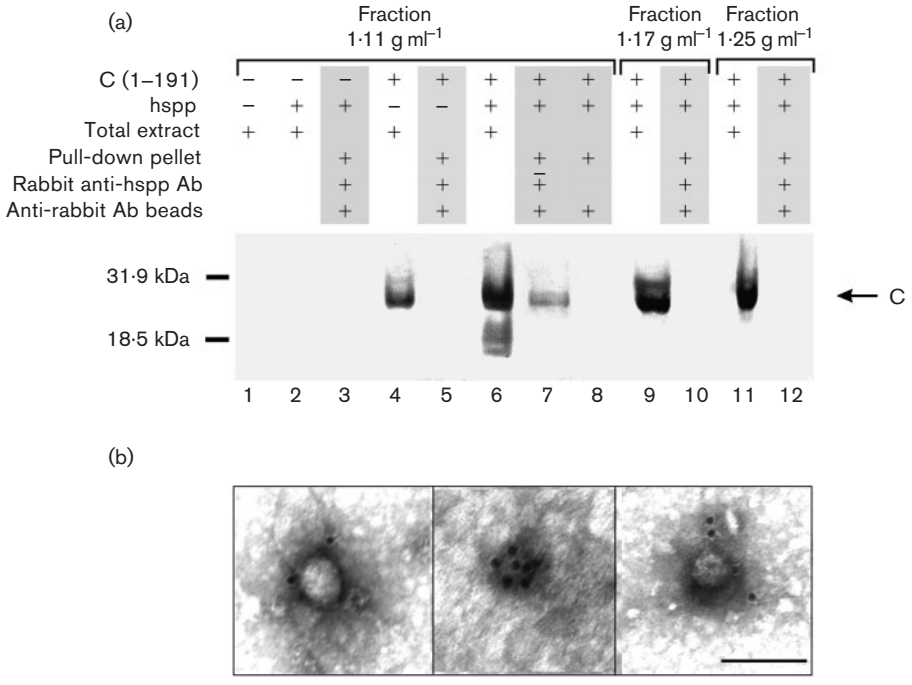

Fig. 7. Immunocapture of HCV particles with antibodies against hspp. (a) Total proteins of yeast extracts were separated by sucrose-gradient sedimentation. An hspp pull-down assay using the anti-hspp rabbit polyclonal antibody and magnetic beads coated with anti-rabbit antibody was done on the sucrose density-gradient fractions. After washing, proteins were denatured and analysed by Western blotting using an anti-C antibody. Yeast extracts with the empty vector are found in lane 1. The pellets of the pull-down assay were loaded in lanes 3, 5, 7, 8, 10 and 12 and the other lanes represent protein samples before the immunoprecipitation. (b) Particles from C (1–191)/hspp-expressing cells present in the 1·11 g ml^{-1} fraction were absorbed onto grids and labelled with an anti-hspp antibody, followed by a gold-conjugated anti-rabbit antibody. Bar, 100 nm.

(Fig. 7a, lane 7), but could not extract C from the sucrose fractions generated with samples of the cells expressing either the C construct (1–191) or hspp (Fig. 7a, lanes 3 and 5). Also, the anti-hspp antibody was unable to pull down C from fractions of 1·17 and 1·25 g ml^{-1} density in C (1–191)/hspp extracts (Fig. 7a, lanes 10 and 12), suggesting that hspp is not associated directly with C, but rather with the ER membranes surrounding NLPs. Enveloped NLPs from cells expressing C (1–191)/hspp were immunolabelled with anti-hspp antibody and labelled with gold with a secondary IgG to be visualized by EM (Fig. 7b). We did not detect any labelling of the HCV NLPs when the secondary IgG (gold anti-rabbit antibody) was used alone. The number of gold particles (two to five) found on the surface of the NLPs was low, suggesting that hspp is not abundant on the surface of the envelope of the NLPs. This is consistent with the data obtained with the immunoprecipitation (Fig. 7a), where only a fraction of NLPs could be pulled down with hspp IgG. However, both experiments show the association of hspp with the envelope of the NLPs.

DISCUSSION

The *P. pastoris* yeast-expression system was shown to be a good system for expressing the HCV C protein and to study the formation of NLPs, as observed by Acosta-Rivero *et al.* (2002). NLPs were present on membranes of the ER and in vacuoles of *P. pastoris*. Anchoring of C into the ER via its C terminus was showed to be essential for accumulation of NLPs (Majeau *et al.*, 2004). Processing and maturation of C from the CE1 polyprotein were also observed in these cells (Acosta-Rivero *et al.*, 2003). In this study, we showed that maturation of full-length C to its matured form (21 kDa) was inefficient in *P. pastoris* cells expressing the C (1–191) construct. Other mammalian proteins expressed in yeast have been reported to be processed poorly by the host spp (Weihofen *et al.*, 2002; Wu & Chang, 2004). However, we observed maturation of C by the yeast-encoded spp (or spp-like protease) when it was expressed as the polyprotein CE1E2. These results suggest that the C terminus of C is presented in a more favourable context for cleavage by spp when produced with the polyprotein.

The resistance of C (1–191) to cleavage by the yeast spp allowed us to show that the maturation of C is not essential for the budding process and that immature C protein is able to recruit membrane around the NLPs. We observed that immature full-length C can generate NLPs of 35–60 nm diameter with a density of 1·11 g ml^{-1}. Treatment with NP-40 removed the envelope and shifted the NLPs to a density of 1·25 g ml^{-1}, as shown for HCV particles isolated from the serum of infected patients (Miyamoto *et al.*, 1992; Kanto *et al.*, 1994; Ishida *et al.*, 2001). However, the C protein detected in the blood of infected patients was present only as the mature 21 kDa form (Yasui *et al.*, 1998). Interestingly, we did not find free C protein associated with the rough ER membrane fraction occurring at a density of 1·18 g ml^{-1} (Walworth *et al.*, 1989), suggesting that encapsidation and NLP formation are processes that occur rapidly after synthesis.

We observed that the yield of C in yeast was higher when the protein was co-expressed with hspp. It is likely that overexpression of C alone leads to clogging in the ER, which affects the fitness of the yeast and thus the yield of protein. The maturation of C with hspp promotes the release of C from this compartment, probably releasing the physiological stress on the cell.

Co-expression of hspp in yeast led to efficient processing of C into its mature form. The processed C protein was found at three different densities: 1·11 g ml^{-1} (enveloped NLPs), 1·17 g ml^{-1} (free C protein) and 1·25 g ml^{-1} (non-enveloped NLPs). It has previously been proposed that C, after hspp cleavage, travels along the ER membrane and reaches the surface of lipid droplets that are formed between the two layers of the ER membrane (McLauchlan *et al.*, 2002) or diffuses to the surface of MAM (Schwer *et al.*, 2004). Yeast produces a small number of cytosolic lipid bodies (Murphy & Vance, 1999). This shortage in lipid droplets may favour accumulation of C on the surface of mitochondrial membranes. The MAM fraction is described as fractionating at a density of 1·17–1·20 g ml^{-1} after sucrose-gradient centrifugation (Rusiñol *et al.*, 1994). The presence of C protein in the 1·17 g ml^{-1} fraction probably reflects association with these structures.

Non-enveloped particles have been detected in blood and in hepatocytes (Miyamoto *et al.*, 1992). The degree of liver inflammation also influences the number of non-enveloped virions circulating in the blood (Kanto *et al.*, 1994). Here, we demonstrate that maturation of the C protein by hspp is essential for the production of free nucleocapsid in the cells. C protein was only present in the 1·25 g ml^{-1} fraction when hspp was co-expressed. The accumulation of NLPs also correlated with the amount of hspp expressed in yeast. These results suggest that, after maturation by hspp, C protein is free to move from the ER membranes and becomes organized in NLPs without any lipid envelope. This capacity to self-assemble without ER membranes has been observed in *in vitro* experiments where the purified protein mixed with structured RNA was sufficient for producing particles (Kunkel *et al.*, 2001; Lorenzo *et al.*, 2001; Majeau *et al.*, 2004).

Protein concentration is important in the initiation of NLP formation. C needs to be expressed at a very high level to trigger the formation of NLPs in cellular systems (Baumert *et al.*, 1998; Blanchard *et al.*, 2002; Ezelle *et al.*, 2002; Greive *et al.*, 2002). As the C protein is first trapped in enveloped NLPs, only a small amount of mature protein is available for the formation of non-enveloped NLPs. This can account for the late production of non-enveloped NLPs after expression.

Several viruses carry the proteases necessary for maturation of their nucleocapsid within the virus particle itself (Greber *et al.*, 1996; Andrés *et al.*, 2001). Similarly, our experiments

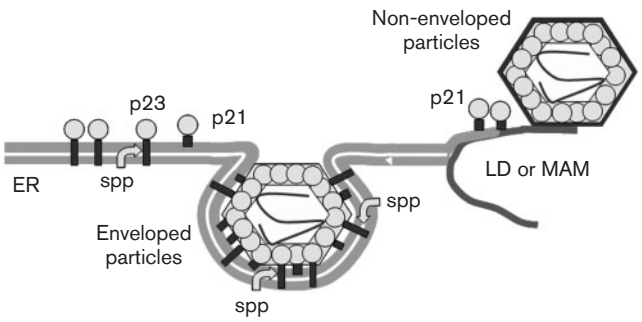

Fig. 8. Model for HCV particle formation. After accumulation at the ER membrane, immature C proteins (p23) initiate particle formation with adjacent protein (mature or immature) and recruit ER membrane containing spp protein. Maturation of the C protein then occurs within the well-formed particle. Mature proteins that are not taken on by the particles migrate to lipid droplets (LD) or MAM structures, where they accumulate; the nucleocapsid formed thereof is free of ER membranes.

show that hspp is sequestered within the membrane of enveloped NLPs. In sedimentation experiments, we observed a shift of hspp toward the fraction of enveloped particles. C was also pulled down when samples were incubated with anti-spp antibody. It was shown previously that hspp is not associated directly with the C protein (Okamoto *et al.*, 2004). We propose that immature C protein triggers the formation of a virion-like structure, incorporating ER membrane via its C-terminal anchoring domain, thus capturing hspp associated with the ER (Fig. 8). The capsid possibly comprises both mature and immature protein. Completion of the maturation of the virus particle can then occur after the budding process.

For several viruses, including *Human immunodeficiency virus 1*, the maturation of the capsid protein by a protease activity is important for infectivity of the virus (Kiernan *et al.*, 1998; Alejo *et al.*, 2003). It is likely that this also applies to HCV. Consistently, Kato *et al.* (2003) provided evidence that a mutation in the HCV C protein that decreases the efficiency of maturation of the 23 kDa precursor to the 21 kDa mature form by the hspp also affects the infectivity of the virus. As previously proposed by Martoglio & Golde (2003), this observation suggests that if we could inhibit the activity of the hspp with a specific drug, we could maintain the C protein in its 23 kDa precursor form and decrease the infectivity of the virus. This approach could help chronically infected patients to clear the infection.

The amount of hspp protein is probably an important factor in the infection process. It is likely that a large proportion of hspp is sequestered in budding virions, simultaneously reducing the amount of the enzyme in the ER compartment of the infected cells. The lack of hspp can be toxic for the cells (Wu & Chang, 2004) and can impair the immune response by affecting the maturation and migration to the surface of major histocompatibility complex (MHC) class I antigens (Bland *et al.*, 2003). Consistent with this observation, it was shown recently that MHC class I presentation by dendritic cells was impaired in transgenic mice expressing HCV structural proteins (Hiasa *et al.*, 2004).

In summary, we have shown that the level of hspp protein influences the number of non-enveloped particles. The major HCV populations are described as changing from virions to nucleocapsids with the progression of liver disease or inflammation (Kanto *et al.*, 1995; Watson *et al.*, 1996). It is possible that a change in hspp expression occurs during HCV infection and that this contributes to an increase in non-enveloped particles in infected cells. We are currently investigating the effect of HCV infection on hspp in infected patients.

ACKNOWLEDGEMENTS

We thank Helen Rothnie for editing of the manuscript. This work was supported by grants from the Canadian Institutes of Health Research of Canada (CIHR) and the 'Réseau SIDAMI' of the Fonds de la Recherche en Santé du Québec (FRSQ).

REFERENCES

Acosta-Rivero, N., Aguilar, J. C., Musacchio, A., Falcón, V., Viña, A., de la Rosa, M. C. & Morales, J. (2001). Characterization of the HCV core virus-like particles produced in the methylotrophic yeast *Pichia pastoris*. *Biochem Biophys Res Commun* **287**, 122–125.

Acosta-Rivero, N., Musacchio, A., Lorenzo, L., Alvarez, C. & Morales, J. (2002). Processing of the hepatitis C virus precursor protein expressed in the methylotrophic yeast *Pichia pastoris*. *Biochem Biophys Res Commun* **295**, 81–84.

Acosta-Rivero, N., Falcón, V., Alvarez, C. & 12 other authors (2003). Structured HCV nucleocapsids composed of P21 core protein assemble primary in the nucleus of *Pichia pastoris* yeast. *Biochem Biophys Res Commun* **310**, 48–53.

Alejo, A., Andrés, G. & Salas, M. L. (2003). African swine fever virus proteinase is essential for core maturation and infectivity. *J Virol* **77**, 5571–5577.

André, P., Komurian-Pradel, F., Deforges, S. & 7 other authors (2002). Characterization of low- and very-low-density hepatitis C virus RNA-containing particles. *J Virol* **76**, 6919–6928.

Andrés, G., Alejo, A., Simón-Mateo, C. & Salas, M. L. (2001). African swine fever virus protease, a new viral member of the SUMO-1-specific protease family. *J Biol Chem* **276**, 780–787.

Barba, G., Harper, F., Harada, T. & 8 other authors (1997). Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. *Proc Natl Acad Sci U S A* **94**, 1200–1205.

Baumert, T. F., Ito, S., Wong, D. T. & Liang, T. J. (1998). Hepatitis C virus structural proteins assemble into viruslike particles in insect cells. *J Virol* **72**, 3827–3836.

Blanchard, E., Brand, D., Trassard, S., Goudeau, A. & Roingeard, P. (2002). Hepatitis C virus-like particle morphogenesis. *J Virol* **76**, 4073–4079.

Bland, F. A., Lemberg, M. K., McMichael, A. J., Martoglio, B. & Braud, V. M. (2003). Requirement of the proteasome for the trimming of signal peptide-derived epitopes presented by the

nonclassical major histocompatibility complex class I molecule HLA-E. *J Biol Chem* **278**, 33747–33752.

Choo, Q. L., Kuo, G., Weiner, A. J., Overby, L. R., Bradley, D. M. & Houghton, M. (1989). Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **244**, 359–362.

Choo, S.-H., So, H.-S., Cho, J. M. & Ryu, W.-S. (1995). Association of hepatitis C virus particles with immunoglobulin: a mechanism for persistent infection. *J Gen Virol* **76**, 2337–2341.

Dubuisson, J., Penin, F. & Moradpour, D. (2002). Interaction of hepatitis C virus proteins with host cell membranes and lipids. *Trends Cell Biol* **12**, 517–523.

Ezelle, H. J., Markovic, D. & Barber, G. N. (2002). Generation of hepatitis C virus-like particles by use of a recombinant vesicular stomatitis virus vector. *J Virol* **76**, 12325–12334.

Falcón, V., García, C., de la Rosa, M. C., Menéndez, I., Seoane, J. & Grillo, J. M. (1999). Ultrastructural and immunocytochemical evidences of core-particle formation in the methylotrophic *Pichia pastoris* yeast when expressing HCV structural proteins (core-E1). *Tissue Cell* **31**, 117–125.

Falcón, V., Acosta-Rivero, N., Chinea, G. & 12 other authors (2003). Ultrastructural evidences of HCV infection in hepatocytes of chronically HCV-infected patients. *Biochem Biophys Res Commun* **305**, 1085–1090.

Golde, T. E. & Younkin, S. G. (2001). Presenilins as therapeutic targets for the treatment of Alzheimer's disease. *Trends Mol Med* **7**, 264–269.

Greber, U. F., Webster, P., Weber, J. & Helenius, A. (1996). The role of the adenovirus protease in virus entry into cells. *EMBO J* **15**, 1766–1777.

Greive, S. J., Webb, R. I., Mackenzie, J. M. & Gowans, E. J. (2002). Expression of the hepatitis C virus structural proteins in mammalian cells induces morphology similar to that in natural infection. *J Viral Hepat* **9**, 9–17.

Hiasa, Y., Takahashi, H., Shimizu, M., Nuriya, H., Tsukiyama-Kohara, K., Tanaka, T., Horiike, N., Onji, M. & Kohara, M. (2004). Major histocompatibility complex class-I presentation impaired in transgenic mice expressing hepatitis C virus structural proteins during dendritic cell maturation. *J Med Virol* **74**, 253–261.

Hijikata, M., Shimizu, Y., Kato, H., Iwamoto, A., Shih, J. W., Alter, H. J., Purcell, R. H. & Yoshikura, H. (1993). Equilibrium centrifugation studies of hepatitis C virus: evidence for circulating immune complexes. *J Virol* **67**, 1953–1958.

Hüssy, P., Langen, H., Mous, J. & Jacobsen, H. (1996). Hepatitis C virus core protein: carboxy-terminal boundaries of two processed species suggest cleavage by a signal peptide peptidase. *Virology* **224**, 93–104.

Ishida, S., Kaito, M., Kohara, M., Tsukiyama-Kohora, K., Fujita, N., Ikoma, J., Adachi, Y. & Watanabe, S. (2001). Hepatitis C virus core particle detected by immunoelectron microscopy and optical rotation technique. *Hepatol Res* **20**, 335–347.

Kaito, M., Watanabe, S., Tsukiyama-Kohara, K. & 7 other authors (1994). Hepatitis C virus particle detected by immunoelectron microscopic study. *J Gen Virol* **75**, 1755–1760.

Kanto, T., Hayashi, N., Takehara, T., Hagiwara, H., Mita, E., Naito, M., Kasahara, A., Fusamoto, H. & Kamada, T. (1994). Buoyant density of hepatitis C virus recovered from infected hosts: two different features in sucrose equilibrium density-gradient centrifugation related to degree of liver inflammation. *Hepatology* **19**, 296–302.

Kanto, T., Hayashi, N., Takehara, T., Hagiwara, H., Mita, E., Naito, M., Kasahara, A., Fusamoto, H. & Kamada, T. (1995). Density analysis of hepatitis C virus particle population in the circulation of infected hosts: implications for virus neutralization or persistence. *J Hepatol* **22**, 440–448.

Kato, T., Miyamoto, M., Furusaka, A., Date, T., Yasui, K., Kato, J., Matsushima, S., Komatsu, T. & Wakita, T. (2003). Processing of hepatitis C virus core protein is regulated by its C-terminal sequence. *J Med Virol* **69**, 357–366.

Kiernan, R. E., Ono, A., Englund, G. & Freed, E. O. (1998). Role of matrix in an early postentry step in the human immunodeficiency virus type 1 life cycle. *J Virol* **72**, 4116–4126.

Kunkel, M., Lorinczi, M., Rijnbrand, R., Lemon, S. M. & Watowich, S. J. (2001). Self-assembly of nucleocapsid-like particles from recombinant hepatitis C virus core protein. *J Virol* **75**, 2119–2129.

Lauer, G. M. & Walker, B. D. (2001). Hepatitis C virus infection. *N Engl J Med* **345**, 41–52.

Lee, T. C., Swartzendruber, D. C. & Snyder, F. (1969). Zonal centrifugation of microsomes from rat liver: resolution of rough- and smooth-surfaced membranes. *Biochem Biophys Res Commun* **36**, 748–755.

Lemberg, M. K. & Martoglio, B. (2003). Analysis of polypeptides by sodium dodecyl sulfate–polyacrylamide gel electrophoresis alongside in vitro-generated reference peptides. *Anal Biochem* **319**, 327–331.

Lin, C., Lindenbach, B. D., Prágai, B. M., McCourt, D. W. & Rice, C. M. (1994). Processing in the hepatitis C virus E2-NS2 region: identification of p7 and two distinct E2-specific products with different C termini. *J Virol* **68**, 5063–5073.

Lorenzo, L. J., Dueñas-Carrera, S., Falcón, V., Acosta-Rivero, N., González, E., de la Rosa, M. C., Menéndez, I. & Morales, J. (2001). Assembly of truncated HCV core antigen into virus-like particles in *Escherichia coli*. *Biochem Biophys Res Commun* **281**, 962–965.

Maillard, P., Krawczynski, K., Nitkiewicz, J. & 7 other authors (2001). Nonenveloped nucleocapsids of hepatitis C virus in the serum of infected patients. *J Virol* **75**, 8240–8250.

Majeau, N., Gagné, V., Boivin, A., Bolduc, M., Majeau, J.-A., Ouellet, D. & Leclerc, D. (2004). The N-terminal half of the core protein of hepatitis C virus is sufficient for nucleocapsid formation. *J Gen Virol* **85**, 971–981.

Martoglio, B. & Golde, T. E. (2003). Intramembrane-cleaving aspartic proteases and disease: presenilins, signal peptide peptidase and their homologs. *Hum Mol Genet* **12**, R201–R206.

McLauchlan, J., Lemberg, M. K., Hope, G. & Martoglio, B. (2002). Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. *EMBO J* **21**, 3980–3988.

Miyamoto, H., Okamoto, H., Sato, K., Tanaka, T. & Mishiro, S. (1992). Extraordinarily low density of hepatitis C virus estimated by sucrose density gradient centrifugation and the polymerase chain reaction. *J Gen Virol* **73**, 715–718.

Moradpour, D., Brass, V., Gosert, R., Wölk, B. & Blum, H. E. (2002). Hepatitis C: molecular virology and antiviral targets. *Trends Mol Med* **8**, 476–482.

Murphy, D. J. & Vance, J. (1999). Mechanisms of lipid-body formation. *Trends Biochem Sci* **24**, 109–115.

Nyborg, A. C., Kornilova, A. Y., Jansen, K., Ladd, T. B., Wolfe, M. S. & Golde, T. E. (2004). Signal peptide peptidase forms a homodimer that is labeled by an active site-directed γ-secretase inhibitor. *J Biol Chem* **279**, 15153–15160.

Ogino, T., Fukuda, H., Imajoh-Ohmi, S., Kohara, M. & Nomoto, A. (2004). Membrane binding properties and terminal residues of the mature hepatitis C virus capsid protein in insect cells. *J Virol* **78**, 11766–11777.

Okamoto, K., Moriishi, K., Miyamura, T. & Matsuura, Y. (2004). Intramembrane proteolysis and endoplasmic reticulum retention of hepatitis C virus core protein. *J Virol* **78**, 6370–6380.

Prince, A. M., Huima-Byron, T., Parker, T. S. & Levine, D. M. (1996). Visualization of hepatitis C virions and putative defective interfering particles isolated from low-density lipoproteins. *J Viral Hepat* **3**, 11–17.

Roingeard, P., Hourioux, C., Blanchard, E., Brand, D. & Ait-Goughoulte, M. (2004). Hepatitis C virus ultrastructure and morphogenesis. *Biol Cell* **96**, 103–108.

Rusiñol, A. E., Cui, Z., Chen, M. H. & Vance, J. E. (1994). A unique mitochondria-associated membrane fraction from rat liver has a high capacity for lipid synthesis and contains pre-Golgi secretory proteins including nascent lipoproteins. *J Biol Chem* **269**, 27494–27502.

Sakuragi, S., Goto, T., Sano, K. & Morikawa, Y. (2002). HIV type 1 Gag virus-like particle budding from spheroplasts of *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A* **99**, 7956–7961.

Santolini, E., Migliaccio, G. & La Monica, N. (1994). Biosynthesis and biochemical properties of the hepatitis C virus core protein. *J Virol* **68**, 3631–3641.

Schwartz, M., Chen, J., Lee, W.-M., Janda, M. & Ahlquist, P. (2004). Alternate, virus-induced membrane rearrangements support positive-strand RNA virus genome replication. *Proc Natl Acad Sci U S A* **101**, 11263–11268.

Schwer, B., Ren, S., Pietschmann, T., Kartenbeck, J., Kaehlcke, K., Bartenschlager, R., Yen, T. S. B. & Ott, M. (2004). Targeting of hepatitis C virus core protein to mitochondria through a novel C-terminal localization motif. *J Virol* **78**, 7958–7968.

Suzuki, R., Tamura, K., Li, J., Ishii, K., Matsuura, Y., Miyamura, T. & Suzuki, T. (2001). Ubiquitin-mediated degradation of hepatitis C virus core protein is regulated by processing at its carboxyl terminus. *Virology* **280**, 301–309.

Thomssen, R. & Bonk, S. (2002). Virolytic action of lipoprotein lipase on hepatitis C virus in human sera. *Med Microbiol Immunol (Berl)* **191**, 17–24.

Walworth, N. C., Goud, B., Ruohola, H. & Novick, P. J. (1989). Fractionation of yeast organelles. *Methods Cell Biol* **31**, 335–356.

Watson, J. P., Bevitt, D. J., Spickett, G. P., Toms, G. L. & Bassendine, M. F. (1996). Hepatitis C virus density heterogeneity and viral titre in acute and chronic infection: a comparison of immunodeficient and immunocompetent patients. *J Hepatol* **25**, 599–607.

Weihofen, A., Binns, K., Lemberg, M. K., Ashman, K. & Martoglio, B. (2002). Identification of signal peptide peptidase, a presenilin-type aspartic protease. *Science* **296**, 2215–2218.

Wu, C.-M. & Chang, M. D.-T. (2004). Signal peptide of eosinophil cationic protein is toxic to cells lacking signal peptide peptidase. *Biochem Biophys Res Commun* **322**, 585–592.

Yasui, K., Wakita, T., Tsukiyama-Kohara, K., Funahashi, S.-I., Ichikawa, M., Kajita, T., Moradpour, D., Wands, J. R. & Kohara, M. (1998). The native form and maturation process of hepatitis C virus core protein. *J Virol* **72**, 6048–6055.