Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 325 (2004) 68–74
BBRC
www.elsevier.com/locate/ybbrc

In vitro assembly into virus-like particles is an intrinsic quality of *Pichia pastoris* derived HCV core protein

Nelson Acosta-Rivero[a,*,1], Armando Rodriguez[b,*,1], Alexis Musacchio[a], Viviana Falcón[b], Viana M. Suarez[c], Gillian Martinez[a], Ivis Guerra[a], Dalila Paz-Lago[a], Yanelys Morera[a], María C. de la Rosa[b], Juan Morales-Grillo[a], Santiago Dueñas-Carrera[a]

[a] *Hepatitis C Department, Center for Genetic Engineering and Biotechnology, P.O. Box 6162, C.P. 10600, Cuba*
[b] *Physical Chemistry Department, Center for Genetic Engineering and Biotechnology, P.O. Box 6162, C.P. 10600, Cuba*
[c] *Molecular Biology Department, Neurosciences Center, Havana, Cuba*

Received 10 September 2004

Abstract

Different variants of hepatitis C virus core protein (HCcAg) have proved to self-assemble in vitro into virus-like particles (VLPs). However, difficulties in obtaining purified mature HCcAg have limited these studies. In this study, a high degree of monomeric HCcAg purification was accomplished using chromatographic procedures under denaturing conditions. Size exclusion chromatography and sucrose density gradient centrifugation of renatured HCcAg (in the absence of structured RNA) under reducing conditions suggested that it assembled into empty capsids. The electron microscopy analysis of renatured HCcAg showed the presence of spherical VLPs with irregular shapes and an average diameter of 35 nm. Data indicated that HCcAg monomers assembled in vitro into VLPs in the absence of structured RNA, suggesting that recombinant HCcAg used in this work contains all the information necessary for the assembly process. However, they also suggest that some cellular factors might be required for the proper in vitro assembly of capsids.

Keywords: Hepatitis C; Core antigen; Virus-like particles; *Pichia pastoris*

The hepatitis C virus (HCV) is an RNA virus that replicates via a virally encoded RNA polymerase [1]. It is thought that the viral particle consists of a nucleocapsid containing a positive-sense, single-stranded RNA genome of approximately 9500 nucleotides (nt) [2]. The HCV nucleocapsid is surrounded by an envelope derived from host membranes into which are inserted the virally encoded glycoproteins (E1 and E2) [3]. The viral products (core, E1, E2, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) are processed from a 3000-amino acid (aa) polyprotein expressed from a single open reading frame [4,5].

The biogenesis of HCV core protein (HCcAg) is dependent on the interaction of the signal sequence of nascent polypeptide with the endoplasmic reticulum (ER) membrane [5,6]. In the ER lumen, the cleavage between aa 191 and 192 by cellular signalase generates the immature form of HCcAg (P23). Additional processing of P23 by the intramembrane protease signal peptide peptidase (SPP) between aa 173 and 182 produces the mature HCcAg (termed P21) (Note that some authors

* Corresponding authors. Fax: +53 7 2714764 (N. Acosta-Rivero); +53 7 33 6008, +53 7 21 8070 (N. Acosta-Rivero and A. Rodriguez).
E-mail addresses: nelson.acosta@cigb.edu.cu (N. Acosta-Rivero), armando.rodriguez@cigb.edu.cu (A. Rodriguez).
[1] Both authors have contributed equally to this work and should be considered as first authors.

0006-291X/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2004.10.012

referred P23 and P21 as P21 and P19, respectively) [7–9]. In addition, it has been shown that HCcAg processing in yeast cells is similar to that observed in mammalian cells, suggesting that processing of HCcAg by a signal peptidase occurs in yeast [10,11].

It has been suggested that P21 represents the native HCcAg found in viral particles present in the sera of HCV-infected patients [12,13]. Non-enveloped HCV nucleocapsids present in the plasma and hepatocytes of HCV-infected individuals showed diameters between 33 and 62 nm [14–16]. Besides, optical rotational EM techniques have revealed that the structure of the HCV capsid exhibits sixfold symmetry with a regular hexagon side of 20 nm [15]. These particles showed a buoyant density of 1.22–1.25 g/ml in sucrose gradients and of 1.32–1.34 g/ml in CsCl gradients [15–17]. In addition, nucleocapsid-like particles (NLPs) produced in yeast, *Escherichia coli*, and insect cells showed similar size and buoyant density to naturally occurring nucleocapsids [16,18–22]. Moreover, different variants of HCcAg have proved to self-assemble in vitro into NLPs [22–24]. However, difficulties in obtaining purified mature HCcAg have limited these studies [23].

The fact that structures resembling HCV nucleocapsid particles in a mature stage assembled in *Pichia pastoris* cells and also in vitro could facilitate studies concerning structural composition and assembly mechanisms [19,20,22,23]. In this study, the intrinsic ability of the mature form of processed HCcAg produced in *P. pastoris* cells to assemble in vitro into virus-like particles (VLPs) was investigated.

Materials and methods

Pichia pastoris strains and growth conditions. The *P. pastoris* strain MP-36/C-E1.339, transformed with pNAO.COE1.339 plasmid coding for the entire HCcAg and the first 148 aa of the HCV E1 protein has been previously described [18]. The MP-36 strain was used as a negative control [18]. MP-36/C-E1.339 and MP-36 strains were grown using conditions already established [18]. At the end of the yeast cell culture (24 h after the induction of HCcAg expression), the cells were harvested and washed twice in TEN buffer (50 mM Tris–HCl, pH 8.0, 1mM EDTA, and 150 mM NaCl).

Proteins and antibodies. Trypsin inhibitor from soybean (TPS) (molecular mass of 21,500 Da) was obtained from Sigma Chemical (St. Louis, USA). Particulate hepatitis B surface antigen (HBsAg) (molecular mass of 10^6 Da) was obtained from CIGB, Cuba [25]. A mouse monoclonal antibody against the residues 5–35 of HCcAg (mAb SS-HepC.1) was used to detect HCcAg in immunoblotting experiments [18].

Purification of HCcAg. Cell disruption was performed using glass beads in TEN buffer containing or not 5 mM dithiothreitol (DTT) and PMSF 1 mM. The lysate was clarified by centrifugation at 12,000*g* for 20 min, and supernatant and pellet fractions were obtained. After cell disruption, the pellet fraction was treated with 2 M urea in TEN buffer containing or not 5 mM DTT (a ratio of 10 ml for each gram of cellular debris was used) and homogenized by sonication. The suspension was incubated for 120 min at room temperature with gentle agitation and centrifuged at 12,000*g* for 20 min. Then the pellet fraction was treated with 8 M urea in TEN buffer containing or not 5 mM DTT (a ratio of 10 ml for each gram of cellular debris was used) and homogenized by sonication. The suspension was incubated overnight at 4 °C and then clarified by centrifugation at 12,000*g* for 20 min. The extracted HCcAg was applied to a cation-exchange column (Fractogel EMD SO3 M Merck, Germany) previously equilibrated with 8 M urea in TEN buffer containing 5 mM DTT. HCcAg was eluted with a linear NaCl gradient. Fractions containing HCcAg with more than 50% of purity were pooled and subsequently applied to a reverse-phase high-pressure liquid CNpropyl chromatography column (J.T. Baker, USA) and eluted with a linear gradient from 0.1% trifluoroacetic acid in water to 0.1% TFA in acetonitrile. Afterwards, eluted HCcAg (0.5 mg/ml) was dialyzed against TEN buffer and 1% Glycerol (TENG) containing or not 1 mM DTT as previously described [23]. Nucleic acids were analyzed by agarose gel electrophoresis [24].

Analysis in Sepharose CL-6B gel filtration chromatography. One milliliter of either denatured or renatured HCcAg, or HBsAg [25] and TPS was applied to a column (60 × 1.6 cm diameter) of Sepharose CL-6B (Pharmacia, Sweden) equilibrated with TEN buffer containing 5 mM DTT. In the case of denatured HCcAg the column was equilibrated with TEN buffer containing 5 mM DTT and 8 M urea. They were run at a flow rate of 0.5 ml/min.

Equilibrium sucrose density gradient centrifugation. Five hundred microliters of either denatured or renatured HCcAg was applied to a 5–50% (w/v) sucrose density gradient in TEN buffer containing 5 mM DTT, centrifuged at 100,000*g* for 16 h in a Beckmann SW40Ti rotor, and fractionated. Five hundred microliter aliquot of each fraction was collected from the bottom of the tube. The refractive index of each fraction was measured using an Abbe-3L refractometer (Milton Roy). To detect HCcAg, fractions were assayed by Dot blot as previously reported [18]. The intensity of the resultant bands was quantified by measuring the optical density with the Eagle Eye II still video system (Stratagene).

Protein analysis. Samples were directly mixed with an equal volume of 2× Laemmli sample buffer in the presence or absence of reducing agent (5 mM DTT) and heated for 10 min. Then protein samples were separated in a 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and stained with Coomassie brilliant blue R250 (CBB, Sigma, St. Louis, USA) [26]. The purity was determined using the software 1-D Manager for Windows 95 (Ver. 2.0, TDI, sa, Madrid, Spain). HBsAg and HCcAg were quantitated using Dc Protein assay (Bio-Rad).

Immunoblotting assay. For immunoblotting the samples were either directly applied or electrotransferred to a nitrocellulose membrane and binding of antibodies was detected as previously described [18].

Electron microscopy. Denatured HCcAg or renatured HCcAg containing or not 1 mM DTT was fixed in glutaraldehyde and negatively stained with uranyl acetate prior to analysis by transmission electron microscopy as previously described [23].

Results and discussion

HCcAg purification

It is known that the presence of the C-terminal hydrophobic domain in HCcAg leads to problems with purification on chromatographic support, even with the addition of chaotropic molecules and/or detergents enabling total or partial solubilization [23,24,27]. Previously, the low soluble HCcAg obtained in *P. pastoris* cells was purified using electrophoresis under denaturing conditions [23]. However, this procedure enables small

quantities of protein to be obtained [23,27]. To purify more significant amounts of HCcAg as low-molecular weight species and avoid interaction with any nucleotides, HCcAg was extracted from pellet fractions using 8 M urea as denaturing agent under reducing conditions. Thus, HCcAg was kept under strong denaturing conditions. Reducing conditions were analyzed since the HCcAg sequence corresponding to matured processed protein contains two Cys residues at aa 128 and 172 [28]. The effect of reducing agent in the multimerization of HCcAg was analyzed by SDS–PAGE under reducing and non-reducing conditions, followed by immunoblotting (Fig. 1). When the sample was analyzed under non-reducing conditions, most of the HCcAg was observed in concentrating gel as disulfide-linked aggregates (Fig. 1, lane 2). However, under reducing conditions, HCcAg migrated as a monomer of expected molecular mass (P21) (Fig. 1, lane 1).

Therefore, the solubilization procedure under reducing conditions led to a high yield of released soluble monomeric HCcAg and enabled it to be injected onto a cation exchange column under strong denaturing conditions. Fractions from this purification step were collected and analyzed using SDS–PAGE (Fig. 2). Fractions containing eluted HCcAg with purity higher than 50% (Fig. 2, lanes 5, 6, and 7) were pooled and applied to a RP-HPLC CNpropyl column as a second chromatographic purification step. The data in Fig. 3 demonstrate the high degree of purification accomplished with CNpropyl chromatography. HCcAg was purified into a homogeneous peak with purity higher than 90% (Fig. 3). In addition, no contaminant nucleic acids were detected in the highly purified HCcAg sample (not shown).

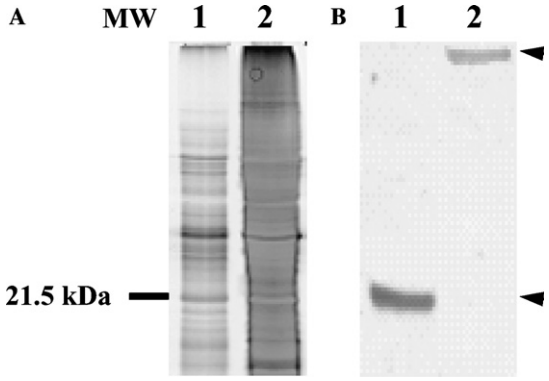

Fig. 1. SDS–PAGE and Western blot analysis of HCcAg under reducing or non-reducing conditions. Proteins from HCcAg samples extracted with 8M urea in the presence or not of 5 mM DTT were separated by SDS–PAGE (A) followed by Western blot analysis (B). The lanes contain molecular weight markers (MW), proteins mixed with an equal volume of 2× Laemmli sample buffer under reducing conditions (5 mM DTT) (1), proteins mixed with an equal volume of 2× Laemmli sample buffer under non-reducing conditions (2). The arrows on the right indicate the position of HCcAg.

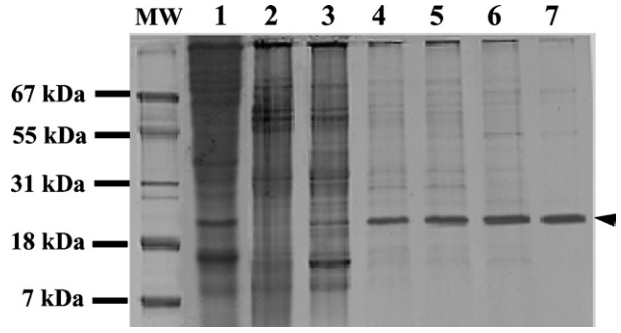

Fig. 2. Purification of HCcAg from the denatured protein sample on cation-exchange column (Fractogel EMD SO3). Proteins from fractions of the HCcAg purification procedures were separated by SDS–PAGE. The lanes contain molecular weight markers (MW), the unfractionated material loaded onto the cation-exchange column (1), unbound proteins (2), and elution of proteins from the cation-exchange column (3–7). The arrow on the right indicates the position of HCcAg.

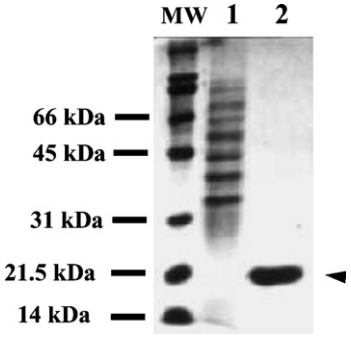

Fig. 3. Purification of HCcAg on CNpropyl column. Eluted HCcAg from the cation-exchange column (fractions 5, 6, and 7) was applied to the CNpropyl column. Proteins from fractions of the HCcAg purification procedure were separated by SDS–PAGE. The lanes contain molecular weight markers (MW), unbound proteins (1), elution of HCcAg from the CNpropyl column (2). The arrow on the right indicates the position of HCcAg.

In vitro assembly of HCcAg into VLPs

It has been suggested that multimerization of HCcAg may occur while the protein is associated with the ER membrane [29,30]. Several studies have provided evidences that the C-terminal domain of HCcAg mediates interaction with cellular membranes and lipids [6,9,31]. In addition, the presence of the C-terminal hydrophobic sequence may modulate the efficiency of HCcAg multimerization [30,32–34]. Previously, it has been demonstrated that the mature form of processed HCcAg (P21) produced in *P. pastoris* cells could assemble in vitro into VLPs, presumably in the absence of nucleic acids [23]. Due to HCcAg associated with *P. pastoris* cellular membranes, this protein was solubilized with detergents [18,23]. Therefore, it is possible that the use of detergents both in the purification and the renaturalization process has modulated the assembly properties of

HCcAg [18,23]. So, no detergents were used in this work.

Previously, it has been shown that structured RNA (range 0.1–10 μm) is required for in vitro assembly of HCcAg into NLPs [24]. In this study, the capacity of purified monomeric HCcAg to assemble into high-molecular weight structures in the absence of structured RNA was studied. First, HCcAg was renatured under reducing conditions, and both denatured and renatured HCcAg were analyzed on a Sepharose CL-6B size exclusion chromatography column. As controls, HBsAg and TPS were analyzed at the same conditions. Fig. 4 reveals that dialyzed HCcAg eluted as a uniform peak close to particulate HBsAg that corresponds to a molecular mass over 10^6 Da (Figs. 4B and C). On the other hand, denatured HCcAg migrated like a monomer close to TPS (Figs. 4A and C). This indicates that, after dialysis, HCcAg monomers assembled into high-molecular weight structures, which is consistent with HCcAg multimerization or capsid formation. This result also demonstrated the homogeneity of these high-molecular weight structures.

Later on, HCcAg was analyzed using a 5–50% (w/v) sucrose density gradient centrifugation. The sucrose gradient was fractionated and the individual fractions were assayed for the presence of HCcAg (Fig. 5). A peak fraction containing the denatured HCcAg (fraction 6) that migrated to a position in the gradient corresponding to a buoyant density of 1.11 g/ml was observed (Fig. 5A). This is in agreement with a recent work in which purified HCcAg was detected in low-density fractions collected from CsCl gradients [22]. HCcAg present in these fractions corresponded with unassembled protein or small oligomers complexes.

On the other hand, a higher density HCcAg fraction (fraction 10) with 1.19 g/ml was observed for renatured HCcAg (Fig. 5B). This value is lower than that obtained for NLPs containing nucleic acids isolated from *P. pastoris* cells [19]. This is consistent with the fact that the full and empty particles should be similar in size and shape but should differ in buoyant density according to their nucleic acid content. So, it is likely that in vitro assembled VLPs containing HCcAg correspond to empty capsids.

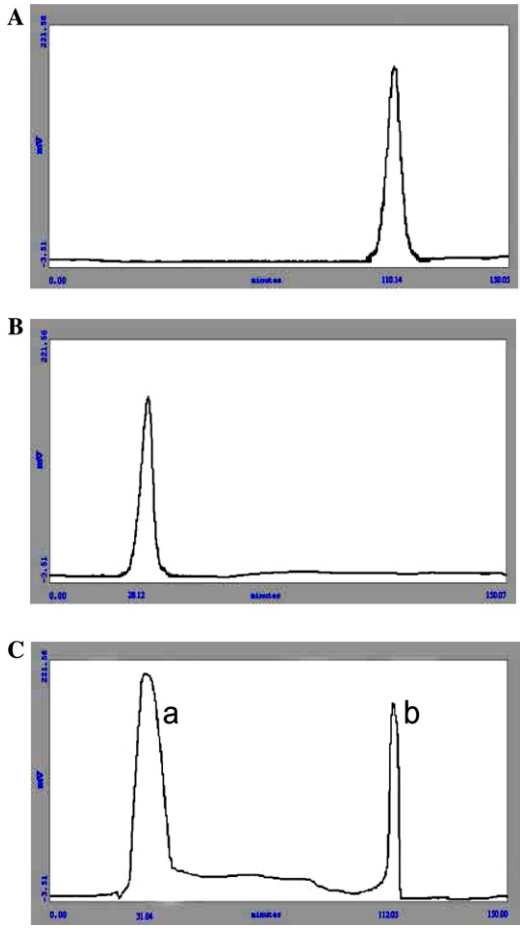

Fig. 4. Size exclusion chromatography on Sepharose CL-6B. (A) Chromatography lot of the denatured HCcAg. (B) Chromatography lot of the renatured HCcAg under reducing conditions. (C) Chromatography lot of the HBsAg (a) and TPS (b) used as controls.

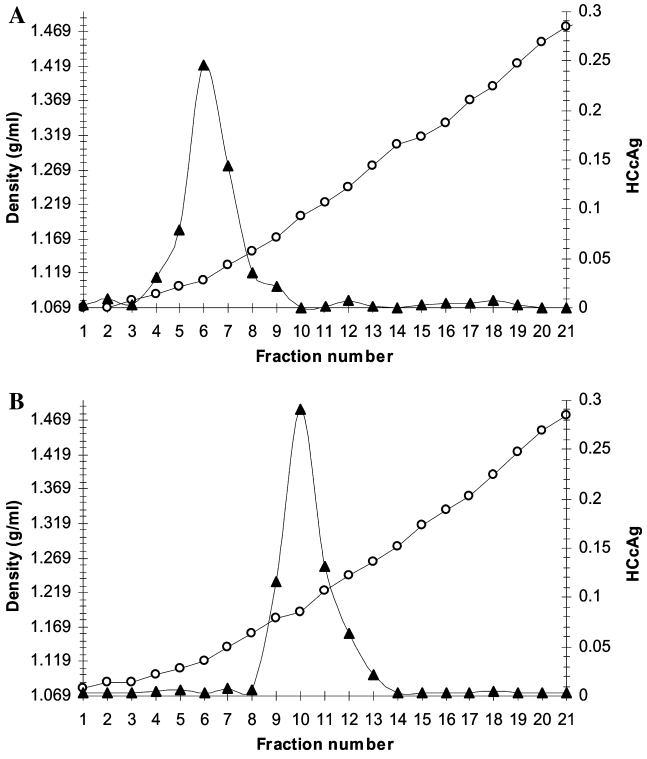

Fig. 5. Five to fifty percentage (w/v) of sucrose density gradient centrifugation analysis. The sucrose density of each fraction is shown [open circles; density (left ordinate) expressed in grams per milliliter, –○– density (g/ml)]. Detection of HCcAg was analyzed by densitometry of dot blot [closed triangles; HCcAg (right ordinate) expressed as signal (OD) at 620 nm –▲– HCcAg]. (A) Analysis of denatured HCcAg. (B) Analysis of renatured HCcAg.

When dialyzed HCcAg was analyzed by electron microscopy the presence of spherical particles with an average diameter of ~35 nm (34.88 nm, SD 6.45) was observed (Fig. 6A). However, these VLPs were not observed in the fully denatured HCcAg (Fig. 6B). Fig. 7 shows the frequency distribution of particle diameters. The diameters of these particles ranged from 26 to 46 nm. Interestingly, most of these VLPs appeared as irregular particles.

However, when HCcAg was renatured under non-reducing conditions, irregular aggregates of particles were observed (Fig. 6C). These irregular protein aggregates were similar to those previously observed inside recombinant *P. pastoris* cells expressing HCcAg [20] and those detected in the hepatocytes from HCV-infected patients [14].

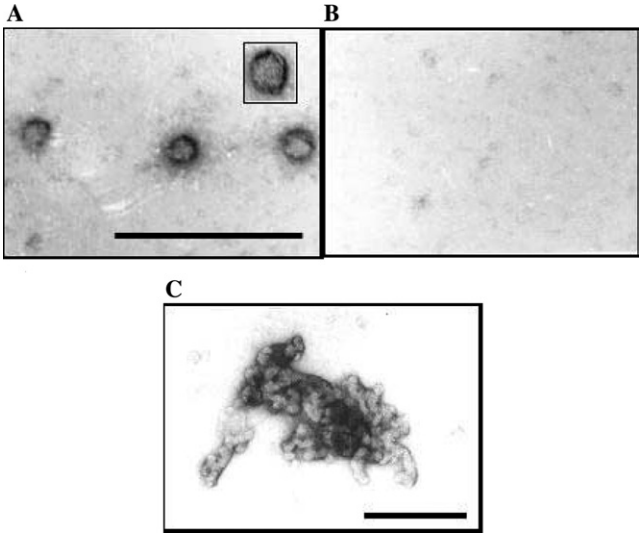

Fig. 6. Electron micrograph of negatively stained samples containing HCcAg. (A) Renatured HCcAg under reducing conditions. The inset contains a magnification of individual capsid. (B) Denatured HCcAg. (C) Renatured HCcAg under non-reducing conditions. Bar = 200 nm.

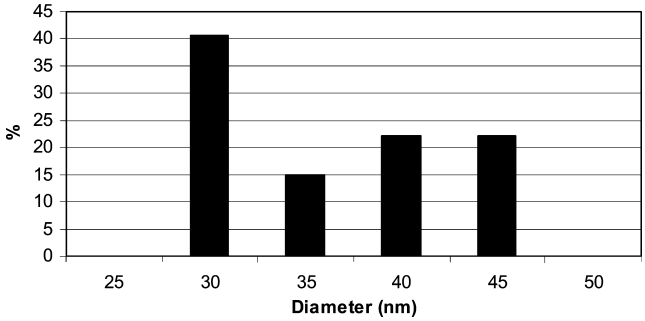

Fig. 7. Histogram representing the frequency distribution of particle diameters observed by EM in the assembly experiments of HCcAg. The diameter of particles was precisely measured in at least 10 fields (from 350 to 500 VLPs) from electron micrographs. Diameters of VLPs derived from HCcAg are shown.

Previously, some authors had demonstrated that HCcAg can undergo specific homotypic interaction directly [30,33,34]. Using the yeast two-hybrid system they tentatively suggested the homotypic interacting domains at aa 36–91 and aa 82–102 [30,33]. In addition, Yan et al. [34] reported that the HCcAg segment residues 123–191 interacted intermolecularly. By using a biochemical approach, a region from aa 123–162 containing two predicted α helices and a leu-zipper-like structure was shown to self-associate non-covalently [34].

On the other hand, HCcAg has been reported to bind oligomeric DNAs and RNAs in vitro [6,35–37]. In addition, other studies showed that structured RNA played a critical role for in vitro assembly of HCcAg into NLPs [22,24]. They also suggested that HCcAg can undergo extensive conformational changes upon binding to structured RNA and assembly into NLPs [38]. However, a recombinant mature HCcAg variant self-associated in vitro in the absence of nucleic acids into a homogeneous population of uniformly sized particles, with diameters of 15 nm [32]. As these particles were not consistent with the morphology and size of previously observed nucleocapsids [24] they were considered as intermediate species in the assembly pathway [32].

Results presented in this work showed that HCcAg assembled in vitro into VLPs in the absence of structured RNA, suggesting that recombinant HCcAg used in this work contains all the information necessary for the self-assembly process. In addition, the VLPs observed in this study were more homogeneous and shorter in size than those obtained by Kunkel and colleagues. Differences in recombinant cellular host (*E. coli* vs. *P. pastoris*), HCcAg sequences (different genotypes), and experimental conditions may partly explain the apparent discrepancy between this study and those published by other groups [22,24]. On the other hand, postraductional changes might also modulate the in vitro assembly capacity of HCcAg. Indeed, HCcAg has proved to be phosphorylated in yeast cells [39]. Although the role of phosphorylation in HCcAg multimerization remains to be elucidated, one of the proposed homotypic interacting domains includes putative phosphorylation sites at Ser-53 and Ser-99 [40].

The use of a reducing agent was critical for in vitro VLP assembly, since non-reducing conditions led to unstructured aggregate formation. These aggregates were similar to those previously described in *P. pastoris* cells composed of both P21 and P23 [19], and those observed in the hepatocytes from HCV-infected patients [14]. Possibly, the use of reducing conditions contributed to the in vitro HCcAg folding and assembly of VLPs in the absence of structured RNA. The irregular aggregates seen under non-reducing conditions may be unproductive assembly.

The VLPs observed in this work showed similar size and lower density values than those described for native particles found in sera and hepatocytes from HCV-in-

fected patients [14–16] and to recombinant NLPs obtained in *E. coli*, *P. pastoris*, and SF9 cells [16,18–22]. Thus, they resemble the size of HCV nucleocapsid particles in a mature stage. This result is also consistent with that obtained using HCcAg solubilized with detergents in in vitro assembly experiments [23]. However, most of VLPs appeared as irregular particles. This is in agreement with a previous work where a recombinant mature HCcAg variant assembled in vitro into particles with irregular shapes in the presence of structured RNA [24]. In that work, modification of the self-assembly pathway by the carboxy-terminal domain of HCcAg was suggested. Interestingly, those particles were heterogeneous in size and larger than the expected native capsids [24].

In contrast to the in vitro capsid assembly of full-length HCcAg, intracellular assembly of HCcAg leads to regularly shaped NLPs that resemble HCV capsids from infected individuals [19,22,29]. In addition, structured NLPs assemble in cell-free systems containing rabbit reticulocyte or wheat germ extracts [41]. One explanation would be that in vitro assembly of full-length HCcAg in solution may differ from in vivo assembly in association with cellular membranes or other elements of the assembly machinery (Fig. 8). Presumably, HCV nucleocapsid assembly in vivo is rigorously controlled, such that NLPs of uniform size and regular morphology are formed. Besides, the absence of cellular factors that are required for the proper NLPs assembly may lead to irregular particle formation in vitro. This would allow changes in HCcAg conformation without affecting its contact sites but prevent it from conforming to the geometry of the native capsid. So, it is important to remain cautious in the interpretation of the assembly mechanisms of HCV nucleocapsid based solely on in vitro systems that do not contain putative cellular factors required for proper HCV assembly.

Although the biological role of HCcAg-containing aggregates and its relationship with the pathogenesis induced by HCV infection are unknown we speculate that under pathological conditions (e.g., unbalanced redox potential of the cell) HCcAg may form unstructured aggregates and/or irregular particles instead of structured capsids, thus affecting the HCV virion assembly and viral replication. On the other hand, under normal conditions P21 may assemble into either irregular or structured capsids. Production of both unstructured aggregates of HCcAg and irregular capsids could lead to production of many fewer competent progeny viruses. However, testing of this hypothesis requires additional evidences.

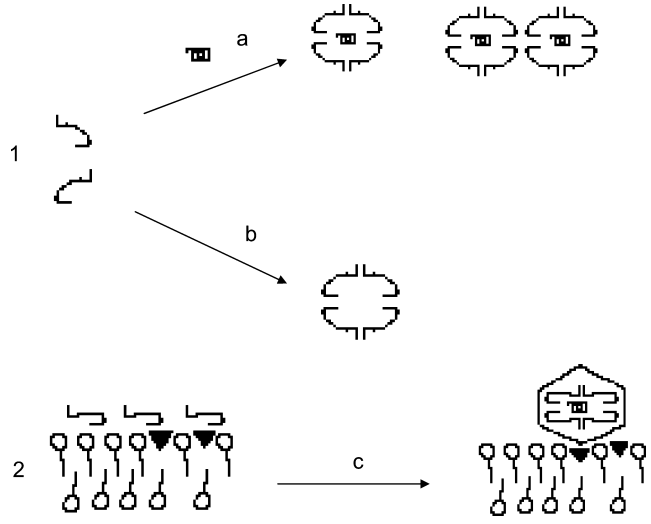

Fig. 8. Assembly of mature HCcAg into VLPs. (1) In vitro systems would allow changes in HCcAg conformation without affecting its contact sites but prevent it from conforming to the geometry of the native capsid. Depending on recombinant HCcAg sources, HCcAg sequences, and experimental conditions, HCcAg assembles either in the presence of structured RNA into irregular particles heterogeneous in size and larger than the expected native capsids (a) or in the absence of structured RNA into irregular particles with similar size to the expected native capsids (b). (2) In vivo assembly of HCcAg in association with intracellular membranes or other elements of the assembly machinery is rigorously controlled leading to the formation of capsids of uniform size and regular morphology (c). ⌐┘, full-length HCcAg folded in vivo; ⌐┘, full-length HCcAg folded in vitro; ▭, structured RNA; ⌇, lipids; ▼, factors of assembly machinery; ⬡, structured capsid; ⬭, irregular particle containing RNA; and ⬭, irregular empty particle.

Acknowledgments

The authors thank Jesus Seone and Nilda Tamayo for their excellent technical assistance, and Dr. Rafael F. Sanchez-Betancourt and Prof. Orlando J. Alvarez Guerrero for critical reading of the manuscript and for many helpful suggestions. Dr. Stanley J. Watowich for his value advice on HCV core purification.

References

[1] R. Bartenschlager, In vitro models for hepatitis C, Virus Res. 82 (2002) 25–32.
[2] Q.L. Choo, G. Kuo, A.J. Weiner, L.R. Overby, D.W. Bradley, M. Houghton, Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome, Science 244 (1989) 359–362.
[3] D.B. Op, L. Cocquerel, J. Dubuisson, Biogenesis of hepatitis C virus envelope glycoproteins, J. Gen. Virol. 82 (2001) 2589–2595.
[4] A. Grakoui, C. Wychowski, C. Lin, S.M. Feinstone, C.M. Rice, Expression and identification of hepatitis C virus polyprotein cleavage products, J. Virol. 67 (1993) 1385–1395.
[5] M. Hijikata, N. Kato, Y. Ootsuyama, M. Nakagawa, K. Shimotohno, Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis, Proc. Natl. Acad. Sci. USA 88 (1991) 5547–5551.
[6] E. Santolini, G. Migliaccio, N. La Monica, Biosynthesis and biochemical properties of the hepatitis C virus core protein, J.Virol. 68 (1994) 3631–3641.
[7] P. Hussy, H. Langen, J. Mous, H. Jacobsen, Hepatitis C virus core protein: carboxy-terminal boundaries of two processed

species suggest cleavage by a signal peptide peptidase, Virology 224 (1996) 93–104.
[8] Q. Liu, C. Tackney, R.A. Bhat, A.M. Prince, P. Zhang, Regulated processing of hepatitis C virus core protein is linked to subcellular localization, J. Virol. 71 (1997) 657–662.
[9] J. McLauchlan, M.K. Lemberg, G. Hope, B. Martoglio, Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets, EMBO J. 21 (2002) 3980–3988.
[10] N. Acosta-Rivero, A. Musacchio, L. Lorenzo, C. Alvarez, J. Morales, Processing of the Hepatitis C virus precursor protein expressed in the methylotrophic yeast Pichia pastoris, Biochem. Biophys. Res. Commun. 295 (2002) 81–84.
[11] T. Isoyama, S. Kuge, A. Nomoto, The core protein of hepatitis C virus is imported into the nucleus by transport receptor Kap123p but inhibits Kap121p-dependent nuclear import of yeast AP1-like transcription factor in yeast cells, J. Biol. Chem. 277 (2002) 39634–39641.
[12] S.U. Nielsen, M.F. Bassendine, A.D. Burt, D.J. Bevitt, G.L. Toms, Characterization of the genome and structural proteins of hepatitis C virus resolved from infected human liver, J. Gen.Virol. 85 (2004) 1497–1507.
[13] K. Yasui, T. Wakita, K. Tsukiyama-Kohara, S.I. Funahashi, M. Ichikawa, T. Kajita, D. Moradpour, J.R. Wands, M. Kohara, The native form and maturation process of hepatitis C virus core protein, J. Virol. 72 (1998) 6048–6055.
[14] V. Falcon, N. Acosta-Rivero, G. Chinea, J. Gavilondo, M.C. de la Rosa, I. Menendez, S. Duenas-Carrera, A. Vina, W. Garcia, B. Gra, M. Noa, E. Reytor, M.T. Barcelo, F. Alvarez, J. Morales-Grillo, Ultrastructural evidences of HCV infection in hepatocytes of chronically HCV-infected patients, Biochem. Biophys. Res. Commun. 305 (2003) 1085–1090.
[15] S. Ishida, M. Kaito, M. Kohara, K. Tsukiyama-Kohora, N. Fujita, J. Ikoma, Y. Adachi, S. Watanabe, Hepatitis C virus core particle detected by immunoelectron microscopy and optical rotation technique, Hepatol. Res. 20 (2001) 335–347.
[16] P. Maillard, K. Krawczynski, J. Nitkiewicz, C. Bronnert, M. Sidorkiewicz, P. Gounon, J. Dubuisson, G. Faure, R. Crainic, A. Budkowska, Nonenveloped nucleocapsids of hepatitis C virus in the serum of infected patients, J. Virol. 75 (2001) 8240–8250.
[17] K. Takahashi, S. Kishimoto, H. Yoshizawa, H. Okamoto, A. Yoshikawa, S. Mishiro, p26 protein and 33-nm particle associated with nucleocapsid of hepatitis C virus recovered from the circulation of infected hosts, Virology 191 (1992) 431–434.
[18] N. Acosta-Rivero, J.C. Aguilar, A. Musacchio, V. Falcon, A. Vina, M.C. de la Rosa, J. Morales, Characterization of the HCV core virus-like particles produced in the methylotrophic yeast Pichia pastoris, Biochem. Biophys. Res. Commun. 287 (2001) 122–125.
[19] N. Acosta-Rivero, V. Falcon, C. Alvarez, A. Musacchio, G. Chinea, d.l.R. Cristina, A. Rodriguez, S. Duenas-Carrera, V. Tsutsumi, M. Shibayama, I. Menendez, J. Luna-Munoz, M.M. Miranda-Sanchez, J. Kouri, J. Morales-Grillo, Structured HCV nucleocapsids composed of P21 core protein assemble primary in the nucleus of Pichia pastoris yeast, Biochem. Biophys. Res. Commun. 310 (2003) 48–53.
[20] V. Falcon, C. Garcia, M.C. de la Rosa, I. Menendez, J. Seoane, J.M. Grillo, Ultrastructural and immunocytochemical evidences of core-particle formation in the methylotrophic Pichia pastoris yeast when expressing HCV structural proteins (core-E1), Tissue Cell 31 (1999) 117–125.
[21] L.J. Lorenzo, S. Duenas-Carrera, V. Falcon, N. Acosta-Rivero, E. Gonzalez, M.C. de la Rosa, I. Menendez, J. Morales, Assembly of truncated HCV core antigen into virus-like particles in Escherichia coli, Biochem. Biophys. Res. Commun. 281 (2001) 962–965.
[22] N. Majeau, V. Gagne, A. Boivin, M. Bolduc, J.A. Majeau, D. Ouellet, D. Leclerc, The N-terminal half of the core protein of hepatitis C virus is sufficient for nucleocapsid formation, J. Gen. Virol. 85 (2004) 971–981.
[23] N. Acosta-Rivero, J.C. Alvarez-Obregon, A. Musacchio, V. Falcon, S. Duenas-Carrera, J. Marante, I. Menendez, J. Morales, In vitro self-assembled HCV core virus-like particles induce a strong antibody immune response in sheep, Biochem. Biophys. Res. Commun. 290 (2002) 300–304.
[24] M. Kunkel, M. Lorinczi, R. Rijnbrand, S.M. Lemon, S.J. Watowich, Self-assembly of nucleocapsid-like particles from recombinant hepatitis C virus core protein, J. Virol. 75 (2001) 2119–2129.
[25] E. Hardy, E. Martínez, D. Diago, R. Díaz, D. González, L. Herrera, Large-scale production of recombinant hepatitis B surface antigen from Pichia pastoris, Journal of Biothecnology 77 (2000) 157–167.
[26] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[27] S. Yvon, D. Rolland, J.P. Charrier, M. Jolivet, An alternative for purification of low soluble recombinant hepatitis C virus core protein: preparative two-dimensional electrophoresis, Electrophoresis 19 (1998) 1300–1305.
[28] J. Morales, A. Viña, C. Garcia, N. Acosta-Rivero, S. Dueñas-Carrera, O.Garcia, I. Guerra, V. Falcon, Sequences derived from the genome of the hepatitis C virus, and use thereof, WO 98/25960, (1998).
[29] E. Blanchard, C. Hourioux, D. Brand, M. Ait-Goughoulte, A. Moreau, S. Trassard, P.Y. Sizaret, F. Dubois, P. Roingeard, Hepatitis C virus-like particle budding: role of the core protein and importance of its Asp111, J. Virol. 77 (2003) 10131–10138.
[30] M. Matsumoto, S.B. Hwang, K.S. Jeng, N. Zhu, M.M. Lai, Homotypic interaction and multimerization of hepatitis C virus core protein, Virology 218 (1996) 43–51.
[31] R.G. Hope, D.J. Murphy, J. McLauchlan, The domains required to direct core proteins of hepatitis C virus and GB virus-B to lipid droplets share common features with plant oleosin proteins, J. Biol. Chem. 277 (2002) 4261–4270.
[32] M. Kunkel, S.J. Watowich, Biophysical characterization of hepatitis C virus core protein: implications for interactions within the virus and host, FEBS Lett. 557 (2004) 174–180.
[33] O. Nolandt, V. Kern, H. Muller, E. Pfaff, L. Theilmann, R. Welker, H.G. Krausslich, Analysis of hepatitis C virus core protein interaction domains, J. Gen. Virol. 78 (1997) 1331–1340.
[34] B.S. Yan, M.H. Tam, W.J. Syu, Self-association of the C-terminal domain of the hepatitis-C virus core protein, Eur. J. Biochem. 258 (1998) 100–106.
[35] Z. Fan, Q.R. Yang, J.S. Twu, A.H. Sherker, Specific in vitro association between the hepatitis C viral genome and core protein, J. Med. Virol. 59 (1999) 131–134.
[36] T. Shimoike, S. Mimori, H. Tani, Y. Matsuura, T. Miyamura, Interaction of hepatitis C virus core protein with viral sense RNA and suppression of its translation, J. Virol. 73 (1999) 9718–9725.
[37] Y. Tanaka, T. Shimoike, K. Ishii, R. Suzuki, T. Suzuki, H. Ushijima, Y. Matsuura, T. Miyamura, Selective binding of hepatitis C virus core protein to synthetic oligonucleotides corresponding to the 5' untranslated region of the viral genome, Virology 270 (2000) 229–236.
[38] M. Kunkel, S.J. Watowich, Conformational changes accompanying self-assembly of the hepatitis C virus core protein, Virology 294 (2002) 239–245.
[39] H. Aoki, J. Hayashi, M. Moriyama, Y. Arakawa, O. Hino, Hepatitis C virus core protein interacts with 14-3-3 protein and activates the kinase Raf-1, J. Virol. 74 (2000) 1736–1741.
[40] W. Lu, J.H. Ou, Phosphorylation of hepatitis C virus core protein by protein kinase A and protein kinase C, Virology 300 (2002) 20–30.
[41] K.C. Klein, S.J. Polyak, J.R. Lingappa, Unique features of hepatitis C virus capsid formation revealed by de novo cell-free assembly, J. Virol. 78 (2004) 9257–9269.