

Expression of Hepatitis C Virus Structural Proteins in *Saccharomyces cerevisiae*

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Received: October 14, 2004

Accepted: December 28, 2004

Abstract Expression in yeast may prove more amenable to generating large amounts of viral antigens for a vaccine candidate. We, therefore, cloned the gene encoding the Hepatitis C virus (HCV) structural proteins (C-E1-E2, c740) fused in-frame with, and immediately 3' to, the chicken-lysozyme signal peptide (C-SIG) gene and under the control of the yeast glyceraldehyde-3-phosphate dehydrogenase gene promoter. In yeast, the HCV structural proteins were expressed in two different forms: a processed and a nonprocessed aggregated form. Biophysical characterization by sucrose linear gradient centrifugation revealed that both forms were present in the same fractions with a buoyant density of 1.127–1.176 g/cm³. These findings suggest that the efficient synthesis of HCV structural proteins in yeast may be an important tool to study virus assembly and may lead to the development of an HCV vaccine.

Key words: HCV structural proteins, yeast expression, vaccine

Hepatitis C virus (HCV), a major cause of chronic hepatitis and hepatocellular carcinoma, is a Hepacivirus of the family Flaviviridae [17]. Its positive-stranded viral RNA genome encodes a single polyprotein of about 3,010 amino acids, which is cleaved by cellular and viral proteases into at least 10 viral proteins [10, 17]. The structural proteins of HCV consist of the core protein and the two putative envelope proteins, E1 and E2. In eukaryotic cells, the core protein is processed into two different forms with molecular weights of 21 kDa and 18 kDa [18]. By analogy with other flaviviruses, the core protein encapsidates the viral genome and forms a

nucleocapsid particle [2, 20]. The glycoproteins E1 and E2 constitute the virion envelope. E1 can associate noncovalently with E2 in a heterodimeric complex, representing the properly folded polypeptides, or as a disulfide-linked aggregate, representing misfolded proteins. E2 may be involved in virus-cell attachment [4, 5, 11]. Despite the high level of expression of E1 and E2, the efficiency of formation of HCV virus-like particles (VLPs) is rather low, and even those particles generated are not transported well out of mammalian cells.

Research on the mechanism of viral replication and the development of a vaccine, however, is hampered by low virus titers in serum and by the lack of a tissue culture system. In methylotrophic yeast, the formation of VLPs composed of only HCV core protein has been observed without any envelope protein [1, 9]. For the production of a vaccine candidate, it is necessary to produce and purify the whole structural proteins of HCV. We fused the gene encoding chicken-lysozyme signal peptide (C-SIG), which was recognized by the yeast secretory apparatus, to the 5'-end of DNA encoding whole HCV structural proteins. We expressed and purified the HCV whole structural proteins from yeast cells for a future vaccine candidate.

MATERIALS AND METHODS

Plasmid Construct

The structural protein gene of HCV (c740), encoding the core, E1 and E2 proteins, was amplified from HCV genotype 1b and subcloned into the yeast expression vector, pGLD, in which the cloned gene is under the control of the GLD promoter and the yeast 3-phosphoglycerate kinase (PGK) terminator. The vector also contained the C-SIG gene,

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which is recognized by the yeast secretory apparatus [16, 19].

Transformation of Yeast Cells

Yeast transformation was performed by the method of Hinnen *et al.* [12, 13]. Briefly, the protease-deficient strain, *Saccharomyces cerevisiae* AH22R- (a *leu2 his4 can1 cir+ pho80*), was cultured in YPDA broth (polypeptone 20 g/l, yeast extract 10 g/l, L-adenine hemisulfate salt 0.1 g/l, 40% glucose 50 ml) until the cells reached an A_{600} of approximately 1.0–1.5. Cells were harvested and resuspended in solution A (0.1 M sodium citrate, 10 mM EDTA, 1.2 M sorbitol), following the addition of zymolase, and spheroplast formation was monitored under a microscope. After centrifugation at $750 \times g$, the cell pellet was resuspended in 0.1 ml of solution B (10 mM Tris-HCl, pH 7.0, 1.2 M sorbitol, 10 mM CaCl_2), plasmid DNA was added, and the suspension was incubated at 25°C for 15 min. Two ml of solution C (20% PEG 4000, 10 mM Tris-HCl, pH 7.0, 10 mM CaCl_2) were added, and the suspension was incubated for another 15 min at 25°C. The cells were harvested and incubated in sorbitol-containing broth for 20 min at 30°C. Transformants were selected by the *Leu2+* phenotype, cultured as described previously [14, 15] and assayed for the expression of HCV structural proteins.

Purification and Analysis of HCV Proteins

The expressed hepatitis B viral proteins were purified as previously described [14], with slight modifications. Yeast cells in stationary growth phase (72 h) were harvested and suspended in 100 ml of buffer A [7.5 M urea, 0.1 M sodium phosphate, pH 7.2, 15 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, 0.1 mM (p-aminophenyl) methanesulfonyl fluoride, 0.1% Tween 80]. The cells were disrupted with glass beads (diameter 0.5 mm) using a BEAD-Breaker (Biospec Products, OK, U.S.A.), and the cell extract was mixed with 0.75 volumes of 33% (w/v) polyethylene glycol (PEG) 6000. Precipitated materials were collected by centrifugation, resuspended, and subjected to CsCl gradient ultracentrifugation and sucrose gradient ultracentrifugation to purify the proteins. The positive fractions for the respective proteins were selected by silver staining (Silver Staining Kit, Wako, Japan) or Western blotting (Fig. 3) and dialyzed at 4°C against buffer A without urea and Tween 80, but containing 0.85% NaCl, for 5–8 h, and concentrated with an Amicon disc filter (Millipore, MA, U.S.A.) to a concentration of about 200–300 $\mu\text{g ml}^{-1}$ (volume 10–15 ml), as measured with a BCA kit (Pierce, IL, U.S.A.). The purified proteins were resolved by SDS-PAGE and transferred to PVDF (polyvinylidene difluoride) membranes (BioRad, CA, U.S.A.), which were incubated with rabbit polyclonal antisera against recombinant HCV core protein and E2 protein, monoclonal antibody (mAb) against core protein, or mAbs against E1 and E2 (Biogenesis, England).

Sucrose Density Gradient Ultracentrifugation and Protease Digestion

The purified HCV structural proteins were separated by centrifugation on a 10–60% sucrose density gradient, containing 20 mM Tris-HCl [pH 7.4] and 150 mM NaCl, at $100,000 \times g$ on an SW Ti 41 rotor (Beckman, Germany) for 12–14 h at 4°C. One ml of each fraction was dialyzed against 0.85% NaCl, transferred to a fresh tube and centrifuged at $100,000 \times g$ for 3 h. Each pellet was resuspended in 50 μl of TNE buffer (50 mM Tris-Cl, pH 7.4, 100 mM NaCl, 0.5 mM EDTA) and stored at 4°C until use. Purified proteins were incubated for 30 min at 37°C with 25 $\mu\text{g}/\mu\text{l}^{-1}$ trypsin (Sigma, St. Louis, U.S.A.) in the presence or absence of 1% (w/v) Triton X-100.

RESULTS AND DISCUSSION

Construction and Purification of HCV Structural Protein from Yeast

There are many impediments for the development of an effective vaccine against hepatitis C virus infection, including the lack of a suitable small animal model, the high degree of HCV genomic diversity, and the inability to produce the HCV in culture system. Attempts have been made to produce VLPs by expressing the structural proteins in heterologous systems, with limited success. For example, in mammalian and insect cells, the full-length HCV coding region was only transiently expressed, and the expression level and efficiency of VLP formation were rather low for the generation of an HCV vaccine [3]. To produce a large amount of whole structural proteins (core, E1, and E2), we have cloned the whole structural gene of HCV (c740) into the 3' end of the chicken-lysozyme signal peptide (C-SIG) gene in the yeast expression vector pGLD (Fig. 1). The resulting plasmid, pGLD-c740, was used to transform the yeast cells. Upon reaching the stationary growth phase, whole-cell extracts were prepared by disrupting with glass beads and fractionating with PEG 6000. Following CsCl density equilibrium centrifugation (10–40%, w/v), we found by silver staining that the proteins were primarily present in fractions 2 through 5 (Fig. 2A).

These fractions were mixed and subjected to sucrose gradient ultracentrifugation (10–50% w/v), and the proteins

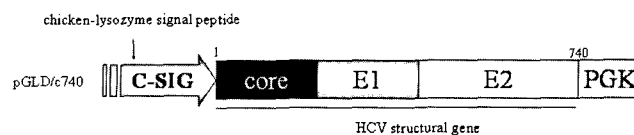


Fig. 1. Schematic diagram of the expression plasmid.

The HCV structural gene (c740) was cloned into the yeast expression vector pGLD immediately 3' to, and under the control of, the chicken-lysozyme signal peptide (C-SIG), and 5' to the PGK terminator, used as the polyadenylation signal. The numbers refer to the corresponding amino acid residues of the HCV structural proteins.

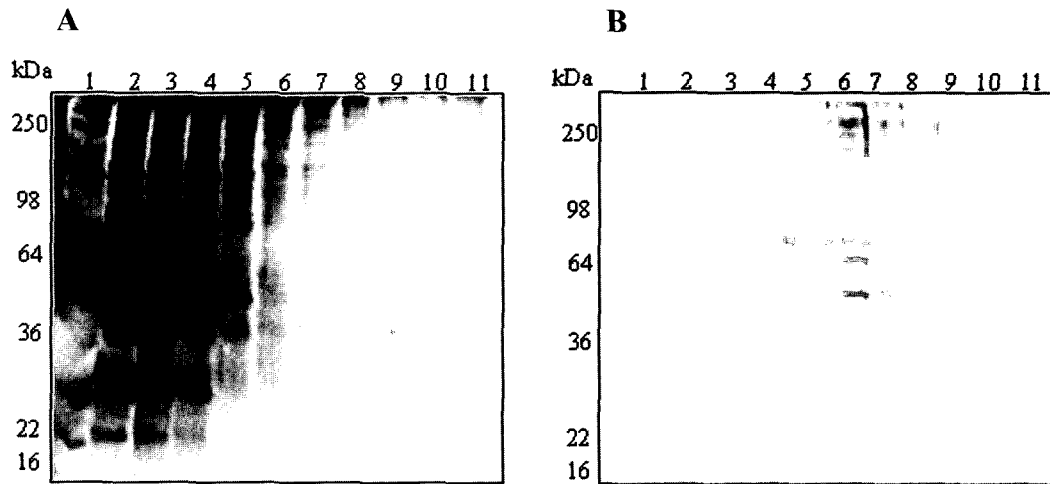


Fig. 2. The profiles of first CsCl density centrifugation (A) and second sucrose density centrifugation (B) in the purification steps. Panels A and B are silver-stained gel (lanes 1–11: Fraction number).

were resolved by SDS-PAGE. The four fractions (Fig. 2B, lanes 5 through 8) containing the major proteins, as determined by silver staining, were subsequently dialyzed overnight against 0.85% saline at 4°C. To determine the properties of the HCV structural proteins expressed in yeast, the proteins were separated by SDS-PAGE under reducing conditions and assayed by Western blotting (Fig. 3). When reacted with rabbit polyclonal antibody to E2 (Fig. 3A), two major bands of approximately 65 and 250 kDa were observed. From their molecular sizes, it is likely that the 65 kDa protein represents the processed form of E2. The

250 kDa protein was reacted with anti-E2, anti-E1, and anti-core antibodies (Figs. 3A, 3B and 3C, respectively). This large protein was assumed to be a nonprocessed aggregated form. The E1 protein was also detected as a slightly smeared form of 35 kDa in size by Western blot (Fig 3B). When we used monoclonal antibody to the core protein to assay the purified proteins (Fig. 3C), we detected bands of 18, 22, 35, and 250 kDa. These results showed that purification of the HCV structural proteins yields both the processed and aggregated forms of each protein. The 18 kDa and 22 kDa protein bands correspond to the processed HCV core protein expressed in yeast, in which the C-terminal hydrophobic domain (a.a. 154-191) is truncated [18, 20], and nontruncated core protein, respectively. The 35 kDa protein seems to correspond to a core protein dimer. These findings showed that the core protein in yeast was cleaved by a signal peptidase, later forming a multimer by homotypic interactions, especially between hydrophilic domains, to form a nucleocapsid [1, 15]. We have shown here that addition of the C-SIG gene to the 5' end of cDNA encoding the HCV structural proteins and expression in yeast enhanced the translational efficiency of the HCV structural proteins, enabling us to purify these proteins. The efficient recognition of the C-SIG peptide by the yeast secretory apparatus may increase the transport of the HCV structural proteins into the ER lumen, similar to the L particle expression of hepatitis B virus in yeast [14]. However, we found that there were at least two different forms of the HCV structural proteins, the processed form and nonprocessed aggregates, in agreement with the data of the expression of HCV structural proteins in insect and COS-7 cells. The aggregates of HCV glycoproteins were observed in both high and low levels of expression. HCV glycoproteins have a tendency to aggregate. Because there is no tissue culture system that allows

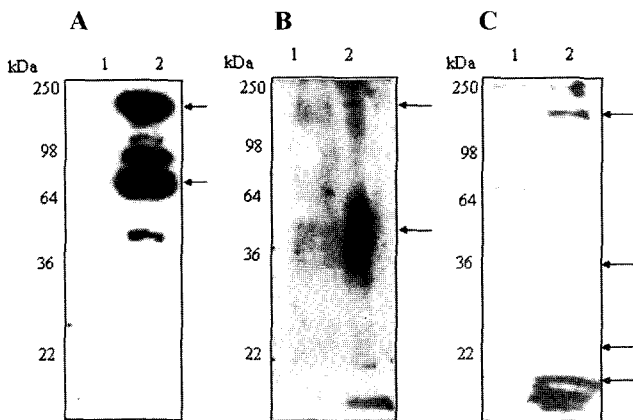


Fig. 3. Western blot analyses of the expressed HCV structural proteins in the yeast cells.

Two different forms of HCV structural proteins were detected (A: anti-E2 polyclonal Ab; B: anti-E1 monoclonal Ab; C: anti-core monoclonal Ab). The lower arrow indicates the processed form and the upper arrow the nonprocessed form of E2 and E1 proteins (panels A and B, respectively). The upper arrow is the aggregate form with envelope protein, the middle arrow indicates the dimer form of core protein, and the lower two arrows indicate core protein in panel C (lane 1: cell extract of the yeast harboring pGLD; lane 2: purified HCV structural proteins from the yeast harboring pGLD/c740).

efficient replication of HCV, it is hard to prove that the aggregates can be formed in the course of infection. However, this tendency to form aggregates is not an artifact linked to the expression system, but probably an intrinsic property of these proteins [6–8]. The inefficient folding of HCV structural proteins might reduce the virion formation to minimize the viral antigens to the immune system and/or induce other pathogenic effects in the long duration of disease.

Biophysical Characterization of Purified HCV Structural Protein

To investigate the conformation of HCV structural protein from yeast, we fractionated the purified HCV structural protein sample on a 10–60% sucrose linear density gradient. When assayed with anti-E2 polyclonal (Fig. 4A) and monoclonal (Fig. 4B) antibodies, the HCV structural proteins were found in fractions with a buoyant density of 1.127–1.176 g/cm³ (Fig. 4A, lanes 5 and 6). Both the processed and aggregated forms of E2 were observed in the same fractions, which also reacted with antibody to core protein (data not shown). We found that the processed E2 protein was in the monomeric form of about 64 kDa, a size corresponding to the expected molecular mass of correctly processed and glycosylated E2 protein in mammalian cells [10]. We also observed the nonprocessed oligomeric E2 protein band (250 kDa in size) with antibodies against all three HCV structural proteins. E1 and E2 proteins have been shown to noncovalently associate to form a heterodimer, which may be the precursor for viral assembly. When this oligomeric band was digested with trypsin, the 120 kDa protease-resistant protein band was recognized by the E2 polyclonal antibody, but not by the E1 monoclonal antibody, indicating that this band probably represents the

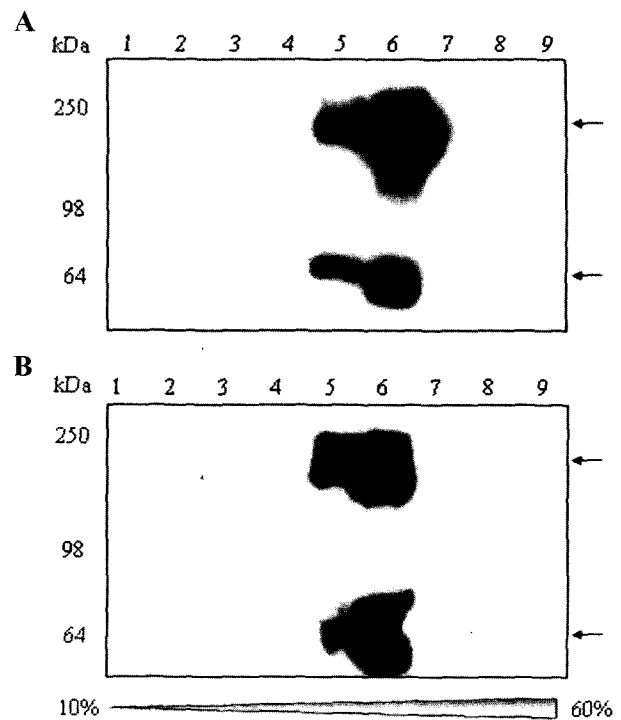


Fig. 4. Sucrose linear gradient analyses. Panels A and B were detected by E2 polyclonal Ab and E2 monoclonal Ab, respectively. The lower arrow indicates the monomeric form and the upper arrow is the aggregated form (lanes 1–9: Fraction number). HCV structural proteins were in fractions with a buoyant density of 1.127–1.176 g/cm³.

E2 dimer and suggesting that the original oligomeric protein contains E2 homodimers as well as E1-E2 heterodimers. However, it is necessary to confirm whether E2 has proper

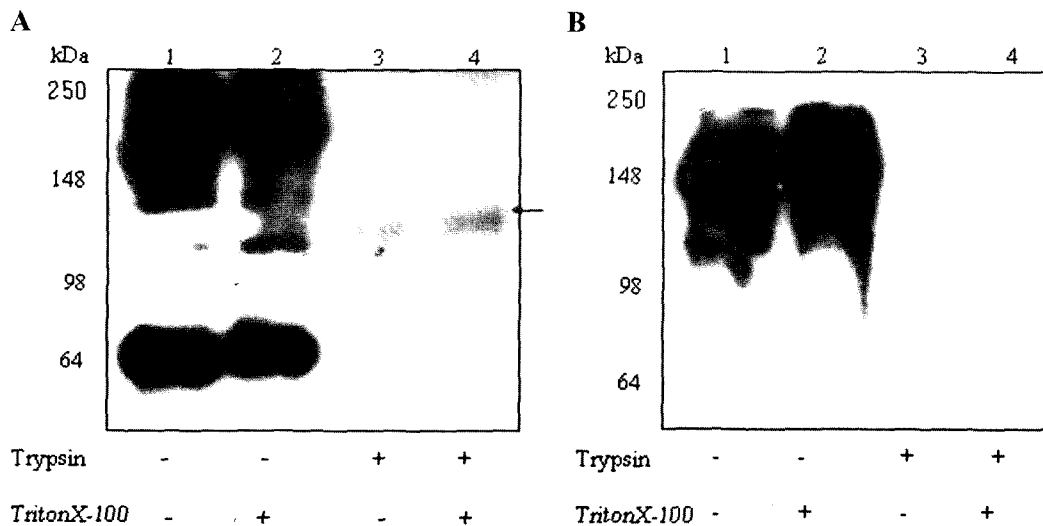


Fig. 5. Trypsin digestions of HCV structural proteins in the presence or absence of detergent. The arrow indicates the protease-resistant fragment. Panels A and B are Western blot analyses with E2 polyclonal Ab and E1 monoclonal Ab, respectively.

conformation using monoclonal antibodies that can discriminate the structure of E2.

The above findings suggest that the efficient expression of whole structural proteins of HCV (C-E1-E2) in yeast may be an important tool to study virus assembly and may lead to the development of an HCV vaccine candidate, similar to currently available yeast-derived HBV vaccine.

Acknowledgments

This work was supported by National Research Laboratory Program grant (2000-N-NL-01-C-171) from the Korean Ministry of Science and Technology and by a grant (R01-1999-000-00144-0) from the Korea Science & Engineering Foundation.

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