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Processing of the Hepatitis C virus precursor protein expressed in the methylotrophic yeast *Pichia pastoris*

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Abstract

The expression and processing of the Hepatitis C virus core protein (HCcAg) were analyzed in the methylotrophic yeast *Pichia pastoris*. Two proteins with 21 (p21) and 23 kDa (p23) were detected in immunoblot with a serum from a chronic carrier patient, as the major products for HCcAg. Both proteins, p21 and p23, produced by proteolytic processing in *P. partoris*, share the same N-terminal region and reacted with a monoclonal antibody towards the first 35 amino acids of HCcAg. The proteolytic processing of the recombinant polypeptide, having the HCcAg and the first 148 aa of E1 protein, was also confirmed by immunoblot analysis using mAbs with HCcAg and E1 specificities, respectively. The 32 kDa glycosilated E1 protein was then immuno-identified, as well as the processed HCcAg. These data demonstrated the usefulness of *P. pastoris*, as expression system, to study the processing of HCV structural proteins.

Keywords: Hepatitis C; Core antigen; Yeast; *P. pastoris*

Hepatitis C virus (HCV) is a major causative agent of non-A non-B hepatitis [1], which often leads to hepatocellular carcinoma [2]. Due to the lack of an efficient in vitro propagation system for HCV, most understanding of the structure and function of the HCV genome have arisen from analysis of viral cDNA. The entire HCV genome showed that the positive-stranded RNA genome is 9400 nucleotides long and contains a single large open reading frame (ORF) [3]. The viral structural and nonstructural proteins are produced by cleavage of a large polyprotein precursor by both host cell signal peptidases and viral proteinases [4,5]. Among the viral structural proteins, the core protein (HCcAg) is derived from the amino terminus of the polyprotein and it likely forms the nucleocapsid of the virion. HCcAg is highly basic and similar in many biological properties to the nucleocapsid proteins found in other flaviviruses [6,7]. The processing of the HCcAg has been studied in in vitro translation studies, in recombinant Semliki forest virus replicon system and transfected mammalian cells [8–11]. In this study, we report the processing of HCcAg in the methylotrophic yeast *Pichia pastoris*.

Materials and methods

Plasmids and strains. The expression vectors used in this study are shown in Fig. 1. The pNAOC-E1.339 vector carries the gene-fusion fragment coding for the HCcAg and the first 148 amino acids (aa) of the HCV E1 protein, under the transcriptional control of the Alcohol Oxidase 1 promoter (pAOX1). It was used for the expression of both recombinant proteins (HCcAg and HCV E1 protein-E1.148) in the methylotrophic yeast *P. pastoris* [12,13]. The strain MP-36 (*his3*) [14] was used for transformation in *P. pastoris*.

The pN12 plasmid, containing the gene-fragment coding for the first 176 aa of the HCcAg under the transcriptional control of the tryptophan promoter, was used for the expression of this protein (Co.176) in *Escherichia coli* strain W3110 [15].

The plasmid $pQE1_{340}$ contains the gene encoding for the first 148 aa of HCV E1 protein fused to poly-Hys tag fusion system (E1.148-H) under the transcriptional control of T5 promoter. *E. coli* strain SG13009 was used for E1.148-H expression [16].

Antibodies. Murine monoclonal antibodies against the residues 5–35 of the HCV core protein (mAb SS-HepC.1) [12] and the residues 190–219 of the HCV E1 protein (mAb SS-HepC.2) [16] were used in immunobloting experiments. A human serum from an infected person

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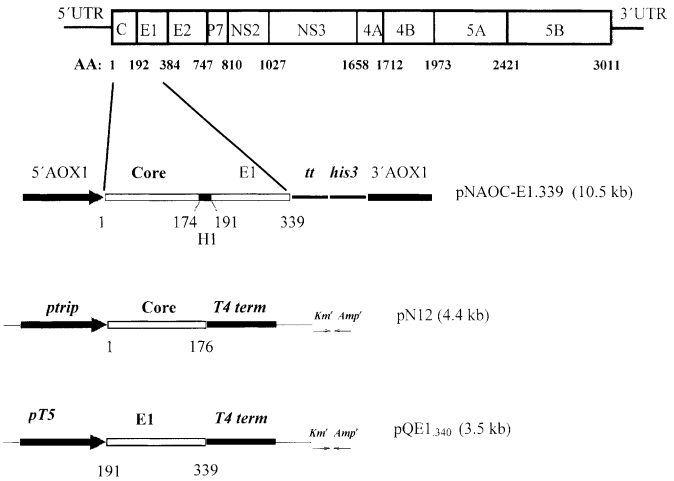

Fig. 1. Schematic representation of the expression vectors used in this study. The pNAOC-E1.339 vector was used to transform *P. pastoris* strain MP-36 for the expression of HCcAg–E1 antigen. The pN12 vector was used to transform *E. coli* strain W3110 for HCcAg expression, under the transcriptional control of tryptophan promoter. The $pQE1_{340}$ plasmid was used to transform *E. coli* strain SG 13009 for E1.148-H expression, under the transcriptional control of T5 promoter. 5′ AOX1, methanol oxidase promoter. *tt*, transcription terminator. AA, amino acid. 5′UTR and 3′UTR, 5′ and 3′ untranslated regions, respectively. C, E1, E2, and P7, HCV structural Core, Envelope 1, Envelope 2, and P7 proteins, respectively. NS2, NS3, 4A, 4B, 5A, 5B, HCV non-structural proteins.

(positive for HCcAg and negative for E1 antigen) was also used in these experiments [15].

Yeast transformation. The pNAOC-E1.339 vector was linearized by *Sal*I–*Cla*I digestion and used to transform the *P. pastoris* strain MP-36 by colony electroporation procedure [17]. The stable transformant MP36/CE1.339.5 was obtained using the procedures described by Cregg and coworkers [18].

Growth conditions for P. pastoris strains. The MP36/CE1.339.5 transformant was grown in minimal glycerol medium (MYG) (1.3% yeast nitrogen base, 1% glycerol, and 0.4 μg/ml biotin) at 30 °C for 48 h. The HCcAg expression was induced by replacing MYG with minimal methanol medium (MM) (1.3% yeast nitrogen base; 0.5% methanol, and 0.4 μg/ml biotin) and further incubation at 30 °C for 96 h. *P. pastoris* strain MP-36 was used as a negative control under the same growth conditions. At the end of the yeast cell culture, the cells were harvested and washed twice in TEN buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, and 150 mM de NaCl).

Growth conditions for E. coli strains. The recombinant SG 13009 cells were grown overnight at 37 °C in Luria Bertani broth (LB), containing 50 μg/mL ampicillin and 50 μg/mL kanamycin (LBKA medium). The E1.148-H protein expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma, St. Louis, USA) at the early exponential growth phase [16]. Five hours after induction, the cells were harvested by centrifugation and washed with TEN buffer.

The recombinant W3110 cells were grown in LB containing 5% of glucose and 50 μg/mL ampicillin. For the expression of Co.176, β-indoleacrylic acid (Sigma, St. Louis, USA) at 50 μg/mL was added to the cell culture in the middle of the exponential growth phase [15]. Cells were harvested later by centrifugation and washed with TEN buffer.

Immunoblotting assay. SDS–PAGE was performed in 12% gel [19]. The proteins were electroblotted onto 0.45 μm pore size nitrocellulose filters (BioRad, CA, USA) for 1 h in a solution containing 0.025 M Tris/HCl, 0.15 M glycine, 20% methanol, pH 8.3. Binding of IgG antibodies was detected as previously described [20].

Results and discussion

The expression of recombinant HCcAg was detected by Western blot analysis using a human serum from a chronic carrier patient (positive for HCcAg and negative for E1 antigen) [15], as shown in Fig. 2. This study revealed the presence of two recombinant HCcAg molecular species with 21 (p21) and 23 kDa (p23) expressed in *P. pastoris* after methanol induction. Both antigens were also recognized by the mAb SS-HepC.1 towards aa Pro^{5}–Tyr^{35} of HCcAg [12]

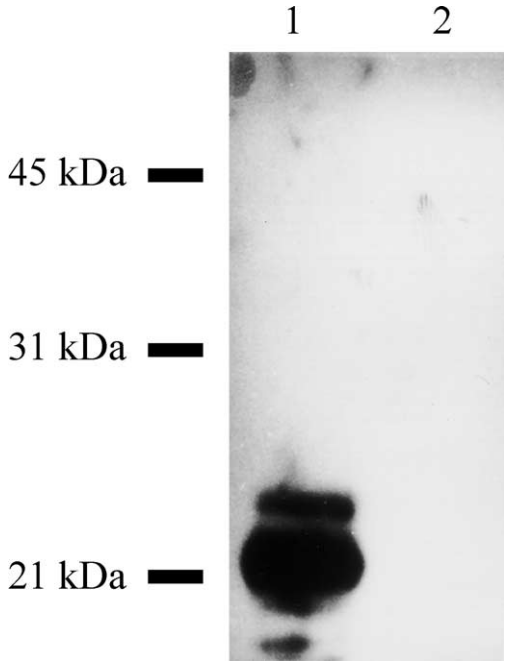

Fig. 2. Detection of HCcAg produced in *P. pastoris*, with a serum from a HCV chronic carrier patient. (Lane1) *P. pastoris* MP36/CE1.339.5 transformant, expressing HCcAg, (lane 2) *P. pastoris* strain MP-36, as negative control. Molecular weight markers are indicated.

(Fig. 3), suggesting that differences in molecular weight of p21 and p23 are located in their carboxyl-terminal regions.

Differences in molecular weight of immunodetected HCcAgs (p21 and p23) would be explained by: (1) Changes in the nucleotide sequence coding for HCcAg–E1 polyprotein that may introduce stop codons. (2) Processing of the expected 34 kDa HCcAg–E1 precursor. (3) The transcription of a truncated mRNA, produced as a consequence of either degradation or transcriptional stops associated with the heterologous gene in *P. pastoris*.

The cloned gene, which codifies for HCcAg–E1 protein in *P. pastoris*, was sequenced and no stop codons were found, other than the original one at 1017 bp position. In addition when the same gene (which encodes for HCcAg–E1 protein) was cloned in *E. coli*, the expected 34 kDa protein was immuno-detected by specific mAb (data not shown), showing the integrity of the selected gene.

The results reported herein are similar to those obtained by others [8–11]. In those studies, two molecular species of HCcAg were co-detected after expression using in vitro translation system, transfected mammalian cells, and the recombinant Semliki forest virus replicon system. The HCV core polypeptides were processed at two cleavage sites, located between Ser^{173}–Phe^{174} and Ala^{191}–Tyr^{192}, and catalyzed by a host signal peptidase on the endoplasmic reticulum (ER). This process rendered a 191-aa HCcAg, which is further processed to a 173-aa HCcAg. The cleavages at these positions resulted in the removal of the H1 fragment, spanning residues 173–191 (Fig. 1) [9]. Then, p23 is composed of 191 aa while p21 is a truncated form of p23, composed of 173 aa.

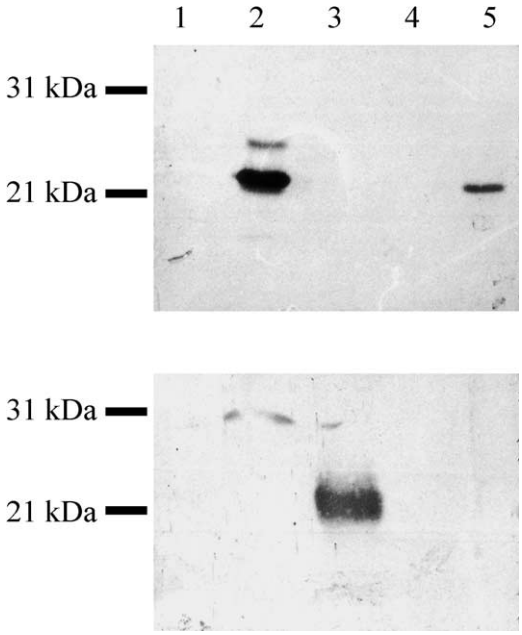

Fig. 3. Detection of processed HCcAg–E1 expressed by the recombinant yeast *P. pastoris*. (Upper) Immunoblot with the mAb SS-HepC.1 against HCcAg. (Lower) Immunoblot with the mAb SS-HepC.2 agasint E1 antigen. (Lane 1) *P. pastoris* MP-36 strain, as a negative control (lane 2), *P. pastoris* MP36/CE1.339.5 transformant (lane 3), E1.148-H, truncated E1 antigen (148 aa), expressed in *E. coli* strain SG 13009, (lane 4), *E. coli* strain W3110, as negative control (lane 5) *E. coli* strain W3110/Co176, expressing HCcAg (176 aa).

The HCV sequence used in this study contains the H1 fragment (Fig. 1). In this way, similar to higher eucariotic cells the *P. pastoris* signal peptidase could account for the observed HCcAg processing. To study the nature of p21, a protein containing the first 176 aa of HCcAg (Co.176) produced in *E. coli*, was used (Fig. 1). Fig. 3 shows the immunodetection in Western blot of p21 (produced in *P. Pastoris*) and Co.176 (produced in *E. coli*) by the mAb SS-HepC.1. This analysis revealed that both immunodetected antigens [p21 (lane 2) and Co.176 (lane 5)] migrated at the same molecular weight, suggesting that p21 is similar to the correctly processed HCV core antigen (173-aa HcAg) [8–11]. This 173-aa HCcAg protein has shown to represent the native HCV core protein found in viral particles present in sera of HCV infected patients [10,21].

The ability of *P. pastoris* to process protein precursors has been previously demonstrated for other antigens [22]. In our case, if the effective processing of HCcAg–E1 protein takes place, the E1 protein must be detected. The results of this analysis are shown in Fig. 3. After 96 h of methanol induction, the presence of a 32 kDa antigenic band (lane 2), detected by the mAb SS-HepC.2 (specific to E1 antigen), was observed in the HCcAg–E1 preparation. This specific signal corresponds to the expected molecular weight of correctly processed highly glycosylated E1.148 [23]. In this study, *E. coli* strain SG 13009, expressing the first 148 aa of E1 protein (E1.148-H) was used as a control. The molecular weight difference between the detected 32 kDa–E1 protein (lane 2) and the E1.148-H (16 kDa, lane 3) is consistent with the modification of four to five potential glycosilation sites in the E1.148 antigen, produced in yeast. This is in accordance with previous studies performed by Fournillier-Jacob and coworkers [24], where removal of N-linked carbohydrates from HCV E1 glycoprotein resulted in the loss of approximately half of the molecular mass, as determined by SDS–PAGE.

In conclusion, the expression of the HCV structural proteins (HCcAg and E1) in *P. pastoris* leads to processing of core protein in a similar manner to that in transfected higher eucariotic cells and in vitro translation studies. These data justify the usefulness of this system to process structural viral proteins. This is the first report of correctly processed HCcAg, produced in *P. pastoris*.

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