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Expression and Characterization of Hepatitis C Virus Core Proteins: Effects of Single Amino Acid Substitution on Protein Conformation and Subcellular Localization

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Hepatitis C virus (HCV) core proteins from two different isolates (HCV-1 and HCV-RH) were expressed in *Spodoptera frugiperda* (Sf9) insect cells. The RH core consisted of two major species of proteins (21 kDa and 19 kDa). On the other hand, the HCV-1 core was approximately 16 kDa in a SDS-PAGE gel. Both core proteins were phosphorylated *in vivo* on serine residues. Furthermore, the RH core but not HCV-1 core formed dimers, indicating that the protein conformation of the core in these two isolates is dfferent from one another. Immunofluorescence studies showed that the RH core was present in the cytoplasm, whereas the HCV-1 core was localized predominantly to the nucleus in recombinant baculovirus-infected insect cells. Since the major difference between the two isolates is the codon 9 of the core protein, a single amino acid substitution appears to play a major role in the protein conformation and these properties may reflect the different biological functions of core proteins in HCV-infected cells.

Keywords: Baculovirus expression system, Core protein, Hepatitis C virus, Phosphorylation, Protein conformation.

Introduction

Hepatitis C virus (HCV) is the major causative agent of blood-borne non-A, non-B hepatitis (Choo *et al.*, 1989; Kuo *et al.*, 1989). HCV infection is associated with liver cirrhosis and hepatocellular carcinoma (Saito *et al.*, 1990). The genome of HCV was first identified in 1989 (Choo *et al.*, 1989). To date, the mechanisms of viral RNA replication and pathogenesis of HCV are poorly understood due to the lack of an *in vitro* cell culture system. HCV contains a positive-strand RNA genome of approximately 9.5 kb in length (Kato *et al.*, 1990; Inchauspe *et al.*, 1991). The genome encodes one large polyprotein precursor of 3010 amino acids which is then cleaved into the mature viral structural and nonstructural proteins (Hjikata *et al.*, 1991; Grakoui *et al.*, 1993; Lin *et al.*, 1994; Manabe *et al.*, 1994). The sequence information from various strains of HCV shows that the virus is a member of the Flaviviridae family (Francki *et al.*, 1991) and is also similar to animal pestiviruses (Miller and Purcell, 1990; Takamizawa *et al.*, 1991). HCV contains viral encoded structural proteins on the N-terminal one third of the protein. These include nucleocapsid (core) protein and envelope proteins. The core, a basic protein, is made up of 191 amino acids which are well conserved among different isolates.

HCV core is a multifunctional protein. It can suppress the gene expression and replication of hepatitis B virus (Shih *et al.*, 1993; 1995), human immnunodeficiency virus type 1 long terminal repeat (LTR), and c-fos promoter activities (Ray *et al.*, 1995). The HCV core protein also activates human c-myc, Rous sarcoma virus LTR, and SV 40 early promoter (Ray *et al.*, 1995). It can sensitize cells to TNF and Fas-mediated cell death (Ruggieri *et al.*, 1997). Furthermore, it can transform primary rat embryo fibroblasts in cooperation with the ras oncogene (Ray *et al.*, 1996). We have recently demonstrated that the HCV core binds to the lymphotoxin β-receptor (Matsumoto *et al.*, 1997) and heterogeneous nuclear ribonucleoprotein K (Hsieh *et al.*, 1998). These findings represent the potential role of the HCV core protein in the regulation of cellular genes.

Previously, we compared core gene products from two different HCV isolates, HCV-1 and HCV-RH (Lo *et al.*, 1994; 1995). Deletion-mapping analysis showed that the HCV-1 core lacked the carboxy-terminal sequence of the RH core. To further characterize HCV core proteins, we expressed both HCV-1 and HCV-RH core proteins in insect

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cells using the baculovirus expression system. Here, we report that the HCV core protein is phosphorylated *in vivo* and the RH core, but not the HCV-1 core, forms dimers. The HCV-1 core was localized to the nucleus, whereas the RH core was detected predominantly in the cytoplasm in insect cells. These properties suggest that the core proteins of these two isolates may have different functions.

Materials and Methods

Construction of HCV core expression vectors The construction procedure has previously been described (Hwang *et al.*, 1992). Briefly, cDNA fragments corresponding to nt #342 to 914 (core open reading frame) flanking the *Bam*HI sites of both HCV-1 and HCV-RH (Lo *et al.*, 1994) were amplified separately by the polymerase chain reaction (PCR). The PCR products were gel-purified, digested with *BamH*I, and cloned into the corresponding site of the transfer vector pVL 941 behind the polyhedrin promoter. The DNA sequences of both recombinant vectors were confirmed by the dideoxynucleotide chain termination sequencing method (Sanger *et al.*, 1977). DNA cotransfection and screening of recombinant viruses were performed as previously described (Hwang *et al.*, 1997).

Cells and viruses Sf9 insect cells were cultured at 27°C using Grace's insect tissue culture medium (GIBCO BRL, Grand Island, USA) supplemented with 10% fetal calf serum (Omega Scientific, USA). Wild-type (AcNPV) and recombinant baculoviruses were maintained as previously described (Hwang *et al.*, 1992).

Metabolic labeling of viral proteins and immunoprecipitation Sf9 cells were infected with recombinant baculoviruses and incubated at 27°C. On day 2 postinfection, cells were incubated with methionine-free Grace's insect cell medium (GIBCO BRL, Grand Island, USA) for 1 h and labeled with 0.1 mCi/ml of [^{35}S]-methionine and -cysteine (NEN, Boston, USA) for 3 h. The labeled cells were washed in PBS, lysed in RIPA buffer [1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.1 mM PMSF], and centrifuged in a microfuge for 15 min. Aliquots of cell lysates were used for immunoprecipitation as previously described (Hwang *et al.*, 1997). For ^{32}P-labeling of the HCV core, recombinant baculovirus-infected Sf9 cells were incubated for 1 h in phosphate-free medium and labeled with [^{32}P]-orthophosphate (0.3 mCi/ml) (ICN, Costa Mesa, USA) for 3 h. Cells were washed in PBS and proteins were immunoprecipitated as described above.

Phosphoamino acid analysis ^{32}P-labeled HCV core protein was immunoprecipitated, eluted from the immunoprecipitate, and hydrolyzed in 6 N HCl at 110°C for 1 h. The hydrolyzed protein was dried and resuspended in a solution containing 1 mg/ml each of phosphoserine, phosphothreonine, and phosphotyrosine and spotted on 3MM Whatman paper. Two-dimentional electrophoresis was performed at 1000 V for 1 h in pH 1.9 buffer containing formic acid and glacial acetic acid, and at 2000 V for 30 min in pH 3.5 buffer containing glacial acetic acid and pyridine. The positions of phosphoamino acids were determined by staining with 0.25% ninhydrin (Sigma, St. Louis, USA) and by autoradiography.

Immunoblot analysis Cell lysates were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to nitrocellulose membrane (Millipore, Bedford, USA). The membrane was incubated in PBS containing 5% nonfat dried milk for 1 h at room temperature and washed three times with TNT buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05% (v/v) Tween 20]. The blot was then incubated for 2 h with rabbit anti-HCV core antibody in PBS containing 3% nonfat dried milk. After several washes with TNT buffer, the membrane was further incubated with HRP-labeled secondary antibody (Amersham Life Science, Buckinghamshire, UK) and proteins were visualized by using the ECL Western blot detection kit (Amersham Life Science) and exposure to X-ray films.

Immunofluorescence analysis Sf9 cells grown on coverslips were infected with the recombinant viruses at an m.o.i. of 8. At 36 h postinfection, both mock- and recombinant virus-infected cells were rinsed in PBS and fixed in cold acetone for 10 min at room temperature. Samples were washed twice in PBS and incubated with rabbit anti-HCV core serum for 1 h at 37°C. After washing in PBS, samples were further incubated at 37°C for 45 min with rhodamine-conjugated goat anti-rabbit IgG (Boehringer-Mannheim Biochemicals, Indianapolis, USA) diluted 1:20 with PBS. Samples were washed in PBS and mounted with Vectashield medium (Vector Lab.) and examined by fluorescence microscopy.

Results

Expression of HCV core proteins in Sf9 cells To understand the biochemical properties of the HCV core protein, a cDNA representing the HCV core open reading frame was cloned into the transfer vector pVL941 (Fig. 1). Recombinant baculoviruses were generated by cotransfection of Sf9 cells with recombinant plasmid and wild-type baculovirus DNA as previously described (Hwang *et al.*, 1992). To analyze the core expression, recombinant baculovirus-infected cells were harvested at day 3 postinfection. Total cell lysates were resolved by SDS-PAGE and proteins were transferred to nitrocellulose membrane, and antisera raised in rabbits were used to detect HCV core proteins. As shown in Fig. 2, the RH core was detected as a major thick protein product. However, this protein consisted of two bands (19 kDa and 21 kDa) at

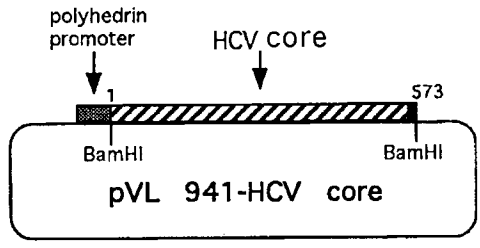

Fig. 1. Schematic diagram of HCV core construct. cDNA encoding either the HCV-1 core or RH core was constructed under the polyhedrin promoter. The construction procedure is detailed in Materials and Methods.

lighter exposure. In contrast, the HCV-1 core was expressed as an apparent molecular mass of 16,000 (data not shown; refer to Fig. 3A). Coomassie blue staining showed very little protein, indicating that the level of HCV core expression is intrinsically lower than other proteins expressed in the baculovirus expression system (data not shown).

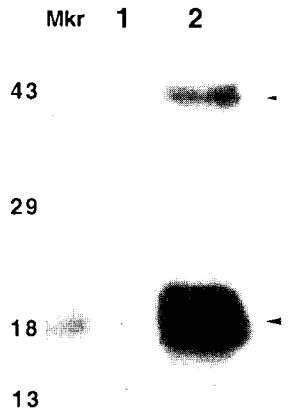

Fig. 2. Expression of HCV-RH core proteins in insect cells. Wild-type (AcNPV) or recombinant baculovirus-infected Sf9 cells were harvested at 3 days postinfection. Cell lysates were separated by electrophoresis in a 15% polyacrylamide gel containing SDS and electrotransferred to a nitrocellulose membrane. HCV core proteins were detected by immunoblotting with rabbit anti-HCV core sera. Lane 1, wild-type-infected cells; Lane 2, recombinant baculovirus-infected cells. The arrows indicate the monomer (thick) and dimer (thin) forms of the HCV-RH core proteins.

Core is phosphorylated *in vivo* on serine residues To examine whether core proteins were phosphorylated *in vivo*, both HCV-1 and RH core proteins expressed in insect cells were analyzed for phosphorylation. Recombinant baculovirus-infected Sf9 cells were labeled with [^{32}P]orthophosphate or [^{35}S]methionine at day 2 postinfection. Core proteins were immunoprecipitated by the rabbit antibodies. Figures 3A and 3B show that both core proteins were phosphorylated *in vivo*. [^{35}S]methionine-labeling was used as a control. It is noteworthy that the dimer form was continuously detected for the RH core but not the HCV-1 core. In order to see which amino acid residues are phosphorylated, [^{32}P]-labeled insect cells were immunoprecipitated and subjected to phosphoamino acid analysis. As shown in Fig. 4, the HCV core was phosphorylated on serine residues.

Localization of HCV core proteins in insect cells To further investigate the subcellular distribution of the HCV nucleocapsid protein, recombinant baculovirus-infected insect cells were stained with rabbit antibody directed against the core protein and analyzed by indirect immunofluorescence. As demonstrated in Fig. 5, the HCV-1 core protein was homogeneously localized in the nuclei of virus-infected insect cells (Fig. 5B). In contrast, the RH core showed a cytoplasmic staining pattern typical to the endoplasmic reticulum-associated proteins (Fig. 5D). No staining was observed in cells reacted with rabbit preimmune sera (Figs. 5A and 5C).

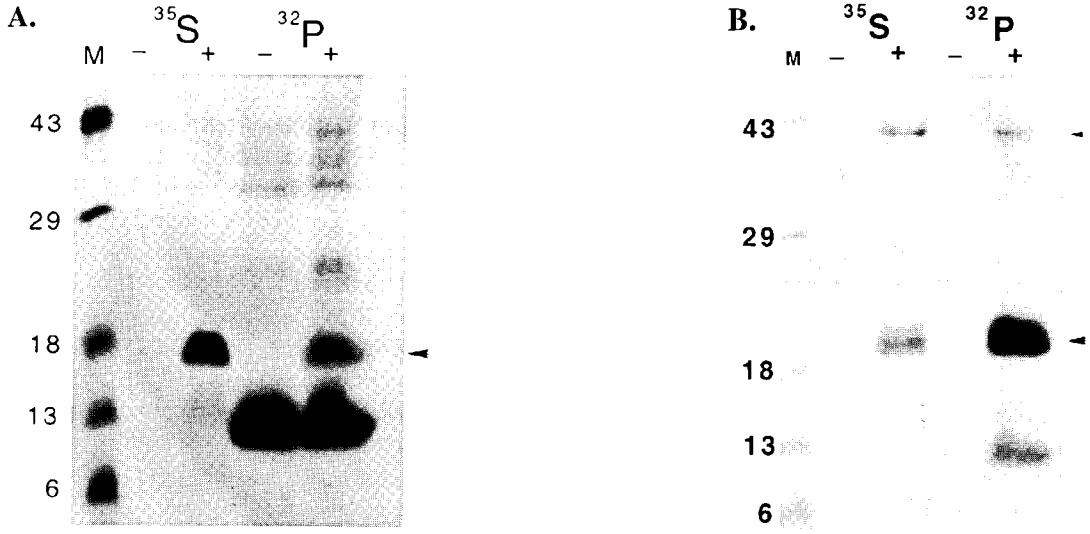

Fig. 3. *In vivo* phosphorylation of HCV core proteins. Sf9 insect cells infected with recombinant baculoviruses expressing either HCV-1 core (A) or RH core (B) were labeled metabolically with either [^{35}S]-methionine and -cysteine or [^{32}P]orthophosphate for 3 h at 2 days postinfection. HCV core proteins were immunoprecipitated with either rabbit preimmune (−) or anti-HCV core antibody (+) in nondenaturing conditions and proteins were resolved on a 15% SDS-polyacrylamide gel. The arrows at the right indicate the monomer (thick) and dimer (thin) forms of the HCV core proteins.

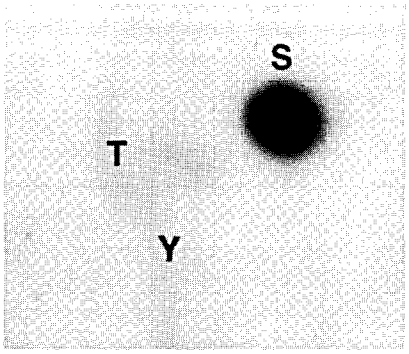

Fig. 4. Phosphoamino acids analysis. Sf9 insect cells infected with recombinant baculovirus were labeled *in vivo* with ^{32}P-orthophosphate and immunoprecipitated with rabbit anti-HCV core antibody. The eluted immunoprecipitates were subjected to hydrolysis in acid and analyzed by two-dimensional electrophoresis as described in Materials and Methods. The positions of unlabeled phosphoamino acid standards were visualized by staining with ninhydrin. S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

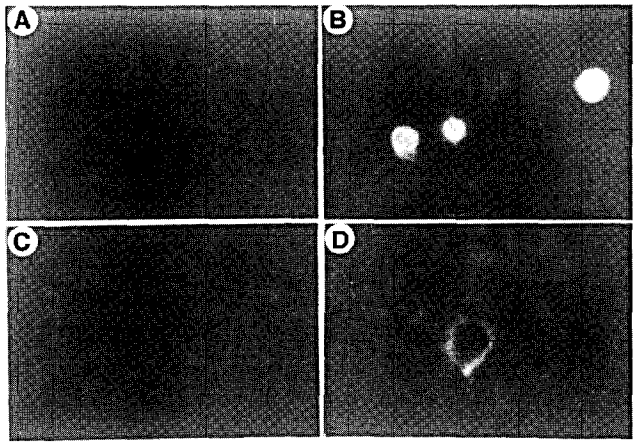

Fig. 5. Subcellular localization of the HCV core in Sf9 cells. Cells were infected with the recombinant baculovirus expressing either HCV-1 core (A and B) or RH core (C and D). At 36 h postinfection, cells were fixed and processed by indirect immunofluorescence using either rabbit preimmune sera (A and C) or anti HCV core antibodies (B and D). Rhodamine-conjugated goat anti-rabbit IgG was used as a secondary antibody.

Discussion

The recombinant baculovirus expression system has been widely used for producing large quantities of foreign proteins. Proteins expressed using the baculovirus expression system preserve the same biological properties of the native counterparts. However, the level of the HCV core expression was lower than other HCV nonstructural proteins that we have previously expressed (Hwang *et al.*, 1997). This was further confirmed by the transient expression of the core protein, in which core protein was hardly detectable in mammalian cells unless cDNA transfection was preceded by a recombinant vaccinia virus (vTF7-3) infection (data not shown).

In a previous work, the HCV core has been claimed to be phosphorylated (Lanford *et al.*, 1993; Shih *et al.*, 1995). However, neither *in vitro* nor *in vivo* studies were able to demonstrate that the ^{32}P-labeled HCV core was immunoprecipitated by the antibody. It was, therefore, not clear whether the core protein was phosphorylated. In this study, we have shown that the HCV core expressed in insect cells was phosphorylated. We believe that this is the first demonstration that core protein is phosphorylated *in vivo* and the ^{32}P-labeled core is immunoprecipitatable by the antibody. It is noteworthy that a dimeric form of the RH core was always detectable in both immunoblot and immunoprecipitation assays. Previously, we have found that the amino-terminal hydrophilic region of the core protein is responsible for the dimerization by a yeast two-hybrid system (Matsumoto *et al.*, 1996). Since the major difference between the HCV-1 and RH cores is the codon 9 of the core protein, which is also localized within the hydrophilic portion, this single amino acid substitution (a lysine of HCV-1 and an arginine of the RH core) may be sufficient to alter the protein conformation. In murine leukemia virus, a single amino acid change in the capsid protein altered a viral tropism, probably due to the change of protein conformation (Kozak and Chakraborti, 1996).

The intracellular localization of the HCV core protein is an intriguing issue. HCV is thought to replicate in the cytoplasm of virus-infected cells similar to Flavivirus. However, our study showed that the HCV-1 core was localized to the nucleus, whereas the RH core was detected in the cytoplasm in association with the endoplasmic reticulum. A previous study demonstrated that the HCV core protein was expressed in the cytoplasm at day 3 and transported to the nucleus at day 6 post-transfection (Shih *et al.*, 1993). The HCV core protein contains a nuclear localization signal at the N-terminus (Chang *et al.*, 1994; Suzuki *et al.*, 1995) and various truncated forms of the core protein were detected in the nucleus (Lo *et al.*, 1995; Liu *et al.*, 1997). The cDNA length of the HCV-1 core is the same as that of other types. However, it encodes a C-terminally truncated core protein (Lo *et al.*,1995). To date, how the HCV core protein is processed is not yet clear. Furthermore, the functional importance of the truncated form of core proteins in the HCV life cycle is not yet known. Although both core proteins contain nuclear localization signals, the difference in subcellular localization may reflect the potential roles in gene regulation. It is also possible that a small portion of the core protein may shuttle between the cytoplasm and the nucleus to control the gene regulation. Taken together, the HCV core protein is a phosphoprotein and the distinct differences in protein conformation and subcellular localization between the two isolates raise the possibililty

that they may have different biological roles in viral replication and pathogenesis of HCV in patients.

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