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Hepatitis C Virus Core Protein Is Efficiently Released into the Culture Medium in Insect Cells

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Hepatitis C virus (HCV) is a causal agent of the chronic liver infection. To understand HCV morphogenesis, we studied the assembly of HCV structural proteins in insect cells. We constructed recombinant baculovirus expression vectors consisting of either HCV core alone, core-E1, or core-E1-E2. These structural proteins were expressed in insect cells and were examined to assemble into particles. Neither core-E1 nor core-E1-E2 was capable of assembling into virus-like particles (VLPs). It was surprising that the core protein alone was assembled into core-like particles. These particles were released into the culture medium as early as 2 days after infection. In our system, HCV structural proteins including envelope proteins did not assemble into VLPs. Instead, the core protein itself has the intrinsic capacity to assemble into amorphous core-like particles. Furthermore, released core particles were associated with HCV RNA, indicating that core proteins were assembled into nucleocapsids. These results suggest that HCV may utilize a unique core release mechanism to evade the hosts defense mechanism, thus contributing to the persistence of HCV infection.

Keywords: Core-like particles, Hepatitis C virus, Nucleocapsid, Virus assembly, Virus-like particles

Introduction

Hepatitis C virus (HCV), a member of the *Flaviviridae* family, is an important human pathogen associated with chronic hepatitis and hepatocellular carcinoma (Saito *et al.*, 1990; Shimotohno, 1993). HCV contains a positive-sense, single-stranded RNA genome of approximately 9.6 kb (Choo *et al*., 1989; Kato *et al*., 1990; Miller and Purcell, 1990; Inchauspe *et al*., 1991; Takamizawa *et al*., 1991). The genome contains a single open reading frame that is flanked by untranslated regions (UTR) at both the 5' and 3' termini. The highly conserved 5' UTR contains an internal ribosome entry site (IRES) that mediates the CAP-independent translation of the viral RNA (Wang and Siddiqui, 1995). The viral genome encodes a single polyprotein precursor of 3,010 to 3,030 amino acids, which is proteolytically processed by both host- and virus-encoded proteases into 10 distinct structural and nonstructural proteins (Hijikata *et al*., 1991; Grakoui *et al*., 1993; Lin *et al*., 1994). The NH_2-terminal portion of the polyprotein encodes structural proteins including core, and two envelope proteins, E1 and E2/p7 and the carboxyl terminus comprises nonstructural proteins. Although both biochemical and functional properties of many structural and nonstructural proteins have been well characterized, studies on virion morphogenesis and viral replication have been hampered by the lack of an efficient tissue culture system.

HCV is an enveloped virus and virion size is between 30 to 60 nm. Virus-like particles have been identified in thin-section electron microscopy of *in vitro* cultured cells. However, the mechanism of HCV virion assembly is not understood because the expression of the HCV structural gene in mammalian cells generates no detectable virion particles. In the present study, we demonstrate that core protein alone undergoes self-assembly into particle-like structure in recombinant baculovirus-infected insect cells. Neither E1 nor E2 envelope protein was required for particle assembly. In our system, core proteins alone were sufficient for assembly into core-like particles. The availability of large amounts of core-like particles will provide us a system to study viral morphogenesis and to develop a serological test for the diagnosis of HCV infection.

Abbreviations: HCV, hepatitis C virus; VLP, virus-like particle; Sf9, *Spodoptera frugiperda*; ER, endoplasmic reticulum; m.o.i., multiplicity of infection

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Materials and Methods

Construction of expression vectors HCV cDNA sequence (genotype 1b) corresponding to the structural proteins (core, E1, E2) was subcloned into the baculovirus shuttle vector, pVL941 as described previously (Hwang *et al*., 1997). Briefly, cDNAs corresponding to the both wild type and mutant forms of HCV structural genes were amplified by polymerase chain reaction (PCR) using *Taq* DNA polymerase (Roche, Mannheim, Germany). Each primer contains a *Bam*HI or a *Bgl*II site and a protein initiation codon (ATG) at the front, and a stop codon (TGA) plus a *Bam*H1 or a *Bgl*II endonuclease site at the end. The amplified PCR product was gel-purified and digested with either *Bam*H1 or *Bgl*II and inserted into the *Bam*HI site of the pVL941 vector behind polyhedrin promoter.

Production of recombinant baculoviruses *Spodoptera frugiperda* (Sf9) insect cells were cotransfected with wild type baculovirus (*Autographa Californica* nuclear polyhedrosis virus, AcNPV) DNA and each recombinant transfer vector DNA as described previously (Hwang *et al*., 1997). Culture supernatants were harvested at day 4 posttransfection and used for plaque assays. Each virus isolated from a plaque was used to infect Sf9 cells to amplify recombinant viruses. Protein expressions were examined either by SDS-PAGE and Coomassie blue staining or by Western blot analysis.

Purification of core-like particles Sf9 cells were infected with recombinant baculoviruses at a multiplicity of infection (m.o.i.) of 3 and incubated at 27°C. The culture supernatant was collected at 3 days after infection and cell debris were removed by centrifugation at 3,500 rpm for 15 min. Supernatant was further subjected to centrifugation at 12,000 rpm for 30 min to eliminate the baculoviruses. The supernatant was pelleted through 30% sucrose cushion (wt/vol in PBS) for 90 min at 27,000 rpm using a SW 28 rotor. For equilibrium density gradient sedimentation, the pellet was resuspened in a solution of CsCl (1.32 g/ml) and centrifuged at 48,000 rpm for 24 h in a SW 50.1 rotor. Ten fractions were diluted in PBS, pelleted, and subjected to Western blot analysis using HCV patient sera.

Western blot analysis Recombinant baculovirus-infected Sf9 cells were harvested at 3 days after infection and washed twice in PBS. Either cell lysates or purified particles were separated by SDS-PAGE and transferred to a nitrocellulose membrane for 1 h. The membrane was incubated with HCV patient serum and proteins were visualized as previously described (Park *et al*., 2000; Kim *et al*., 2004).

HCV RNA detection by RT-PCR Culture supernatants were harvested from cells infected with recombinant viruses expressing either HCV core or HCV core-E1-E2 proteins. Culture supernatants were subjected to centrifugation using 30% sucrose cushion as described above. RNA was extracted from the pellet and reverse transcribed by AMV reverse transcriptase (Promega, Madison, USA) using the primer 5'-GGAAGATCTTCAAGCAGAAACTGG GGT-3', which corresponds the C-terminal end of the core. The cDNA was amplified with this primer and the sense primer 5'-CCGCTCGAGGATGAGCACAAATCC-3' of the HCV core.

Results

Expression of HCV structural proteins in insect cells In order to understand the HCV morphogenesis, we generated recombinant baculoviruses encoding various parts or portions of HCV structural proteins (Fig. 1) and used them to infect insect cells with low m.o.i. (10^3). Cell lysates were prepared at 3 days after infection and analyzed for protein expression. As shown in Fig. 2A, all recombinant baculovirus-infected cells expressed the corresponding HCV structural proteins. As reported previously, core protein consisted of two bands, 19 kDa and 21 kDa (Hwang, 1998). Protein expressions of both E1 and E2 were verified by immunoblotting with monoclonal anti-E1 and E2 antibodies, respectively (data not shown). It was interesting that C-terminus of E1 and p7 exerted the inhibitory effect on overall expression levels of structural proteins (Fig. 2A, lanes 4 and 6). Furthermore, E1 protein was barely detectable when it was expressed as a fusion to the E2 envelope protein (Fig. 2A, lane 5). It is uncertain why E1 expression is so low in this construct. In fact, many HCV patient sera showed the similar results (data not shown).

Core-like particles but not VLPs were produced from the structural gene Since all constructs were verified to express correct proteins, we examined if any construct would produce VLP. We first examined intracellular VLPs using electron microscopy. However, no VLPs were detected in recombinant baculovirus-infected cells. Based on other group's findings (Luo *et al*., 1990; Jiang *et al*., 1992; Crawford *et al*., 1994), we then examined cell culture supernatant to test whether HCV structural proteins were released into the medium. Sf9 cells were infected with recombinant baculoviruses and cell culture supernatant was harvested at day 3 postinfection. The supernatant was pelleted through a 30% sucrose cushion and

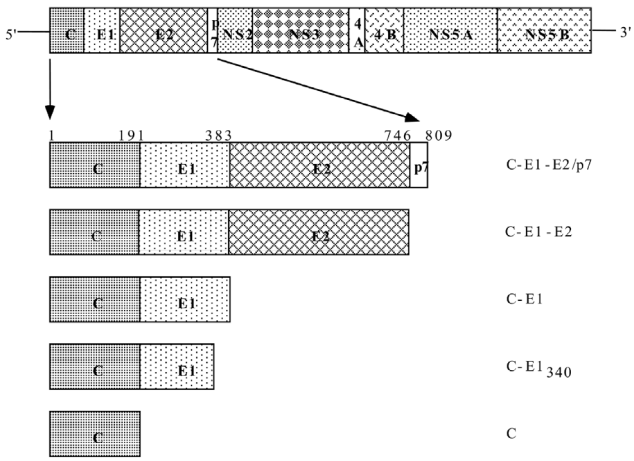

Fig. 1. Schematic diagram of HCV structural protein constructs used in baculovirus expression system. cDNA corresponding to the HCV structural gene was subcloned into the *Bam*HI site of the transfer vector pVL941 behind polyhedrin promoter. Recombinant baculoviruses expressing HCV structural proteins were produced as described in Materials and Methods.

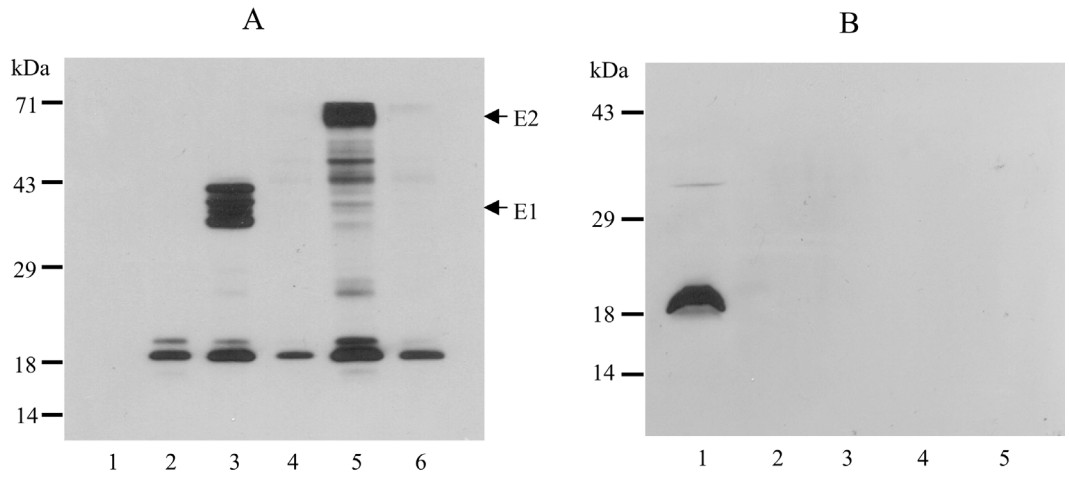

Fig. 2. Core-like particles but not VLPs were produced from the structural gene. (A) Expression of HCV structural proteins in insect cells. Sf9 cells were infected with recombinant baculoviruses expressing HCV structural proteins and were harvested at day 3 postinfection. Cell lysates were separated by SDS-containing polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane. Proteins were detected by Western blotting using HCV patient sera. Lane 1, mock-infected; lane 2, core; lane 3, C-E1 340; lane 4, C-E1; lane 5, C-E1-E2; lane 6, C-E1-E2 (p7). The results shown are representative of three independent experiments. (B) The culture medium from recombinant baculovirus-infected insect cells was centrifuged at 3,500 rpm for 15 min to remove cell debris. Supernatant was further centrifuged at 12,000 rpm for 30 min to remove recombinant baculoviruses. The resultant supernatant was then pelleted through a 30% sucrose cushion for 90 min at 27,000 rpm. The pellet was dissolved in sample buffer and analyzed by Western blotting using HCV patient sera. Lane 1, core; lane 2, C-E1340; lane 3, C-E1; lane 4, C-E1-E2; lane 5, C-E1-E2 (p7).

examined for VLPs using an HCV patient serum. The result showed that only the supernatant collected from the core-expressing cells contained the particles that reacted with an HCV patient serum (Fig. 2B, lane 1) and thus might represent core-like particles. However, neither core-E1- nor core-E1-E2-expressing culture medium contained VLPs. We further looked for VLPs in the culture supernatant harvested from envelope-expressing cells at day 4 postinfection. Although cytolysis started to occur at day 4 postinfection (Hwang *et al*., 1998), we were unable to detect VLPs. To further examine the presence of VLPs, we used 20% sucrose cushion, instead of 30%, to pellet the culture supernatant. Nevertheless, only core protein, without envelope proteins, was released into the culture medium as a core-like particle. We then examined how early core-like particles were released after recombinant virus infection. Sf9 cells were infected with recombinant baculovirus expressing HCV core protein and particle production was examined. As shown in Fig. 3, released core-like particles were detected as early as 2 days after infection, and efficiently released into the culture medium until 4 days after infection. We have not examined particle release more than 4 days after virus infection because wild type baculovirus-induced cytolysis occurs thereafter (Hwang *et al*., 1998).

Characterization of core-like particles To characterize the core-like particles, culture supernatants were harvested from recombinant baculovirus-infected cells and pelleted through the 30% sucrose cushion. The pellet was then further subjected to CsCl density gradient centrifugation. As shown in Fig. 4, core-like particles were detected in a specific fraction.

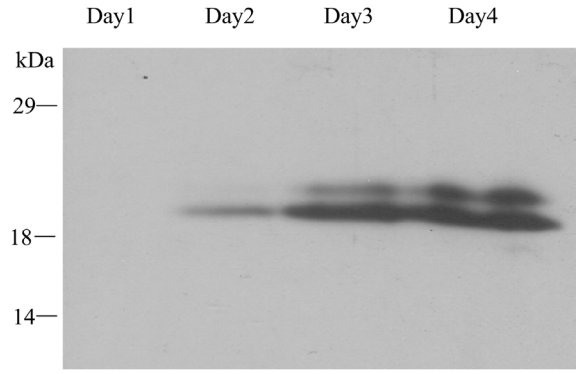

Fig. 3. Kinetics of core-like particle release from recombinant baculovirus-infected insect cells. Sf9 cells were infected with low m.o.i. of recombinant baculoviruses expressing core protein. Culture supernatants were harvested from day 1 to day 4 postinfection and partially purified core-like particles were detected by Western blot analysis.

We found that some particles were pelleted to the bottom in a CsCl gradient centrifugation (data not shown) but this pellet was no longer present after RNAse treatment (0.1 mg/ml), suggesting that some particles were associated with RNA. The density of CsCl fraction containing the core-like particles was approximately 1.25 g/ml. These particles were not observed in preparations from either core-E1-E2 recombinant virus- or wild type baculovirus-infected cells (data not shown). When the peak fractions were examined by electron microscopy, most of the released core proteins were heterogeneous in size with amorphous particle-like structure (data not shown). We

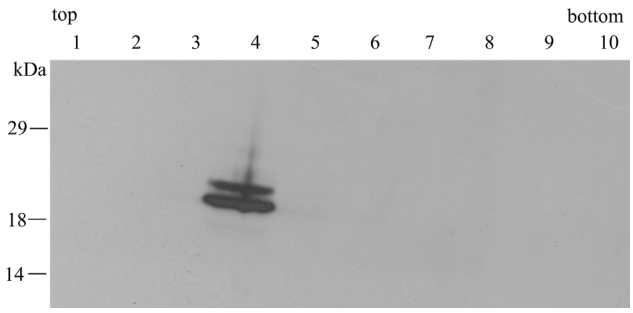

Fig. 4. Isolation of core-like particles by equilibrium density gradient. Sf9 cells were infected with recombinant baculoviruses expressing HCV core protein. The culture supernatant was collected at day 3 postinfection. Following removal of cell debris and recombinant baculoviruses, the particles were partially purified through a sucrose cushion and were subjected to equilibrium density gradient sedimentation. Ten fractions were collected and proteins were detected by Western blot analysis using HCV patient sera.

observed that some of the core proteins were easily aggregated during preparation for electron microscopy. However, we were unable to detect intracellular core-like particles.

Core-like particles contain HCV RNA To verify whether the core particles contain HCV RNA, RNA was extracted from supernatants purified from either the core- or the core-E1-E2-expressing cells. To eliminate the possible DNA contamination, all RNA samples were treated with DNAse I for 30 min at 37ºC. RNA was then reverse transcribed and the cDNA was amplified by PCR. As shown in Fig. 5, cDNA corresponding to the HCV core RNA (573 nt) was detected (lane 2), indicating that core-like particles are associated with HCV RNA. However, the culture medium isolated from the core-E1-E2 construct did not contain HCV RNA (Fig. 5, lane 4).

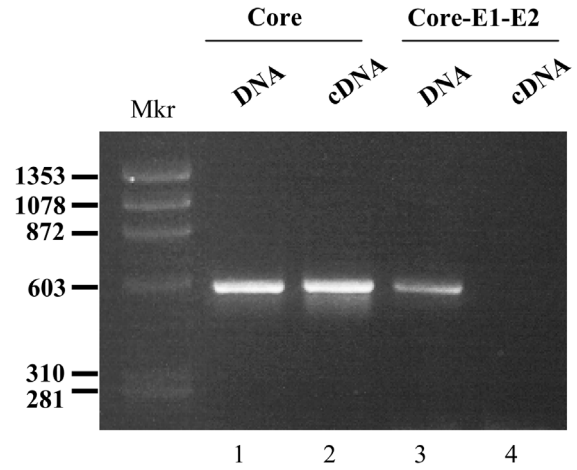

Fig. 5. Core-like particles contain HCV RNA. Sf9 insect cells were infected with recombinant baculoviruses expressing either HCV core or HCV core-E1-E2 protein. Culture supernatants were harvested at 60 h postinfection and were partially purified as described in Materials and Methods. RNA was extracted and reverse transcribed by AMV reverse transcriptase. The cDNA was amplified and then separated by an agarose gel. Mkr, molecular mass marker; lane 1, positive control for RT-PCR of core; lane 2, RT-PCR product from core-like particles; lane 3, positive control for RT-PCR of core-E1-E2 construct; lane 4, RT-PCR product from culture supernatant from core-E1-E2.

Discussion

To understand the HCV morphogenesis, we studied the assembly of structural proteins in insect cells. Recombinant baculoviruses expressing HCV structural proteins were constructed and were used to infect Sf9 insect cells. Both virus-infected cells and culture supernatants were examined to see whether HCV structural proteins were capable of assembling into VLPs. VLPs were not detected in cells and supernatant. However, HCV core protein was released as core-like particles into the culture supernatant from recombinant virus expressing core protein alone. These core-like particles are associated with HCV RNA and amorphous in structure. We further found that these core-like particles were released into the medium as a membrane complex (data not shown). When core protein was expressed as a polyprotein with envelope protein(s), it was clear that particles were no longer released into the culture medium. Previously, it had been demonstrated that VLPs produced from recombinant baculoviruses expressing a part of the 5' UTR and structural proteins were retained in intracellular membrane vesicles and were not released into the culture medium (Baumert *et al*., 1998). At present, it is not clear why two systems show the discrepancy in particle formation. It has been reported that transmembrane domains of E1 and E2 function as retension signals in the endoplasmic reticulum (ER). Since core protein is colocalized with E2 protein (Santolini *et al*., 1994), it is possible that envelope proteins might have inhibitory effect on core particle release. Indeed, if envelope proteins were coexpressed, particle assembly was interrupted. The biological significance of HCV core release is not yet understood. HCV seems to employ a similar assembly mechanism to those of retroviruses and rhabdoviruses because assembly of gag protein of HIV-1 and simian immunodeficiency virus (Delchambre *et al*., 1989; Gheysen *et al*., 1989) and budding of rabies virus particle (Mebatsion et al., 1996) occurred in the absence of envelope proteins. Nevertheless, it is uncertain how virions are assembled in HCV-infected patients.

How HCV core protein itself can be assembled into core-like particles in insect cells is not clear. Previously it has been reported that E1 and E2 hetrodimers do not leave the ER (Deleersnyder *et al*., 1997) and C-terminal 29 amino acids of E2 are responsible for the retention of E2 in the ER (Cocquerel *et al*., 1998). Based on this report, we made

mutants consisting of either core-E1 (C-E1) or core-E1 but lacking C-terminal hydrophobic region (C-$E1_{340}$). Therefore, these constructs do not contain E2 envelope protein. When these mutants were expressed in Sf9 cells, VLPs were still not released into the culture medium. In contrast, core protein alone was efficiently released out of the cells as early as 2 days after infection. These results suggest that HCV core has the intrinsic capacity to assemble into core-like particles. Since most of the cells were alive throughout the experimental period, the core-like particle release is not due to cytolysis. We also infected Sf9 cells with low titer of virus (m.o.i. of 3) to prevent cells from baculovirus-induced cytolysis (Hwang *et al*., 1998). Since HCV core protein was secreted from mammalian cell lines in culture (Sabile *et al*., 1999), and nonenveloped HCV nucleocapsids were overproduced in the plasma of HCV patients and released into the bloodstream (Maillard *et al*., 2001), our finding may represent a unique mechanism of nucleocapsid assembly of HCV. These data hence may represent a novel mechanism of HCV to evade the hosts immunity, thus contributing to the persistence of HCV infection.

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