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C-terminal domain of hepatitis C virus core protein is essential for secretion

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Abstract

AIM: We have previously demonstrated that hepatitis C virus (HCV) core protein is efficiently released into the culture medium in insect cells. The objective of this study is to characterize the HCV core secretion in insect cells.

METHODS: We constructed recombinant baculoviruses expressing various-length of mutant core proteins, expressed these proteins in insect cells, and examined core protein secretion in insect cells.

RESULTS: Only wild type core was efficiently released into the culture medium, although the protein expression level of wild type core was lower than those of other mutant core proteins. We found that the shorter form of the core construct expressed the higher level of protein. However, if more than 18 amino acids of the core were truncated at the C-terminus, core proteins were no longer secreted into the culture medium. Membrane flotation data show that the secreted core proteins are associated with the cellular membrane protein, indicating that HCV core is secreted as a membrane complex.

CONCLUSION: The C-terminal 18 amino acids of HCV core were crucial for core secretion into the culture media. Since HCV replication occurs on lipid raft membrane structure, these results suggest that HCV may utilize a unique core release mechanism to escape immune surveillance, thereby potentially representing the feature of HCV morphogenesis.

Key words: Hepatitis C virus; Core secretion; Morphogenesis; Virus assembly

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INTRODUCTION

Hepatitis C virus (HCV) is the major etiologic agent of transfusion-associated hepatitis[1-3] and is associated with a chronic infection that leads to liver cirrhosis and hepatocellular carcinoma[4,5]. HCV is an enveloped virus and its virion size has been estimated at a diameter of 30-60 nm[6]. The virion contains a single-stranded, positive-sense RNA genome of approximately 9 600 nucleotides[7-10]. HCV belongs to the *Flaviviridae* family[11,12] and its genomic sequence is related to the flaviviruses and the pestiviruses[9,13]. The viral genome encodes a polyprotein precursor of 3 010-3 030 amino acids from one long open reading frame and is further processed into multiple viral proteins[14-16]. The structural proteins are processed by a host signal peptidase into a core protein, envelope proteins E1 and E2, and p7[13,16,17]. The viral nonstructural proteins are cleaved by viral proteinase into serine protease, helicase, RNA polymerase, and functionally undefined proteins. Biochemical properties of many structural and nonstructural proteins have been extensively characterized. However, studies on virion morphogenesis and viral replication have been hampered by the inability to propagate the viruses in a cell culture system.

The mechanism of HCV virion assembly is not yet known because the expression of the HCV structural gene in mammalian cells generates no detectable virion particles. However, it has been reported that either virus-like particles (VLPs) were produced in insect cells infected with recombinant baculoviruses expressing HCV structural proteins[18] or nucleocapsid-like particles were self-assembled from recombinant proteins purified in *E. coli*[19]. Furthermore, previous study showed that HCV core proteins were secreted in insect cells[20] and in mammalian cell culture[21]. In the present study, we have further characterized the HCV core secretion using recombinant baculovirus expression system in insect cells. We found that C-terminal 18 amino acids of the core were necessary for the secretion of core protein into the culture media. Furthermore, HCV core is efficiently released into the culture medium as a membrane-associated protein, which may represent a unique mechanism of HCV core assembly.

MATERIALS AND METHODS

Construction of recombinant baculovirus shuttle vector

HCV cDNA sequence corresponding to the core protein of the Korean isolate (genotype 1b)[22] was subcloned into the baculovirus shuttle vector, pVL941, as described previously[23]. Briefly, cDNAs corresponding to the both wild type and mutant forms of HCV core protein were amplified by polymerase chain reaction (PCR) using *Taq*

DNA polymerase (Boehringer Mannheim). 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*HI or a *Bgl*II endonuclease site at the end. The amplified PCR product was gel-purified and digested with either *Bam*HI 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 co-transfected with wild type baculovirus (*Autographa californica* nuclear polyhedrosis virus, AcNPV) DNA and each recombinant transfer vector DNA as described previously[23]. The supernatant was harvested at 4 d after transfection and used for plaque assays. Each virus isolated from a plaque was used to infect Sf9 cells to obtain high titer of recombinant viruses. Protein expression was examined either by SDS-PAGE and Coomassie blue staining or by Western blot analysis.

Purification of secreted core protein

Sf9 cells were infected with recombinant baculoviruses at a multiplicity of infection (m.o.i.) of 3 and incubated at 27 ℃. The culture supernatant was collected at 3 d after infection and cell debris were removed by centrifugation at 3 500 r/min for 15 min. Supernatant was further subjected to centrifugation at 12 000 *g* for 30 min to eliminate the baculoviruses. The supernatant was pelleted through 300 g/L sucrose cushion for 90 min at 27 000 *g* using a SW 28 rotor. For velocity centrifugation, the pellet was resuspended in PBS and layered onto 200-600 g/L sucrose gradient and centrifuged at 38 000 *g* for 12 h at 4 ℃ using a SW 41 rotor. Twelve fractions were collected from the top, diluted in PBS, and subjected to centrifugation at 48 000 *g* for 90 min using a SW 55 Ti rotor. The peak fractions were pooled and pelleted as described above. The pellet was dissolved in sample buffer and analyzed by Western blot using HCV patient sera.

Western blot analysis

Recombinant baculovirus-infected Sf9 cells were harvested at 3 d after infection and washed twice in PBS. Either cell lysates or secreted proteins were separated by electrophoresis in 10-15% polyacrylamide gel containing 0.5% SDS and transferred to a nitrocellulose membrane for 1 h. The membrane was incubated with either HCV patient serum or rabbit anti-HCV core antibody and proteins were visualized as previously described[23].

Membrane flotation analysis

Sf9 insect cells were infected with recombinant baculoviruses expressing full-length of HCV core protein. At 60 h after infection, the culture supernatant was collected and cell debris was removed by centrifugation at 3 500 r/min for 20 min. Culture supernatant was further subjected to centrifugation at 12 000 *g* for 30 min to eliminate the baculoviruses. The supernatant was pelleted through 300 g/L sucrose cushion for 90 min at 27 000 *g* using a SW 28 rotor. The secreted HCV core proteins were subjected to equilibrium density centrifugation and 1 mL of each fraction was analyzed by immunoblotting using rabbit anti-HCV core antibody as previously described[23].

RESULTS

Expression of HCV core protein in insect cells

In order to understand the mechanism of HCV core secretion in culture media, we generated recombinant baculoviruses encoding full-length of HCV core protein and used them to infect insect cells with low m.o.i. (10^3). Cell lysates were prepared at 3 d after infection and analyzed for protein expression by immunoblotting with HCV patient sera. As shown in Figure 1A, recombinant baculovirus-infected cells expressed the corresponding HCV core protein (lane 3). Both wild type baculovirus (AcNPV)-infected and recombinant baculovirus expressing small hepatitis delta antigen (SHDAg)-infected cells[24] were compared as controls. To examine whether HCV core proteins are released into the culture medium, Sf9 cells were infected with either wild type or recombinant baculoviruses and cell culture supernatants were harvested at d 3 postinfection. The supernatants were pelleted through a 300 g/L sucrose cushion and examined for core secretion using HCV patient serum. The result showed that the supernatant collected from the core-expressing cells contained the core protein reacting with HCV patient serum (Figure 1B, lane 3). Although SHDAg protein was highly expressed in insect cells[24], this protein was not released into the culture medium (data not shown). We further looked for SHDAg release in the culture supernatant harvested from SHDAg-expressing cells at d 4 postinfection. Although cytolysis started to occur at d 4 postinfection in insect cells[25], we were unable to detect SHDAg in supernatant collected from SHDAg-expressing cells (data not shown).

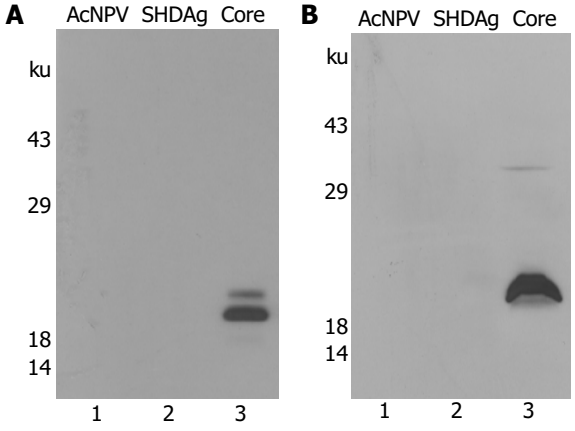

Figure 1 Core proteins are secreted into the culture medium in insect cells. **A**: Expression of HCV core protein in insect cells. cDNA corresponding to the HCV core was subcloned into the *Bam*HI site of the transfer vector pVL941 behind polyhedrin promoter. Recombinant baculoviruses expressing HCV core protein were produced as described in Materials and methods. Sf9 cells were infected with either wild type (AcNPV) or recombinant baculoviruses expressing SHDAg, or recombinant baculoviruses expressing HCV core protein and were harvested at d 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, Sf9 cells infected with wild type baculoviruses; lane 2, Sf9 cells infected with recombinant baculovirus expressing SHDAg; lane 3, Sf9 cells infected with recombinant baculovirus expressing HCV core; **B**: The culture medium from (*A*) was centrifuged at 3 500 r/min for 15 min to remove cell debris. Supernatant was further centrifuged at 12 000 *g* for 30 min to remove baculoviruses. The resultant supernatant was then pelleted through a 300 g/L sucrose cushion for 90 min at 27 000 *g*. The pellet was dissolved in sample buffer and analyzed by Western blotting using HCV patient sera.

Characterization of secreted core proteins

To characterize the secreted core proteins, culture supernatant harvested from recombinant baculovirus-infected cells was pelleted through the 300 g/L sucrose cushion. The pellet was then further subjected to sucrose velocity gradient centrifugation. As shown in Figure 2, secreted core proteins were located in specific fractions. When the peak fractions were examined by electron microscopy, most of the released core proteins were heterogeneous in size as reported[19] with amorphous structure (Choi *et al*.[20], and data not shown). We observed that some of the core proteins were easily aggregated during preparation for electron microscopy. As we previously reported, these secreted proteins have a buoyant density of 1.25 g/mL in CsCl gradient separation[20].

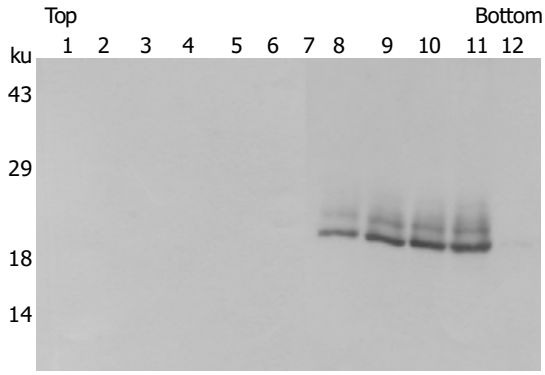

Figure 2 Isolation of core proteins from cell culture supernatant. Sf9 cells were infected with recombinant baculoviruses expressing HCV core protein. The culture supernatant was collected at d 3 postinfection. Following removal of cell debris and recombinant baculoviruses, the released core proteins were partially purified through a sucrose cushion and were subjected to velocity gradient centrifugation. Twelve fractions were collected and proteins were detected by Western blot analysis using HCV patient sera.

Kinetics of core secretion

The HCV core secretion in recombinant baculovirus-infected cells was examined over a 4-d period following infection. Sf9 cells were infected with recombinant baculovirus expressing full-length HCV core and kinetics of protein expression in cells and core secretion in culture media were examined. Cell lysates and secreted core protein were prepared from d 1 to d 4 after infection as described above. Two species of HCV core proteins, M_r 19 000 and M_r 21 000, were expressed in cells as early as d 1 (Figures 3A and C). Two days after virus infection, intracellular core levels reached plateau and gradually decreased thereafter. In contrast, secreted core proteins were detected 2 d after the infection, efficiently released into the culture medium and reached plateau on the 3rd d, and maintained the similar level for an additional day (Figures 3B and D). Trypan blue staining result indicated that most of the recombinant baculovirus-infected cells were viable for 4 d (data not shown).

C-terminal 18 amino acids of core protein are essential for core secretion

To examine which domain of the HCV core is required for core secretion in insect cells, we constructed a series of C-terminal-deleted mutants (Figure 4A) and generated

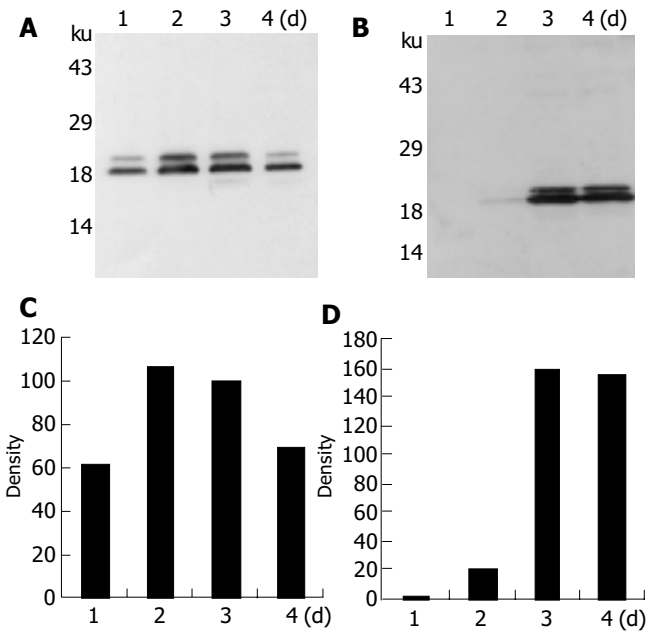

Figure 3 Kinetics of core protein secretion in culture medium of the recombinant baculovirus-infected insect cells. Sf9 insect cells were infected with recombinant baculoviruses expressing full-length core protein (**A**). Cell lysates were prepared from d 1 to d 4 postinfection, and separated by SDS-PAGE on a 15% gel and Western blotted with an HCV patient serum (**B**); Culture supernatants were harvested from d 1 to d 4 postinfection and secreted core proteins were detected by Western blot analysis. Kinetics of intracellular core protein (**C**) and extracellular core protein (**D**) productions were quantified using a densitometric scanner (Molecular Dynamics).

recombinant baculoviruses by co-transfecting insect cells with each mutant DNA and wild type baculovirus DNA. Using low titer (10^3 PFU/mL) of these recombinant baculoviruses, Sf9 insect cells were infected and protein expressions were determined using cell lysates. As shown in Figure 4B, all mutant viruses expressed the expected molecular mass of proteins. This was further confirmed by Western blot analysis (Figure 4C). It is noteworthy that the shorter form of the core construct expressed the higher level of protein. We proceeded to purify the secreted core from each culture supernatant in the same way as described above. Figure 4D showed that full-length core (C191) was efficiently released into the culture medium, although the protein expression level of C191 was lower than those of other mutant core proteins. However, if C-terminal 18 amino acids of the core were deleted, core proteins were no longer secreted into the culture medium, indicating that C-terminal 18 amino acids of HCV core were crucial for core secretion into the culture media. We next examined whether the C-terminal domain of HCV core could be replaced with the comparable domains of other related hepatitis viruses. For this purpose, we replaced C-terminal 18 aa of HCV core with either C-terminal 18 aa of hepatitis B virus surface antigen (HBsAg) or C-terminal 19 aa of large delta antigen of hepatitis delta virus[24]. Although these chimera proteins were highly expressed in insect cells, none of these proteins were released into the culture medium (data not shown), suggesting that authentic core sequence is necessary for secretion.

Membrane association of the secreted core protein

Recently, we showed that HCV core and NS5A protein are

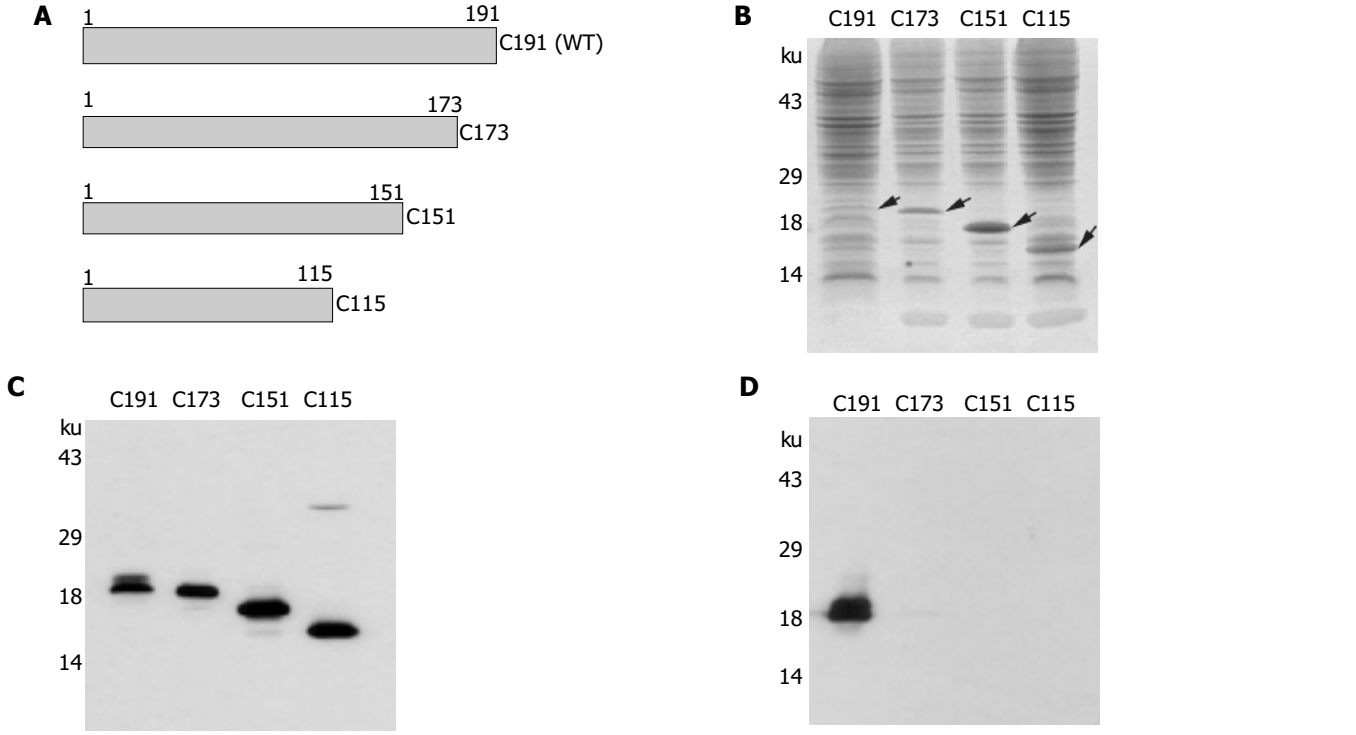

Figure 4 Effects of mutant core proteins on core secretion. (**A**) Schematic diagram illustrating the recombinant baculoviruses expressing truncated forms of core protein. Mutant core constructs were generated by PCR and the subsequent recombinant baculoviruses were made as described in Materials and methods. Protein expression of each mutant was confirmed by SDS-PAGE and Coomassie Brilliant Blue staining (**B**) and Western blot analysis by using HCV patient serum (**C**). (**D**) Determination of extracellular core release among wild type and mutant core proteins. Insect cells were infected with recombinant baculoviruses expressing wild type and various mutant forms of core proteins and harvested at d 3 postinfection. Culture supernatants were partially purified and determined for core secretion by Western blot analysis as described in the legend to Figure 1B.

associated with cellular membrane[26] and HCV replication occurs on lipid raft membrane structure[27]. To investigate whether extracellular form of core proteins were secreted as a membrane complex, we separated the released core proteins into membrane and cytosol fractions using the membrane flotation method as previously described[23]. The presence of the core protein in each fraction was determined by immunoblotting using a rabbit anti-core antibody. As shown in Figure 5, the secreted HCV core protein was found in both membranous and cytosolic fractions. This result indicates that the secreted core proteins are associated with some membranous materials.

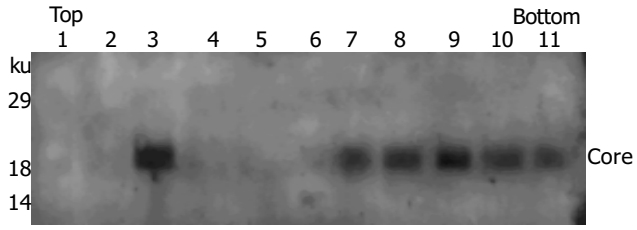

Figure 5 Membrane flotation analysis of secreted core proteins. Sf9 insect cells were infected with recombinant baculoviruses expressing full-length HCV core protein. Culture supernatant was collected at 60 h postinfection and secreted core proteins were partially purified as described in Materials and methods. The sample was then subjected to fractionation by equilibrium sucrose gradient centrifugation. Eleven fractions collected from the top were analyzed by Western blotting using rabbit anti-HCV core antibody.

DISCUSSION

It has been previously reported that HCV core without envelope proteins could form a capsid in an acellular assay[19,28] and secreted core protein has also been detected in mammalian cells[21]. Recently, we have demonstrated that HCV core protein is efficiently released into the culture medium in insect cells[20]. To further understand the mechanisms of core assembly and HCV morphogenesis, we studied the HCV core secretion in insect cells using mutant forms of core protein. We constructed recombinant baculoviruses expressing various-length of HCV core proteins and were used to infect Sf9 insect cells. Culture supernatants harvested from recombinant baculovirus-infected cells were examined to see which domain of core protein is required for core secretion. As we previously reported[20], full-length HCV core protein was efficiently released in cell culture media. However, C-terminal-truncated mutant core proteins were not able to be released into culture media although protein expression levels were higher than that of wild type core protein. This result suggests that C-terminal 18 amino acids are essential for core protein secretion in insect cells. We further showed that secreted core proteins are amorphous in structure and are released into the medium as a membrane complex. This result is consistent with the finding that both core and NS5A are associated with intracellular membranes[27], which may play a role in the pathogenesis of HCV. Previously, it has 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[18]. In fact, transmembrane domains of E1 and E2 function as retention signals in the endoplasmic reticulum (ER) compartment. It has been reported that E1 and E2 of HCV formed a complex and were retained to the ER[29,30]. Moreover, core protein co-localized with the E2 protein[31]. This may be the reason why core protein alone, if envelope proteins were not present, was efficiently released into the culture media.

Previously, many capsid proteins of non-enveloped viruses were reported to assemble into VLPs, including B19 parvovirus[32], Norwalk virus[33], papillomavirus[34], rotavirus[35,36], and rabbit hemorrhagic disease virus[37]. Similarly, HCV core protein without envelope proteins may be assembled into particle-like structure. HCV is an enveloped virus. How HCV core alone, in the absence of envelope proteins, could be assembled into particles is an intriguing question. Nevertheless, there are similar bodies of evidence that gag protein precursor of HIV-1[38], HIV-2[39], or simian immunodeficiency virus[40] self-assembled into VLPs in recombinant baculovirus-infected insect cells. Budding of rabies virus particles also occurred in the absence of glycoprotein[41]. Therefore, HCV seems to employ a similar assembly mechanism to those of retroviruses and rhabdoviruses. One study showed that VLPs were not produced from the Huh-7 cells carrying the full-length HCV genome[42]. To date, it is uncertain how virions are assembled in HCV-infected patients.

It is not clear how HCV core protein itself can be efficiently secreted into insect culture media. In this study, core protein was released out of the cells as early as 2 d after infection. This result suggests that HCV core has the intrinsic capacity to be secreted in culture media. Kunkel *et al*.[19], reported that N-terminal 124 aa residues of the core (genotype 1a) were sufficient for self-assembly into nucleocapsid-like particles. In contrast, our data suggest that full-length of core should be necessary for core assembly. The discrepancy between the two systems may be due to the different genotypes or different expression systems. However, it is consistent that C-terminal hydrophobic sequence (E1 peptidase signal) inhibits high level of protein expression in both prokaryotic and eukaryotic cell culture systems. In mammalian cells, the C-terminally truncated core 173 is translocated into the nucleus, whereas intact core is destined to the ER[43,44]. This is why core 173 could not be released into the medium although its intracellular expression level was high. In this study we showed that only the full-length core was efficiently released into the culture medium, although the protein expression level of the full-length core was lower than those of other mutant core proteins. Furthermore, the comparable domains of other hepatitis viruses were unable to replace the function of C-terminal region of HCV core. It is hence conceivable that the C-terminal domain of HCV core, in addition to being a signal sequence for E1 protein, has intrinsic function in secretion. It is also possible that C-terminal domain of HCV core may interact with cellular proteins specifically.

We have compared the viability of cells infected with recombinant baculovirus expressing HCV core and those infected with wild type or recombinant baculoviruses expressing HCV 5A or 5B. All of these cells showed the similar level of viability after virus infection, indicating that HCV core was not toxic to the insect cells (data not shown). We also used low titer of virus (m.o.i. of 3) to infect cells in order to prevent cells from baculovirus-induced cytolysis[25]. Since most of the cells were alive at the time of harvest, the release of core protein in culture media is not due to cytolysis. It may represent a unique mechanism of the HCV core secretion. Indeed, it has been shown that HCV core protein was also secreted from mammalian cell lines in culture[21]. Recently, Maillard *et al*.[45], reported that nonenveloped HCV nucleocapsids were overproduced in the plasma of HCV patients and released into the bloodstream. They also found that nucleocapsid-like particles but not VLPs were produced in insect cells infected with recombinant baculovirus expressing entire structural proteins. Our study together with these reports strongly suggests that the production of nonenveloped HCV capsids may represent the feature of HCV morphogenesis. HCV may utilize a unique core release mechanism to escape immune surveillance and hence may play a role in HCV pathogenesis.

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