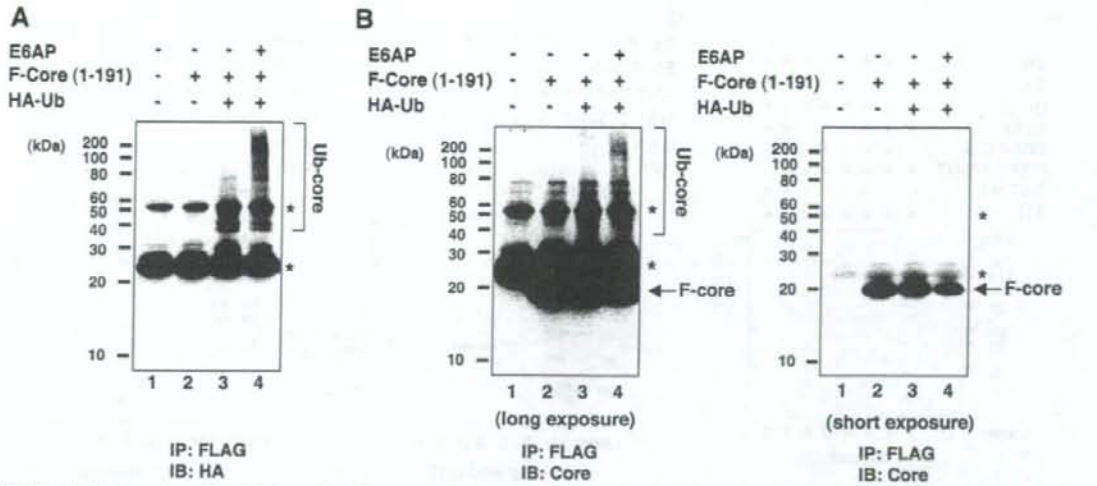

FIG. 7. E6AP-dependent ubiquitylation of HCV core protein in vivo. 293T cells (1×10^6 cells/10-cm dish) were transfected with 1 μg of pCAG FLAG-core (1–191) together with 2 μg of plasmid encoding E6AP as indicated. Each transfection also included 2 μg of plasmid encoding HA-ubiquitin. The cell lysates were immunoprecipitated with FLAG beads and analyzed by immunoblotting with anti-HA PAb (A) or anticore PAb (B). A shorter exposure of the core blot shows immunoprecipitated FLAG-core protein (B, right panel). A longer exposure of the core blot shows the presence of a ubiquitin smear (B, left panel). Asterisks indicate cross-reacting immunoglobulin light chain or heavy chain. Arrows indicate FLAG-core. IB, immunoblot; IP, immunoprecipitation.

jugated with HA-ubiquitin were indeed ubiquitylated forms of the core protein (Fig. 7B, lanes 3 and 4, long exposure).

E6AP mediates ubiquitylation of HCV core protein in vitro. To rule out the possibility that E6AP contributes to core protein degradation by inducing degradation of inhibitors of core turnover, we determined whether E6AP functions directly as a ubiquitin ligase by testing the ability of purified MEF-E6AP to mediate in vitro ubiquitylation of the purified recombinant HCV core protein. HCV core protein was expressed as a fusion protein containing N-terminal GST tag and C-terminal His tag and purified as described in Materials and Methods. GST-C173HT (aa 1–173) and GST-C152HT (aa 1–152) (see Materials and Methods) were used to determine whether the mature core protein and the C-terminally truncated core protein are targeted for ubiquitylation in vitro. The validity of this assay was established by demonstrating that E6AP but not E6AP C-A induced ATP-dependent ubiquitylation of GST-core protein. When in vitro ubiquitylation reactions were carried out either in the absence of MEF-E6AP or in the presence of MEF-E6AP C-A, no ubiquitylation signal was detected (Fig. 8A, lanes 4 and 5). However, inclusion of purified MEF-E6AP in the reaction mixture resulted in marked ubiquitylation of GST-C173HT (Fig. 8A, lane 6), while no ubiquitylation was observed in the absence of ATP (Fig. 8A, lane 7). No signal was detected when GST-HT was used as a substrate (Fig. 8A, lane 8). The higher-molecular-weight species of GST-core proteins were reactive with both anti-ubiquitin MAb (Fig. 8B, right panel, lanes 2 and 4) and anti-GST MAb (Fig. 8B, left panel, lanes 2 and 4). Both GST-C152HT and GST-C173HT were polyubiquitylated by E6AP in vitro (Fig. 8B), indicating that both the C-terminally truncated core and the mature core are polyubiquitylated by E6AP in vitro. These results revealed that E6AP directly mediated ubiquitylation of HCV core proteins in an ATP-dependent manner.

Exogenous expression of E6AP reduces intracellular HCV core protein levels and supernatant infectivity titers in HCV-infected Huh-7 cells. We used a recently developed system for the production of infectious HCV particles using the HCV JFH1 strain (28, 56, 61) to examine whether E6AP can promote degradation of HCV core protein expressed from infectious HCV. E6AP-dependent core degradation was assessed in Huh-7 cells inoculated with the culture supernatant containing HCV JFH1. Levels of HCV core protein were detectable at day 3 postinfection and increased with time. Immunofluorescence staining for the core protein indicated that the percentage of HCV core-positive cells in the Huh-7 cells was almost 100 at day 7 postinfection. Transfection efficiency was 50 to 60% as measured with GFP-expressing plasmid. At day 7 postinfection, exogenous expression of E6AP reduced the intracellular core protein level by about 60% compared to the empty plasmid-transfected control cells (Fig. 9A). Inactive E6AP had little effect on the core protein levels. Total protein levels in the cells (Fig. 9B) and intracellular HCV RNA levels (Fig. 9C) did not change after transfection of wild-type E6AP or inactive E6AP. The immunofluorescence study revealed that HCV core protein was variably detected and the intensity of core staining was reduced in the cells staining positive for wild-type E6AP compared with neighboring cells staining negative for E6AP (Fig. 9E). Using inactive E6AP revealed colocalization of the core protein and E6AP in the perinuclear region (Fig. 9F) of HCV-infected cells. These results suggest that E6AP enhanced degradation of HCV core protein expressed from infectious HCV. Then we titrated HCV infectivity in the culture supernatant at day 7 postinfection by limiting

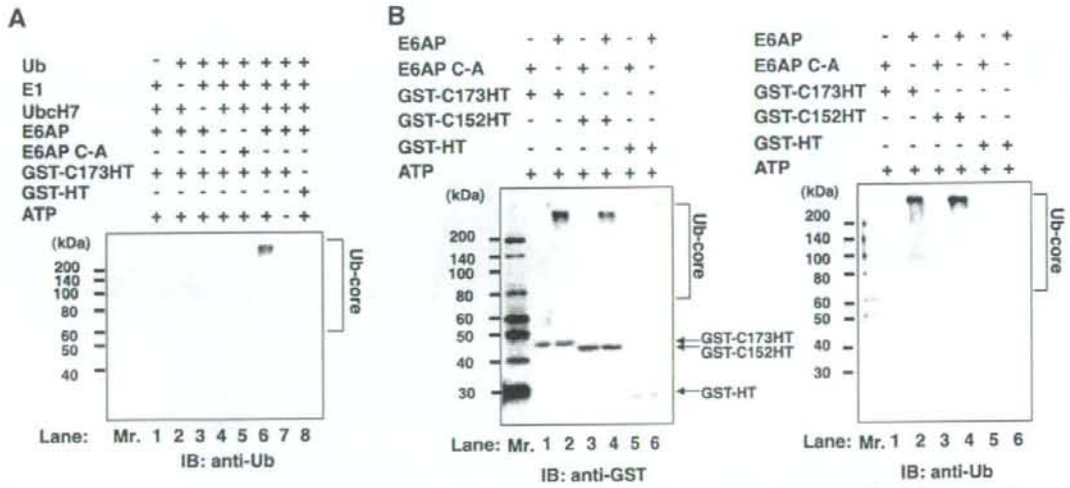

FIG. 8. In vitro ubiquitylation of HCV core protein by recombinant E6AP. For in vitro ubiquitylation of HCV core protein, purified GST-C173HT and GST-C152HT were used as substrates. Purified GST-HT was used as a negative control. Assays were done in 40-μl volumes containing each component as indicated. The reaction mixture is described in Materials and Methods. The reaction was carried out at 37°C for 120 min followed by purification with glutathione-Sepharose beads and analysis by immunoblotting with the indicated antibodies. Arrows indicate GST-C173HT, GST-C152HT, and GST-HT, respectively. Ubiquitylated species of GST-core proteins are marked by brackets. IB, immunoblot.

dilution assays. Exogenous expression of E6AP reduced the supernatant infectivity titer, whereas inactive E6AP had no effect on its infectivity titer (Fig. 9D), suggesting that the E6AP-dependent ubiquitin proteasome pathway affects the production of HCV particles through downregulation of the core protein.

E6AP silencing increases the levels of intracellular HCV core protein and supernatant infectivity titers in HCV-infected Huh-7 cells. Finally, to further validate the role of E6AP in HCV production, expression of endogenous E6AP was knocked down by siRNA and the HCV infectivity titers released from HCV JFH1-infected cells were examined. Knockdown of E6AP by siRNA led to an increase in intracellular core protein levels (Fig. 10A) and supernatant HCV infectivity titers (Fig. 10B). Taken together, our results suggest that E6AP mediates ubiquitylation and degradation of HCV core protein in HCV-infected cells, thereby affecting the production of HCV particles.

DISCUSSION

HCV core protein is a major component of viral nucleocapsid, plays a central role in viral assembly (25, 40), and contributes to viral pathogenesis and hepatocarcinogenesis (9). Therefore, it is important to clarify the molecular mechanisms that govern the cellular stability of this viral protein. We have previously reported that processing at the C-terminal hydrophobic domain of the core protein leads to efficient polyubiquitylation of the core protein (52). In this study, we identified E6AP as an HCV core-binding protein and showed that HCV core protein interacts with E6AP in vivo and in vitro, that E6AP enhances ubiquitylation and degradation of the mature core protein as well as the C-terminally truncated core protein, and that HCV core protein expressed from infectious HCV is degraded via E6AP-dependent proteolysis. HCV core protein and E6AP were found to colocalize in the cytoplasm, especially in the perinuclear region. Moreover, exogenous expression of E6AP reduces intracellular core protein levels and supernatant HCV infectivity titers in HCV-infected Huh-7 cells. Knockdown of endogenous E6AP by siRNA increases intracellular core protein levels and supernatant infectivity titers in HCV-infected cells. These findings suggest that E6AP mediates ubiquitylation and degradation of HCV core protein, thereby affecting the production of HCV particles.

HCV core protein interacts with E6AP through the region of the core protein between aa 58 and aa 71. These 14 amino acids are highly conserved, with the first nine amino acids (PRGRRQPIP) present in the core protein of all the HCV genotypes (3). This result suggests that E6AP-dependent degradation of HCV core protein is common to all HCV genotypes and plays an important role in the HCV life cycle or viral pathogenesis. Our data indicated that HCV core proteins of genotypes 1b and 2a are subjected to proteolysis through an E6AP-mediated degradation pathway. We are currently examining whether E6AP promotes degradation of HCV core proteins of other genotypes.

Studies in addition to ours have reported that other HCV proteins, such as NS5B (8), the unglycosylated cytosolic form of E2 (39), NS2 (7), and F protein (58), are degraded through the ubiquitin-proteasome pathway. These studies suggest that the ubiquitin-proteasome pathway plays a role in the HCV life cycle or viral pathogenesis. To our knowledge, the present study is the first to demonstrate that the ubiquitin-proteasome pathway affects the HCV life cycle.

PA28γ was found to interact with HCV core protein in hepatocytes and promote proteasomal degradation of HCV core protein (30). PA28γ, however, has been shown to function

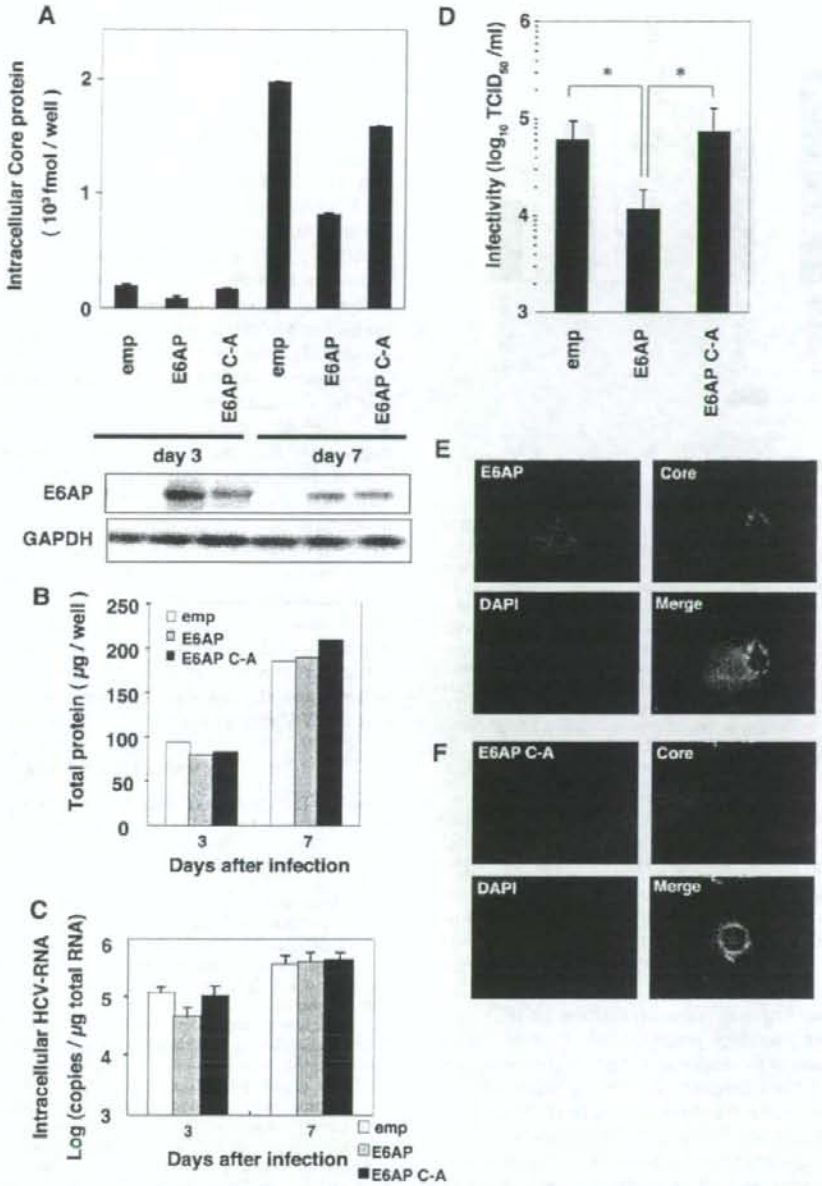

FIG. 9. Exogenous expression of E6AP reduces intracellular HCV core protein levels and supernatant infectivity titers in HCV-infected Huh-7 cells. Naïve Huh-7 cells were seeded as described in Materials and Methods; inoculated with 2.5 ml of the inoculum including infectious HCV JFH1 (6.5×10^3 $TCID_{50}$/ml); and transfected with 6 μg of empty plasmid, pCAG-HA-E6AP, or pCAG-HA-E6AP C-A. The culture supernatant and the cells were collected at days 3 and 7 postinfection. (A) Intracellular HCV core protein levels. (B) Levels of total protein. (C) Levels of intracellular HCV RNA in HCV-infected Huh-7 cells. Data represent the averages of three experiments with error bars. (D) Supernatant infectivity titers. At day 7 postinfection, culture supernatants were collected and assayed for $TCID_{50}$ determinations. The difference between empty vector and E6AP or between E6AP and E6AP C-A was significant (*, $P < 0.05$, Student's t test). (E and F) HCV JFH1-infected Huh-7 cells were transfected with either MEF-E6AP plasmid or MEF-E6AP C-A plasmid, grown on coverslips, fixed, and processed for double-label immunofluorescence for HCV core and MEF-E6AP (E) or MEF-E6AP C-A (F). Anticore MAb (2H9) and anti-FLAG PAb were used as primary antibodies. Nuclei were visualized by staining the cells with DAPI. All the samples were examined with a BZ-8000 microscope. Representative images of individual cells are shown with merge images. emp, empty vector.

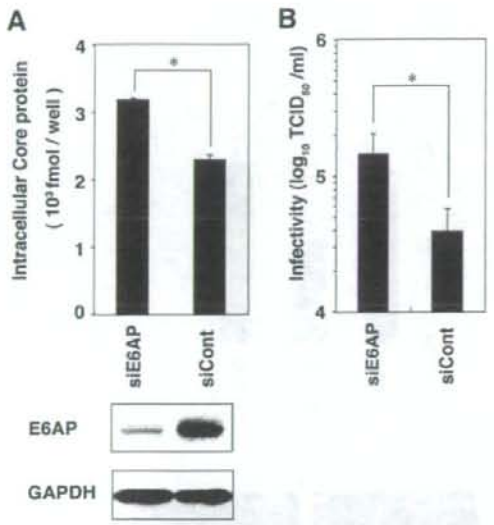

FIG. 10. E6AP silencing leads to an increase in the level of intracellular HCV core protein and supernatant infectivity titer in HCV-infected Huh-7 cells. (A) HCV JFH1-infected cells were replated in a six-well plate at 3×10^5 cells/well and transfected with 40 pmol of E6AP siRNA or control siRNA. The culture medium was changed at 24 h after transfection. The cells were harvested at day 2 after transfection, and the intracellular core protein levels were quantitated using the HCV core antigen ELISA. Equivalent amounts of the whole-cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-E6AP MAb or anti-GAPDH MAb. (B) Culture supernatants were collected at day 2 after transfection and assayed for $TCID_{50}$ determinations. For both panels, the difference between E6AP siRNA and control siRNA was significant (*, $P < 0.05$, Student's t test).

in a ubiquitin-independent, ATP-independent, and 20S proteasome-dependent pathway (27). There have been reports that several cellular factors, such as p53 (2), p73 (2), and RPN4 (18), are degraded through two alternative pathways, the ubiquitin-dependent 26S proteasome-dependent pathway and the ubiquitin-independent 20S proteasome-dependent pathway. Here we provide evidence that E6AP mediates ubiquitylation of HCV core protein. Still unclear is whether the PA28γ-dependent pathway requires polyubiquitylation of HCV core protein. HCV core protein is predominantly localized in the cytoplasm, especially at the endoplasmic reticulum membrane, on the surface of lipid droplets, and on mitochondria and mitochondrion-associated membranes (51). In HCV JFH1-infected cells, HCV core was found to localize in the cytoplasm and frequently to accumulate in the perinuclear region and the lipid droplets (44). Our results indicated that E6AP colocalized with HCV core protein especially in the perinuclear region. PA28γ was found to colocalize with HCV core protein in the nucleus. Functional differences may exist between the E6AP-dependent pathway and the PA28γ-dependent pathway in the stability control of HCV core protein. The functional role of the E6AP-dependent pathway and the PA28γ-dependent pathway remains to be elucidated.

The HCV core-binding region of E6AP was mapped to the region between aa 418 and aa 517. The multicopy maintenance protein 7, Mcm7, interacts with E6AP through a short motif, termed the L2G box (aa 412 to 414), that lies within the E6 binding site of E6AP (23). Our data indicated that the E6 binding region containing the L2G motif is not required for interaction between HCV core protein and E6AP (Fig. 2C, lane M).

We propose here that E6AP may affect the production of HCV particles through controlling the amounts of HCV core protein. This mechanism may contribute to persistent infection. The E6AP binding domain of the core protein resides in the RNA-binding domain and binding domains for many host factors (40). These factors may affect the binding between E6AP and HCV core protein, resulting in control of E6AP-dependent core degradation. Another possibility is that HCV core protein may affect the normal function of E6AP, thereby contributing to pathogenesis. It will be intriguing to investigate whether HCV core protein has any effect on E6AP-dependent degradation of host factors. The other intriguing possibility is that HCV core-E6AP complex may function as an E3 ligase-like E6-E6AP complex to target host factors for proteasomal degradation and contribute to viral pathogenesis.

In conclusion, we have demonstrated that E6AP interacts with HCV core protein in vitro and in vivo and mediates ubiquitin-dependent degradation of the core protein, leading to downregulation of HCV particles. We propose that the E6AP-mediated ubiquitin-proteasome pathway may play a role in affecting the production of HCV particles through controlling the amounts of viral nucleocapsid protein. Identification of the specific E3 ubiquitin ligase may contribute to gaining a better understanding of the biology of the HCV life cycle as well as molecular details of the ubiquitin-dependent degradation of HCV core protein.

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Review

Molecular biology of hepatitis C virus

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Infection with hepatitis C virus (HCV), which is distributed worldwide, often becomes persistent, causing chronic hepatitis, cirrhosis, and hepatocellular carcinoma. For many years, the characterization of the HCV genome and its products has been done by heterologous expression systems because of the lack of a productive cell culture system. The development of the HCV replicon system is a highlight of HCV research and has allowed examination of the viral RNA replication in cell culture. Recently, a robust system for production of recombinant infectious HCV has been established, and classical virological techniques are now able to be applied to HCV. This development of reverse genetics-based experimental tools in HCV research can bring a greater understanding of the viral life cycle and pathogenesis of HCV-induced diseases. This review summarizes the current knowledge of cell culture systems for HCV research and recent advances in the investigation of the molecular virology of HCV.

Key words: hepatitis C virus, translation, polyprotein processing, RNA replication, viral assembly, ubiquitin

Introduction

Hepatitis C virus (HCV), discovered in 1989, is a major etiologic agent of posttransfusion- and sporadic non-A, non-B hepatitis[1] and at present infects approximately 200 million people worldwide.[2,3] Persistent infection with HCV is associated with the development of chronic hepatitis, hepatic steatosis, cirrhosis, and hepatocellular carcinoma.[3,4–8] HCV is a small, enveloped RNA virus that belongs to the *Hepacivirus* genus of the *Flaviviridae* family.[9,10] Its genome consists of a single-strand of positive-sense RNA of approximately 9.6 kb, which contains an open reading frame (ORF) coding for a polyprotein precursor of approximately 3000 residues.[11] The precursor is cleaved into at least ten different proteins: the structural proteins core, E1, E2, and p7, and the nonstructural proteins NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Fig. 1).

To date, six major genotypes of HCV have been identified that differ by 31%–34% in their nucleotide sequence and by about 30% in their amino acid sequence. It has been shown that HCV, like many other RNA viruses, circulates in infected individuals as a population of diverse but closely related variants referred to as quasispecies.[12] This quasispecies model of mixed virus populations may confer a significant survival advantage, because the simultaneous presence of multiple variant genomes and the high rate of generation of new variants allows rapid selection of mutants better suited to new environmental conditions.[13]

Specific anti-HCV drugs that efficiently block virus production are not yet available. The current standard care is combination therapy with interferon (IFN)-α and the nucleoside analog ribavirin, which cures about 40% of hepatitis C patients infected by HCV genotype 1, the most prevalent genotype in industrialized countries, and about 80% of those infected by genotype 2 or 3.[14,15] Since many patients still do not benefit from the treatment and IFN therapy is associated with undesirable side effects such as headache, fever, severe depression, myalgia, arthralgia, and hemolytic anemia, development of innovative treatment alternatives for hepatitis C patients is immediately needed. Studies of HCV life cycle in cell cultures have been greatly facilitated by the development of genetically engineered viral genomes that are capable of self-amplifying to high levels (replicon system), and by recent establishment of a production system for recombinant infectious HCV. Such progress will aid in the development of significantly improved HCV antiviral agents.

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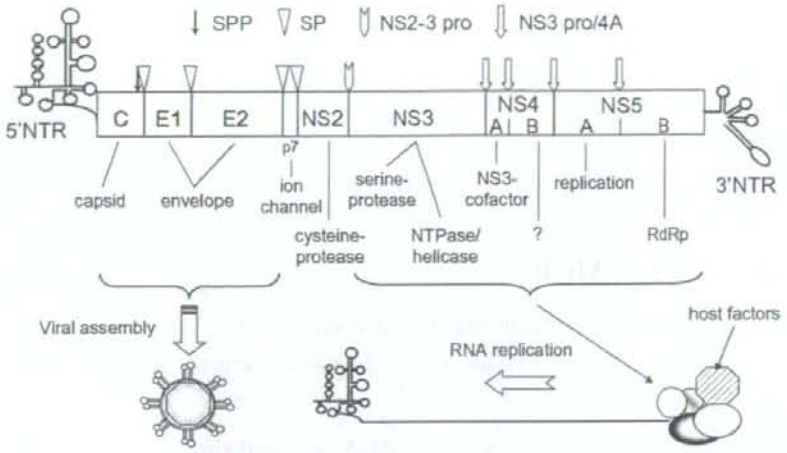

Fig. 1. Hepatitis C virus (HCV) genome organization and polyprotein processing. Posttranslational cleavages by signal peptide peptidase (*SPP*), signal peptidase (*SP*), NS2–NS3 protease (*NS2–3 pro*), and NS3 protease and NS4A complex (*NS3 pro/4A*) lead to the production of functional HCV proteins. *NTR*, nontranslated region

Cell culture systems for HCV research

Although substantial information on HCV protein structure and function has been obtained from the use of a variety of cell culture and in vitro expression systems, for many years, HCV research has been hampered by the restricted host range and the inefficiency of cell culture models for viral infection and propagation. The development of the HCV replicon system, therefore, is a milestone in HCV research and has allowed examination of viral RNA replication in cell culture.[16] Expression systems of heterologous virus genes based on RNA replicons have been established in a variety of positive-strand RNA viruses such as polio virus,[17–20] the alphavirus Semliki Forest virus,[21] Sindbis virus,[22–25] Kunjin virus,[26] human rhinovirus 14,[27] and bovine viral diarrhea virus.[28] In general, advantages of replicon systems are (1) a high level of gene expression and RNA replication, (2) easy construction of recombinants, and (3) a wide permissible host range.

The HCV replicons are typically composed of selectable, bicistronic RNA, with the first cistron containing the HCV 5′ nontranslated region (NTR), which directs translation of the gene encoding the neomycin phosphotransferase, and the second cistron containing the internal ribosome entry site (IRES) of the encephalomyocarditis virus, which directs translation of HCV NS3 through NS5B region, and the 3′ NTR. The prototype subgenomic replicon utilized a particular HCV genotype 1b clone termed Con1. Following transfection of RNA generated by in vitro transcription of the cloned replicon sequences into a human hepatoma cell line Huh-7, antibiotic G418-resistant cells could be obtained in which the subgenomic RNA replicated autonomously. RNA replication was first detected at relatively low frequency, followed by the identification of replicons harboring cell culture-adaptive mutations, which increased the efficiency of replication initiation by several orders of magnitude.[29–31]

Adaptive mutations were found primarily at the N-terminus of the NS3 helicase, in NS4B, and in the center of NS5A, which is upstream of the region putatively involved in IFN sensitivity. Most of the mutations in NS5A are located at highly conserved serine residues and lead to change in the phosphorylation state of NS5A.[32,33] A combination of adaptive mutation in NS3 and NS5A resulted in the highest level of replication of a particular HCV genotype 1b isolate.[31] Later work, however, has indicated that adaptive mutations can arise in most of the viral nonstructural proteins.[34,35] The mechanisms by which adaptive mutations increase RNA replication efficiency are not well understood.

In the last 7 years, a variety of different replicons have been generated, including replicons with reporters or markers such as luciferase and green fluorescent protein, replicons from genotype 1a and 2a, and genome-length dicistronic HCV RNAs (genomic HCV replicons). HCV replicons with reporter genes allow us to execute fast and reproducible screening of large series of compounds for antivirals.[36–38] Huh-7 cells are the most permissive for HCV replicons. However, variability in the permissiveness for replicons has been observed for a given Huh-7 cell pool, and the cells that are able to support efficient replication of the viral genome are enriched during selection such as G418 treatment. A so-called "cured" cell clone, which can be prepared by removing the replicons by treatment with IFN, supports viral replication to a much higher level in many cases and is useful for introducing genome-length HCV RNAs.[39,40]

An HCV genotype 2a replicon with the JFH-1 strain, which was first isolated from the serum of a Japanese patient with fulminant hepatitis C by our group,[41] replicates efficiently in not only Huh-7 cells but also other

hepatocyte-derived cell lines, HepG2 and IMY-N9, and nonhepatocyte-derived cell lines, HeLa and 293.[42–44] Interestingly, the JFH-1 replicon does not require adaptive mutations for replicating in these cell lines, and enormously efficient RNA replication is detected by transient replication assay as well as by colony formation assay with G418 selection,[42] suggesting that the JFH-1 genome can replicate autonomously without the help of drug selection or the requirement of adaptive mutations. This observation laid the basis for a breakthrough in HCV research.

Transfection of the full-length JFH-1 genome into Huh-7 cells leads to the production of HCV particles that are infectious both for naïve cells and for animal models.[45] As a first attempt, an in vitro transcribed full-length JFH-1 RNA was introduced into naïve Huh-7 cells, which is the original cell line used for subgenomic replicon studies. Efficient RNA replication in the transfected cells was detectable by Northern blot analysis, and the viral-enveloped particles, which are spherical structures with an outer diameter of approximately 55 nm, were secreted to the culture medium.[45] Secreted virus was found to be infectious, although at low efficiency, for naïve Huh-7 cells, and its infectivity can be neutralized by anti-CD81 antibody and hepatitis C patients' sera.[45] Subsequently, to increase the infection efficiency, "cured" Huh-7 cell lines such as Huh7.5, Huh7.5.1, and Huh7-Lunet were used. Infectivity of these cured cell lines with JFH-1 became more intense compared with standard Huh-7 cells, and the virus titers released from cells freshly transfected with the JFH-1 genome were markedly increased by continuous passage of the cells carrying persistent replicating viral RNA.[46] Further, chimeric constructs with the core to NS2 region of another genotype 2a clone, J6, improved the infectivity.[47] Thus, this recombinant infectious HCV cell culture system opens avenues of biochemical and genetic studies of the HCV life cycle.

Besides isolating functional molecular clones of HCV that replicate to high levels, to generate a cell culture model that mimics natural host cell environments may be advantageous for improving HCV production systems suitable for studying the virus–host interaction. It is likely that HCV morphogenesis occurs in a complex cellular environment in which host factors may either enhance or reduce the assembly and budding process. Generally, the interaction of viruses with polarized epithelia in the host is one of the key steps in the viral life cycle. A variety of enveloped viruses mature and bud from distinct membrane domains of the host cells.[48–51] We found that a dicistronic HCV genome of genotype 1b supports the production and secretion of infectious HCV particles in two independent three-dimensional (3D) culture systems, the radial-flow bioreactor (RFB) and the thermoreversible gelation polymer (TGP), but not in monolayer cultures, although its productivity is much lower than that observed in the JFH-1 system[52] (Fig. 2). The RFB system was initially aimed at the

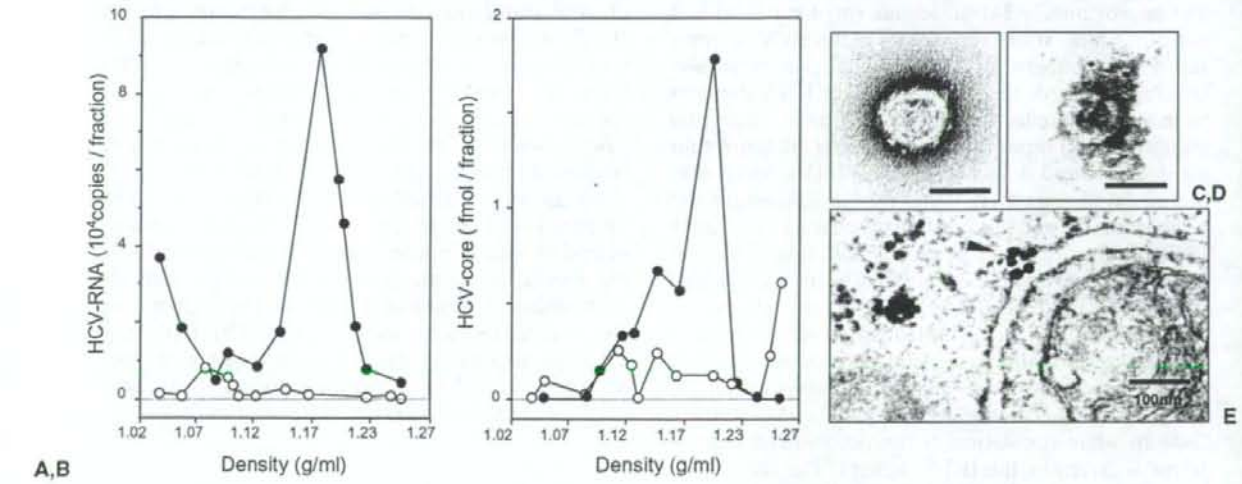

Fig. 2A–E. Production of HCV particles in the three-dimensional, thermoreversible gelatin polymer (TGP) culture of the Huh-7 cell line (RCYM1) carrying genome-length dicistronic HCV RNA of genotype 1b. **A, B** Sucrose density gradient analysis of culture supernatants of RCYM1 cells. The culture supernatants were fractionated and then HCV RNA (**A**) and core protein (**B**) in each fraction were determined by enzyme-linked immunosorbent assay and real-time reverse transcription polymerase chain reaction, respectively. *Closed circles*, TGP culture; *open circles*, monolayer culture. **C, D** Electron microscopy of HCV particles in the supernatants of TGP-cultured RCYM1 cells. **C** Negative staining. **D** Immunogold labeling with an anti-E2 antibody. Gold particles, 5 nm; bars, 50 nm. **E** Silver-intensified immunogold staining with anti-E1 antibody. The *arrowhead* indicates virus-like particles reacting with anti-E1 antibody

development of artificial liver tissue, and the bioreactor column consists of a vertically extended cylindrical matrix through which liquid medium flows continuously from the periphery toward the center of the reactor.[53] In RFB culture, human hepatocyte-derived cells can grow spherically or cubically, and they retain liver functions such as albumin synthesis[53-55] and drug-metabolizing activity mediated by cytochrome P450 3A4.[56] TGP is a chemically synthesized biocompatible polymer which has a sol–gel transition temperature, thus enabling us to culture cells three-dimensionally in the gel phase at 37°C and to harvest them in the sol phase at 4°C, without enzyme digestion.[57] In contrast to other matrix gels made from conventional natural polymers, TGP has several advantages that allow us to investigate the functional characteristics of epithelial cells, their tissue-like morphology, and their potential clinical applications. For example, the use of 3D culture materials other than TGP requires treatment with appropriate digestive enzymes or heating to collect cells grown as spheroids from the culture media, and the matrices may damage the cultured cells to some extent. A 3D culture system based on RFB and TGP, in which human hepatoma cells can assemble into spheroids with potentially polarized morphology, is a valuable tool in studies of HCV morphogenesis.

Translation

The approximately 341-nucleotide (nt)-long 5′ NTR is one of the most conserved regions of the HCV genome, and the secondary structural model, which is also largely conserved, reveals four distinct RNA domains in the region, reflecting its importance in both viral translation and replication.[58-61] The 5′ NTR forms four highly structured domains (domains I–IV), which may be conserved among HCV and related flaviviruses and pestiviruses,[59,60] and it is functionally characterized as an IRES to direct cap-independent translation of the genome.[62,63] To determine the minimal sequence required for HCV IRES-dependent translation, as in the earlier studies of picornaviruses, the bicistronic RNAs in which two reporter protein-coding sequences are separated by an IRES sequence were analyzed. Translation of the upstream reading frame occurs in a 5′ end-dependent fashion, while translation of the downstream reading frame is driven by the IRES element. The IRES comprises nearly the entire 5′ UTR of the genome. There is evidence to suggest that the first 12 to 30 nt of the coding sequence are also important for IRES activity.[64-66] The first 40 nt of the 5′ NTR, which includes a single stem–loop (domain I), is not essential for the translation; the 5′ border of the IRES was mapped between nt 38 and 46.[61,67,68] Domains II and III are relatively more complex and contain multiple stems and loops.[60,69] Domain IV consists of a small stem–loop containing the polyprotein start codon at nt 342 and forms a pseudoknot via base-pairing with a loop in domain III.

Recruitment of the 43S ribosomal complex, containing a small 40S ribosomal subunit, eukaryotic initiation factor (eIF) 3, and a tRNA–eIF2–GTP ternary complex, to mRNA molecules is critical for initiation of eukaryotic protein synthesis. The 40S subunit and eIF3 can bind independently to the HCV IRES.[64,70-72] However, it appears that interaction between IRES RNA and the 40S subunit drives formation of an IRES–40S subunit–eIF3 complex, since HCV IRES RNA demonstrates similar affinity to both the 40S subunit and the 40S–eIF complex.[71] Other cellular factors such as La autoantigen,[73-75] heterogeneous ribonucleoprotein L,[76] poly-C binding protein,[77,78] and pyrimidine tract-binding protein,[79,80] also bind to the IRES element and modulate translation.

Regulation of IRES-dependent translation of HCV is also likely to involve viral factors. We found that the core protein specifically inhibits HCV translation, possibly by binding to a stem–loop IIId domain, particularly a GGG triplet within the hairpin loop structure of the domain, within the IRES (Fig. 3).[79-81] Although a conflicting report has suggested that inhibition of HCV translation is due to an RNA–RNA interaction, rather than to an interaction between RNA and the core protein,[82] later studies support the role of a core protein sequence spanning amino acids (aa) 34–44 in inhibition of viral translation through its interaction with the IRES.[83] Furthermore, the N-terminal 20 residues of the core protein have been shown to selectively inhibit translation mediated by HCV IRES in a cell type-specific manner.[84] We propose a model in which competitive binding of the core protein to the IRES and 40S ribosomal subunit regulates HCV translation.

By analogy with other RNA viruses with IRES-mediated expression, the HCV 5′ NTR has been expected to contain not only determinants for translation but also *cis*-acting elements for RNA replication. Recent studies demonstrated that (1) the sequence upstream of the IRES is essential for viral RNA replication, (2) sequences within the IRES are required for high-level HCV replication, and (3) the stem–loop domain II of the IRES is crucial for the replication.[85]

Polyprotein processing

IRES-mediated translation of the HCV ORF yields a polyprotein precursor that is subsequently processed by cellular and viral proteases into mature structural and nonstructural proteins (Fig. 1). As deduced from the hydrophobicity profile and the dependence on micro-

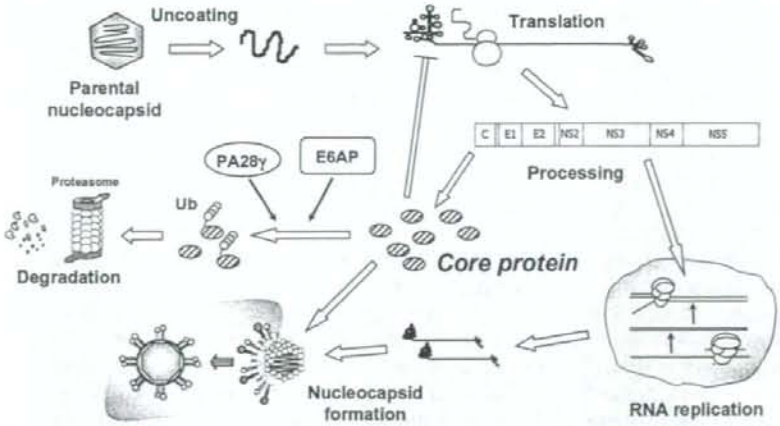

Fig. 3. The role and fate of HCV core protein in the postulated HCV life cycle. See text for further explanation and details

somal membranes, junctions at core/E1, E1/E2, E2/p7, and p7/NS2 are processed by host signal peptidases. For instance, secondary structure analysis of the core protein reveals that all major alpha helices are located in the C-terminal half of the protein. A predicted alpha helix encoded by aa 174–191 is extremely hydrophobic and resembles typical signal peptide sequences. Further posttranslational cleavage close to the C terminus of the core protein takes place, removing the E1 signal sequence by the signal peptide peptidase.[86–89] This peptidase has recently been identified[90] and exhibits protease activity within cellular membranes, resulting in cleavage of peptide bonds in the plane of lipid bilayers.

The viral nonstructural proteins are processed by two viral proteases: processing between NS2 and NS3 is a rapid intramolecular reaction that is accomplished by the NS2–3 protease, which spans NS2 and the N-terminal domain of NS3, whereas the remaining four junctions are cleaved by the serine protease located at the N-terminal 180 residues of NS3 protein. Efficient cleavage at the NS2/3 site requires the 130 C-terminal residues and the first 180 aa of NS3. Recombinant proteins lacking the N-terminal membrane domain of NS2 were found to be enzymatically active, allowing further characterization of this activity.[91,92] Deletion of NS2 from the nonstructural polyprotein did not abolish the replication of HCV RNA in cell cultures, indicating that NS2 is not essential for vial RNA replication.[16,29]

The NS3–NS5B region is processed presumably with the following preferred order of cleavage: NS3/4A→NS5A/5B→NS4A/4B→NS4B/5A.[93–98] Processing at the NS3/4A site is an intramolecular reaction, whereas cleavage at the other sites can be mediated intermolecularly. NS3 is a multifunctional molecule. Besides its N-terminal protease activity, the helicase and nucleotide triphosphatase (NTPase) activities reside in the C-terminal 500 residues of the NS3 protein.[97–101] NS4A functions as a cofactor of the NS3 serine protease and is required for efficient polyprotein processing. There are significant differences in the stability and activity of the NS3 protease in the presence or absence of NS4A. NS3 protein is relatively unstable when expressed in cells in the absence of NS4A.[102] Structural studies by nuclear magnetic resonance and X-ray methods show that the NS3–4A complex has a more highly ordered N-terminal domain and NS4A binding leads the NS3 protease to a rearrangement of the active site triad to a canonical conformation.[103] It has been predicted that the N-terminus of NS4A forms a transmembrane helix, which presumably anchors the NS3–4A complex to the cellular membrane.[104]

RNA replication

HCV is assumed to replicate its genome through the synthesis of a full-length negative-strand RNA. Positive-strand RNA is then produced from the negative-strand template; it is several-fold more abundant than the negative-stranded RNA and is utilized for translation, replication, and packaging into progeny viruses. RNA replication of most RNA viruses involves certain intracellular membrane structures, including the endoplasmic reticulum (ER),[105–107] Golgi,[108] endosomes, and lysosomes.[109] HCV RNA replication is also believed to occur in the cytoplasm of the virus-infected cells.

Although NS5B protein has RNA-dependent RNA polymerase (RdRp) activity in vitro, its recombinant product alone is presumably short of strict template specificity and fidelity, which are essential for viral RNA synthesis. It is highly likely that other viral or host factors are important for conferring proper RNA replication and that the replication complexes (RCs), which are composed of NS5B and additional components re-

quired for modulating polymerase activity, are involved in catalyzing HCV RNA synthesis during the replication process. NS3 is directly involved in RNA synthesis, possibly through its helicase/NTPase activities. The helicase activity is presumed to be involved in unwinding a putative double-stranded replication intermediate or to remove regions of secondary structure so that MS5B RdRp can copy both strands of the viral RNA. It is likely that the NTPase activity is coupled with the helicase function, supplying the energy required for disrupting RNA duplexes. Although little is known about the function of NS4B in the HCV life cycle to date, NS4B protein can induce a membranous web, consisting of small vesicles embedded in a membranous matrix,[110] and it has been reported that the newly synthesized HCV RNA and most of the viral nonstructural proteins occur in these membrane webs or speckle-like structures.[111–113] NS4B may play an important role in the formation of the HCV RNA replication complex.[114] Evidence indicating an involvement of NS5A in viral RNA replication is now accumulating. As described above, a hot spot of the cell culture-adaptive mutations that increase replication efficiency of HCV RNA is located in the central region of NS5A.[29–31] The membrane association of NS5A through its amino-terminal transmembrane domain[115] and the interaction between NS5A and 5B[116] are essential for RNA replication. Several cellular proteins interacting with NS5A have been identified, and human vesicle-associated membrane protein-associated proteins (hVAP-A and -B) are likely to play a key role in RNA replication through the interaction with NS5A.[114,117] The 3′ NTR also contains a significant predicted RNA structure with three distinct domains: a variable region of about 40 nt, a variable length poly(U/UC) tract, and a highly conserved, 98-nt 3′ terminal segment (3′X) that putatively forms three stem–loop structures.[118–120] Viral RNA replication was not detected when any of the three putative stem–loop structures within the 3′X region or the entire poly(U/UC) was deleted.[121] The variable region segment also contributes to efficient RNA replication.[122]

Several groups have succeeded in demonstrating the in vitro replication activities of HCV RCs in crude membrane fractions of cells harboring the subgenomic replicons.[123–126] These cell-free systems provide a valuable complement to the in vitro RdRp assays for biochemical dissection of HCV RNA replication and are a useful source for isolation of viral RCs. From the in vitro replication studies, it appears that RNA synthesis can be initiated in the absence of added negative-strand template RNA, suggesting that preinitiated template RNA copurifies with the RC.[124,125,127] Although the newly synthesized single-strand RNA can be used as a template for a further round of double-strand RNA synthesis, no exogenous RNA serves as a template for HCV RC preparation.[125] Added RNA templates might not access the active site of the HCV RCs owing to sequestration by membranes. The HCV RCs contain both positive- and negative-strand RNAs.[124,127] It has also been reported that cell-free replication activity increases at temperatures ranging from 25° to 40°C, and divalent cations (Mn^{2+} and Mg^{2+}) can be used in the reaction.[125,127]

Membrane flotation analysis and a replication assay have shown that viral RNA and proteins are present in detergent-resistant membrane structures, most likely a lipid-raft structure, and RNA replication activity was detected even after treatment with detergent.[123,128] Lipid rafts are cholesterol- and sphingolipid–rich microdomains characterized by detergent insolubility.[129–131] These structures are known to play a critical role in a number of biological processes, such as as regulators and organizing centers of signal transduction and membrane traffic pathways, including virus entry and assembly of, for example, influenza virus,[132–134] human immunodeficiency virus type-1,[27,135,136] Ebola virus, Marburg virus,[137] enterovirus,[138] avian sarcoma and leukosis virus,[139] Coxsackie B virus, adenovirus,[140] measles virus,[16] and respiratory syncytial virus.[141] However, HCV may be the first example of the association of a lipid raft with viral RNA replication.

On the other hand, it has been widely believed that most of the HCV life cycle, including protein processing and genome replication, takes place in the ER, where cholesterol–sphingolipid rafts are not assembled.[110,142–144] Several studies using the replicon system have indicated that the nonstructural proteins are associated with the ER.[143,145] Nevertheless, it is still possible that HCV nonstructural proteins synthesized at the ER relocalize to lipid-raft membranes when they are actively engaged in RNA replication. It has been shown by membrane separation analysis that HCV nonstructural proteins are present both in the ER and the Golgi, but the activity of viral RNA replication was detected mainly in the Golgi fraction.[123,146] Further studies to elucidate where and how the HCV genome replicates in infected cells are needed.

Viral assembly

The assembly of HCV and the virion structure remains largely unknown. By analogy with related viruses, the mature HCV virion presumably possesses a nucleocapsid and outer envelope composed of a lipid membrane and envelope proteins. HCV virions are thought to have a diameter of 40–70 nm.[147,148] These observations were recently confirmed by immunoelectron microscopy of infectious HCV particles produced in cell cultures.[45,52] It has been reported that HCV circulates in various forms

in the sera of infected hosts, for example, as (1) free mature virions, (2) virions bound to low-density lipoproteins and very low density lipoproteins, (3) virions bound to immunoglobulins, and (4) nonenveloped nucleocapsids, which exhibit physicochemical and antigenic properties.[147–150]

The HCV structural proteins (core, E1, and E2) are located in the N-terminal one-third of the precursor polyprotein (Fig. 1). A crucial function of the core protein, which is derived from the N-terminus of the viral polyprotein, is assembly of the viral nucleocapsid. The aa sequence of this protein is well conserved among different HCV strains, compared with other HCV proteins. The N-terminal domain of the core protein is highly basic, while its C-terminus is hydrophobic. When expressed in mammalian cells and transgenic mice, the core protein is found on membranes of the ER, on the surface of lipid droplets, on the mitochondrial outer membrane, and, to some extent, in the nucleus.[151–156] The core protein is likely multifunctional and is not only involved in formation of the HCV virion but also has a number of regulatory functions, including modulation of lipid metabolism and hepatocarcinogenesis.[153,157–159] The envelope proteins E1 and E2 are extensively glycosylated and have an apparent molecular weight of 30–35 and 70–75 kDa, respectively. Predictive algorithms and genetic analyses of deletion mutants and glycosylation-site variants of the E1 protein suggest that E1 can adopt two topologies in the ER membrane: the conventional type I membrane topology and a polytopic topology in which the protein spans the ER membrane twice with an intervening cytoplasmic loop.[160] E1 and E2 proteins form a noncovalent complex, which is believed to be the building block of the viral envelope.

Several expression systems have been used to investigate HCV capsid assembly using mammalian, insect, yeast, bacteria, and reticulocyte lysates, as well as purified recombinant proteins.[148,161–170] The results suggest that immunogenic nucleocapsid-like particles are heterologous in size and range from 30 to 80 nm in diameter. The N-terminal half of the core protein is important for nucleocapsid formation.[163,169,170] HCV capsid formation occurs in the presence or absence of ER-derived membrane, which supports cleavage of the signal peptide at the C-terminus.[170]

Nucleocapsid assembly generally involves oligomerization of the capsid protein and encapsidation of genomic RNA. In fact, study of a recombinant mature core protein has shown it to exist as a large multimer in solution under physiological conditions, within which stable secondary structures have been observed.[171] Studies using yeast two-hybrid systems have identified a potential homotypic interaction domain within the N-terminal region of the core protein (aa 1–115 or –122), with particular emphasis on the region encompassing aa 82–102.[172,173] However, other studies have identified two C-terminal regions, extending from aa 123 to 191 and from 125 to 179, as responsible for self-interaction. Furthermore, Pro substitution within these C-terminal regions has been observed to abolish core protein self-interaction.[171,174] Circular dichroism spectroscopy has further shown that a Trp-rich region spanning aa 76–113 is largely solvent-exposed and unlikely to play a role in multimerization.[171] Recently, our group demonstrated that self-oligomerization of the core protein is promoted by aa 72–91 in the core.[160]

Once a HCV nucleocapsid is formed in the cytoplasm, it acquires an envelope as it buds through intracellular membranes. Interactions between the core and E1/E2 proteins are considered to determine viral morphology. Expression of HCV structural proteins using recombinant virus vectors has led to successful generation of virus-like particles with similar ultrastructural properties to HCV virions. Packaging of these HCV-like particles into intracellular vesicles as a result of budding from the ER has been noted.[161,175,176] Mapping studies to determine the nature of interaction between core and E1 proteins have demonstrated the importance of C-terminal regions in this interaction.[177,178] Since corresponding sequences are not well conserved among various HCV isolates, interactions between core and E1 proteins might depend more on hydrophobicity than on specific sequences. By contrast, it has been shown that interaction between the self-oligomerized HCV core and the E1 glycoprotein is mediated through the cytoplasmic loop present in a polytopic form of the E1 protein.[160]

Implication of the ubiquitin–proteasome pathway in core protein maturation

The ubiquitin–proteasome pathway is the major route by which selective protein degradation occurs in eukaryotic cells and is now emerging as an essential mechanism of cellular regulation.[179,180] This pathway is also involved in the posttranslational regulation of the core protein.[158,181–183] We have reported that processing at the carboxyl-terminal hydrophobic domain of the core protein leads to its efficient polyubiquitylation and proteasomal degradation.[181] Recently, our group identified the ubiquitin ligase E6AP as an HCV core-binding protein and showed that E6AP enhances ubiquitylation and degradation of the mature as well as the carboxyl-terminally truncated core proteins, and that the core protein produced from infectious HCV is degraded via an E6AP-dependent pathway (Fig. 3).[183] E6AP, the prototype of HECT domain ubiquitin ligases,[184] was initially identified as the cellular factor that stimulates ubiquitin-dependent degradation of the tumor suppres-

sor p53 in conjunction with E6 protein of cancer-associated human papillomavirus types 16 and 18.[185,186] Exogenous expression of E6AP reduces intracellular core protein levels and supernatant viral infectivity in infected cell cultures. Knockdown of exogenous E6AP by siRNA increases intracellular core protein levels and virus titers in the culture supernatants. The core protein interacts with E6AP through the aa 58–71 region of the core, which is highly conserved among all HCV genotypes, suggesting that E6AP-dependent degradation of the core protein is common to a variety of HCV isolates and plays a critical role in the HCV life cycle or viral pathogenesis.

A role for the proteasome activator PA28γ core-binding protein in degradation of the core protein has also been demonstrated (Fig. 3).[158,182] Overexpression of PA28γ promotes proteolysis of the core protein. PA28γ predominates in the nucleus and forms a homopolymer, which associates with the 20S proteasome,[187] thereby enhancing proteasomal activity.[188] Both nuclear retention and core protein stability are regulated via a PA28γ-dependent pathway.

In eukaryotic cells, targeted protein degradation is increasingly understood to be an important mechanism by which cells regulate levels of specific proteins, and thereby regulate their function. The core protein is believed to play a key role in viral replication and pathogenesis since it forms the viral particle and regulates a number of host cell functions. Although the biological significance of ubiquitylation and proteasomal degradation of the core protein is not fully understood, E6AP possibly affects the production of HCV particles through controlling the amount of core protein (Fig. 3). This mechanism may contribute to virus persistence by maintaining a (moderately) low level of the viral nucleocapsid. The E6AP binding domain within the core protein resides in the region that is considered to be important for binding to the viral RNA and several host factors.[189] These factors may affect the interaction between the core and E6AP, resulting in control of E6AP-dependent core degradation. A recent study demonstrated that a knockdown of the *PA28γ* gene induces the accumulation of the core protein in the nucleus of hepatocytes of HCV core gene-transgenic mice and disrupts development of both hepatic steatosis and hepatocellular carcinoma.[158] Upregulation of several genes related to fatty acid biosynthesis and lipid homeostasis by the core protein was observed in the cells and the mouse liver in the PA28γ-dependent manner. Thus, it is likely that PA28γ plays an important role in the development of liver pathology induced by HCV infection.

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Dynamic behavior of hepatitis C virus quasispecies in a long-term culture of the three-dimensional radial-flow bioreactor system

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Abstract

Hepatitis C virus (HCV) exists in infected individuals as quasispecies, usually consisting of a dominant viral isolate and a variable mixture of related, yet genetically distinct, variants. A prior HCV infection system was developed using human hepatocellular carcinoma cells cultured in the three-dimensional radial-flow bioreactor (RFB), in which the cells retain morphological appearance and their differentiated hepatocyte functions for an extended period of time. This report studies the selection and alteration of the viral quasispecies in the RFB system inoculated with pooled serum derived from HCV carriers. Monitoring the viral RNA and core protein in the culture supernatants, together with nucleotide sequencing of hypervariable region 1 of the HCV genome, demonstrated that (1) the virus production intermittently fluctuated in the cultures, (2) the viral genetic diversity was markedly reduced 3 days post-infection (p.i.), and (3) dominant species changed on days 19–33 p.i., suggesting that the virus populations can be selected according to susceptibility to the viral infection and replication. A therapeutic effect of interferon-α also demonstrated the inhibition of HCV expression. Thus, this HCV infection model in the RFB system should be useful for investigating the dynamic behavior of HCV quasispecies in cultured cells and evaluating anti-HCV compounds.

Keywords: Hepatitis C virus; Three-dimensional culture; Radial-flow bioreactor; Dynamics; Quasispecies

1. Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver diseases (Choo et al., 1989; Kuo et al., 1989; Saito et al., 1990) and has been estimated to infect more than 170 million people throughout the world (Poynard et al., 2003). Symptoms of persistent HCV infection extend from chronic hepatitis to cirrhosis and ultimately hepatocellular carcinoma (Choo et al., 1989; Kuo et al., 1989; Saito et al., 1990). HCV belongs to the genus *Hepacivirus*, included in the family of Flaviviridae, and possesses a viral genome of a single, positive-stranded RNA with a nucleotide (nt) length of approximately 9.6 kb (Choo et al., 1991; Grakoui et al., 1993; Hijikata et al., 1991). It has been shown that HCV, like many other RNA viruses, circulates within infected individuals as a diverse population and closely related variants are referred to as quasispecies (Martell et al., 1992). This quasispecies model of mixed virus populations may imply a significant survival advantage because the simultaneous presence of multiple variant genomes and/or high rate of generation of new variants allow rapid selection of the mutants are better suited to new environmental conditions (Pawlotsky, 2006).

Studies on HCV replication and development of selective antiviral drugs have been hampered primarily by the lack of efficient cell culture systems. Establishment of selectable dicistronic HCV RNAs that are capable of autonomous replication to high levels in human hepatoma Huh-7 cells was a

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