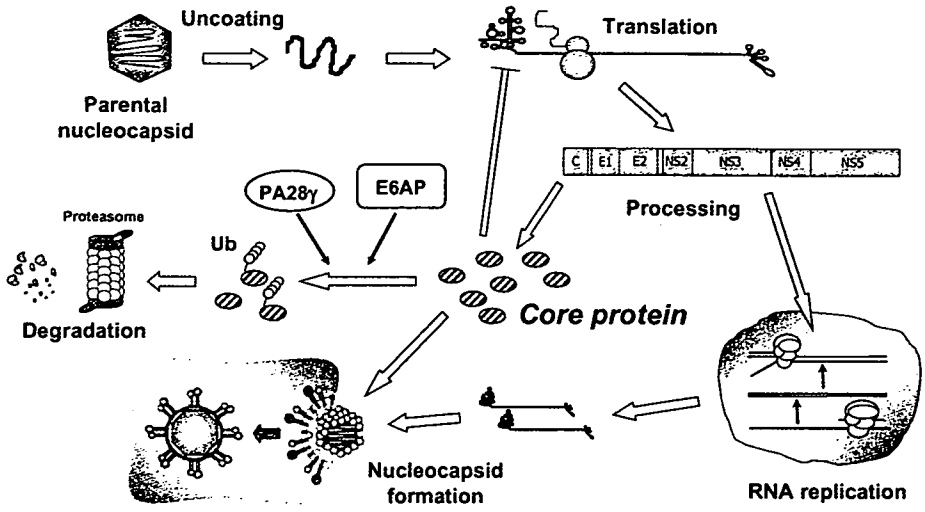

Fig. 3. The role and fate of HCV core protein in the postulated HCV life cycle. See text for further explanation and details

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.[93–96] 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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References

1. Kuo G, Choo QL, Alter HJ, Gitnick GL, Redeker AG, Purcell RH, et al. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science 1989;244: 362–4.
2. Grakoui A, Hanson HL, Rice CM. Bad time for Bonzo? Experimental models of hepatitis C virus infection, replication, and pathogenesis. Hepatology 2001;33:489–95.
3. Lauer GM, Walker BD. Hepatitis C virus infection. N Engl J Med 2001;345:41–52.
4. Saito I, Miyamura T, Ohbayashi A, Harada H, Katayama T, Kikuchi S, et al. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proc Natl Acad Sci USA 1990;87:6547–9.
5. Alter MJ. Epidemiology of hepatitis C in the West. Semin Liver Dis 1995;15:5–14.
6. Di Bisceglie AM. Hepatitis C and hepatocellular carcinoma. Hepatology 1997;26:34S–8S.
7. Poynard T, Yuen MF, Ratziu V, Lai CL. Viral hepatitis C. Lancet 2003;362:2095–100.
8. Pawlotsky JM. Pathophysiology of hepatitis C virus infection and related liver disease. Trends Microbiol 2004;12:96–102.
9. Houghton M, Weiner A, Han J, Kuo G, Choo Q-L. Molecular biology of the hepatitis C viruses: implications for diagnosis, development and control of viral disease. Hepatology 1991;14: 381–8.
10. Robertson B, Myers G, Howard C, Brettin T, Bukh J, Gaschen B, et al. Classification, nomenclature, and database development for hepatitis C virus (HCV) and related viruses: proposals for standardization. International Committee on Virus Taxonomy. Arch Virol 1998;143:2493–503.
11. Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 1989;244: 359–62.
12. Martell M, Esteban JI, Quer J, Genesca J, Weiner A, Esteban R, et al. Hepatitis C virus (HCV) circulates as a population of different but closely related genomes: quasispecies nature of HCV genome distribution. J Virol 1992;66:3225–9.
13. Pawlotsky JM. Hepatitis C virus population dynamics during infection. Curr Top Microbiol Immunol 2006;299:261–84.
14. Zeuzem S, Feinman SV, Rasenack J, Heathcote EJ, Lai MY, Gane E, et al. Peginterferon alpha-2a in patients with chronic hepatitis C. N Engl J Med 2000;343:1666–72.
15. Heathcote EJ, Shiffman ML, Cooksley WG, Dusheiko GM, Lee SS, Balart L, et al. Peginterferon alpha-2a in patients with chronic hepatitis C and cirrhosis. N Engl J Med 2000;343: 1673–80.
16. Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 1999;285:110–3.
17. Andino R, Rieckhof GE, Achacoso PL, Baltimore D. Poliovirus RNA synthesis utilizes an RNP complex formed around the 5′-end of viral RNA. EMBO J 1993;12:3587–98.
18. Collis PS, O'Donnell BJ, Barton DJ, Rogers JA, Flanegan JB. Replication of poliovirus RNA and subgenomic RNA transcripts in transfected cells. J Virol 1992;66:6480–8.
19. Hagino-Yamagishi K, Nomoto A. In vitro construction of poliovirus defective interfering particles. J Virol 1989;63:5386–92.

20. Kaplan G, Racaniello VR. Construction and characterization of poliovirus subgenomic replicons. J Virol 1988;62:1687–96.
21. Liljestrom P, Garoff H. A new generation of animal cell expression vectors based on the Semliki Forest virus replicon. Biotechnology (NY) 1991;9:1356–61.
22. Bredenbeek PJ, Frolov I, Rice CM, Schlesinger S. Sindbis virus expression vectors: packaging of RNA replicons by using defective helper RNAs. J Virol 1993;67:6439–46.
23. Johanning FW, Conry RM, LoBuglio AF, Wright M, Sumerel LA, Pike MJ, et al. A Sindbis virus mRNA polynucleotide vector achieves prolonged and high level heterologous gene expression in vivo. Nucleic Acids Res 1995;23:1495–501.
24. Kamrud KI, Powers AM, Higgs S, Olson KE, Blair CD, Carlson JO, et al. The expression of chloramphenicol acetyltransferase in mosquitoes and mosquito cells using a packaged Sindbis replicon system. Exp Parasitol 1995;81:394–403.
25. Xiong C, Levis R, Shen P, Schlesinger S, Rice CM, Huang HV. Sindbis virus: an efficient, broad host range vector for gene expression in animal cells. Science 1989;243:1188–91.
26. Khromykh AA, Westaway EG. Subgenomic replicons of the flavivirus Kunjin: construction and applications. J Virol 1997;71:1497–505.
27. McKnight KL, Lemon SM. Capsid coding sequence is required for efficient replication of human rhinovirus 14 RNA. J Virol 1996;70:1941–52.
28. Behrens SE, Grassmann CW, Thiel HJ, Meyers G, Tautz N. Characterization of an autonomous subgenomic pestivirus RNA replicon. J Virol 1998;72:2364–72.
29. Blight KJ, Kolykhalov AA, Rice CM. Efficient initiation of HCV RNA replication in cell culture. Science 2000;290:1972–4.
30. Krieger N, Lohmann V, Bartenschlager R. Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations. J Virol 2001;75:4614–24.
31. Lohmann V, Korner F, Dobierzewska A, Bartenschlager R. Mutations in hepatitis C virus RNAs conferring cell culture adaptation. J Virol 2001;75:1437–49.
32. Evans MJ, Rice CM, Goff SP. Phosphorylation of hepatitis C virus nonstructural protein 5A modulates its protein interactions and viral RNA replication. Proc Natl Acad Sci USA 2004;101: 13038–43.
33. Appel N, Pietschmann T, Bartenschlager R. Mutational analysis of hepatitis C virus nonstructural protein 5A: potential role of differential phosphorylation in RNA replication and identification of a genetically flexible domain. J Virol 2005;79:3187–94.
34. Lohmann V, Hoffmann S, Herian U, Penin F, Bartenschlager R. Viral and cellular determinants of hepatitis C virus RNA replication in cell culture. J Virol 2003;77:3007–19.
35. Yi M, Lemon SM. Adaptive mutations producing efficient replication of genotype 1a hepatitis C virus RNA in normal Huh7 cells. J Virol 2004;78:7904–15.
36. Bartenschlager R. Hepatitis C virus molecular clones: from cDNA to infectious virus particles in cell culture. Curr Opin Microbiol 2006;9:416–22.
37. Brass V, Moradpour D, Blum HE. Molecular virology of hepatitis C virus (HCV): 2006 update. Int J Med Sci 2006;3:29–34.
38. Bartenschlager R. The hepatitis C virus replicon system: from basic research to clinical application. J Hepatol 2005;43:210–6.
39. Blight KJ, McKeating JA, Rice CM. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. J Virol 2002;76:13001–14.
40. Friebe P, Boudet J, Simorre JP, Bartenschlager R. Kissing-loop interaction in the 3′ end of the hepatitis C virus genome essential for RNA replication. J Virol 2005;79:380–92.
41. Kato T, Furusaka A, Miyamoto M, Date T, Yasui K, Hiramoto J, et al. Sequence analysis of hepatitis C virus isolated from a fulminant hepatitis patient. J Med Virol 2001;64:334–9.
42. Kato T, Date T, Miyamoto M, Furusaka A, Tokushige K, Mizokami M, et al. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. Gastroenterology 2003;125: 1808–17.
43. Date T, Kato T, Miyamoto M, Zhao Z, Yasui K, Mizokami M, et al. Genotype 2a hepatitis C virus subgenomic replicon can replicate in HepG2 and IMY-N9 cells. J Biol Chem 2004;279: 22371–6.
44. Kato T, Date T, Miyamoto M, Zhao Z, Mizokami M, Wakita T. Nonhepatic cell lines HeLa and 293 support efficient replication of the hepatitis C virus genotype 2a subgenomic replicon. J Virol 2005;79:592–6.
45. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005;11:791–6.
46. Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, et al. Robust hepatitis C virus infection in vitro. Proc Natl Acad Sci USA 2005;102:9294–9.
47. Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, et al. Complete replication of hepatitis C virus in cell culture. Science 2005;309:623–6.
48. Compans RW. Virus entry and release in polarized epithelial cells. Curr Top Microbiol Immunol 1995;202:209–19.
49. Garoff H, Hewson R, Opstelten DJ. Virus maturation by budding. Microbiol Mol Biol Rev 1998;62:1171–90.
50. Schmitt AP, Lamb RA. Escaping from the cell: assembly and budding of negative-strand RNA viruses. Curr Top Microbiol Immunol 2004;283:145–96.
51. Takimoto T, Portner A. Molecular mechanism of paramyxovirus budding. Virus Res 2004;106:133–45.
52. Murakami K, Ishii K, Ishihara Y, Yoshizaki S, Tanaka K, Gotoh Y, et al. Production of infectious hepatitis C virus particles in three-dimensional cultures of the cell line carrying the genome-length dicistronic viral RNA of genotype 1b. Virology 2006;351: 381–92.
53. Kawada M, Nagamori S, Aizaki H, Fukaya K, Niiya M, Matsuura T, et al. Massive culture of human liver cancer cells in a newly developed radial flow bioreactor system: ultrafine structure of functionally enhanced hepatocarcinoma cell lines. In Vitro Cell Dev Biol Anim 1998;34:109–15.
54. Matsuura T, Kawada M, Hasumura S, Nagamori S, Obata T, Yamaguchi M, et al. High density culture of immortalized liver endothelial cells in the radial-flow bioreactor in the development of an artificial liver. Int J Artif Organs 1998;21:229–34.
55. Aizaki H, Nagamori S, Matsuda M, Kawakami H, Hashimoto O, Ishiko H, et al. Production and release of infectious hepatitis C virus from human liver cell cultures in the three-dimensional radial-flow bioreactor. Virology 2003;314:16–25.
56. Iwahori T, Matsuura T, Maehashi H, Sugo K, Saito M, Hosokawa M, et al. CYP3A4 inducible model for in vitro analysis of human drug metabolism using a bioartificial liver. Hepatology 2003;37:665–73.
57. Yoshioka H, Mikami M, Mori Y, Tsuchida E. A synthetic hydrogel with thermoreversible gelation. J Macromol Sci 1994; A31:113–20.
58. Bukh J, Purcell RH, Miller RH. Sequence analysis of the 5′ noncoding region of hepatitis C virus. Proc Natl Acad Sci USA 1992;89:4942–6.
59. Brown EA, Zhang H, Ping LH, Lemon SM. Secondary structure of the 5′ nontranslated regions of hepatitis C virus and pestivirus genomic RNAs. Nucleic Acids Res 1992;20:5041–5.
60. Honda M, Beard MR, Ping LH, Lemon SM. A phylogenetically conserved stem–loop structure at the 5′ border of the internal ribosome entry site of hepatitis C virus is required for cap-independent viral translation. J Virol 1999;73:1165–74.
61. Honda M, Brown EA, Lemon SM. Stability of a stem–loop involving the initiator AUG controls the efficiency of internal initiation of translation on hepatitis C virus RNA. RNA 1996;2: 955–68.

62. Tsukiyama-Kohara K, Iizuka N, Kohara M, Nomoto A. Internal ribosome entry site within hepatitis C virus RNA. J Virol 1992; 66:1476–83.
63. Wang C, Sarnow P, Siddiqui A. Translation of human hepatitis C virus RNA in cultured cells is mediated by an internal ribosome-binding mechanism. J Virol 1993;67:3338–44.
64. Hellen CU, Pestova TV. Translation of hepatitis C virus RNA. J Viral Hepat 1999;6:79–87.
65. Lu HH, Wimmer E. Poliovirus chimeras replicating under the translational control of genetic elements of hepatitis C virus reveal unusual properties of the internal ribosomal entry site of hepatitis C virus. Proc Natl Acad Sci USA 1996;93:1412–7.
66. Reynolds JE, Kaminski A, Kettinen HJ, Grace K, Clarke BE, Carroll AR, et al. Unique features of internal initiation of hepatitis C virus RNA translation. EMBO J 1995;14:6010–20.
67. Rijnbrand R, Bredenbeek P, van der Straaten T, Whetter L, Inchauspe G, Lemon S, et al. Almost the entire 5′ non-translated region of hepatitis C virus is required for cap-independent translation. FEBS Lett 1995;365:115–9.
68. Yoo BJ, Spaete RR, Geballe AP, Selby M, Houghton M, Han JH. 5′ end-dependent translation initiation of hepatitis C viral RNA and the presence of putative positive and negative translational control elements within the 5′ untranslated region. Virology 1992;191:889–99.
69. Lemon S, Honda M. Internal ribosome entry sites within the RNA genomes of hepatitis C virus and other flaviviruses. Semin Virol 1997;8:274–88.
70. Buratti E, Tisminetzky S, Zotti M, Baralle FE. Functional analysis of the interaction between HCV 5′UTR and putative subunits of eukaryotic translation initiation factor eIF3. Nucleic Acids Res 1998;26:3179–87.
71. Kieft JS, Zhou K, Jubin R, Doudna JA. Mechanism of ribosome recruitment by hepatitis C IRES RNA. RNA 2001;7:194–206.
72. Sizova DV, Kolupaeva VG, Pestova TV, Shatsky IN, Hellen CU. Specific interaction of eukaryotic translation initiation factor 3 with the 5′ nontranslated regions of hepatitis C virus and classical swine fever virus RNAs. J Virol 1998;72:4775–82.
73. Ali N, Pruijn GJ, Kenan DJ, Keene JD, Siddiqui A. Human La antigen is required for the hepatitis C virus internal ribosome entry site-mediated translation. J Biol Chem 2000;275:27531–40.
74. Ali N, Siddiqui A. The La antigen binds 5′ noncoding region of the hepatitis C virus RNA in the context of the initiator AUG codon and stimulates internal ribosome entry site-mediated translation. Proc Natl Acad Sci USA 1997;94:2249–54.
75. Isoyama T, Kamoshita N, Yasui K, Iwai A, Shiroki K, Toyoda H, et al. Lower concentration of La protein required for internal ribosome entry on hepatitis C virus RNA than on poliovirus RNA. J Gen Virol 1999;80:2319–27.
76. Hahm B, Kim YK, Kim JH, Kim TY, Jang SK. Heterogeneous nuclear ribonucleoprotein L interacts with the 3′ border of the internal ribosomal entry site of hepatitis C virus. J Virol 1998; 72:8782–8.
77. Fukushi S, Okada M, Kageyama T, Hoshino FB, Nagai K, Katayama K. Interaction of poly(rC)-binding protein 2 with the 5′-terminal stem loop of the hepatitis C-virus genome. Virus Res 2001;73:67–79.
78. Anwar A, Ali N, Tanveer R, Siddiqui A. Demonstration of functional requirement of polypyrimidine tract-binding protein by SELEX RNA during hepatitis C virus internal ribosome entry site-mediated translation initiation. J Biol Chem 2000;275: 34231–5.
79. Shimoike T, Mimori S, Tani H, Matsuura Y, Miyamura T. Interaction of hepatitis C virus core protein with viral sense RNA and suppression of its translation. J Virol 1999;73:9718–25.
80. Tanaka Y, Shimoike T, Ishii K, Suzuki R, Suzuki T, Ushijima H, et al. Selective binding of hepatitis C virus core protein to synthetic oligonucleotides corresponding to the 5′ untranslated region of the viral genome. Virology 2000;270:229–36.
81. Shimoike T, Koyama C, Murakami K, Suzuki R, Matsuura Y, Miyamura T, et al. Down-regulation of the internal ribosome entry site (IRES)-mediated translation of the hepatitis C virus: critical role of binding of the stem–loop IIId domain of IRES and the viral core protein. Virology 2006;345:434–45.
82. Wang TH, Rijnbrand RC, Lemon SM. Core protein-coding sequence, but not core protein, modulates the efficiency of cap-independent translation directed by the internal ribosome entry site of hepatitis C virus. J Virol 2000;74:11347–58.
83. Zhang J, Yamada O, Yoshida H, Iwai T, Araki H. Autogenous translational inhibition of core protein: implication for switch from translation to RNA replication in hepatitis C virus. Virology 2002;293:141–50.
84. Li D, Takyar ST, Lott WB, Gowans EJ. Amino acids 1–20 of the hepatitis C virus (HCV) core protein specifically inhibit HCV IRES-dependent translation in HepG2 cells, and inhibit both HCV IRES- and cap-dependent translation in HuH7 and CV-1 cells. J Gen Virol 2003;84:815–25.
85. Friebe P, Lohmann V, Krieger N, Bartenschlager R. Sequences in the 5′ nontranslated region of hepatitis C virus required for RNA replication. J Virol 2001;75:12047–57.
86. Hüssy P, Langen H, Mous J, Jacobsen H. Hepatitis C virus core protein: carboxy-terminal boundaries of two processed species suggest cleavage by a signal peptide peptidase. Virology 1996;224: 93–104.
87. McLauchlan J, Lemberg MK, Hope G, Martoglio B. Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. EMBO J 2002;21:3980–8.
88. Lemberg MK, Martoglio B. Requirements for signal peptide peptidase-catalyzed intramembrane proteolysis. Mol Cell 2002; 10:735–44.
89. Okamoto K, Moriishi K, Miyamura T, Matsuura Y. Intramembrane proteolysis and endoplasmic reticulum retention of hepatitis C virus core protein. J Virol 2004;78:6370–80.
90. Weihofen A, Binns K, Lemberg MK, Ashman K, Martoglio B. Identification of signal peptide peptidase, a presenilin-type aspartic protease. Science 2002;296:2215–8.
91. Pallaoro M, Lahm A, Biasiol G, Brunetti M, Nardella C, Orsatti L, et al. Characterization of the hepatitis C virus NS2/3 processing reaction by using a purified precursor protein. J Virol 2001;75: 9939–46.
92. Thibeault D, Maurice R, Pilote L, Lamarre D, Pause A. In vitro characterization of a purified NS2/3 protease variant of hepatitis C virus. J Biol Chem 2001;276:46678–84.
93. Bartenschlager R, Ahlborn-Laake L, Mous J, Jacobsen H. Kinetic and structural analyses of hepatitis C virus polyprotein processing. J Virol 1994;68:5045–55.
94. Failla C, Tomei L, De Francesco R. An amino-terminal domain of the hepatitis C virus NS3 protease is essential for interaction with NS4A. J Virol 1995;69:1769–77.
95. Lin C, Pragai BM, Grakoui A, Xu J, Rice CM. Hepatitis C virus NS3 serine proteinase: *trans*-cleavage requirements and processing kinetics. J Virol 1994;68:8147–57.
96. Tanji Y, Hijikata M, Hirowatari Y, Shimotohno K. Hepatitis C virus polyprotein processing: kinetics and mutagenic analysis of serine proteinase-dependent cleavage. J Virol 1994;68:8418–22.
97. Jin L, Peterson DL. Expression, isolation, and characterization of the hepatitis C virus ATPase/RNA helicase. Arch Biochem Biophys 1995;323:47–53.
98. Kim DW, Gwack Y, Han JH, Choe J. C-terminal domain of the hepatitis C virus NS3 protein contains an RNA helicase activity. Biochem Biophys Res Commun 1995;215:160–6.
99. Suzich JA, Tamura JK, Palmer-Hill F, Warrener P, Grakoui A, Rice CM, et al. Hepatitis C virus NS3 protein polynucleotide-stimulated nucleoside triphosphatase and comparison with the related pestivirus and flavivirus enzymes. J Virol 1993;67: 6152–8.

100. Tai CL, Chi WK, Chen DS, Hwang LH. The helicase activity associated with hepatitis C virus nonstructural protein 3 (NS3). J Virol 1996;70:8477–84.
101. Tai CL, Pan WC, Liaw SH, Yang UC, Hwang LH, Chen DS. Structure-based mutational analysis of the hepatitis C virus NS3 helicase. J Virol 2001;75:8289–97.
102. Wolk B, Sansonno D, Krausslich HG, Dammacco F, Rice CM, Blum HE, et al. Subcellular localization, stability, and trans-cleavage competence of the hepatitis C virus NS3–NS4A complex expressed in tetracycline-regulated cell lines. J Virol 2000;74:2293–304.
103. Barbato G, Cicero DO, Nardi MC, Steinkuhler C, Cortese R, De Francesco R, et al. The solution structure of the N-terminal proteinase domain of the hepatitis C virus (HCV) NS3 protein provides new insights into its activation and catalytic mechanism. J Mol Biol 1999;289:371–84.
104. Tanji Y, Hijikata M, Satoh S, Kaneko T, Shimotohno K. Hepatitis C virus-encoded nonstructural protein NS4A has versatile functions in viral protein processing. J Virol 1995;69:1575–81.
105. Restrepo-Hartwig MA, Ahlquist P. Brome mosaic virus helicase- and polymerase-like proteins colocalize on the endoplasmic reticulum at sites of viral RNA synthesis. J Virol 1996;70: 8908–16.
106. Schaad MC, Jensen PE, Carrington JC. Formation of plant RNA virus replication complexes on membranes: role of an endoplasmic reticulum-targeted viral protein. EMBO J 1997;16:4049–59.
107. van der Meer Y, van Tol H, Locker JK, Snijder EJ. ORF1a-encoded replicase subunits are involved in the membrane association of the arterivirus replication complex. J Virol 1998;72: 6689–98.
108. Shi ST, Schiller JJ, Kanjanahaluethai A, Baker SC, Oh JW, Lai MM. Colocalization and membrane association of murine hepatitis virus gene 1 products and de novo-synthesized viral RNA in infected cells. J Virol 1999;73:5957–69.
109. Froshauer S, Kartenbeck J, Helenius A. Alphavirus RNA replicase is located on the cytoplasmic surface of endosomes and lysosomes. J Cell Biol 1988;107:2075–86.
110. Egger D, Wolk B, Gosert R, Bianchi L, Blum HE, Moradpour D, et al. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. J Virol 2002;76:5974–84.
111. Gosert R, Egger D, Lohmann V, Bartenschlager R, Blum HE, Bienz K, et al. Identification of the hepatitis C virus RNA replication complex in Huh-7 cells harboring subgenomic replicons. J Virol 2003;77:5487–92.
112. Shi ST, Lee KJ, Aizaki H, Hwang SB, Lai MM. Hepatitis C virus RNA replication occurs on a detergent-resistant membrane that cofractionates with caveolin-2. J Virol 2003;77:4160–8.
113. Piccininni S, Varaklioti A, Nardelli M, Dave B, Raney KD, McCarthy JE. Modulation of the hepatitis C virus RNA-dependent RNA polymerase activity by the non-structural (NS) 3 helicase and the NS4B membrane protein. J Biol Chem 2002;277: 45670–9.
114. Gao L, Aizaki H, He JW, Lai MM. Interactions between viral nonstructural proteins and host protein hVAP-33 mediate the formation of hepatitis C virus RNA replication complex on lipid raft. J Virol 2004;78:3480–8.
115. Brass V, Bieck E, Montserret R, Wolk B, Hellings JA, Blum HE, et al. An amino-terminal amphipathic alpha-helix mediates membrane association of the hepatitis C virus nonstructural protein 5A. J Biol Chem 2002;277:8130–9.
116. Shimakami T, Hijikata M, Luo H, Ma YY, Kaneko S, Shimotohno K, et al. Effect of interaction between hepatitis C virus NS5A and NS5B on hepatitis C virus RNA replication with the hepatitis C virus replicon. J Virol 2004;78:2738–48.
117. Hamamoto I, Nishimura Y, Okamoto T, Aizaki H, Liu M, Mori Y, et al. Human VAP-B is involved in hepatitis C virus replication through interaction with NS5A and NS5B. J Virol 2005;79: 13473–82.
118. Ito T, Lai MM. An internal polypyrimidine-tract-binding protein-binding site in the hepatitis C virus RNA attenuates translation, which is relieved by the 3'-untranslated sequence. Virology 1999;254:288–96.
119. Kolykhalov AA, Agapov EV, Blight KJ, Mihalik K, Feinstone SM, Rice CM. Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA. Science 1997;277:570–4.
120. Tanaka T, Kato N, Cho M-J, Shimotohno K. A novel sequence found at the 3' terminus of hepatitis C virus genome. Biochem Biophys Res Commun 1995;215:744–9.
121. Yi M, Lemon SM. 3' nontranslated RNA signals required for replication of hepatitis C virus RNA. J Virol 2003;77:3557–68.
122. Friebe P, Bartenschlager R. Genetic analysis of sequences in the 3' nontranslated region of hepatitis C virus that are important for RNA replication. J Virol 2002;76:5326–38.
123. Aizaki H, Lee KJ, Sung VM, Ishiko H, Lai MM. Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts. Virology 2004;324:450–61.
124. Ali N, Tardif KD, Siddiqui A. Cell-free replication of the hepatitis C virus subgenomic replicon. J Virol 2002;76:12001–7.
125. Lai VC, Dempsey S, Lau JY, Hong Z, Zhong W. In vitro RNA replication directed by replicase complexes isolated from the subgenomic replicon cells of hepatitis C virus. J Virol 2003;77: 2295–300.
126. Miyanari Y, Hijikata M, Yamaji M, Hosaka M, Takahashi H, Shimotohno K. Hepatitis C virus non-structural proteins in the probable membranous compartment function in viral genome replication. J Biol Chem 2003;278:50301–8.
127. Hardy RW, Marcotrigiano J, Blight KJ, Majors JE, Rice CM. Hepatitis C virus RNA synthesis in a cell-free system isolated from replicon-containing hepatoma cells. J Virol 2003;77: 2029–37.
128. Waris G, Sarker S, Siddiqui A. Two-step affinity purification of the hepatitis C virus ribonucleoprotein complex. RNA 2004;10: 321–9.
129. Simons K, Ikonen E. Functional rafts in cell membranes. Nature 1997;387:569–72.
130. Simons K, Ikonen E. How cells handle cholesterol. Science 2000;290:1721–6.
131. Simons K, Toomre D. Lipid rafts and signal transduction. Nat Rev Mol Cell Biol 2000;1:31–9.
132. Barman S, Ali A, Hui EK, Adhikary L, Nayak DP. Transport of viral proteins to the apical membranes and interaction of matrix protein with glycoproteins in the assembly of influenza viruses. Virus Res 2001;77:61–9.
133. Scheiffele P, Rietveld A, Wilk T, Simons K. Influenza viruses select ordered lipid domains during budding from the plasma membrane. J Biol Chem 1999;274:2038–44.
134. Zhang J, Pekosz A, Lamb RA. Influenza virus assembly and lipid raft microdomains: a role for the cytoplasmic tails of the spike glycoproteins. J Virol 2000;74:4634–44.
135. Ding L, Derdowski A, Wang JJ, Spearman P. Independent segregation of human immunodeficiency virus type 1 Gag protein complexes and lipid rafts. J Virol 2003;77:1916–26.
136. Ono A, Freed EO. Plasma membrane rafts play a critical role in HIV-1 assembly and release. Proc Natl Acad Sci USA 2001;98: 13925–30.
137. Bavari S, Bosio CM, Wiegand E, Ruthel G, Will AB, Geisbert TW, et al. Lipid raft microdomains: a gateway for compartmentalized trafficking of Ebola and Marburg viruses. J Exp Med 2002;195:593–602.
138. Stuart AD, Eustace HE, McKee TA, Brown TD. A novel cell entry pathway for a DAF-using human enterovirus is dependent on lipid rafts. J Virol 2002;76:9307–22.
139. Narayan S, Barnard RJ, Young JA. Two retroviral entry pathways distinguished by lipid raft association of the viral receptor and differences in viral infectivity. J Virol 2003;77: 1977–83.

140. Ashbourne Excoffon KJ, Moninger T, Zabner J. The Coxsackie B virus and adenovirus receptor resides in a distinct membrane microdomain. J Virol 2003;77:2559–67.
141. Brown DA, Rose JK. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. Cell 1992;68:533–44.
142. Hijikata M, Mizushima H, Tanji Y, Komoda Y, Hirowatari Y, Akagi T, et al. Proteolytic processing and membrane association of putative nonstructural proteins of hepatitis C virus. Proc Natl Acad Sci USA 1993;90:10773–7.
143. Mottola G, Cardinali G, Ceccacci A, Trozzi C, Bartholomew L, Torrisi MR, et al. Hepatitis C virus nonstructural proteins are localized in a modified endoplasmic reticulum of cells expressing viral subgenomic replicons. Virology 2002;293:31–43.
144. Tu H, Gao L, Shi ST, Taylor DR, Yang T, Mircheff AK, et al. Hepatitis C virus RNA polymerase and NS5A complex with a SNARE-like protein. Virology 1999;263:30–41.
145. Pietschmann T, Lohmann V, Rutter G, Kurpanek K, Bartenschlager R. Characterization of cell lines carrying self-replicating hepatitis C virus RNAs. J Virol 2001;75:1252–64.
146. Choi J, Lee KJ, Zheng Y, Yamaga AK, Lai MM, Ou JH. Reactive oxygen species suppress hepatitis C virus RNA replication in human hepatoma cells. Hepatology 2004;39:81–9.
147. Kaito M, Watanabe S, Tsukiyama-Kohara K, Yamaguchi K, Kobayashi Y, Konishi M, et al. Hepatitis C virus particle detected by immunoelectron microscopic study. J Gen Virol 1994; 75:1755–60.
148. Shimizu YK, Feinstone SM, Kohara M, Purcell RH, Yoshikura H. Hepatitis C virus: detection of intracellular virus particles by electron microscopy. Hepatology 1996;23:205–9.
149. Maillard P, Krawczynski K, Nitkiewicz J, Bronnert C, Sidorkiewicz M, Gounon P, et al. Nonenveloped nucleocapsids of hepatitis C virus in the serum of infected patients. J Virol 2001;75: 8240–50.
150. Andre P, Komurian-Pradel F, Deforges S, Perret M, Berland JL, Sodoyer M, et al. Characterization of low- and very-low-density hepatitis C virus RNA-containing particles. J Virol 2002;76: 6919–28.
151. Moradpour D, Wakita T, Tokushige K, Carlson RI, Krawczynski K, Wands JR. Characterization of three novel monoclonal antibodies against hepatitis C virus core protein. J Med Virol 1996;48: 234–41.
152. Barba G, Harper F, Harada T, Kohara M, Goulinet S, Matsuura Y, et al. Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. Proc Natl Acad Sci USA 1997;94:1200–5.
153. Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, et al. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat Med 1998;4: 1065–7.
154. Hope RG, Murphy DJ, McLauchlan J. The domains required to direct core proteins of hepatitis C virus and GB virus-B to lipid droplets share common features with plant oleosin proteins. J Biol Chem 2002;277:4261–70.
155. Suzuki R, Sakamoto S, Tsutsumi T, Rikimaru A, Tanaka K, Shimoike T, et al. Molecular determinants for subcellular localization of hepatitis C virus core protein. J Virol 2005;79: 1271–81.
156. Suzuki T, Suzuki R. Maturation and assembly of hepatitis C virus core protein. In: Kalitzky M, Borowski P, editors. Molecular biology of the Flavivirus. Norfolk, UK: Horizon Bioscience; 2006. p. 295–311.
157. Shintani Y, Fujie H, Miyoshi H, Tsutsumi T, Tsukamoto K, Kimura S, et al. Hepatitis C virus infection and diabetes: direct involvement of the virus in the development of insulin resistance. Gastroenterology 2004;126:840–8.
158. Moriishi K, Mochizuki R, Moriya K, Miyamoto H, Mori Y, Abe T, et al. Critical role of PA28γ in hepatitis C virus-associated steatogenesis and hepatocarcinogenesis. Proc Natl Acad Sci U S A 2007;104:1661–6.
159. Miyamoto H, Moriishi K, Moriya K, Murata S, Tanaka K, Suzuki T, et al. Involvement of the PA28γ-dependent pathway in insulin resistance induced by hepatitis C virus core protein. J Virol 2007;81:1727–35.
160. Nakai K, Okamoto T, Kimura-Someya T, Ishii K, Lim CK, Tani H, et al. Oligomerization of hepatitis C virus core protein is crucial for interaction with the cytoplasmic domain of E1 envelope protein. J Virol 2006;80:11265–73.
161. Baumert TF, Ito S, Wong DT, Liang TJ. Hepatitis C virus structural proteins assemble into viruslike particles in insect cells. J Virol 1998;72:3827–36.
162. Falcon V, Garcia C, de la Rosa MC, Menendez I, Seoane J, Grillo JM. Ultrastructural and immunocytochemical evidences of core-particle formation in the methylotrophic *Pichia pastoris* yeast when expressing HCV structural proteins (core-E1). Tissue Cell 1999;31:117–25.
163. Kunkel M, Lorinczi M, Rijnbrand R, Lemon SM, Watowich SJ. Self-assembly of nucleocapsid-like particles from recombinant hepatitis C virus core protein. J Virol 2001;75:2119–29.
164. Lorenzo LJ, Duenas-Carrera S, Falcon V, Acosta-Rivero N, Gonzalez E, de la Rosa MC, et al. Assembly of truncated HCV core antigen into virus-like particles in *Escherichia coli*. Biochem Biophys Res Commun 2001;281:962–5.
165. Acosta-Rivero N, Aguilar JC, Musacchio A, Falcon V, Vina A, de la Rosa MC, et al. Characterization of the HCV core virus-like particles produced in the methylotrophic yeast *Pichia pastoris*. Biochem Biophys Res Commun 2001;287:122–5.
166. Kunkel M, Watowich SJ. Conformational changes accompanying self-assembly of the hepatitis C virus core protein. Virology 2002;294:239–45.
167. Acosta-Rivero N, Falcon V, Alvarez C, Musacchio A, Chinea G, Cristina de la Rosa M, et al. Structured HCV nucleocapsids composed of P21 core protein assemble primary in the nucleus of *Pichia pastoris* yeast. Biochem Biophys Res Commun 2003; 310:48–53.
168. Blanchard E, Hourioux C, Brand D, Ait-Goughoulte M, Moreau A, Trassard S, et al. Hepatitis C virus-like particle budding: role of the core protein and importance of its Asp111. J Virol 2003; 77:10131–8.
169. Majeau N, Gagne V, Boivin A, Bolduc M, Majeau JA, Ouellet D, et al. The N-terminal half of the core protein of hepatitis C virus is sufficient for nucleocapsid formation. J Gen Virol 2004; 85:971–81.
170. Klein KC, Polyak SJ, Lingappa JR. Unique features of hepatitis C virus capsid formation revealed by de novo cell-free assembly. J Virol 2004;78:9257–69.
171. Kunkel M, Watowich SJ. Biophysical characterization of hepatitis C virus core protein: implications for interactions within the virus and host. FEBS Lett 2004;557:174–80.
172. Matsumoto M, Hwang SB, Jeng KS, Zhu N, Lai MM. Homotypic interaction and multimerization of hepatitis C virus core protein. Virology 1996;218:43–51.
173. Nolandt O, Kern V, Muller H, Pfaff E, Theilmann L, Welker R, et al. Analysis of hepatitis C virus core protein interaction domains. J Gen Virol 1997;78(Pt 6):1331–40.
174. Yan BS, Tam MH, Syu WJ. Self-association of the C-terminal domain of the hepatitis-C virus core protein. Eur J Biochem 1998;258:100–6.
175. Ezelle HJ, Markovic D, Barber GN. Generation of hepatitis C virus-like particles by use of a recombinant vesicular stomatitis virus vector. J Virol 2002;76:12325–34.
176. Clayton RF, Owsianka A, Aitken J, Graham S, Bhella D, Patel AH. Analysis of antigenicity and topology of E2 glycoprotein present on recombinant hepatitis C virus-like particles. J Virol 2002;76:7672–82.
177. Lo S-Y, Selby MJ, Ou J-H. Interaction between hepatitis C virus core protein and E1 envelope protein. J Virol 1996;70:5177–82.

178. Ma HC, Ke CH, Hsieh TY, Lo SY. The first hydrophobic domain of the hepatitis C virus E1 protein is important for interaction with the capsid protein. J Gen Virol 2002;83:3085–92.
179. Hershko A, Ciechanover A. The ubiquitin system. Annu Rev Biochem 1998;67:425–79.
180. Finley D, Ciechanover A, Varshavsky A. Ubiquitin as a central cellular regulator. Cell 2004;116:S29–32, 2 p following S.
181. Suzuki R, Tamura K, Li J, Ishii K, Matsuura Y, Miyamura T, et al. Ubiquitin-mediated degradation of hepatitis C virus core protein is regulated by processing at its carboxyl terminus. Virology 2001;280:301–9.
182. Moriishi K, Okabayashi T, Nakai K, Moriya K, Koike K, Murata S, et al. Proteasome activator PA28γ-dependent nuclear retention and degradation of hepatitis C virus core protein. J Virol 2003;77:10237–49.
183. Shirakura M, Murakami K, Ichimura T, Suzuki R, Shimoji T, Fukuda K, et al. E6AP ubiquitin ligase mediates ubiquitylation and degradation of hepatitis C virus core protein. J Virol 2007; 81:1174–85.
184. Huibregtse JM, Scheffner M, Beaudenon S, Howley PM. A family of proteins structurally and functionally related to the E6-AP ubiquitin–protein ligase. Proc Natl Acad Sci USA 1995;92: 2563–7.
185. Huibregtse JM, Scheffner M, Howley PM. Cloning and expression of the cDNA for E6-AP, a protein that mediates the interaction of the human papillomavirus E6 oncoprotein with p53. Mol Cell Biol 1993;13:775–84.
186. Scheffner M, Huibregtse JM, Vierstra RD, Howley PM. The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell 1993;75:495–505.
187. Tanahashi N, Yokota K, Ahn JY, Chung CH, Fujiwara T, Takahashi E, et al. Molecular properties of the proteasome activator PA28 family proteins and gamma-interferon regulation. Genes Cells 1997;2:195–211.
188. Realini C, Jensen CC, Zhang Z, Johnston SC, Knowlton JR, Hill CP, et al. Characterization of recombinant REGalpha, REGbeta, and REGgamma proteasome activators. J Biol Chem 1997;272:25483–92.
189. Polyak SJ, Klein KC, Shoji I, Miyamura T, Lingappa JR. Assemble and interact pleiotropic functions of the HCV core protein. In: Tan S-L, editor. Hepatitis C viruses: genomes and molecular biology. Norwich, UK: Horizon Bioscience; 2006. p. 89–119.

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E6AP Ubiquitin Ligase Mediates Ubiquitylation and Degradation of Hepatitis C Virus Core Protein▽

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Hepatitis C virus (HCV) core protein is a major component of viral nucleocapsid and a multifunctional protein involved in viral pathogenesis and hepatocarcinogenesis. We previously showed that the HCV core protein is degraded through the ubiquitin-proteasome pathway. However, the molecular machinery for core ubiquitylation is unknown. Using tandem affinity purification, we identified the ubiquitin ligase E6AP as an HCV core-binding protein. E6AP was found to bind to the core protein in vitro and in vivo and promote its degradation in hepatic and nonhepatic cells. Knockdown of endogenous E6AP by RNA interference increased the HCV core protein level. In vitro and in vivo ubiquitylation assays showed that E6AP promotes ubiquitylation of the core protein. Exogenous expression of E6AP decreased intracellular core protein levels and supernatant HCV infectivity titers in the HCV JFH1-infected Huh-7 cells. Furthermore, knockdown of endogenous E6AP by RNA interference increased intracellular core protein levels and supernatant HCV infectivity titers in the HCV JFH1-infected cells. Taken together, our results provide evidence that E6AP mediates ubiquitylation and degradation of HCV core protein. We propose that the E6AP-mediated ubiquitin-proteasome pathway may affect the production of HCV particles through controlling the amounts of viral nucleocapsid protein.

Hepatitis C virus (HCV; a single-stranded, positive-sense RNA virus that is classified in the family *Flaviviridae*) is the main cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (5, 26, 45). More than 170 million people worldwide are chronically infected with HCV (41). The approximately 9.6-kb HCV genome encodes a unique open reading frame that is translated into a polyprotein (5, 54). The polyprotein is cleaved cotranslationally into at least 10 proteins by viral proteases and cellular signalases (6, 10).

The HCV core protein represents the first 1 to 191 amino acids (aa) of the polyprotein and is followed by two glycoproteins, E1 and E2 (6). The core protein plays a central role in the packaging of viral RNA (25, 40); modulates various cellular processes, including signal transduction pathways, transcriptional control, cell cycle progression, apoptosis, lipid metabolism, and the immune response (9, 40); and has transforming potential in certain cells (43). Mice transgenic for the HCV core gene develop steatosis (32) and later hepatocellular carcinoma (31). These findings suggest that HCV core protein plays a crucial role in hepatocarcinogenesis.

Two major forms of the HCV core protein, p21 (mature form) and p23 (immature form), can be generated in cultured cells (60). Cellular signal peptidase cleaves at the junction of the core/E1, releasing the immature form of the core protein from the polypeptide (12, 46). Signal peptide peptidase cleaves just before the signal sequence, liberating the mature form of the HCV core protein at the cytoplasmic face of the endoplasmic reticulum (29). Several different sites have been proposed as potential cleavage sites of signal peptide peptidase, such as Leu-179 (15, 29), Phe-177 (36, 37), Leu-182 (15), and Ser-173 (46). Further processing of the HCV core protein yields a 17-kDa product with a C terminus at around amino acid 152. A truncated form of the core protein, p17, was found in transfected cells (42, 52) and liver tissues from humans with hepatocellular carcinoma (59). The majority of this protein translocates to the nucleus. The C terminus of the core protein is important for regulating the stability of the protein (20, 52).

We previously showed that the C-terminally truncated forms of the core protein are degraded through the ubiquitin-proteasome pathway (52). We found that the mature form of the core protein, p21, also links to a few ubiquitin moieties, suggesting that the ubiquitin-proteasome pathway involves proteolysis of heterologous species of the core protein (52). Overexpression of PA28γ (a REG family proteasome activator also known as REGγ or Ki antigen) enhances the proteasomal degradation of the HCV core protein (30). A recent study has shown that

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PA28γ is involved in the degradation of the steroid receptor coactivator 3 (SRC-3) in an ATP- and ubiquitin-independent manner (27). It is still unclear what E3 ubiquitin ligase is responsible for ubiquitylation of the HCV core protein.

E6AP was initially identified as the cellular factor that stimulates ubiquitin-mediated degradation of the tumor suppressor p53 in conjunction with the E6 protein of cancer-associated human papillomavirus types 16 and 18 (14, 48). The E6-E6AP complex functions as a E3 ubiquitin ligase in the ubiquitylation of p53 (49). E6AP is the prototype of a family of ubiquitin ligases called HECT domain ubiquitin ligases, all of which contain a domain *h*omologous to the *E6AP c*arboxyl *t*erminus (13). Interestingly, E6AP is not involved in the regulation of p53 ubiquitylation in the absence of E6 (55). Several potential E6-independent substrates for E6AP have been identified, such as hHR23A, Blk, and Mcm7 (23, 24, 35). E6AP is also a candidate gene for Angelman syndrome, which is a severe neurological disorder characterized by mental retardation (21).

This study aimed to identify endogenous ubiquitin-proteasome pathway proteins that are associated with HCV core protein. Tandem affinity purification and mass spectrometry analysis identified E6AP as an HCV core-binding protein. Here we present evidence that E6AP associates with HCV core protein in vitro and in vivo and is involved in ubiquitylation and degradation of HCV core protein. We propose that an E6AP-mediated ubiquitin-proteasome pathway may affect the production of HCV particles through controlling the amounts of HCV core protein.

MATERIALS AND METHODS

Cell culture and transfection. Human embryonic kidney 293T cells, human hepatoblastoma HepG2 cells, and human hepatoma Huh-7 cells were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin (Invitrogen), and 10% (vol/vol) fetal bovine serum (JRH Biosciences) at 37°C in a 5% CO_2 incubator. 293T cells and HepG2 cells were transfected with plasmid DNA using FuGene 6 transfection reagents (Roche). Huh-7 cells were transfected with plasmid DNA using *Trans*IT LT1 transfection reagents (Mirus).

Plasmids and recombinant baculoviruses. MEF tag cassette (containing *myc* tag, the tobacco *e*tch virus protease cleavage site, and *F*LAG tag) (16) was fused to the N terminus of the cDNA encoding core protein of HCV NIHJ1 (genotype 1b) (1). To express MEF-tagged core protein in mammalian cells, the genome coding for HCV core protein (amino acids 1 to 191) was amplified by PCR using pBR HCV NIHJ1 as a template. Sense oligonucleotide containing a Kozak consensus translation initiation codon and antisense oligonucleotide containing an in-frame translation stop codon were synthesized by PCR. The amplified PCR product was purified, digested with EcoRI and EcoRV, and then inserted into the EcoRI-EcoRV site of pcDNA3-MEF. FLAG-tagged HCV core expression plasmids based upon pCAGGS (34) were described previously (30). To express E6AP and the active-site cysteine-to-alanine mutant of E6AP in mammalian cells, pCMV4-HA-E6AP isoform II and pCMV4-HA-E6AP C-A were utilized (19). The C-A mutation was introduced at the site of E6AP C843. To express E6AP and E6AP C-A under the CAG promoter, the E6AP fragment and the E6AP C-A fragment were amplified by PCR, purified, digested with SmaI and NotI, and blunt ended using a DNA blunting kit (Takara). These PCR fragments were subcloned into pCAGGS.

To make a fusion protein consisting of glutathione *S*-transferase (GST) fused to the N terminus of E6AP in *Escherichia coli*, the E6AP fragment was amplified by PCR and the resultant product was cloned into the SmaI-NotI site of pGEX4T-1 vector (Amersham Biosciences). To express a series of E6AP truncation mutants as GST fusion proteins, each fragment was amplified by PCR and cloned into the SmaI-NotI site of pGEX4T-1. To purify GST core protein efficiently by two-step affinity purification, we fused hexahistidine (His) tag to the C terminus of GST fusion proteins. To bacterially express HCV core (aa 1 to 173) protein as a fusion protein containing N-terminal GST tag and C-terminal His tag, core fragment was amplified by PCR and the resultant product was cloned into the EcoRI-NotI site of pGEX4T-1 vector. The resultant plasmid was designated pGEX GST-C173HT. To express GST core (1–152)-His and GST-His in *E. coli*, pGEX core (1–152)-His and pGEX-His were constructed similarly. The resultant plasmids were designated pGEX GST-C152HT and pGEX GST-HT, respectively.

To generate recombinant baculoviruses expressing GST-E6AP, GST-E6AP fragment was excised from pGEX E6AP by digestion with SmaI and Tth111I and ligated into the SmaI-Tth111I site of pVL1392 (Invitrogen). To express GST-E6AP C-A, pVLGST-E6AP C-A was constructed similarly. To generate recombinant baculovirus expressing HCV core (aa 1 to 173) protein as a fusion protein containing N-terminal GST tag and C-terminal His tag, GST-C173HT fragment was amplified by PCR using pGEX GST-C173HT as a template, digested with BglII-XbaI, and subcloned into the BglII-XbaI site of pVL1392. To generate recombinant baculoviruses expressing GST-C152HT and GST-HT, cDNA fragments corresponding to GST-C152HT and GST-HT were amplified by PCR and subcloned into pVL1392, respectively. The resultant plasmids were designated pVLGST-C173HT, pVLGST-C152HT, and pVLGST-HT. To generate recombinant baculovirus expressing MEF-tagged E6AP, cDNA fragment encoding MEF-E6AP was subcloned into pVL1392. To express HCV core protein in the TNT-coupled wheat germ lysate system (Promega), HCV core cDNA was inserted in the EcoRI site of pCMVTNT (Promega). The primer sequences used in this study are available from the authors upon request. The sequences of the inserts were extensively verified using an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). Recombinant baculoviruses were recovered using a BaculoGold transfection kit (Pharmingen) according to the manufacturer's instructions.

Antibodies. The mouse monoclonal antibodies (MAbs) used in this study were anti-hemagglutinin (anti-HA) MAb (12CA5; Roche), anti-FLAG (M2) MAb (Sigma), anti-c-*myc* MAb (9E10; Santa Cruz), anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) MAb (Chemicon), anti-GST MAb (Santa Cruz), anti-ubiquitin MAb (Chemicon), anti-E6AP MAb (E6AP-330) (Sigma), anticore MAb (B2; Anogen), and another anti-core MAb (2H9) (56). Polyclonal antibodies (PAbs) used in this study were anti-HA rabbit PAb (Y-11; Santa Cruz), anti-FLAG rabbit PAb (F7425; Sigma), anti-E6AP rabbit PAb (H-182; Santa Cruz), anti-DDX3 rabbit PAb (47), anti-PA28γ rabbit PAb (Affiniti), and anti-GST goat PAb (Amersham). Anticore rabbit PAb (TS1) was raised against the recombinant GST core protein.

MEF purification procedure. 293T cells were transfected with the plasmid expressing MEF core by the calcium phosphate precipitation method (4). After the cells were lysed, the expressed MEF core and its binding proteins were recovered following the procedure described previously (16). 293T cells transfected with pcDNA3-MEF core in four 10-cm dishes were lysed in 2 ml of lysis buffer: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% (wt/vol) glycerol, 100 mM NaF, 1 mM Na_3VO_4, 1% (wt/vol) Triton X-100, 5 μM $ZnCl_2$, 2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 1 μg/ml leupeptin. The lysate was centrifuged at 100,000 × *g* for 20 min at 4°C. The supernatant was passed through a 5-μm filter, incubated with 100 μl of Sepharose beads for 60 min at 4°C, and then passed through a 0.65-μm filter. The filtered supernatant was mixed with 100 μl of anti-myc-conjugated Sepharose beads for the first immunoprecipitation. After incubation for 90 min at 4°C, the beads were washed five times with 1 ml of TNTG buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% [wt/vol] glycerol, and 1% [wt/vol] Triton X-100), twice with 1 ml of buffer A (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1% [wt/vol] Triton X-100), and finally once with 1 ml of TNT buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% [wt/vol] Triton X-100). The washed beads were incubated with 10 U of tobacco etch virus protease (Invitrogen) in TNT buffer (100 μl) to release bound protein complexes from the beads. After incubation for 60 min at room temperature, the supernatant was pooled and the beads were washed twice with 70 μl of buffer A. The resulting supernatants were combined and incubated with 12 μl of FLAG-Sepharose beads for the second immunoprecipitation. After incubation for 60 min at room temperature, the beads were washed three times with 240 μl of buffer A, and proteins bound to the immobilized HCV core protein on the FLAG beads were dissociated by incubation with 80 μg/ml FLAG peptide (NH_2-Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys-COOH) (Sigma).

MS/MS. Proteins were separated by 9% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by silver staining. The stained bands were excised and digested in the gel with lysylendoprotease-C (Lys-C), and the resulting peptide mixtures were analyzed using a direct nanoflow liquid chromatography-tandem mass spectrometry (MS/MS) system (33), equipped with an electrospray interface reversed-phase column, a nanoflow gradient device, a high-resolution Q-time of flight hybrid mass spectrometer (Q-TOF2; Micromass), and an automated data analysis system. All the MS/MS

spectra were searched against the nonredundant protein sequence database maintained at the National Center for Biotechnology Information using the Mascot program (Matrixscience) to identify proteins. The MS/MS signal assignments were also confirmed manually.

Expression and purification of recombinant proteins. *E. coli* BL21(DE3) cells were transformed with plasmids expressing GST fusion protein or His-tagged protein and grown at 37°C. Expression of the fusion protein was induced by 1 mM isopropyl-β-D-thiogalactopyranoside at 37°C for 4 h. Bacteria were harvested, suspended in lysis buffer (phosphate-buffered saline [PBS] containing 1% Triton X-100), and sonicated on ice.

Hi5 cells were infected with recombinant baculoviruses to produce GST-C173HT, GST-C152HT, GST-HT, MEF-E6AP, and His-tagged mouse E1 (17). GST and GST fusion proteins were purified on glutathione-Sepharose beads (Amersham Bioscience) according to the manufacturer's protocols. His-tagged proteins were purified on nickel-nitrilotriacetic acid beads (QIAGEN) according to the manufacturer's protocols. MEF-E6AP and MEF-E6AP C-A were purified on anti-FLAG M2 agarose beads (Sigma) according to the manufacturer's protocols.

Immunoblot analysis. Immunoblot analysis was performed essentially as described previously (11). The membrane was visualized with SuperSignal West Pico chemiluminescent substrate (Pierce).

HCV core protein and E6AP binding assays. To map the E6AP binding site on HCV core protein, 2.5 μg of purified recombinant GST-E6AP expressed in Hi5 cells was mixed with 1,000 μg of 293T cell lysates transfected with a series of FLAG-tagged HCV core deletion mutants as indicated. The protein concentration of the cells was determined using the bicinchoninic acid protein assay kit (Pierce). The mixtures were immunoprecipitated with anti-FLAG M2 agarose beads (Sigma), and proteins bound to the immobilized HCV core protein on anti-FLAG beads were dissociated with FLAG peptide (Sigma). The eluates were analyzed by immunoblotting with anti-GST PAb. To map the HCV core-binding site on E6AP, GST pull-down assays were performed as described previously (51).

In vivo ubiquitylation assay. In vivo ubiquitylation assays were performed essentially as described previously (57). FLAG-core was immunoprecipitated with anti-FLAG beads. Immunoprecipitates were analyzed by immunoblotting, using either anti-HA PAb or anticore PAb (TS1) to detect ubiquitylated core proteins.

In vitro ubiquitylation assay. 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 20 mM Tris-HCl, pH 7.6, 50 mM NaCl, 5 mM ATP, 10 mM $MgCl_2$, 8 μg of bovine ubiquitin (Sigma), 0.1 mM dithiothreitol, 200 ng mouse E1, 200 ng E2 (UbcH7), and 0.5 μg each of MEF-E6AP or MEF-E6AP C-A. The reaction mixtures were incubated at 37°C for 120 min followed by purification with glutathione-Sepharose beads and immunoblotting with the indicated antibodies.

siRNA transfection. 293T cells or Huh-7 cells at 3×10^5 cells in a six-well plate were transfected with 40 pmol of either E6AP-specific short interfering RNA (siRNA; Sigma) or scramble negative-control siRNA duplexes (Sigma) using HiPerFect transfection reagent (QIAGEN) following the manufacturer's instructions. The siRNA target sequences were as follows: E6AP (sense), 5′-GGGUC UACACCAGAUUGCUTT-3′; scramble negative control (sense), 5′-UUGCG GGUCUAAUCACCGATT-3′.

CHX half-life experiments. To examine the half-life of HCV core protein, transfected 293T cells were treated with 50 μg/ml cycloheximide (CHX) at 44 h posttransfection. The cells at zero time points were harvested immediately after treatment with CHX. Cells from subsequent time points were incubated in medium containing CHX at 37°C for 3, 6, and 9 h as indicated.

Infection of Huh-7 cells with secreted HCV. Infectious HCV JFH1 was produced in Huh-7.5.1 cells (61) as described previously (56). Culture supernatant containing infectious HCV JFH1 was collected and passed through a 0.22-μm filter. Naïve Huh-7 cells were seeded 24 h before infection at a density of 1×10^6 in a 10-cm dish. The cells were incubated with 2.5 ml of the inoculum (6.5×10^3 50% tissue culture infectious dose [$TCID_{50}$]/ml) for 3 h, washed three times with PBS, and supplemented with fresh complete Dulbecco's modified Eagle's medium. Then the cells were transfected with 6 μg each of pCAGGS, pCAG-HA-E6AP, or pCAG-HA-E6AP C-A by using TransIT LT1 (Mirus). The cells were trypsinized and replated in six-well plates at 1 day postinfection. The culture medium was changed every 2 days. The culture supernatants and the cells were collected at days 3 and 7 postinfection.

Quantitation of HCV RNA and core protein. We quantitated HCV core protein in cell lysate using the HCV core antigen enzyme-linked immunosorbent assay (ELISA) (Ortho-Clinical Diagnostics). Total RNA was extracted from cells using TRIzol reagent (Invitrogen). To quantitate HCV RNAs, real-time reverse transcription-PCR was performed as described previously (53).

Infectivity assay. The $TCID_{50}$ was calculated essentially based on the method described previously (28). Virus titration was performed by seeding Huh-7 cells in 96-well plates at 1×10^4 cells/well. Samples were serially diluted fivefold in complete growth medium and used to infect the seeded cells (six wells per dilution). Following 3 days of incubation, the cells were immunostained for core with anticore MAb (2H9). Wells that expressed at least one core-expressing cell were counted as positive, and the $TCID_{50}$ was calculated.

Immunocytochemistry and fluorescence microscopy. Cells on collagen-coated coverslips were washed with PBS, fixed with 4% paraformaldehyde for 30 min at 4°C, and permeabilized with PBS containing 0.2% Triton X-100. Cells were preincubated with BlockAce (Dainippon Pharmaceuticals), incubated with specific antibodies as primary antibodies, washed, and incubated with rhodamine-conjugated goat anti-rabbit immunoglobulin G (ICN Pharmaceuticals, Inc.) and Qdot 565-conjugated goat anti-mouse immunoglobulin G (Quantumdot) as secondary antibody. Then the cells were washed with PBS, counterstained with DAPI (4′,6′-diamidino-2-phenylindole) solution (Sigma) for 3 min, mounted on glass slides, and examined with a BZ-8000 microscope (Keyence).

Knockdown of endogenous E6AP in HCV JFH1-infected Huh-7 cells. Naïve Huh-7 cells at 10^6 cells/10-cm dish were inoculated with 2.5 ml of the inoculum including infectious HCV JFH1 (6.5×10^3 $TCID_{50}$/ml) and cultured. The cells were replated in a six-well plate at 3×10^5 cells/well at day 11 postinfection 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. The culture supernatants were collected at day 2 after transfection and assayed for $TCID_{50}$ determinations.

RESULTS

Identification of E6AP as an HCV core-binding protein. To identify the molecular machinery for HCV core ubiquitylation, we searched for endogenous ubiquitin-proteasome pathway proteins that associated with HCV core protein. HCV core-binding proteins (i.e., MEF core and its binding proteins, recovered from lysed cells) were purified by a tandem affinity purification procedure using a tandem tag (known as MEF tag) (16). Ten proteins were reproducibly detected (Fig. 1A, lane 2), but none were recovered from lysed control cells transfected with empty vector alone (Fig. 1A, lane 1).

To identify the proteins, silver-stained bands were excised from the gel, digested by Lys-C, and analyzed using a direct nanoflow liquid chromatography-MS/MS system. Nine proteins were identified: two known HCV core-binding proteins, human DEAD box protein DDX3 (38) and proteasome activator PA28γ (30), and seven potential HCV core-binding proteins. E6AP was identified (Fig. 1A, lane 2) on the basis of five independent MS/MS spectra (Table 1). Immunoblot analyses confirmed the proteomic identification of E6AP, DDX3, PA28γ, and MEF-core (Fig. 1B to E).

E6AP binding domain for HCV core protein. The E6AP binding domain for HCV core protein was investigated. Figure 2A is a schematic representation of E6AP and known motifs in E6AP. A series of deletion mutants of E6AP as GST fusion proteins were expressed in *E. coli*. GST pull-down assays found that the carboxyl-terminal deletion mutant E6AP (1–517), but not E6AP (1–418) (Fig. 2C, lanes C and D), and the amino-terminal deletion mutant E6AP (418–875), but not E6AP (517–875) (Fig. 2C, lanes J and K), were able to bind to the core protein. The signal was absent when unprogrammed wheat germ extracts (the negative control) were used as a source of proteins (data not shown). GST pull-down assays (Fig. 2B) found that the region from aa 418 to aa 517 is important for binding to the HCV core protein. An assay of the

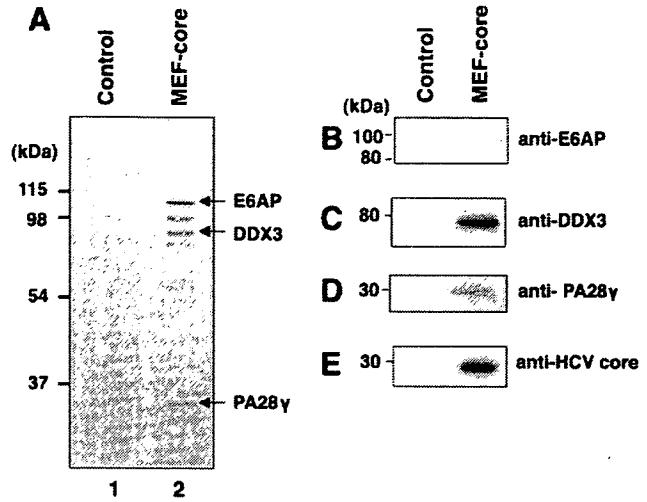

FIG. 1. HCV core protein associates with E6AP in vivo. (A) 293T cells were transfected with pcDNA3-MEF-core or empty plasmid, incubated for 48 h, and then harvested. The expressed MEF-core and binding proteins were recovered using the MEF purification procedure. Proteins bound to the MEF-core immobilized on anti-FLAG beads were dissociated with FLAG peptides, resolved by 9% SDS-PAGE, and visualized by silver staining. Control experiments were performed using 293T cells transfected with vector alone. The positions of E6AP, DDX3, and PA28γ are indicated by arrows. (B to E) The proteins detected in panel A were confirmed by immunoblotting with appropriate antibodies: E6AP (B), DDX3 (C), PA28γ (D), and MEF-core (E).

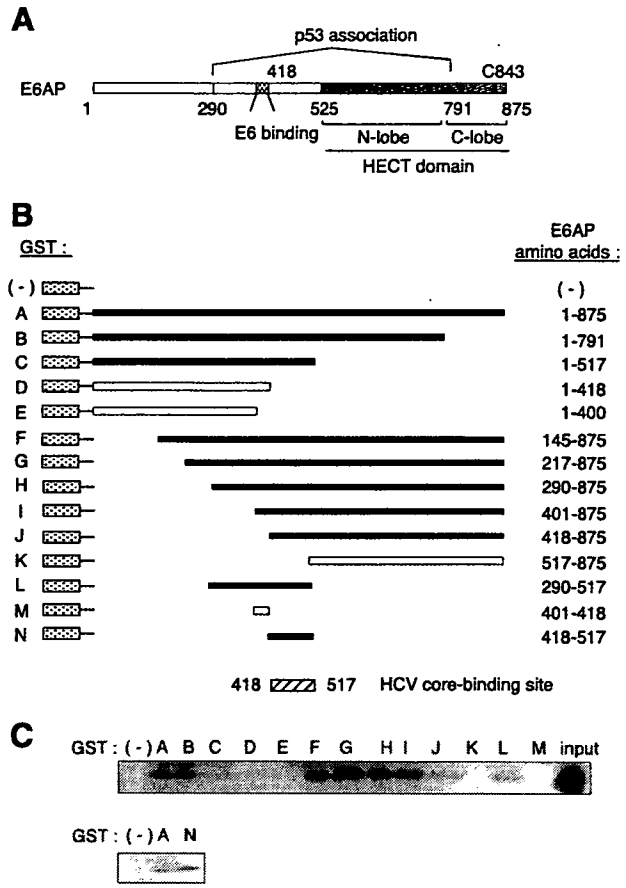

FIG. 2. Mapping of the HCV core-binding domain for E6AP. (A) Structure of E6AP. Shown is a schematic representation of the regions of E6AP isoform II that mediate E6 binding (aa 401 to 418), E6-dependent association with p53 (aa 290 to 791), and the HECT catalytic domain (aa 525 to 875). The catalytic cysteine residue is located at aa 843. (B) Schematic representation of GST-E6AP proteins. GST proteins A through N contain the E6AP amino acids indicated to the right. The shaded region of each represents the GST sequence. Closed boxes represent proteins that are bound specifically to HCV core protein, and open boxes represent those that are not bound. (C) Binding of HCV core protein to GST-E6AP proteins A through N. In vitro-translated core protein (aa 1 to 173) was assayed for association with GST (-) or the GST-E6AP fusion proteins A through N. Association of core protein was detected by immunoblotting with anti-core MAb.

ability of GST-E6AP (418–517) to bind to the HCV core protein was confirmatory (Fig. 2C, lane N) and led to the conclusion that the HCV core-binding domain of E6AP was aa 418 to aa 517.

The HCV core-binding domain for E6AP. By use of a panel of HCV core deletion mutants (Fig. 3A), GST-E6AP was found to coimmunoprecipitate with all of the FLAG-core proteins (Fig. 3A, lanes A to H) except FLAG-core (72–191) or FLAG-core (92–191) (Fig. 3A, lanes I and J). No association of control GST protein with any FLAG-core proteins was observed (data not shown). These data suggest that the aa-58-to-aa-71 segment of the HCV core binds to E6AP. The ability of GST-core (58–71) to associate with purified MEF-E6AP confirmed that the core (aa 58–71) was the site for E6AP binding on the HCV core protein (Fig. 3B).

E6AP decreases steady-state levels of HCV core protein in 293T cells and HepG2 cells. One of the features of HECT domain ubiquitin ligases is direct association with their substrates (50). Thus, we hypothesized that E6AP would function as an E3 ubiquitin ligase for the HCV core protein. We assessed the effects of E6AP on the HCV core protein in 293T cells. FLAG-core (1–191) together with HA-tagged wild-type E6AP, catalytically inactive mutant E6AP, E6AP C-A (19), or WWP1 (another HECT domain ubiquitin ligase) (22) was introduced into 293T cells, and the levels of the core protein were examined by immunoblotting. The steady-state levels of the core protein decreased with an increase in the amount of E6AP plasmids (Fig. 4A and B). However, neither E6AP C-A mutant nor WWP1 decreased the steady-state levels of the core protein, suggesting that E6AP enhances degradation of the core protein.

To verify the critical need for endogenous E6AP in the core degradation, expression of E6AP was knocked down by siRNA and the expression of the core protein and E6AP was assayed by immunoblotting. Transfection of the E6AP-specific siRNA

TABLE 1. Identification of E6AP by tandem mass spectrometry[a]

Peptide *m/z*	Sequence determined	Residues
720.9	VFSSAEALVQSFR	156–168
922.4	AACSAAAMEEDSEASSSR	196–213
774.9	MMETFQQLITYK	339–350
1,053.1	ITVLYSLVQGQQLNPYLR	507–524
809.4	EFVISYSDYILNK	712–724

[a] The protein was ubiquitin protein ligase E3A (E6AP) isoform 2 (GenBank accession no. NP_000453).

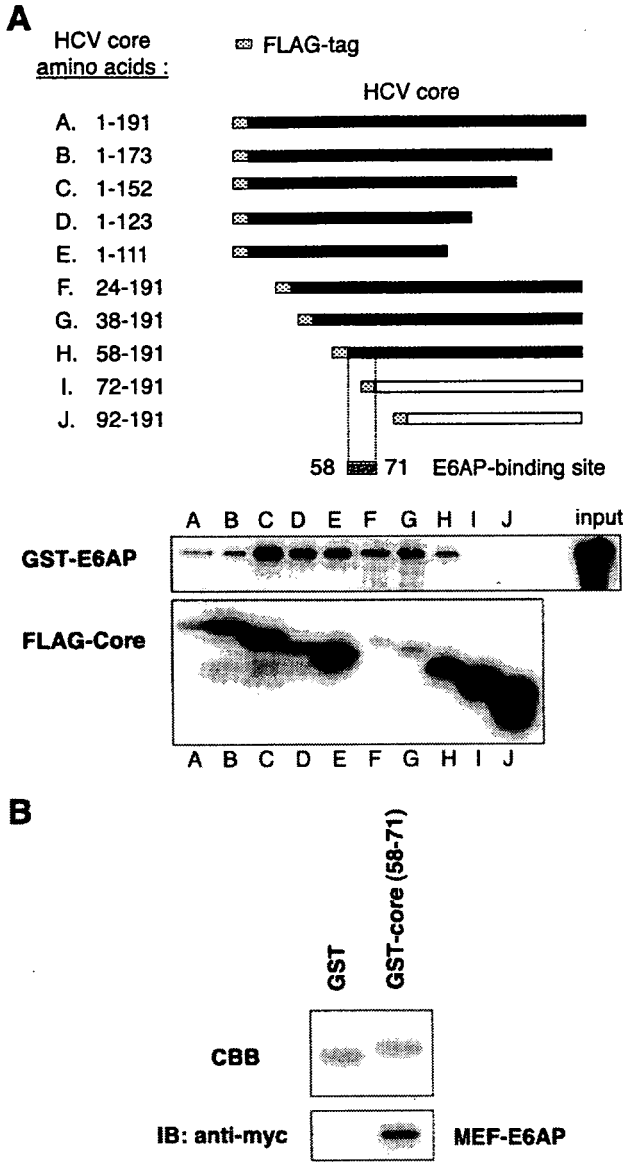

FIG. 3. Mapping of the E6AP binding domain for HCV core protein. (A) In vitro binding of E6AP to HCV core protein. 293T cells were transfected with each plasmid indicated in the upper panel. At 48 h posttransfection, cell lysates were mixed with purified GST-E6AP, immunoprecipitated with anti-FLAG beads, and then immunoblotted with anti-GST PAb (middle panel) or anti-FLAG MAb (bottom panel). The last lane (input) represents GST-E6AP used in this assay (middle panel). (B) Binding of GST-core (aa 58 to aa 71) to purified MEF-E6AP. GST served as a negative control for binding. Upper panel, Coomassie blue-stained SDS-PAGE of GST and GST-core (58–71). Lower panel, results of the GST pull-down assay. MEF-E6AP was detected by anti-myc MAb. CBB, Coomassie brilliant blue; IB, immunoblot.

duplex reduced the protein level of E6AP by 90% at 48 h posttransfection (Fig. 4C, middle panel). Immunoblotting revealed a 4.1-fold increase in the level of the core protein in the cells transfected with E6AP siRNA (Fig. 4C, top panel), suggesting that endogenous E6AP plays a role in the proteolysis of the HCV core protein.

Then we examined whether E6AP reduces the steady-state

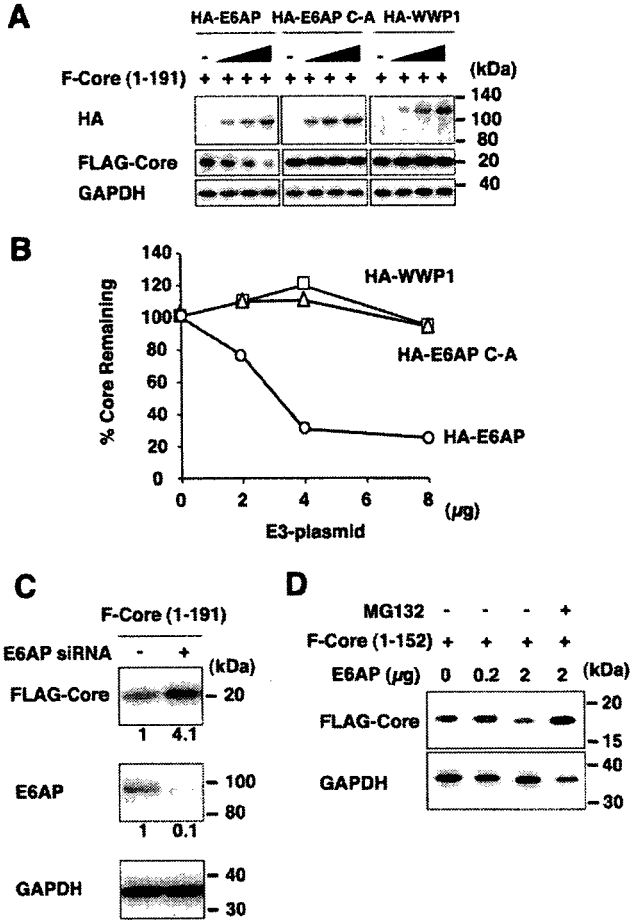

FIG. 4. E6AP decreases steady-state levels of HCV core protein in 293T cells and in HepG2 cells. (A) 293T cells (1×10^6 cells/10-cm dish) were transfected with 1 μg of pCAG FLAG-core (1–191) along with either pCAG-HA-E6AP, pCAG-HA-E6AP C-A, or pCAG-HA-WWP1 as indicated. At 48 h posttransfection, protein extracts were separated by SDS-PAGE and analyzed by immunoblotting with anti-HA PAb (top panel), anti-FLAG MAb (middle panel), and anti-GAPDH MAb (bottom panel). (B) Quantitation of data shown in panel A. Intensities of the gel bands were quantitated using the NIH Image 1.62 program. The level of GAPDH served as a loading control. Circles, E6AP; triangles, E6AP C-A; squares, WWP1. (C) Knockdown of endogenous E6AP by siRNA inhibits degradation of HCV core protein in 293T cells. 293T cells (3×10^5 cells/six-well plate) were transfected with 40 pmol of E6AP-specific duplex siRNA (or control siRNA) as described in Materials and Methods. The cells were transfected with 2 μg of FLAG-core (1–191) expression plasmid and cultured for 24 h, harvested, and analyzed by immunoblotting. Shown is immunoblot detection of FLAG-tagged core protein (top panel), E6AP protein (middle panel), and GAPDH (bottom panel) in control siRNA-treated 293T cells or E6AP-siRNA-treated 293T cells. The relative levels of protein expression were quantitated by densitometry and indicated below in the respective lanes. GAPDH served as a loading control. (D) HepG2 cells (2×10^5 cells/six-well plate) were transfected with pCAG FLAG-core (1–152) along with either empty vector or pCMV E6AP as indicated. The cells were harvested at 44 h posttransfection. Where indicated, cells were treated with 25 μM MG132 or with dimethyl sulfoxide control 14 h prior to collection. Equivalent amounts of the whole-cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-FLAG MAb (upper panel) or anti-GAPDH MAb (lower panel).

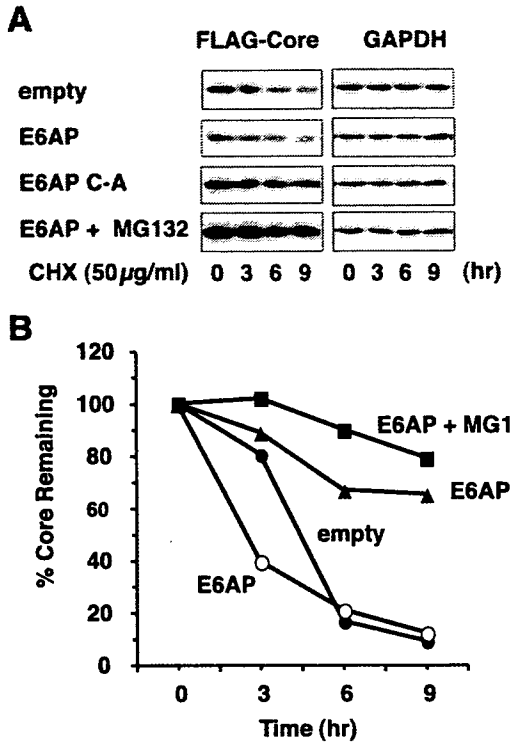

FIG. 5. Kinetic analysis of E6AP-dependent degradation of HCV core protein. (A) 293T cells (1×10^6 cells/10-cm dish) were transfected with 1 μg of pCAG-FLAG core (1–152) plus 4 μg of empty vector, pCMV-HA-E6AP, or pCMV-HA-E6AP C-A. The cells were treated with 50 μg/ml CHX at 44 h after transfection. Cell extracts were collected at 0, 3, 6, and 9 h after treatment with CHX, followed by immunoblotting. (B) Specific signals were quantitated by densitometry, and the percent remaining core at each time was compared with that at the starting point. The level of GAPDH served as a loading control. Open circles, E6AP; closed circles, empty plasmid; closed triangles, E6AP C-A; closed squares, E6AP with MG132 treatment. Data are representative of three independent experimental determinations.

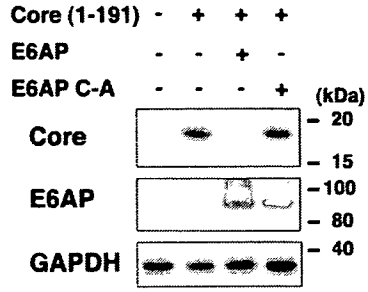

FIG. 6. E6AP promotes degradation of full-length HCV core protein in Huh-7 cells. Huh-7 cells (2×10^5 cells/six-well plate) were transfected with 0.5 μg of pCAG-core (1–191) together with 2 μg of pCMV-HA-E6AP or pCMV-HA-E6AP C-A. At 48 h posttransfection, cells were harvested and analyzed by immunoblotting with anticore MAb (top panel), anti-E6AP PAb (middle panel), or anti-GAPDH MAb (bottom panel).

levels of the core protein in hepatic cells as well as in 293T cells. Exogenous expression of E6AP resulted in reduction of the core protein in human hepatoblastoma HepG2 cells (Fig. 4D). Treatment of the cells with the proteasome inhibitor MG132 increased the core protein level, suggesting that the core protein was degraded through the ubiquitin-proteasome pathway. These results indicate that E6AP enhances proteasomal degradation of the HCV core protein in both hepatic cells and nonhepatic cells.

Kinetic analysis of E6AP-dependent degradation of HCV core protein. To determine whether the E6AP-induced reduction of the core protein is due to an increase in the rate of core degradation, we performed kinetic analysis using the protein synthesis inhibitor CHX. HCV core protein together with wild-type E6AP or inactive mutant E6AP C-A was expressed in 293T cells. At 44 h after transfection, cells were treated with either 50 μg/ml CHX alone or 50 μg/ml CHX plus 25 μM MG132 to inhibit proteasome function. Cells were collected at 0, 3, 6, and 9 h following treatment and analyzed by immunoblotting (Fig. 5A). Overexpression of E6AP resulted in rapid degradation of the core protein, whereas inactive mutant E6AP C-A increased the half-life of the core protein (Fig. 5B), suggesting that the inactive E6AP inhibited degradation of the core protein in a dominant-negative manner, which is in agreement with previous studies (19, 55). Treatment of the cells with MG132 inhibited the degradation of the core protein (Fig. 5B). Reverse transcription-PCR to determine mRNA levels of the HCV core gene and GAPDH gene found that neither wild-type E6AP nor inactive E6AP changed mRNA levels of the HCV core gene and GAPDH gene (data not shown). These results indicate that E6AP enhances proteasomal degradation of the core protein.

E6AP promotes degradation of the full-length core protein in Huh-7 cells. To determine whether the full-length HCV core protein expressed in hepatic cells is degraded through an E6AP-dependent pathway, human hepatoma Huh-7 cells were transfected with pCAG HCV core (1–191) along with either E6AP or E6AP C-A. To rule out the effects of N-terminal FLAG tag on the core degradation, HCV core protein was expressed as untagged protein. Expression of wild-type E6AP resulted in reduction of the core protein (Fig. 6). On the other hand, HCV core protein was not decreased after transfection of inactive E6AP, indicating that the full-length core protein expressed in Huh-7 cells is also degraded through an E6AP-dependent pathway.

E6AP mediates ubiquitylation of HCV core protein in vivo. To determine whether E6AP can induce ubiquitylation of HCV core protein in cells, we performed in vivo ubiquitylation assays. 293T cells were cotransfected with FLAG-core (1–191) and either E6AP or empty plasmid, together with a plasmid encoding HA-tagged ubiquitin to facilitate detection of ubiquitylated core protein. Cell lysates were immunoprecipitated with anti-FLAG MAb and immunoblotted with anti-HA PAb to detect ubiquitylated core protein (Fig. 7A). Only a little ubiquitin signal was observed on the core protein in the absence of cotransfected E6AP (Fig. 7A, lane 3). In contrast, coexpression of E6AP led to readily detectable ubiquitylated forms of the core protein as a ladder and a smear of higher-molecular-weight bands (Fig. 7A, compare lane 3 with lane 4). Immunoblot analysis with anticore PAb confirmed that FLAG-core proteins were immunoprecipitated (Fig. 7B, lanes 2 to 4, short exposure) and that higher-molecular-weight bands con-

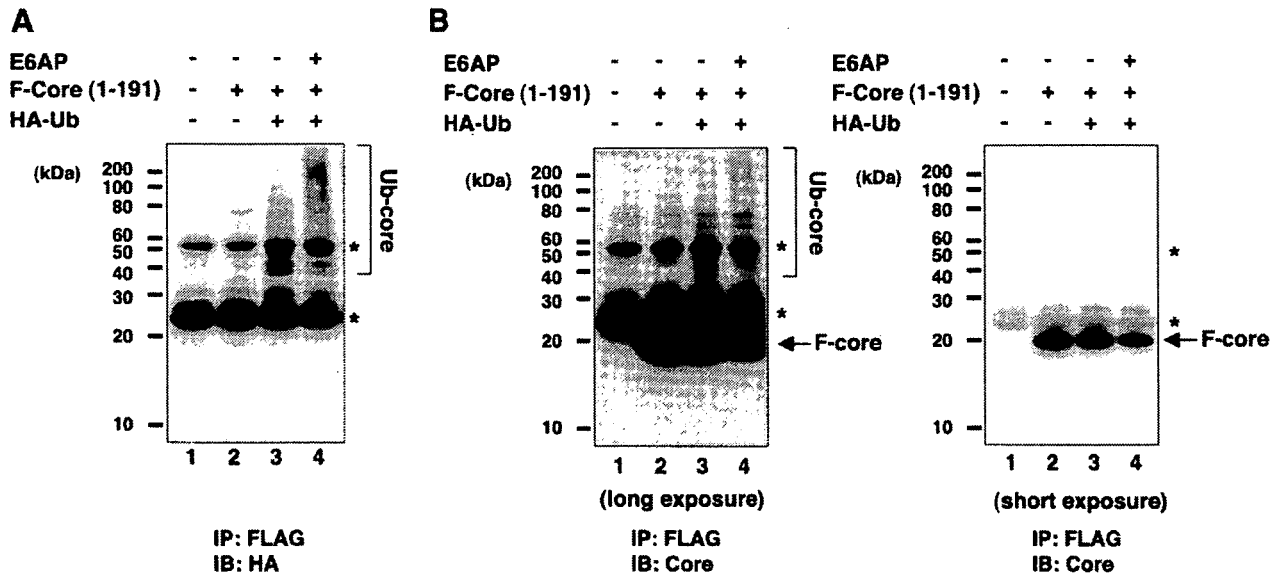

FIG. 7. E6AP-dependent ubiquitylation of HCV core protein in vivo. 293T cells (1×10^6 cells/10-cm dish) were transfected with 1 μg of pCAG FLAG-core (1–191) together with 2 μg of plasmid encoding E6AP as indicated. Each transfection also included 2 μg of plasmid encoding HA-ubiquitin. The cell lysates 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

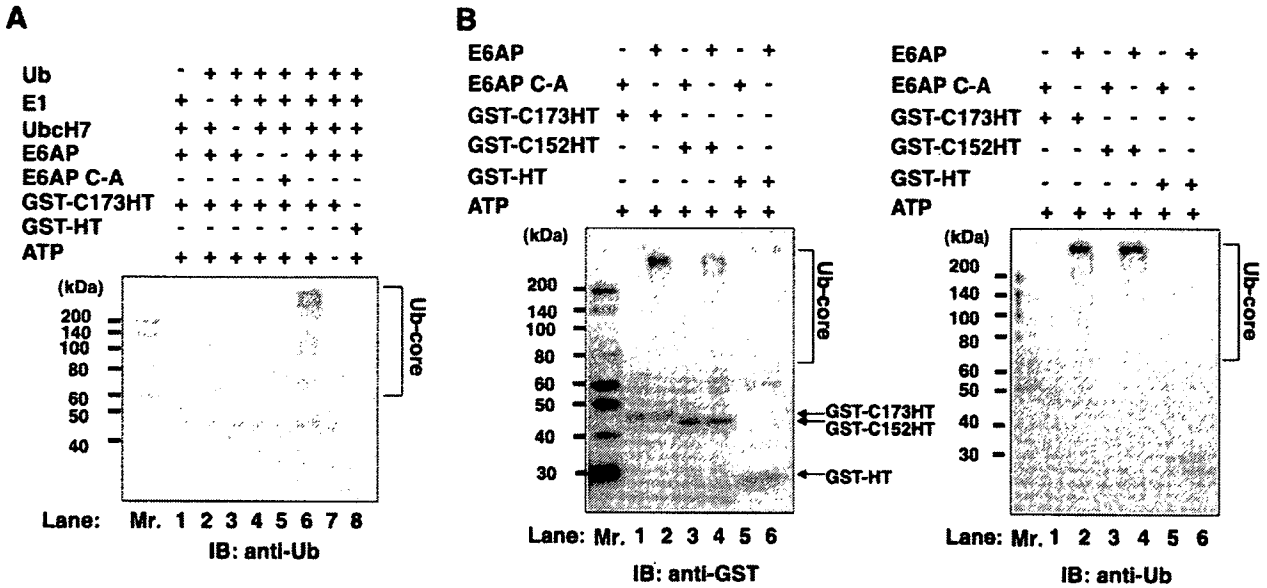

FIG. 8. In vitro ubiquitylation of HCV core protein by recombinant E6AP. For in vitro ubiquitylation of HCV core protein, purified GST-C173HT and GST-C152HT were used as substrates. Purified GST-HT was used as a negative control. Assays were done in 40-μl volumes containing each component as indicated. 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

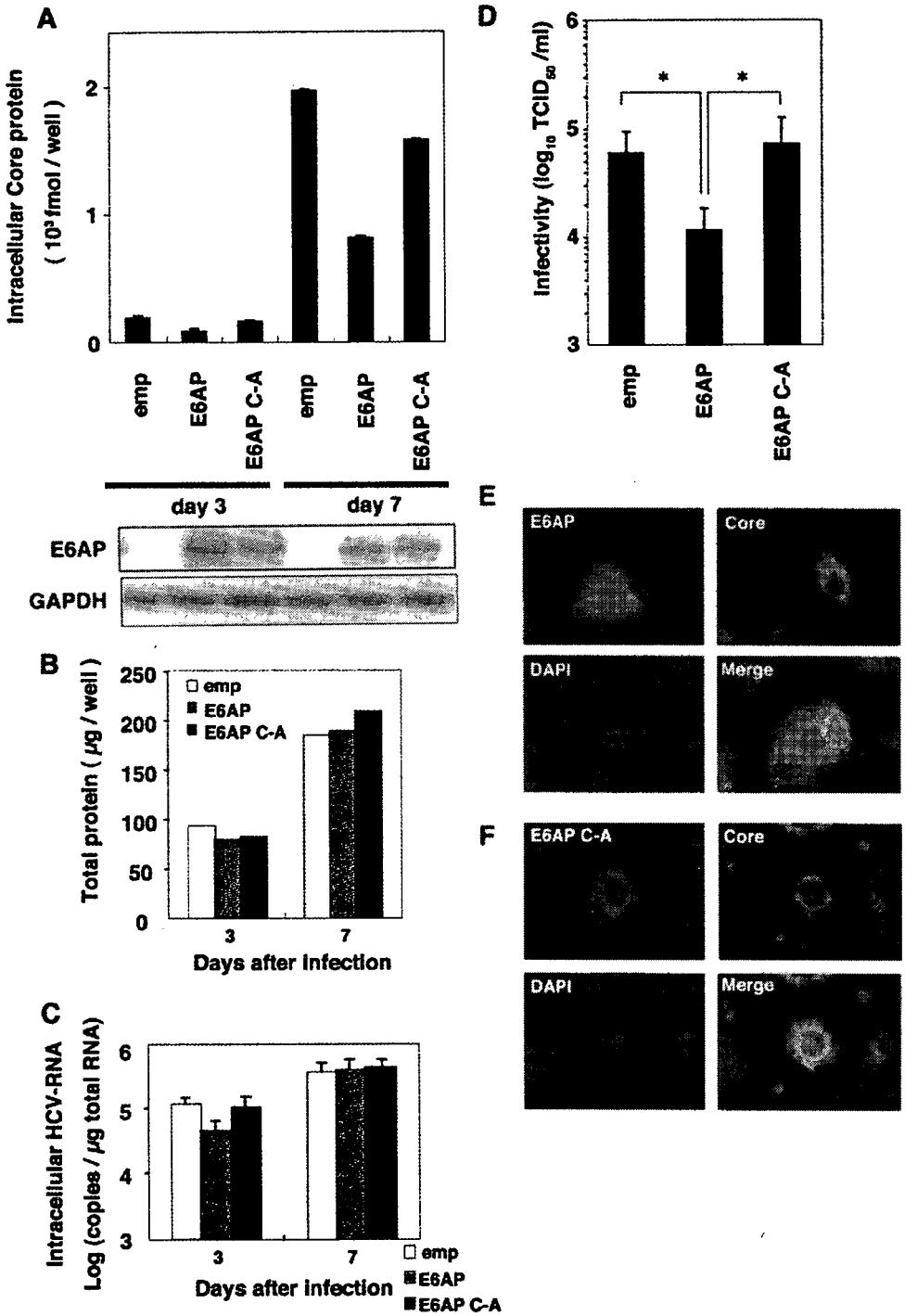

FIG. 9. Exogenous expression of E6AP reduces intracellular HCV core protein levels and supernatant infectivity titers in HCV-infected Huh-7 cells. Naïve Huh-7 cells were seeded as described in Materials and Methods; inoculated with 2.5 ml of the inoculum including infectious HCV JFH1 (6.5×10^3 $TCID_{50}$/ml); 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.

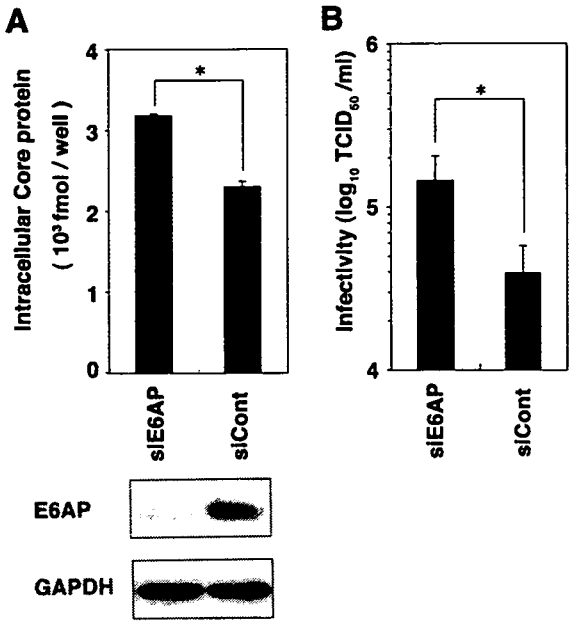

FIG. 10. E6AP silencing leads to an increase in the level of intracellular HCV core protein and supernatant infectivity titer in HCV-infected Huh-7 cells. (A) HCV JFH1-infected cells were replated in a six-well plate at 3×10^5 cells/well and transfected with 40 pmol of E6AP siRNA or control siRNA. The 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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REFERENCES

1. **Aizaki, H., Y. Aoki, T. Harada, K. Ishii, T. Suzuki, S. Nagamori, G. Toda, Y. Matsuura, and T. Miyamura.** 1998. Full-length complementary DNA of hepatitis C virus genome from an infectious blood sample. Hepatology **27:** 621–627.
2. **Asher, G., P. Tsvetkov, C. Kahana, and Y. Shaul.** 2005. A mechanism of ubiquitin-independent proteasomal degradation of the tumor suppressors p53 and p73. Genes Dev. **19:**316–321.
3. **Bukh, J., R. H. Purcell, and R. H. Miller.** 1994. Sequence analysis of the core gene of 14 hepatitis C virus genotypes. Proc. Natl. Acad. Sci. USA **91:**8239–8243.

4. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. 7:2745–2752.
5. **Choo, Q. L., G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradley, and M. Houghton.** 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244:359–362.
6. **Choo, Q. L., K. H. Richman, J. H. Han, K. Berger, C. Lee, C. Dong, C. Gallegos, D. Coit, R. Medina-Selby, P. J. Barr, et al.** 1991. Genetic organization and diversity of the hepatitis C virus. Proc. Natl. Acad. Sci. USA 88:2451–2455.
7. **Franck, N., J. Le Seyec, C. Guguen-Guillouzo, and L. Erdtmann.** 2005. Hepatitis C virus NS2 protein is phosphorylated by the protein kinase CK2 and targeted for degradation to the proteasome. J. Virol. 79:2700–2708.
8. **Gao, L., H. Tu, S. T. Shi, K. J. Lee, M. Asanaka, S. B. Hwang, and M. M. Lai.** 2003. Interaction with a ubiquitin-like protein enhances the ubiquitination and degradation of hepatitis C virus RNA-dependent RNA polymerase. J. Virol. 77:4149–4159.
9. **Giannini, C., and C. Brechot.** 2003. Hepatitis C virus biology. Cell Death Differ. 10(Suppl. 1):S27–S38.
10. **Grakoui, A., D. W. McCourt, C. Wychowski, S. M. Feinstone, and C. M. Rice.** 1993. Characterization of the hepatitis C virus-encoded serine proteinase: determination of proteinase-dependent polyprotein cleavage sites. J. Virol. 67:2832–2843.
11. **Harris, K. F., I. Shoji, E. M. Cooper, S. Kumar, H. Oda, and P. M. Howley.** 1999. Ubiquitin-mediated degradation of active Src tyrosine kinase. Proc. Natl. Acad. Sci. USA 96:13738–13743.
12. **Hijikata, M., H. Mizushima, T. Akagi, S. Mori, N. Kakiuchi, N. Kato, T. Tanaka, K. Kimura, and K. Shimotohno.** 1993. Two distinct proteinase activities required for the processing of a putative nonstructural precursor protein of hepatitis C virus. J. Virol. 67:4665–4675.
13. **Huibregtse, J. M., M. Scheffner, S. Beaudenon, and P. M. Howley.** 1995. A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. Proc. Natl. Acad. Sci. USA 92:2563–2567.
14. **Huibregtse, J. M., M. Scheffner, and P. M. Howley.** 1993. Cloning and expression of the cDNA for E6-AP, a protein that mediates the interaction of the human papillomavirus E6 oncoprotein with p53. Mol. Cell. Biol. 13:775–784.
15. **Hussy, P., H. Langen, J. Mous, and H. Jacobsen.** 1996. Hepatitis C virus core protein: carboxy-terminal boundaries of two processed species suggest cleavage by a signal peptide peptidase. Virology 224:93–104.
16. **Ichimura, T., H. Yamamura, K. Sasamoto, Y. Tominaga, M. Taoka, K. Kakiuchi, T. Shinkawa, N. Takahashi, S. Shimada, and T. Isobe.** 2005. 14-3-3 proteins modulate the expression of epithelial Na^+ channels by phosphorylation-dependent interaction with Nedd4-2 ubiquitin ligase. J. Biol. Chem. 280:13187–13194.
17. **Iwai, K., K. Yamanaka, T. Kamura, N. Minato, R. C. Conaway, J. W. Conaway, R. D. Klausner, and A. Pause.** 1999. Identification of the von Hippel-Lindau tumor-suppressor protein as part of an active E3 ubiquitin ligase complex. Proc. Natl. Acad. Sci. USA 96:12436–12441.
18. **Ju, D., and Y. Xie.** 2004. Proteasomal degradation of RPN4 via two distinct mechanisms, ubiquitin-dependent and -independent. J. Biol. Chem. 279: 23851–23854.
19. **Kao, W. H., S. L. Beaudenon, A. L. Talis, J. M. Huibregtse, and P. M. Howley.** 2000. Human papillomavirus type 16 E6 induces self-ubiquitination of the E6AP ubiquitin-protein ligase. J. Virol. 74:6408–6417.
20. **Kato, T., M. Miyamoto, A. Furusaka, T. Date, K. Yasui, J. Kato, S. Matsushima, T. Komatsu, and T. Wakita.** 2003. Processing of hepatitis C virus core protein is regulated by its C-terminal sequence. J. Med. Virol. 69:357–366.
21. **Kishino, T., M. Lalande, and J. Wagstaff.** 1997. UBE3A/E6-AP mutations cause Angelman syndrome. Nat. Genet. 15:70–73.
22. **Komuro, A., T. Imamura, M. Saitoh, Y. Yoshida, T. Yamori, K. Miyazono, and K. Miyazawa.** 2004. Negative regulation of transforming growth factor-beta (TGF-beta) signaling by WW domain-containing protein 1 (WWP1). Oncogene 23:6914–6923.
23. **Kuhne, C., and L. Banks.** 1998. E3-ubiquitin ligase/E6-AP links multicopy maintenance protein 7 to the ubiquitination pathway by a novel motif, the L2G box. J. Biol. Chem. 273:34302–34309.
24. **Kumar, S., A. L. Talis, and P. M. Howley.** 1999. Identification of HHR23A as a substrate for E6-associated protein-mediated ubiquitination. J. Biol. Chem. 274:18785–18792.
25. **Kunkel, M., M. Lorinczi, R. Rijnbrand, S. M. Lemon, and S. J. Watowich.** 2001. Self-assembly of nucleocapsid-like particles from recombinant hepatitis C virus core protein. J. Virol. 75:2119–2129.
26. **Kuo, G., Q. L. Choo, H. J. Alter, G. L. Gitnick, A. G. Redeker, R. H. Purcell, T. Miyamura, J. L. Dienstag, M. J. Alter, C. E. Stevens, et al.** 1989. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science 244:362–364.
27. **Li, X., D. M. Lonard, S. Y. Jung, A. Malovannaya, Q. Feng, J. Qin, S. Y. Tsai, M. J. Tsai, and B. W. O'Malley.** 2006. The SRC-3/AIB1 coactivator is degraded in a ubiquitin- and ATP-independent manner by the REGγ proteasome. Cell 124:381–392.
28. **Lindenbach, B. D., M. J. Evans, A. J. Syder, B. Wolk, T. L. Tellinghuisen, C. C. Liu, T. Maruyama, R. O. Hynes, D. R. Burton, J. A. McKeating, and C. M. Rice.** 2005. Complete replication of hepatitis C virus in cell culture. Science 309:623–626.
29. **McLauchlan, J., M. K. Lemberg, G. Hope, and B. Martoglio.** 2002. Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. EMBO J. 21:3980–3988.
30. **Moriishi, K., T. Okabayashi, K. Nakai, K. Moriya, K. Koike, S. Murata, T. Chiba, K. Tanaka, R. Suzuki, T. Suzuki, T. Miyamura, and Y. Matsuura.** 2003. Proteasome activator PA28γ-dependent nuclear retention and degradation of hepatitis C virus core protein. J. Virol. 77:10237–10249.
31. **Moriya, K., H. Fujie, Y. Shintani, H. Yotsuyanagi, T. Tsutsumi, K. Ishibashi, Y. Matsuura, S. Kimura, T. Miyamura, and K. Koike.** 1998. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat. Med. 4:1065–1067.
32. **Moriya, K., H. Yotsuyanagi, Y. Shintani, H. Fujie, K. Ishibashi, Y. Matsuura, T. Miyamura, and K. Koike.** 1997. Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. J. Gen. Virol. 78:1527–1531.
33. **Natsume, T., Y. Yamauchi, H. Nakayama, T. Shinkawa, M. Yanagida, N. Takahashi, and T. Isobe.** 2002. A direct nanoflow liquid chromatography-tandem mass spectrometry system for interaction proteomics. Anal. Chem. 74:4725–4733.
34. **Niwa, H., K. Yamamura, and J. Miyazaki.** 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 108:193–199.
35. **Oda, H., S. Kumar, and P. M. Howley.** 1999. Regulation of the Src family tyrosine kinase Blk through E6AP-mediated ubiquitination. Proc. Natl. Acad. Sci. USA 96:9557–9562.
36. **Ogino, T., H. Fukuda, S. Imajoh-Ohmi, M. Kohara, and A. Nomoto.** 2004. Membrane binding properties and terminal residues of the mature hepatitis C virus capsid protein in insect cells. J. Virol. 78:11766–11777.
37. **Okamoto, K., K. Moriishi, T. Miyamura, and Y. Matsuura.** 2004. Intramembrane proteolysis and endoplasmic reticulum retention of hepatitis C virus core protein. J. Virol. 78:6370–6380.
38. **Owsianka, A. M., and A. H. Patel.** 1999. Hepatitis C virus core protein interacts with a human DEAD box protein DDX3. Virology 257:330–340.
39. **Pavio, N., D. R. Taylor, and M. M. Lai.** 2002. Detection of a novel unglycosylated form of hepatitis C virus E2 envelope protein that is located in the cytosol and interacts with PKR. J. Virol. 76:1265–1272.
40. **Polyak, S. J., K. C. Klein, I. Shoji, T. Miyamura, and J. R. Lingappa.** 2006. Assemble and interact: pleiotropic functions of the HCV core protein, p. 89–119. *In* S.-L. Tan (ed.), Hepatitis C viruses: genomes and molecular biology. Horizon Bioscience, Norwich, United Kingdom.
41. **Poynard, T., M. F. Yuen, V. Ratziu, and C. L. Lai.** 2003. Viral hepatitis C. Lancet 362:2095–2100.
42. **Ravaggi, A., G. Natoli, D. Primi, A. Albertini, M. Levrero, and E. Cariani.** 1994. Intracellular localization of full-length and truncated hepatitis C virus core protein expressed in mammalian cells. J. Hepatol. 20:833–836.
43. **Ray, R. B., L. M. Lagging, K. Meyer, and R. Ray.** 1996. Hepatitis C virus core protein cooperates with *ras* and transforms primary rat embryo fibroblasts to tumorigenic phenotype. J. Virol. 70:4438–4443.
44. **Rouille, Y., F. Helle, D. Delgrange, P. Roingeard, C. Voisset, E. Blanchard, S. Belouzard, J. McKeating, A. H. Patel, G. Maertens, T. Wakita, C. Wychowski, and J. Dubuisson.** 2006. Subcellular localization of hepatitis C virus structural proteins in a cell culture system that efficiently replicates the virus. J. Virol. 80:2832–2841.
45. **Saito, I., T. Miyamura, A. Ohbayashi, H. Harada, T. Katayama, S. Kikuchi, Y. Watanabe, S. Koi, M. Onji, Y. Ohta, et al.** 1990. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proc. Natl. Acad. Sci. USA 87:6547–6549.
46. **Santolini, E., G. Migliaccio, and N. La Monica.** 1994. Biosynthesis and biochemical properties of the hepatitis C virus core protein. J. Virol. 68: 3631–3641.
47. **Sato, S., M. Fukasawa, Y. Yamakawa, T. Natsume, T. Suzuki, I. Shoji, H. Aizaki, T. Miyamura, and M. Nishijima.** 2006. Proteomic profiling of lipid droplet proteins in hepatoma cell lines expressing hepatitis C virus core protein. J. Biochem. (Tokyo) 139:921–930.
48. **Scheffner, M., J. M. Huibregtse, and P. M. Howley.** 1994. Identification of a human ubiquitin-conjugating enzyme that mediates the E6-AP-dependent ubiquitination of p53. Proc. Natl. Acad. Sci. USA 91:8797–8801.
49. **Scheffner, M., J. M. Huibregtse, R. D. Vierstra, and P. M. Howley.** 1993. The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell 75:495–505.
50. **Scheffner, M., U. Nuber, and J. M. Huibregtse.** 1995. Protein ubiquitination involving an E1–E2–E3 enzyme ubiquitin thioester cascade. Nature 373:81–83.
51. **Suzuki, R., S. Sakamoto, T. Tsutsumi, A. Rikimaru, K. Tanaka, T. Shimoike, K. Moriishi, T. Iwasaki, K. Mizumoto, Y. Matsuura, T. Miyamura, and T. Suzuki.** 2005. Molecular determinants for subcellular localization of hepatitis C virus core protein. J. Virol. 79:1271–1281.
52. **Suzuki, R., K. Tamura, J. Li, K. Ishii, Y. Matsuura, T. Miyamura, and T. Suzuki.** 2001. Ubiquitin-mediated degradation of hepatitis C virus core pro-