[63] R.A. Love, H.E. Parge, J.A. Wickersham, Z. Hostomsky, N. Habuka, E.W. Moomaw, T. Adachi, Z. Hostomska, The crystal structure of hepatitis C virus NS3 proteinase reveals a trypsin-like fold and a structural zinc binding site, Cell 87 (1996) 331–342.
[64] Y. Yan, Y. Li, S. Munshi, V. Sardana, J.L. Cole, M. Sardana, C. Steinkuehler, L. Tomei, R. De Francesco, L.C. Kuo, Z. Chen, Complex of NS3 protease and NS4A peptide of BK strain hepatitis C virus: a 2.2 A resolution structure in a hexagonal crystal form, Protein Sci. 7 (4) (1998) 837–847.
[65] D. Kwong, J.L. Kim, C. Lin, Structure and function of hepatitis C virus NS3 helicase, Curr. Top. Microbiol. Immunol. 242 (2000) 171–196.
[66] L. Kim, K.A. Morgenstern, J.P. Griffith, M.D. Dwyer, J.A. Thomson, M.A. Murcko, C. Lin, P.R. Caron, Hepatitis C virus NS3 RNA helicase domain with a bound oligonucleotide: the crystal structure provides insights into the mode of unwinding, Structure 6 (1) (1998) 89–100.
[67] V. Serebrov, A.M. Pyle, Periodic cycles of RNA unwinding and pausing by hepatitis C virus NS3 helicase, Nature 430 (6998) (2004) 476–480.
[68] M.K. Levin, M. Gurjar, S.S. Patel, A Brownian motor mechanism of translocation and strand separation by hepatitis C virus helicase, Nat. Struct. Mol. Biol. 12 (5) (2005) 429–435.
[69] S. Dumont, W. Cheng, V. Serebrov, R.K. Beran, I. Tinoco Jr., A.M. Pyle, C. Bustamante, RNA translocation and unwinding mechanism of HCV NS3 helicase and its coordination by ATP, Nature 439 (7072) (2006) 105–108.
[70] D.N. Frick, R.S. Rypma, A.M. Lam, B. Gu, The nonstructural protein 3 protease/helicase requires an intact protease domain to unwind duplex RNA efficiently, J. Biol. Chem. 279 (2) (2004) 1269–1280.
[71] M. Lundin, M. Monne, A. Widell, G. Von Heijne, M.A. Persson, Topology of the membrane-associated hepatitis C virus protein NS4B, J. Virol. 77 (9) (2003) 5428–5438.
[72] D. Egger, B. Wolk, R. Gosert, L. Bianchi, H.E. Blum, D. Moradpour, K. Bienz, Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex, J. Virol. 76 (12) (2002) 5974–5984.
[73] L. Gao, H. Aizaki, J.W. He, M.M. Lai, 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. 78 (7) (2004) 3480–3488.
[74] T.L. Tellinghuisen, J. Marcotrigiano, A.E. Gorbalenya, C.M. Rice, The NS5A protein of hepatitis C virus is a zinc metalloprotein, J. Biol. Chem. 279 (47) (2004) 48576–48587.
[75] T.L. Tellinghuisen, J. Marcotrigiano, C.M. Rice, Structure of the zinc-binding domain of an essential component of the hepatitis C virus replicase, Nature 435 (7040) (2005) 374–379.
[76] K.J. Blight, A.A. Kolykhalov, C.M. Rice, Efficient initiation of HCV RNA replication in cell culture, Science 290 (2000) 1972–1974.
[77] N. Krieger, V. Lohmann, R. Bartenschlager, Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations, J. Virol. 75 (10) (2001) 4614–4624.
[78] V. Lohmann, F. Korner, A. Dobierzewska, R. Bartenschlager, Mutations in hepatitis C virus RNAs conferring cell culture adaptation, J. Virol. 75 (3) (2001) 1437–1449.
[79] T. Shimakami, M. Hijikata, H. Luo, Y.Y. Ma, S. Kaneko, K. Shimotohno, S. Murakami, Effect of interaction between hepatitis C virus NS5A and NS5B on hepatitis C virus RNA replication with the hepatitis C virus replicon, J. Virol. 78 (6) (2004) 2738–2748.
[80] Y. Shirota, H. Luo, W. Qin, S. Kaneko, T. Yamashita, K. Kobayashi, S. Murakami, Hepatitis C virus (HCV) NS5A binds RNA-dependent RNA polymerase (RdRP) NS5B and modulates RNA-dependent RNA polymerase activity, J. Biol. Chem. 277 (13) (2002) 11149–11155.
[81] M. Dimitrova, I. Imbert, M.P. Kieny, C. Schuster, Protein–protein interactions between hepatitis C virus nonstructural proteins, J. Virol. 77 (9) (2003) 5401–5414.
[82] D. Moradpour, V. Brass, E. Bieck, P. Friebe, R. Gosert, H.E. Blum, R. Bartenschlager, F. Penin, V. Lohmann, Membrane association of the RNA-dependent RNA polymerase is essential for hepatitis C virus RNA replication, J. Virol. 78 (23) (2004) 13278–13284.
[83] J. Schmidt-Mende, E. Bieck, T. Hugle, F. Penin, C.M. Rice, H.E. Blum, D. Moradpour, Determinants for membrane association of the hepatitis C virus RNA-dependent RNA polymerase, J. Biol. Chem. 276 (47) (2001) 44052–44063.
[84] H. Ago, T. Adachi, A. Yoshida, M. Yamamoto, N. Habuka, K. Yatsunami, M. Miyano, Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus, Struct. Fold. Des. 7 (11) (1999) 1417–1426.
[85] A. Lesburg, M.B. Cable, E. Ferrari, Z. Hong, A.F. Mannarino, P.C. Weber, Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encircled active site, Nat. Struct. Mol. Biol. 6 (10) (1999) 937–943.
[86] S. Bressanelli, L. Tomei, F.A. Rey, R. De Francesco, Structural analysis of the hepatitis C virus RNA polymerase in complex with ribonucleotides, J. Virol. 76 (7) (2002) 3482–3492.
[87] S.J. Butcher, J.M. Grimes, E.V. Makeyev, D.H. Bamford, D.I. Stuart, A mechanism for initiating RNA-dependent RNA polymerization, Nature 410 (6825) (2001) 235–240.
[88] T.F. Baumert, S. Ito, D.T. Wong, T.J. Liang, Hepatitis C virus structural proteins assemble into viruslike particles in insect cells, J. Virol. 72 (5) (1998) 3827–3836.
[89] E. Matsuo, H. Tani, C. Lim, Y. Komoda, T. Okamoto, H. Miyamoto, K. Moriishi, S. Yagi, A.H. Patel, T. Miyamura, Y. Matsuura, Characterization of HCV-like particles produced in a human hepatoma cell line by a recombinant baculovirus, Biochem. Biophys. Res. Commun. 340 (1) (2006) 200–208.
[90] L.M. Lagging, K.M. Randall, J. Owens, R. Ray, Functional Role of Hepatitis C Virus Chimeric Glycoproteins in the Infectivity of Pseudotyped Virus, J. Virol. 72 (1998) 3539–3546.
[91] Y. Matsuura, H. Tani, K. Suzuki, T. Kimura-Someya, R. Suzuki, H. Aizaki, K. Ishii, K. Moriishi, C.S. Robison, M.A. Whitt, T. Miyamura, Characterization of pseudotype VSV possessing HCV envelope proteins, Virology 286 (2) (2001) 263–275.
[92] B. Bartosch, J. Bukh, J.C. Meunier, C. Granier, R.E. Engle, W.C. Blackwelder, S.U. Emerson, F.L. Cosset, R.H. Purcell, In vitro assay for neutralizing antibody to hepatitis C virus: evidence for broadly conserved neutralization epitopes, Proc. Natl. Acad. Sci. U. S. A. 100 (24) (2003) 14199–14204.
[93] M. Hsu, J. Zhang, M. Flint, C. Logvinoff, C. Cheng-Mayer, C.M. Rice, J.A. McKeating, Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles, Proc. Natl. Acad. Sci. U. S. A. 100 (12) (2003) 7271–7276.
[94] T. Wakita, T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H.G. Krausslich, M. Mizokami, R. Bartenschlager, T.J. Liang, Production of infectious hepatitis C virus in tissue culture from a cloned viral genome, Nat. Med. 11 (7) (2005) 791–796.
[95] J. Zhong, P. Gastaminza, G. Cheng, S. Kapadia, T. Kato, D.R. Burton, S.F. Wieland, S.L. Uprichard, T. Wakita, F.V. Chisari, Robust hepatitis C virus infection in vitro, Proc. Natl. Acad. Sci. U. S. A. 102 (26) (2005) 9294–9299.
[96] D. Lindenbach, 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, C.M. Rice, Complete replication of hepatitis C virus in cell culture, Science 309 (5734) (2005) 623–626.
[97] P. Pileri, Y. Uematsu, S. Campagnoli, G. Galli, F. Falugi, R. Petracca, A.J. Weiner, M. Houghton, D. Rosa, G. Grandi, S. Abrignani, Binding of hepatitis C virus to CD81, Science 282 (1998) 938–941.
[98] S. Levy, S.C. Todd, H.T. Maecker, CD81 (TAPA-1): a molecule involved in signal transduction and cell adhesion in the immune system, Annu. Rev. Immunol. 16 (1998) 89–109.
[99] R. Petracca, F. Falugi, G. Galli, N. Norais, D. Rosa, S. Campagnoli, V. Burgio, E. Di Stasio, B. Giardina, M. Houghton, S. Abrignani, G. Grandi, Structure–function analysis of hepatitis C virus envelope-CD81 binding, J. Virol. 74 (10) (2000) 4824–4830.
[100] B. Bartosch, J. Dubuisson, F.L. Cosset, Infectious hepatitis C virus pseudo-particles containing functional E1–E2 envelope protein complexes, J. Exp. Med. 197 (5) (2003) 633–642.
[101] E.G. Cormier, F. Tsamis, F. Kajumo, R.J. Durso, J.P. Gardner, T. Dragic, CD81 is an entry coreceptor for hepatitis C virus, Proc. Natl. Acad. Sci. U. S. A. 101 (19) (2004) 7270–7274.

[102] J. Zhang, G. Randall, A. Higginbottom, P. Monk, C.M. Rice, J.A. McKeating, CD81 is required for hepatitis C virus glycoprotein-mediated viral infection, J. Virol. 78 (3) (2004) 1448–1455.
[103] B. Bartosch, A. Vitelli, C. Granier, C. Goujon, J. Dubuisson, S. Pascale, E. Scarselli, R. Cortese, A. Nicosia, F.L. Cosset, Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR–B1 scavenger receptor, J. Biol. Chem. 278 (43) (2003) 41624–41630.
[104] A. McKeating, L.Q. Zhang, C. Logvinoff, M. Flint, J. Zhang, J. Yu, D. Butera, D.D. Ho, L.B. Dustin, C.M. Rice, P. Balfe, Diverse hepatitis C virus glycoproteins mediate viral infection in a CD81-dependent manner, J. Virol. 78 (16) (2004) 8496–8505.
[105] D. Lavillette, A.W. Tarr, C. Voisset, P. Donot, B. Bartosch, C. Bain, A.H. Patel, J. Dubuisson, J.K. Ball, F.L. Cosset, Characterization of host-range and cell entry properties of the major genotypes and subtypes of hepatitis C virus, Hepatology 41 (2) (2005) 265–274.
[106] E. Scarselli, H. Ansuini, R. Cerino, R.M. Roccasecca, S. Acali, G. Filocamo, C. Traboni, A. Nicosia, R. Cortese, A. Vitelli, The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus, EMBO J. 21 (19) (2002) 5017–5025.
[107] D. Rhainds, L. Brissette, The role of scavenger receptor class B type I (SR–BI) in lipid trafficking. defining the rules for lipid traders, Int. J. Biochem. Cell Biol. 36 (1) (2004) 39–77.
[108] E. Yamada, M. Montoya, C.G. Schuettler, T.P. Hickling, A.W. Tarr, A. Vitelli, J. Dubuisson, A.H. Patel, J.K. Ball, P. Borrow, Analysis of the binding of hepatitis C virus genotype 1a and 1b E2 glycoproteins to peripheral blood mononuclear cell subsets, J. Gen. Virol. 86 (Pt 9) (2005) 2507–2512.
[109] S. Acton, A. Rigotti, K.T. Landschulz, S. Xu, H.H. Hobbs, M. Krieger, Identification of scavenger receptor SR–BI as a high density lipoprotein receptor, Science 271 (5248) (1996) 518–520.
[110] K.T. Landschulz, R.K. Pathak, A. Rigotti, M. Krieger, H.H. Hobbs, Regulation of scavenger receptor, class B, type I, a high density lipoprotein receptor, in liver and steroidogenic tissues of the rat, J. Clin. Invest. 98 (4) (1996) 984–995.
[111] H. Barth, R. Cerino, M. Arcuri, M. Hoffmann, P. Schurmann, M.I. Adah, B. Gissler, X. Zhao, V. Ghisetti, B. Lavezzo, H.E. Blum, F. von Weizsacker, A. Vitelli, E. Scarselli, T.F. Baumert, Scavenger receptor class B type I and hepatitis C virus infection of primary tupaia hepatocytes, J. Virol. 79 (9) (2005) 5774–5785.
[112] B. Bartosch, G. Verney, M. Dreux, P. Donot, Y. Morice, F. Penin, J.M. Pawlotsky, D. Lavillette, F.L. Cosset, An interplay between hypervariable region 1 of the hepatitis C virus E2 glycoprotein, the scavenger receptor BI, and high-density lipoprotein promotes both enhancement of infection and protection against neutralizing antibodies, J. Virol. 79 (13) (2005) 8217–8229.
[113] D.D. Lavillette, Y. Morice, G. Germanidis, P. Donot, A. Soulier, E. Pagkalos, G. Sakellariou, L. Intrator, B. Bartosch, J.M. Pawlotsky, F.L. Cosset, Human serum facilitates hepatitis C virus infection, and neutralizing responses inversely correlate with viral replication kinetics at the acute phase of hepatitis C virus infection, J. Virol. 79 (10) (2005) 6023–6034.
[114] J.C. Meunier, R.E. Engle, K. Faulk, M. Zhao, B. Bartosch, H. Alter, S.U. Emerson, F.L. Cosset, R.H. Purcell, J. Bukh, Evidence for cross-genotype neutralization of hepatitis C virus pseudo-particles and enhancement of infectivity by apolipoprotein C1, Proc. Natl. Acad. Sci. U. S. A. 102 (12) (2005) 4560–4565.
[115] C. Voisset, N. Callens, E. Blanchard, A. Op De Beeck, J. Dubuisson, N. Vu-Dac, High density lipoproteins facilitate hepatitis C virus entry through the scavenger receptor class B type I, J. Biol. Chem. 280 (9) (2005) 7793–7799.
[116] J.P. Gardner, R.J. Durso, R.R. Arrigale, G.P. Donovan, P.J. Maddon, T. Dragic, W.C. Olson, L-SIGN (CD 209L) is a liver-specific capture receptor for hepatitis C virus, Proc. Natl. Acad. Sci. U. S. A. 100 (8) (2003) 4498–4503.
[117] Y. Lozach, H. Lortat-Jacob, A. de Lacroix de Lavalette, I. Staropoli, S. Foung, A. Amara, C. Houles, F. Fieschi, O. Schwartz, J.L. Virelizier, F. Arenzana-Seisdedos, R. Altmeyer, DC-SIGN and L-SIGN are high affinity binding receptors for hepatitis C virus glycoprotein E2, J. Biol. Chem. 278 (22) (2003) 20358–20366.
[118] S. Pohlmann, J. Zhang, F. Baribaud, Z. Chen, G.J. Leslie, G. Lin, A. Granelli-Piperno, R.W. Doms, C.M. Rice, J.A. McKeating, Hepatitis C virus glycoproteins interact with DC-SIGN and DC-SIGNR, J. Virol. 77 (7) (2003) 4070–4080.
[119] B. Saunier, M. Triyatni, L. Ulianich, P. Maruvada, P. Yen, L.D. Kohn, Role of the asialoglycoprotein receptor in binding and entry of hepatitis C virus structural proteins in cultured human hepatocytes, J. Virol. 77 (1) (2003) 546–559.
[120] E.G. Cormier, R.J. Durso, F. Tsamis, L. Boussemart, C. Manix, W.C. Olson, J.P. Gardner, T. Dragic, L-SIGN (CD209L) and DC-SIGN (CD209) mediate transinfection of liver cells by hepatitis C virus, Proc. Natl. Acad. Sci. U. S. A. 101 (39) (2004) 14067–14072.
[121] Y. Lozach, A. Amara, B. Bartosch, J.L. Virelizier, F. Arenzana-Seisdedos, F.L. Cosset, R. Altmeyer, C-type lectins L-SIGN and DC-SIGN capture and transmit infectious hepatitis C virus pseudotype particles, J. Biol. Chem. 279 (31) (2004) 32035–32045.
[122] V. Agnello, G. Abel, M. Elfahal, G.B. Knight, Q.X. Zhang, Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor, Proc. Natl. Acad. Sci. U. S. A. 96 (22) (1999) 12766–12771.
[123] M.J. Evans, T. von Hahn, D.M. Tscherne, A.J. Syder, M. Panis, B. Wolk, T. Hatziioannou, J.A. McKeating, P.D. Bieniasz, C.M. Rice, Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry, Nature 446 (7137) (2007) 801–805.
[124] R. Rijnbrand, P. Bredenbeek, T. van der Straaten, L. Whetter, G. Inchauspe, S. Lemon, W. Spaan, Almost the entire 5′ non-translated region of hepatitis C virus is required for cap-independent translation, FEBS Lett. 365 (2-3) (1995) 115–119.
[125] B.J. Yoo, R.R. Spaete, A.P. Geballe, M. Selby, M. Houghton, J.H. Han, 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 191 (2) (1992) 889–899.
[126] U. Hellen, T.V. Pestova, Translation of hepatitis C virus RNA, J. Viral Hepatitis 6 (2) (1999) 79–87.
[127] H.H. Lu, E. Wimmer, 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. U. S. A. 93 (4) (1996) 1412–1417.
[128] J.E. Reynolds, A. Kaminski, H.J. Kettinen, K. Grace, B.E. Clarke, A.R. Carroll, D.J. Rowlands, R.J. Jackson, Unique features of internal initiation of hepatitis C virus RNA translation, EMBO J. 14 (23) (1995) 6010–6020.
[129] M. Spahn, J.S. Kieft, R.A. Grassucci, P.A. Penczek, K. Zhou, J.A. Doudna, J. Frank, Hepatitis C virus IRES RNA-induced changes in the conformation of the 40s ribosomal subunit, Science 291 (5510) (2001) 1959–1962.
[130] A. Otto, J.D. Puglisi, The pathway of HCV IRES-mediated translation initiation, Cell 119 (3) (2004) 369–380.
[131] N. Ali, G.J. Pruijn, D.J. Kenan, J.D. Keene, A. Siddiqui, Human La antigen is required for the hepatitis C virus internal ribosome entry site-mediated translation, J. Biol. Chem. 275 (36) (2000) 27531–27540.
[132] N. Ali, A. Siddiqui, 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. U. S. A. 94 (6) (1997) 2249–2254.
[133] T. Isoyama, N. Kamoshita, K. Yasui, A. Iwai, K. Shiroki, H. Toyoda, A. Yamada, Y. Takasaki, A. Nomoto, Lower concentration of La protein required for internal ribosome entry on hepatitis C virus RNA than on poliovirus RNA, J. Gen. Virol. 80 (Pt 9) (1999) 2319–2327.
[134] B. Hahm, Y.K. Kim, J.H. Kim, T.Y. Kim, S.K. Jang, Heterogeneous nuclear ribonucleoprotein L interacts with the 3′ border of the internal ribosomal entry site of hepatitis C virus, J. Virol. 72 (11) (1998) 8782–8788.
[135] I.S. Fukushi, M. Okada, T. Kageyama, F.B. Hoshino, K. Nagai, K. Katayama, Interaction of poly(rC)-binding protein 2 with the 5′-terminal stem loop of the hepatitis C-virus genome, Virus Res. 73 (1) (2001) 67–79.

[136] A. Anwar, N. Ali, R. Tanveer, A. Siddiqui, 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. 275 (44) (2000) 34231–34235.
[137] T. Shimoike, S. Mimori, H. Tani, Y. Matsuura, T. Miyamura, Interaction of hepatitis C virus core protein with viral sense RNA and suppression of its translation, J. Virol. 73 (12) (1999) 9718–9725.
[138] Y. Tanaka, T. Shimoike, K. Ishii, R. Suzuki, T. Suzuki, H. Ushijima, Y. Matsuura, T. Miyamura, Selective binding of hepatitis C virus core protein to synthetic oligonucleotides corresponding to the 5′ untranslated region of the viral genome, Virology 270 (1) (2000) 229–236.
[139] T. Shimoike, C. Koyama, K. Murakami, R. Suzuki, Y. Matsuura, T. Miyamura, T. Suzuki, 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 345 (2) (2006) 434–445.
[140] J. Zhang, O. Yamada, H. Yoshida, T. Iwai, H. Araki, Autogenous translational inhibition of core protein: implication for switch from translation to RNA replication in hepatitis C virus, Virology 293 (1) (2002) 141–150.
[141] D. Li, S.T. Takyar, W.B. Lott, E.J. Gowans, 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 84 (Pt 4) (2003) 815–825.
[142] T.H. Wang, R.C. Rijnbrand, S.M. Lemon, 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. 74 (23) (2000) 11347–11358.
[143] J.L. Walewski, T.R. Keller, D.D. Stump, A.D. Branch, Evidence for a new hepatitis C virus antigen encoded in an overlapping reading frame, RNA 7 (5) (2001) 710–721.
[144] Z. Xu, J. Choi, T.S. Yen, W. Lu, A. Strohecker, S. Govindarajan, D. Chien, M.J. Selby, J. Ou, Synthesis of a novel hepatitis C virus protein by ribosomal frameshift, EMBO J. 20 (14) (2001) 3840–3848.
[145] A. Varaklioti, N. Vassilaki, U. Georgopoulou, P. Mavromara, Alternate translation occurs within the core coding region of the hepatitis C viral genome, J. Biol. Chem. 277 (20) (2002) 17713–17721.
[146] P. Hüssy, H. Langen, J. Mous, H. Jacobsen, Hepatitis C virus core protein: carboxy-terminal boundaries of two processed species suggest cleavage by a signal peptide peptidase, Virology 224 (1996) 93–104.
[147] J. McLauchlan, M.K. Lemberg, G. Hope, B. Martoglio, Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets, EMBO J. 21 (15) (2002) 3980–3988.
[148] M.K. Lemberg, B. Martoglio, Requirements for signal peptide peptidase-catalyzed intramembrane proteolysis, Mol. Cell 10 (4) (2002) 735–744.
[149] K. Okamoto, K. Moriishi, T. Miyamura, Y. Matsuura, Intramembrane proteolysis and endoplasmic reticulum retention of hepatitis C virus core protein, J. Virol. 78 (12) (2004) 6370–6380.
[150] A. Weihofen, K. Binns, M.K. Lemberg, K. Ashman, B. Martoglio, Identification of signal peptide peptidase, a presenilin-type aspartic protease, Science 296 (5576) (2002) 2215–2218.
[151] R. Bartenschlager, L. Ahlborn-Laake, J. Mous, H. Jacobsen, Kinetic and structural analyses of hepatitis C virus polyprotein processing, J. Virol. 68 (1994) 5045–5055.
[152] C. Failla, L. Tomei, R. De Francesco, An amino-terminal domain of the hepatitis C virus NS3 protease is essential for interaction with NS4A, J. Virol. 69 (3) (1995) 1769–1777.
[153] C. Lin, B.M. Pragai, A. Grakoui, J. Xu, C.M. Rice, Hepatitis C virus NS3 serine proteinase: *trans*-cleavage requirements and processing kinetics, J. Virol. 68 (1994) 8147–8157.
[154] Y. Tanji, M. Hijikata, Y. Hirowatari, K. Shimotohno, Hepatitis C virus polyprotein processing: kinetics and mutagenic analysis of serine proteinase-dependent cleavage, J. Virol. 68 (1994) 8418–8422.
[155] H. Aizaki, K.J. Lee, V.M. Sung, H. Ishiko, M.M. Lai, Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts, Virology 324 (2) (2004) 450–461.
[156] N. Ali, K.D. Tardif, A. Siddiqui, Cell-free replication of the hepatitis C virus subgenomic replicon, J. Virol. 76 (23) (2002) 12001–12007.
[157] V.C. Lai, S. Dempsey, J.Y. Lau, Z. Hong, W. Zhong, In vitro RNA replication directed by replicase complexes isolated from the subgenomic replicon cells of hepatitis C virus, J. Virol. 77 (3) (2003) 2295–2300.
[158] Y. Miyanari, M. Hijikata, M. Yamaji, M. Hosaka, H. Takahashi, K. Shimotohno, Hepatitis C virus non-structural proteins in the probable membranous compartment function in viral genome replication, J. Biol. Chem. 278 (50) (2003) 50301–50308.
[159] R.W. Hardy, J. Marcotrigiano, K.J. Blight, J.E. Majors, C.M. Rice, Hepatitis C virus RNA synthesis in a cell-free system isolated from replicon-containing hepatoma cells, J. Virol. 77 (3) (2003) 2029–2037.
[160] S.T. Shi, K.J. Lee, H. Aizaki, S.B. Hwang, M.M. Lai, Hepatitis C virus RNA replication occurs on a detergent-resistant membrane that cofractionates with caveolin-2, J. Virol. 77 (7) (2003) 4160–4168.
[161] U. Pfeifer, R. Thomssen, K. Legler, U. Bottcher, W. Gerlich, E. Weinmann, O. Klinge, Experimental non-A, non-B hepatitis: four types of cytoplasmic alteration in hepatocytes of infected chimpanzees, Virchows Arch., B Cell Pathol. Incl. Mol. Pathol. 33 (3) (1980) 233–243.
[162] G. Mottola, G. Cardinali, A. Ceccacci, C. Trozzi, L. Bartholomew, M.R. Torrisi, E. Pedrazzini, S. Bonatti, G. Migliaccio, Hepatitis C virus nonstructural proteins are localized in a modified endoplasmic reticulum of cells expressing viral subgenomic replicons, Virology 293 (1) (2002) 31–43.
[163] T. Pietschmann, V. Lohmann, G. Rutter, K. Kurpanek, R. Bartenschlager, Characterization of cell lines carrying self-replicating hepatitis C virus RNAs, J. Virol. 75 (3) (2001) 1252–1264.
[164] G. Waris, S. Sarker, A. Siddiqui, Two-step affinity purification of the hepatitis C virus ribonucleoprotein complex, RNA 10 (2) (2004) 321–329.
[165] T. Umehara, M. Sudoh, F. Yasui, C. Matsuda, Y. Hayashi, K. Chayama, M. Kohara, Serine palmitoyltransferase inhibitor suppresses HCV replication in a mouse model, Biochem. Biophys. Res. Commun. 346 (1) (2006) 67–73.
[166] H. Sakamoto, K. Okamoto, M. Aoki, H. Kato, A. Katsume, A. Ohta, T. Tsukuda, N. Shimma, Y. Aoki, M. Arisawa, M. Kohara, M. Sudoh, Host sphingolipid biosynthesis as a target for hepatitis C virus therapy, Nat. Chem. Biol. 1 (6) (2005) 333–337.
[167] I. Hamamoto, Y. Nishimura, T. Okamoto, H. Aizaki, M. Liu, Y. Mori, T. Abe, T. Suzuki, M.M. Lai, T. Miyamura, K. Moriishi, Y. Matsuura, Human VAP-B is involved in hepatitis C virus replication through interaction with NS5A and NS5B, J. Virol. 79 (21) (2005) 13473–13482.
[168] T. Okamoto, Y. Nishimura, T. Ichimura, K. Suzuki, T. Miyamura, T. Suzuki, K. Moriishi, Y. Matsuura, Hepatitis C virus RNA replication is regulated by FKBP8 and Hsp90, EMBO J. 25 (20) (2006) 5015–5025.
[169] J. Ye, C. Wang, R. Sumpter Jr., M.S. Brown, J.L. Goldstein, M. Gale Jr., Disruption of hepatitis C virus RNA replication through inhibition of host protein geranylgeranylation, Proc. Natl. Acad. Sci. U. S. A. 100 (26) (2003) 15865–15870.
[170] S.B. Kapadia, F.V. Chisari, Hepatitis C virus RNA replication is regulated by host geranylgeranylation and fatty acids, Proc. Natl. Acad. Sci. U. S. A. 102 (7) (2005) 2561–2566.
[171] T.L. Tan, H. Nakao, Y. He, S. Vijaysri, P. Neddermann, B.L. Jacobs, B.J. Mayer, M.G. Katze, NS5A, a nonstructural protein of hepatitis C virus, binds growth factor receptor-bound protein 2 adaptor protein in a Src homology 3 domain/ligand-dependent manner and perturbs mitogenic signaling, Proc. Natl. Acad. Sci. U. S. A. 96 (10) (1999) 5533–5538.
[172] K. Ghosh, M. Majumder, R. Steele, P. Yaciuk, J. Chrivia, R. Ray, R.B. Ray, Hepatitis C virus NS5A protein modulates transcription through a novel cellular transcription factor SRCAP, J. Biol. Chem. 275 (10) (2000) 7184–7188.
[173] K.M. Chung, J. Lee, J.E. Kim, O.K. Song, S. Cho, J. Lim, M. Seedorf, B. Hahm, S.K. Jang, Nonstructural protein 5A of hepatitis C virus inhibits the function of karyopherin beta3, J. Virol. 74 (11) (2000) 5233–5241.
[174] T. Buckstummer, M. Kriegs, J. Lupberger, E.K. Pauli, S. Schmittel, E. Hildt, Raf-1 kinase associates with Hepatitis C virus NS5A and regulates viral replication, FEBS Lett. 580 (2) (2006) 575–580.
[175] M. Ikeda, K. Abe, M. Yamada, H. Dansako, K. Naka, N. Kato, Different anti-HCV profiles of statins and their potential for combination therapy with interferon, Hepatology 44 (1) (2006) 117–125.

[176] K. Watashi, N. Ishii, M. Hijikata, D. Inoue, T. Murata, Y. Miyanari, K. Shimotohno, Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase, Mol. Cell 19 (1) (2005) 111–122.
[177] P.Y. Goh, Y.J. Tan, S.P. Lim, Y.H. Tan, S.G. Lim, F. Fuller-Pace, W. Hong, Cellular RNA helicase p68 relocalization and interaction with the hepatitis C virus (HCV) NS5B protein and the potential role of p68 in HCV RNA replication, J. Virol. 78 (10) (2004) 5288–5298.
[178] M. Hirano, S. Kaneko, T. Yamashita, H. Luo, W. Qin, Y. Shirota, T. Nomura, K. Kobayashi, S. Murakami, Direct interaction between nucleolin and hepatitis C virus NS5B, J. Biol. Chem. 278 (7) (2003) 5109–5115.
[179] T. Shimakami, M. Honda, T. Kusakawa, T. Murata, K. Shimotohno, S. Kaneko, S. Murakami, Effect of hepatitis C virus (HCV) NS5B-nucleolin interaction on HCV replication with HCV subgenomic replicon, J. Virol. 80 (7) (2006) 3332–3340.
[180] S. Kim, S.K. Seol, O.K. Song, J.H. Park, S.K. Jang, An RNA-binding protein, hnRNP A1, and a scaffold protein, Septin 6, facilitate hepatitis C virus replication, J. Virol. 81 (8) (2007) 3852–3865.
[181] N. Ali, A. Siddiqui, Interaction of polypyrimidine tract-binding protein with the 5′ noncoding region of the hepatitis C virus RNA genome and its functional requirement in internal initiation of translation, J. Virol. 69 (10) (1995) 6367–6375.
[182] T. Ito, M.M. Lai, Determination of the secondary structure of and cellular protein binding to the 3′-untranslated region of the hepatitis C virus RNA genome, J. Virol. 71 (11) (1997) 8698–8706.
[183] K. Murakami, M. Abe, T. Kageyama, N. Kamoshita, A. Nomoto, Down-regulation of translation driven by hepatitis C virus internal ribosomal entry site by the 3′ untranslated region of RNA, Arch. Virol. 146 (4) (2001) 729–741.
[184] K. Tsuchihara, T. Tanaka, M. Hijikata, S. Kuge, H. Toyoda, A. Nomoto, N. Yamamoto, K. Shimotohno, Specific interaction of polypyrimidine tract-binding protein with the extreme 3′-terminal structure of the hepatitis C virus genome, the 3′X, J. Virol. 71 (9) (1997) 6720–6726.
[185] H. Aizaki, K.S. Choi, M. Liu, Y.J. Li, M.M. Lai, Polypyrimidine-tract-binding protein is a component of the HCV RNA replication complex and necessary for RNA synthesis, J. Biomed. Sci. 13 (4) (2006) 469–480.
[186] M. Kaito, S. Wastanabe, K. Tsukiyama-Kohara, K. Yamaguchi, Y. Kobayashi, M. Konishi, M. Yokoi, S. Ishida, S. Suzuki, M. Kohara, Hepatitis C virus particle detected by immunoelectron microscopic study, J. Gen. Virol. 75 (1994) 1755–1760.
[187] Y.K. Shimizu, S.M. Feinstone, M. Kohara, R.H. Purcell, H. Yoshikura, Hepatitis C virus: detection of intracellular virus particles by electron microscopy, Hepatology 23 (1996) 205–209.
[188] P. Maillard, K. Krawczynski, J. Nitkiewicz, C. Bronnert, M. Sidorkiewicz, P. Gounon, J. Dubuisson, G. Faure, R. Crainic, A. Budkowska, Nonenveloped nucleocapsids of hepatitis C virus in the serum of infected patients, J. Virol. 75 (17) (2001) 8240–8250.
[189] P. Andre, F. Komurian-Pradel, S. Deforges, M. Perret, J.L. Berland, M. Sodoyer, S. Pol, C. Brechot, G. Paranhos-Baccala, V. Lotteau, Characterization of low- and very-low-density hepatitis C virus RNA-containing particles, J. Virol. 76 (14) (2002) 6919–6928.
[190] M. Kunkel, M. Lorinczi, R. Rijnbrand, S.M. Lemon, S.J. Watowich, Self-assembly of nucleocapsid-like particles from recombinant hepatitis C virus core protein, J. Virol. 75 (5) (2001) 2119–2129.
[191] L.J. Lorenzo, S. Duenas-Carrera, V. Falcon, N. Acosta-Rivero, E. Gonzalez, M.C. de la Rosa, I. Menendez, J. Morales, Assembly of truncated HCV core antigen into virus-like particles in Escherichia coli, Biochem. Biophys. Res. Commun. 281 (4) (2001) 962–965.
[192] N. Acosta-Rivero, J.C. Aguilar, A. Musacchio, V. Falcon, A. Vina, M.C. de la Rosa, J. Morales, Characterization of the HCV core virus-like particles produced in the methylotrophic yeast Pichia pastoris, Biochem. Biophys. Res. Commun. 287 (1) (2001) 122–125.
[193] E. Blanchard, C. Hourioux, D. Brand, M. Ait-Goughoulte, A. Moreau, S. Trassard, P.Y. Sizaret, F. Dubois, P. Roingeard, Hepatitis C virus-like particle budding: role of the core protein and importance of its Asp111, J. Virol. 77 (18) (2003) 10131–10138.
[194] N. Majeau, V. Gagne, A. Boivin, M. Bolduc, J.A. Majeau, D. Ouellet, D. Leclerc, The N-terminal half of the core protein of hepatitis C virus is sufficient for nucleocapsid formation, J. Gen. Virol. 85 (Pt 4) (2004) 971–981.
[195] C. Klein, S.J. Polyak, J.R. Lingappa, Unique features of hepatitis C virus capsid formation revealed by de novo cell-free assembly, J. Virol. 78 (17) (2004) 9257–9269.
[196] M. Matsumoto, S.B. Hwang, K.S. Jeng, N. Zhu, M.M. Lai, Homotypic interaction and multimerization of hepatitis C virus core protein, Virology 218 (1) (1996) 43–51.
[197] O. Nolandt, V. Kern, H. Muller, E. Pfaff, L. Theilmann, R. Welker, H.G. Krausslich, Analysis of hepatitis C virus core protein interaction domains, J. Gen. Virol. 78 (Pt 6) (1997) 1331–1340.
[198] S. Yan, M.H. Tam, W.J. Syu, Self-association of the C-terminal domain of the hepatitis-C virus core protein, Eur. J. Biochem. 258 (1) (1998) 100–106.
[199] H.J. Ezelle, D. Markovic, G.N. Barber, Generation of hepatitis C virus-like particles by use of a recombinant vesicular stomatitis virus vector, J. Virol. 76 (23) (2002) 12325–12334.
[200] K. Murakami, K. Ishii, Y. Ishihara, S. Yoshizaki, K. Tanaka, Y. Gotoh, H. Aizaki, M. Kohara, H. Yoshioka, Y. Mori, N. Manabe, I. Shoji, T. Sata, R. Bartenschlager, Y. Matsuura, T. Miyamura, T. Suzuki, 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 351 (2) (2006) 381–392.
[201] S.-Y. Lo, M.J. Selby, J.-H. Ou, Interaction between hepatitis C virus core protein and E1 envelope protein, J. Virol. 70 (1996) 5177–5182.
[202] H.C. Ma, C.H. Ke, T.Y. Hsieh, S.Y. Lo, The first hydrophobic domain of the hepatitis C virus E1 protein is important for interaction with the capsid protein, J. Gen. Virol. 83 (Pt 12) (2002) 3085–3092.
[203] A. Serafino, M.B. Valli, F. Andreola, A. Crema, G. Ravagnan, L. Bertolini, G. Carloni, Suggested role of the Golgi apparatus and endoplasmic reticulum for crucial sites of hepatitis C virus replication in human lymphoblastoid cells infected in vitro, J. Med. Virol. 70 (1) (2003) 31–41.
[204] K. Sato, H. Okamoto, S. Aihara, Y. Hoshi, T. Tanaka, S. Mishiro, Demonstration of sugar moiety on the surface of hepatitis C virions recovered from the circulation of infected humans, Virology 196 (1993) 354–357.

J Gastroenterol 2007; 42:411–423
DOI 10.1007/s00535-007-2030-3

Journal of Gastroenterology

Review

Molecular biology of hepatitis C virus

Tetsuro Suzuki, Hideki Aizaki, Kyoko Murakami, Ikuo Shoji, and Takaji Wakita

Department of Virology II, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

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

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

Introduction

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

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

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

Received: February 8, 2007 / Accepted: February 10, 2007
Reprint requests to: T. Suzuki

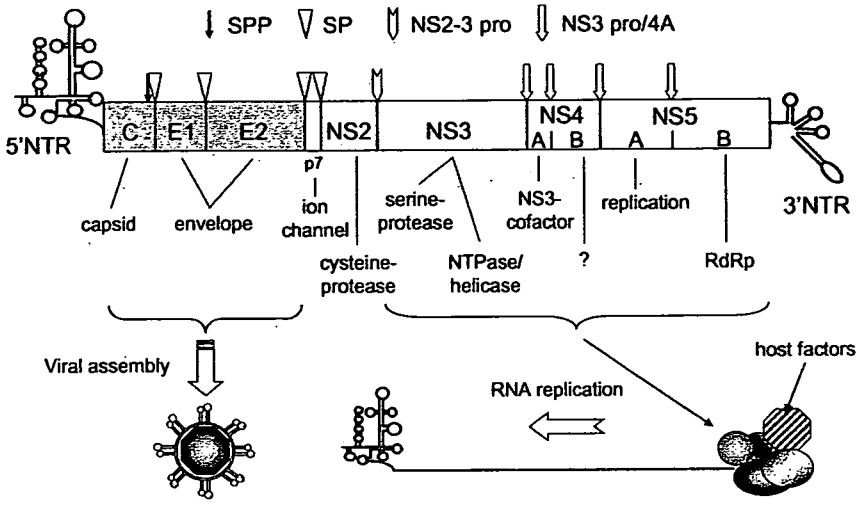

Fig. 1. Hepatitis C virus (HCV) genome organization and polyprotein processing. Posttranslational cleavages by signal peptide peptidase (*SPP*), signal peptidase (*SP*), NS2–NS3 protease (*NS2–3 pro*), and NS3 protease and NS4A complex (*NS3 pro/4A*) lead to the production of functional HCV proteins.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

Cell culture systems for HCV research

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

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

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

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

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

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

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

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

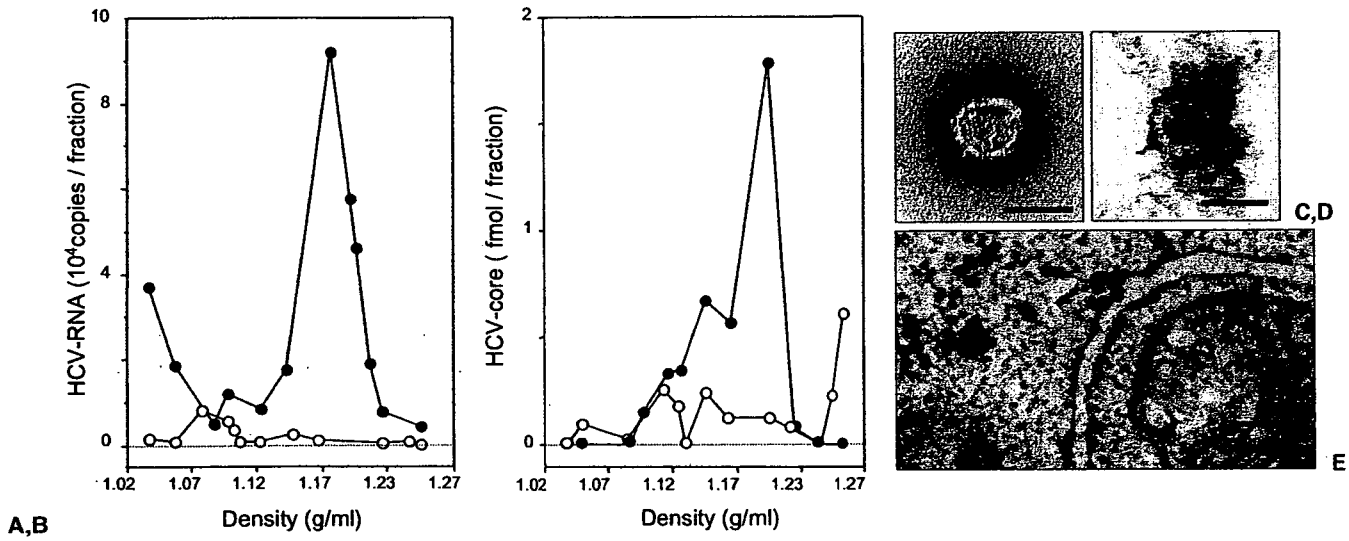

Fig. 2A–E. Production of HCV particles in the three-dimensional, thermoreversible gelatin polymer (TGP) culture of the Huh-7 cell line (RCYM1) carrying genome-length dicistronic HCV RNA of genotype 1b. **A, B** Sucrose density gradient analysis of culture supernatants of RCYM1 cells. The culture supernatants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

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

Translation

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

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

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

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

Polyprotein processing

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

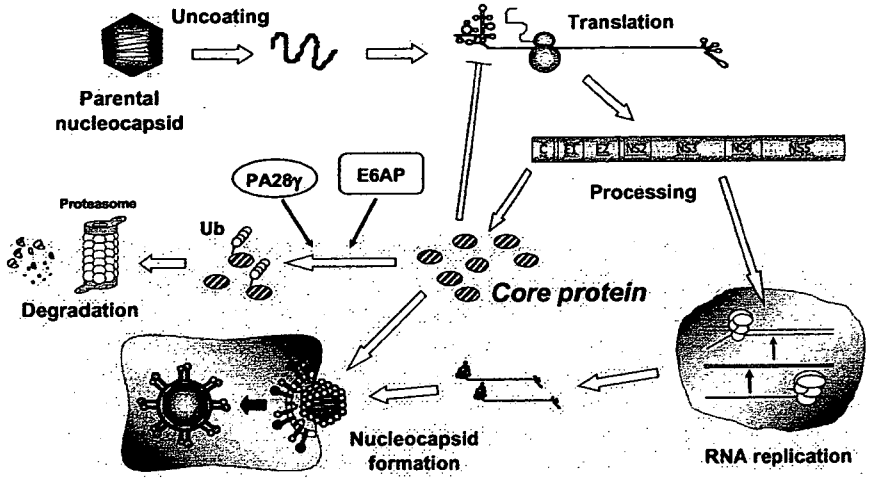

Fig. 3. The role and fate of HCV core protein in the postulated HCV life cycle. See text for further explanation and details

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

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

The NS3–NS5B region is processed presumably with the following preferred order of cleavage: NS3/4A→NS5A/5B→NS4A/4B→NS4B/5A.[93–96] 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.

Acknowledgments. The authors are grateful to all their coworkers who contributed to the studies cited here, most especially Tatsuo Miyamura. We also thank T. Mizoguchi for secretarial work. This work was supported in part by a grant for Research on Health Sciences focusing on Drug Innovation from the Japan Health Sciences Foundation; and by Grants-in-Aid from the Ministry of Health, Labour and Welfare, Japan.

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.

JOURNAL OF VIROLOGY, Feb. 2007, p. 1174–1185 Vol. 81, No. 3
0022-538X/07/$08.00+0 doi:10.1128/JVI.01684-06

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

Masayuki Shirakura,[1] Kyoko Murakami,[1] Tohru Ichimura,[2] Ryosuke Suzuki,[1] Tetsu Shimoji,[1] Kouichirou Fukuda,[1] Katsutoshi Abe,[1] Shigeko Sato,[3] Masayoshi Fukasawa,[3] Yoshio Yamakawa,[3] Masahiro Nishijima,[3] Kohji Moriishi,[4] Yoshiharu Matsuura,[4] Takaji Wakita,[1] Tetsuro Suzuki,[1] Peter M. Howley,[5] Tatsuo Miyamura,[1] and Ikuo Shoji[1]*

Department of Virology II[1] and Department of Biochemistry and Cell Biology,[3] National Institute of Infectious Diseases, Shinjuku-ku, Tokyo 162-8640, Japan; Department of Chemistry, Graduate School of Science, Tokyo Metropolitan University, Hachioji-shi, Tokyo 192-0397, Japan[2]; Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan[4]; and Department of Pathology, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, Massachusetts 02115[5]

Received 4 August 2006/Accepted 8 November 2006

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

* Corresponding author. Mailing address: Department of Virology II, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. Phone: 81 3-5285-1111. Fax: 81 3-5285-1161. E-mail: ishoji@nih.go.jp.
▽ Published ahead of print on 15 November 2006.

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 *etch* virus protease cleavage site, and *FLAG* 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