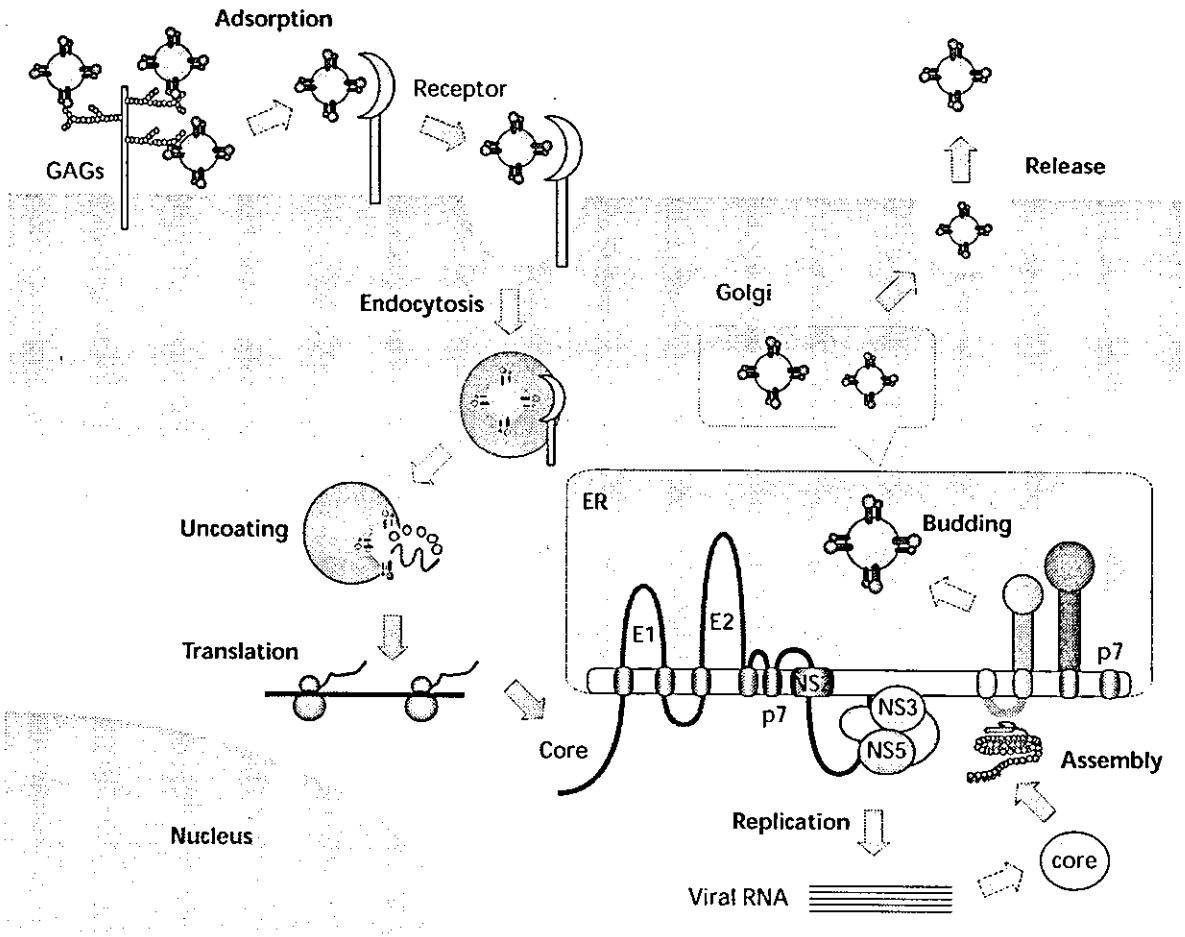
Figure 2. Putative life cycle of hepatitis C virus (HCV)



HCV are trapped by glycosaminoglycans (GAGs) and then transferred to a cell surface receptor and/or co-receptor and internalized into cells through endocytosis. Acidification leads to conformational changes of the HCV envelope proteins into a fusion-competent state and induces the fusion of viral membranes with host membranes. Fusion allows the viral genome to be liberated into cytosol. After uncoating, viral RNA is translated into a precursor polyprotein that is processed into each viral protein by cellular and viral proteases, and replication takes place by the viral polymerase complex on the ER membrane. Core protein binds to the viral sense RNA and forms the nucleocapsid. HCV particles seem to bud into the ER lumen after the interaction of the nucleocapsid with E1 and E2 proteins. It was suggested that the small, hydrophobic peptide p7 is important for viral budding. HCV particles are released from the plasma membrane through the secretory pathway.

was shown to be essential for virus budding (Harada *et al.*, 2000). NS2 is a membrane-spanning protein with four transmembrane regions (Yamaga & Ou, 2002). No functional role for NS2 has yet been described, except for its autoprotease activity cleaving between the junction of NS2 and NS3. NS3 forms a complex with NS4A on the ER membrane. This interaction stabilizes NS3 and retains it on the ER (Wolk *et al.*, 2000). NS3 has serine protease and RNA helicase activities assigned to the N-terminal one-third and remaining two-thirds of the protein, respectively (Dubuisson *et al.*, 2002). Cleavage sites downstream of NS3 are processed by NS3 to yield mature nonstructural proteins (Bartenschlager, 1999; Grakoui *et al.*, 1993; Tomei *et al.*, 1993). NS4B is localized to the ER (Hugle *et al.*, 2001) and was reported to induce membranous web formation together with other non-structural proteins (Egger *et al.*, 2002). Although mutation of NS4B affected the hyperphosphorylation of NS5A (Koch & Bartenschlager, 1999), the function of NS4B in terms of viral replication remains to be defined. NS5A is anchored on the ER through its N-terminal 30 amino acids (Brass *et al.*, 2002). NS5A was found to be a highly phosphorylated polypeptide that may be involved in resistance to the antiviral effects of interferon (IFN) alpha (Enomoto *et al.*, 1995; Enomoto *et al.*, 1996; Gale *et al.*, 1998; Gale *et al.*, 1997). NS5A plays an important role in viral replication, because mutation of the phosphorylation sites of NS5A results in enhanced replication of the HCV replicon in the Huh-7.5 cell line (Blight *et al.*, 2000; Guo *et al.*, 2001; Krieger *et al.*, 2001; Lohmann *et al.*, 2001). NS5B is also anchored in the ER through a C-terminal hydrophobic region, and the main body of the replication complex serves as an RNA-dependent RNA polymerase (Schmidt-Mende *et al.*, 2001).

Recent advances in the treatment of chronic HCV infection and antiviral agents and their targets were recently described in a review by Walker and colleague (Walker *et al.*, 2003). Here, we seek to summarize recent studies investigating candidates for HCV receptor or co-receptor and *in vitro* systems to facilitate our understanding of the life cycle of HCV.

Initial stage of HCV infection

Host surface molecules required for virus entry are classified as either receptors or co-receptors. Several viruses utilize only one molecule as a receptor for entry into host cells, while many viruses require a co-receptor that localizes near the receptors for their entry. Receptors are primarily involved in the attachment of virus to specific host cells and, in some cases, in viral entry. Affinity for a particular receptor may restrict host range, tropism and pathogenicity, although different viruses that have different or partially crossed pathogenicity may utilize the same receptor. Receptors need not be membrane proteins, as carbohydrates and lipids have been identified as receptors for different viruses. For HCV, several molecules have been reported to be candidates for receptor or co-receptor for viral entry. However, the critical determination of receptor or co-receptor requirements for HCV is quite difficult because a reliable *in vitro* cell culture system and a sufficient amount of viral particles are not currently available. Comparisons with other *Flaviviruses* indicate that HCV E2 protein probably plays a role in receptor binding. To identify potential receptor molecules for HCV, soluble envelope protein was used as a probe to screen an expression library. This screen identified several candidates for an HCV receptor that exhibited direct binding to E2 protein and are known membrane proteins. A pseudotype system based on vesicular stomatitis virus (VSV) or retroviruses has also been used to identify putative receptors for difficult- to-culture viruses. This system is a powerful technique for examining binding to cells and also internalization mechanisms.

Human CD81

Following binding of a cell surface receptor, it is thought that HCV enters the cell through endocytosis. Pileri and colleagues (Pileri *et al.*, 1998) first reported human CD81 (hCD81) as a candidate for the HCV receptor. Using a cDNA library derived from a human T cell lymphoma cell line, they used soluble HCV E2 protein as a probe. The soluble extracellular domain of E2 could bind to hCD81, a 25-kDa tetraspan membrane protein widely expressed in haematopoietic and epithermal cells. E2 was reported to lie between amino acids 384–746 in the polyprotein. The second extracelluar loop of hCD81, EC2, is responsible for binding with the E2 protein (Pileri *et al.*, 1998), while amino acids 480–493 and 544-551 of E2 were involved in the hCD81-binding site (Flint *et al.*, 1999). In particular, amino acid 186 of hCD81 is critical for E2 binding and is one of three amino acids that differ from African green monkey CD81, which does not support E2 binding (Higginbottom *et al.*, 2000). In contrast, E2 could bind to tamarin CD81 (the same amino acid at position 186 with hCD81) with higher affinity than hCD81, suggesting that species permissiveness to infection is not due to CD81.

The most important question to address is whether expression of hCD81 alone is sufficient for HCV infection. Replacement of mouse CD81 with hCD81 did not confer susceptibility to HCV infection (Masciopinto *et al.*, 2002). Human CD81 could bind to HCV E2 with a *Kd* value of 1.8 nM; however, it may simply serve as an attachment molecule because hCD81 does not efficiently internalize ligands (Petracca *et al.*, 2000). HCV E2 and a pseudotype VSV expressing chimeric HCV E1 and E2 proteins also bound to the surface of the hepatoma cell line HepG2, a line that does not express detectable levels of hCD81 (Matsuura *et al.*, 2001; Petracca *et al.*, 2000). Recently, a

pseudotype retrovirus carrying authentic HCV envelope proteins was reported by Cosset and colleagues (Bartosch *et al.*, 2003); they indicated that soluble recombinant hCD81 could neutralize the interaction of the pseudotype retrovirus with hepatoma cell line Huh-7. However, expression of hCD81 on nonpermissive cell lines did not confer susceptibility to either the pseudotype retrovirus or the pseudotype VSV (Bartosch *et al.*, 2003). An assay, referred to as neutralization of binding (NOB), has been developed to assess the ability of antibody to inhibit the interaction between soluble truncated E2 and hCD81 (Rosa *et al.*, 1996). The appearance and maintenance of high NOB antibody titres was correlated with clinical resolution of liver disease and viral clearance (Ishii *et al.*, 1998). Thus, other molecules similar to hCD81 may contribute to binding and/or entry of HCV to host cells. Another possibility is that hCD81 is one component of the HCV receptor complex, as described below.

LDL receptor

The low-density lipoprotein (LDL) receptor was reported as a candidate for the HCV receptor. Serum fraction composed of HCV with LDL, or very low-density lipoprotein (VLDL), was involved in binding to the LDL receptor (Agnello *et al.*, 1999). It has been suggested that other members of the *Flavivirus* family also utilize the LDL receptor for viral entry (Agnello *et al.*, 1999). Apolipoprotein B and apolipoprotein E integrated in LDL or VLDL are essential for binding to the LDL receptor, because antibody against apolipoprotein B or apolipoprotein E could inhibit the interaction between HCV and LDL receptor (Agnello *et al.*, 1999). Interestingly, the interaction between HCV particles and the LDL receptor was not dependent on the presence of the E2 envelope protein (Wunschmann *et al.*, 2000). A low-density fraction containing HCV genome has high infectivity. Viral particles containing apolipoprotein B, HCV core and viral RNA could bind to a hepatoma cell line in an apolipoprotein-dependent manner (Andre *et al.*, 2002). However, HCV-like particles prepared from insect cells infected with a recombinant baculovirus exhibited binding to a hepatoma cell line through a pathway independent of the LDL receptor and hCD81 (Triyatni *et al.*, 2002). In addition, VLDL and LDL could not inhibit the interaction between the hepatoma cell line and the pseudotype retrovirus composed of authentic HCV envelope proteins (Bartosch *et al.*, 2003; Hsu *et al.*, 2003b). The role of the LDL receptor in HCV pathogenesis remains obscure.

DC-SIGN and homologues

Dendritic cell-specific intracellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN), a C-type lectin, is reported to serve as a receptor for human cytomegalovirus (Halary *et al.*, 2002), Ebola virus (Alvarez *et al.*, 2002; Colmenares *et al.*, 2002), HIV-type 1 (Geijtenbeek *et al.*, 2000), *Mycobacterium tuberculosis* (Geijtenbeek *et al.*, 2003), and *Leishmania amastigotes* (Colmenares *et al.*, 2002). These pathogens interact with DC-SIGN through high mannose oligosaccharides. Two papers reported that DC-SIGN and related molecules L-SIGN and DC-SIGNR are utilized as HCV receptors (Gardner *et al.*, 2003; Pohlmann *et al.*, 2003). Soluble E2 protein and a pseudotype HIV, bearing chimeric HCV envelope proteins fused with the transmembrane region of Sindbis virus envelope protein, could bind to DC-SIGN and DC-SIGNR (Pohlmann *et al.*, 2003). Their interactions were inhibited by mannose molecules and antibodies against the receptor candidates, suggesting that DC-SIGN and its homologues recognize the oligosaccharides of HCV envelope proteins (Pohlmann *et al.*, 2003). DC-SIGN and DC-SIGNR are expressed in liver sinusoidal endothelial cells but not hepatocytes (Pohlmann *et al.*, 2003). Those receptors may play an important role in hepatocyte infection with HCV through sinusoidal endothelial cells. Interestingly, dengue fever virus also utilizes DC-SIGN as a receptor in dendritic cells (Tassaneetrithep *et al.*, 2003). Although chimeric HCV E2 envelope protein and pseudotype HIV bearing the chimeric HCV envelope proteins could bind to cells expressing DC-SIGN or DC-SIGNR (Pohlmann *et al.*, 2003), the pseudotype HIV carrying authentic HCV envelope proteins was not internalized (Hsu *et al.*, 2003b). DC-SIGN and DC-SIGNR may serve as attachment proteins, but it does not appear that either is the entry receptor for HCV.

Human scavenger receptor, class B type I

Scarselli and colleagues reported another putative receptor using cross-linkers (Scarselli *et al.*, 2002). E2 molecules of HCV types 1a and 1b bind to a hepatoma cell line independently of hCD81. They tried to isolate the protein that binds to soluble E2 protein in the hepatoma cell line HepG2 (Scarselli *et al.*, 2002). Using cross-linkers, soluble E2 was found to associate with lipid raft-localized proteins and, based on expected molecular weights, the scavenger receptor class B type I (SR-B1) was identified as a potential binding partner for HCV E2. E2 of type 1a strain H77 could bind to human SR-B1, but not mouse SR-R1, through its hyper variable region 1 (HVR1). Human SR-B1 is expressed on the hepatoma cell line HepG2, which does not express hCD81, suggesting that human SR-B1 may be involved in the binding of HCV to cells in a hCD81-independent manner. However, HVR1 is not essential; an HCV cDNA clone lacking HVR1 was found to infect chimpanzees (Forns *et al.*, 2000). Overall, the involvement of human SR-B1 in HCV infection is not clear.

Pseudotype viruses for characterization of HCV infection

VSV has a non-segmented 11 kb genome of negative stranded RNA. The VSV genome is transcribed in the cytoplasm and codes five structural proteins. Recombinant VSV, in which native envelope protein G is replaced with other membrane proteins, could contribute to the study of viruses that inefficiently replicate in experimental systems. Additionally, such pseudotype viruses could lead to the induction of cellular and humoral host immunity (Schnell *et al.*, 1996). Several reports describe the characterization of VSV recombinants bearing HCV envelope proteins. HCV E1 and E2 proteins are retained in ER by C-terminal retention signals (Cocquerel *et al.*, 1999; Cocquerel *et al.*, 1998).

In the pseudotype VSV system, envelope proteins should be expressed on cell surface because VSV buds from the plasma membrane. Pseudotype VSVs bearing chimeric proteins, comprised of the ectodomains of HCV envelope proteins with the signal sequence, transmembrane and cytoplasmic regions of VSV G envelope protein, were constructed (Matsuura *et al.*, 2001). The chimeric E1 and E2 proteins were translocated onto the cell surface and incorporated into the released VSV particles, which infect human, but not mouse, rat or hamster, cell lines. CHO cells expressing chimeric HCV envelope proteins could induce membrane fusion with HepG2 cells in a pH-dependent manner (Takikawa *et al.*, 2000). This finding was confirmed by pseudotype HIV carrying authentic HCV envelope proteins (Bartosch *et al.*, 2003; Hsu *et al.*, 2003b). Fab fragments obtained from a chronic HCV-infected patient can neutralize the ability of pseudotype VSV to infect HepG2 cells, suggesting that infection of the VSV pseudotype mimics natural HCV infection (Burioni *et al.*, 2002). However, a recombinant VSV possessing chimeric HCV envelope proteins described by Rose and colleague was not infectious (Buonocore *et al.*, 2002). The molecular size of the chimeric E1 protein incorporated into the pseudotype VSV is larger than that of the recombinant VSV (Buonocore *et al.*, 2002). Different glycosylation patterns or other modifications of the chimeric E1 proteins may affect the infectivity of VSV bearing chimeric HCV envelope proteins.

HCV envelope proteins are statically retained in the ER (Cocquerel *et al.*, 1999). However, Bartosch and colleagues reported that the native form of HCV glycoproteins were partially expressed on cell surface of 293T cells and encapsulated retrovirus nucleocapsid, resulting in the production of infectious particles (Bartosch *et al.*, 2003). This pseudotype retrovirus exhibits the highest infectivity against the hepatoma cell line Huh-7 among the cell lines tested. The glycoproteins E1 and E2 are necessary for the highest degree of infectivity, although the pseudotype bearing either E1 or E2 could infect Huh-7 at two hundred times lower infectivity than those bearing both envelope proteins (Bartosch *et al.*, 2003). Although infection by pseudotype retrovirus was inhibited by the addition of anti-hCD81 antibody or a soluble form of hCD81, expression of hCD81 alone failed to confer mouse NIH3T3 cells susceptible to infection by retrovirus pseudotype (Bartosch *et al.*, 2003). A similar study suggested that pseudotype HIV bearing native forms of HCV envelope proteins infects the Huh-7.5 cell line in a pH-dependent manner and, furthermore, that the expression of one or all of the candidate receptor molecules (hCD81, LDL receptor, human SB-R1 and DC-SIGN) failed to confer permissivity to pseudotype infection (Hsu *et al.*, 2003b).

RNA replicon system

The use of *in vitro* studies of HCV proteins has led to the accumulation of knowledge regarding the pathogenesis of this disease, as well as providing insights into potential therapies. However, the assessment of antiviral drug candidates for HCV patients has been hampered by the lack of a robust and reliable cell culture system and the absence of a small animal model. Viral proteins whose biological activities are already known (such as envelope proteins, helicase and protease, and RNA-dependent RNA polymerase) could be targets for the development of antiviral drugs. Bartenschlager and colleague reported that an RNA replicon comprised of the 5′-UTR, neo gene EMCV IRES (the gene encoding HCV genotype 1b NS2 or NS3 up to NS5B and 3′-UTR) could replicate autonomously in Huh-7 cell line (Lohmann *et al.*, 1999). Stable replication with the highest amount of RNA replication was obtained from an RNA replicon containing the NS3 to NS5B genes. Detection of positive and negative strands of RNA and processed nonstructural proteins in cells indicate that the replicon system partially mimics the HCV replication cycle.

Studies of the replication cycle of HCV have been advanced by improvements of the replicon system. Sequences upstream of HCV IRES in the 5′-UTR are essential for RNA replication (Friebe *et al.*, 2001) and the 3′-UTR plays an important role in the initiation of minus-strand RNA synthesis (Friebe & Bartenschlager, 2002). Replication of replicons was stable for at least 1 year (Pietschmann *et al.*, 2001). Efficient replication of subgenomic replicons carrying type 1b infectious strain Con1 generally required several adaptive mutations. Once RNA replicons acquired adaptive mutations, the efficiency of colony formation was increased by an unknown mechanism (Blight *et al.*, 2000; Lohmann *et al.*, 1999). Adaptive mutations were generally detected in the genes encoding NS3, NS5A and NS5B proteins (Blight *et al.*, 2000; Lohmann *et al.*, 2003; Lohmann *et al.*, 2001). Adaptive mutations within NS5A were found to confer efficient replication *in vitro*

independently of IFN sensitivity (Blight *et al.*, 2000; Guo *et al.*, 2001). Although a full-length HCV replicon released substantial amounts of nuclease-resistant HCV RNA-containing particles, comparable amounts of such RNA-containing particles were detected in the supernatant of cells carrying the subgenomic replicons. These results indicate that the RNA-containing particles are released independently of the presence of HCV structural proteins and that Huh-7 cells may lack host cell factors essential for HCV assembly and release (Pietschmann *et al.*, 2001).

It is not known why adaptive mutation is necessary for the replication of HCV replicons in Huh-7 cells, while viral particles produced under similar conditions are infectious to chimpanzees. Bukh and colleagues demonstrated that adaptive mutations are not necessary for *in vivo* infectivity (Bukh *et al.*, 2002). A full-length clone bearing three adaptive mutations was not infectious to chimpanzees, while a clone bearing one adaptive mutation could infect but the mutation reverted back to wild-type (Bukh *et al.*, 2002). In contrast to the Con1 strain, an RNA replicon construct based on the genome of type 1b infectious clone HCV-N could stably replicate, conserving the wild-type sequence of the polyprotein-coding region (Ikeda *et al.*, 2002). The dicistronic replicon genome encoding neomycin and an entire polyprotein of HCV-N strain was also able to replicate in Huh-7, as detected by Northern blot analysis for genomic RNA (Ikeda *et al.*, 2002). The HCV replicons described above are constructed with genomes of type 1b strains (Con1 and HCV-N). Recently, two groups reported efficient replication of replicons carrying genotype 1a strain H77 (Blight *et al.*, 2003; Gu *et al.*, 2003). Subgenomic and full-length replicons based on type 1a sequences could replicate in Huh-7.5 (Blight *et al.*, 2003; Gu *et al.*, 2003) and exhibited lower sensitivity to IFN alpha than type 1b replicons (Gu *et al.*, 2003). Adaptive mutations appeared in NS3, NS5A, and NS5B (Blight *et al.*, 2003; Gu *et al.*, 2003). The HCV replicon system provides a useful tool to assess antiviral compounds targeting the HCV replication complex.

Virus assembly

HCV core protein is processed by signal peptidase and host intramembrane protease (McLauchlan *et al.*, 2002) and is localized to the ER and lipid droplets (Hope & McLauchlan, 2000; McLauchlan *et al.*, 2002). The intramembrane processing of HCV core protein is inhibited by the chemical compound (Z-LL)2 keton (McLauchlan *et al.*, 2002). Signal peptide peptidase (SPP) was identified as the intramembrane protease that is inhibited by (Z-LL)2 keton (Weihofen *et al.*, 2002). Prolactin, HLA-E and other host proteins are known substrates of SPP (Lemberg *et al.*, 2001). HCV core protein is cleaved by (Z-LL)2 keton-sensitive intramembrane protease, resulting in localization of the processed HCV core protein on lipid droplets (Lemberg & Martoglio, 2002; McLauchlan *et al.*, 2002; Weihofen *et al.*, 2003). Mclauchlan and colleague suggested that Domain II of HCV core protein, a hydrophobic region upstream of the C-terminal transmembrane region, is necessary for stability of HCV core protein and localization to the ER or lipid droplets (Hope & McLauchlan, 2000). There are several homologues to SPP in humans (Grigorenko *et al.*, 2002; Weihofen *et al.*, 2002); these may cleave the transmembrane region of HCV core protein. HCV core protein is a highly basic protein corresponding to the capsid protein found in other members of *Flaviviridae* (Grakoui *et al.*, 1993; Harada *et al.*, 1991; Selby *et al.*, 1993). HCV core protein could interact with the 5′ one-third of positive- strand RNA genome but not negative-strand RNA (Shimoike *et al.*, 1999), suggesting that HCV core protein forms a nucleocapsid with the RNA genome. HCV core protein binds most efficiently to loop IIId and, to a lesser extend, loop I and the region from nt 23–41 (Tanaka *et al.*, 2000).

The putative envelope glycoproteins of HCV are the E1 and E2 proteins, which contain five or six and 11 N-linked glycosylation sites, respectively (Miyamura & Matsuura, 1993). Host signal peptidase processes the polyprotein into core, E1, E2 and p7 (Figure 1). E1 and E2 proteins lie from amino acid 193–383 and 384–746, respectively (Matsuura *et al.*, 1992; Mizushima *et al.*, 1994) and penetrate the ER membrane via C-terminal transmembrane domains, which non-covalently interact with each other (Dubuisson & Rice, 1996; Matsuura *et al.*, 1992; Ralston *et al.*, 1993). The heterodimer is most likely the prebudding form of the functional complex (Deleersnyder *et al.*, 1997).

The structure and function of HCV envelope proteins have been predicted from studies on truncated soluble forms of both envelope proteins (Matsuura *et al.*, 1992; Michalak *et al.*, 1997) and on chimeric proteins in which the wild-type transmembrane domains have been replaced with the transmembrane domains of other proteins (Cocquerel *et al.*, 1998; Flint *et al.*, 1999). E2 protein appears to attach to the ER membrane through its C-terminus, because the deletion of at least 31 amino acids from C-terminus of E2 leads to its secretion (Michalak *et al.*, 1997; Mizushima *et al.*, 1994; Selby *et al.*, 1993). Anchoring of E1 on the ER membrane may depend on a longer C-terminal region of at least 62 amino acids (Michalak *et al.*, 1997), while our data suggest that the hydrophobic domain between positions 262 and 290 is important for anchoring to the ER membrane (Matsuura *et al.*, 1992). Cocquerel and colleagues (Cocquerel *et al.*, 1999) presented the hypothesis that the transmembrane domains of E1 and E2 proteins adopt a transient hairpin structure with both their N- and C-termini facing the ER lumen and subsequently reorient themselves. This data,

taken together with glycosylation studies (Meunier *et al.*, 1999), suggest that E1 is a type I transmembrane protein. However, a physical interaction between the core protein and E1 protein has also been suggested (Lo *et al.*, 1996). The overall topology of E1 and its relation to core protein remains unresolved.

Establishment of persistent infection

It is well-known that 80% of patients have persistent HCV infection, while cure occurs in the remaining patients. Presumably, HCV has the ability to escape from the host immune system and/or to alter or suppress the host immune response. Most patients fail to resolve HCV, despite generating both cellular and humoral immune responses. It is possible that the high mutation rate of HCV allows for escape from the host immune response. The RNA viral genome is highly efficient at mutating and is comprised of several genomes called quasispecies. Different host environments, including the immune response, act as selective pressures on the generation of new viral particles. Sequence diversity of HCV genomes results from the lack of proofreading activity within the viral polymerase (Bukh *et al.*, 1995a; Bukh *et al.*, 1995b).

The role of the host antibody response in protecting the host from the establishment of chronic infection is unclear. For example, antibodies against HCV proteins do not hinder reinfection (Farci *et al.*, 1992; Lai *et al.*, 1994), and humoral responses are decreased in many people who have recovered from HCV infection (Takaki *et al.*, 2000). In contrast, NOB antibody titres are observed in naturally resolved cases of chronic HCV, as described above (Ishii *et al.*, 1998). Whether humoral immune responses against HCV proteins are necessary for protection and recovery has not been clarified.

Chimpanzees, which are known to be the most faithful model for HCV infection (Shimizu *et al.*, 1990), develop persistent infection and hepatocellular carcinoma after infection with HCV (Muchmore *et al.*, 1988). HCV-specific T-cell responses were observed in the blood of patients (Ferrari *et al.*, 1994; Lechner *et al.*, 2000; Thimme *et al.*, 2001) and in the livers of chimpanzees (Cooper *et al.*, 1999; Erickson *et al.*, 2001) during acute HCV infection and are maintained for decades after recovery. Strong responses of intrahepatic cytotoxic T lymphocyte (CTL) were detected in two chimpanzees who resolved acute HCV infection despite a poor antibody response, although weak responses of CTL were found in four chimpanzees who suffered from chronic infection (Cooper *et al.*, 1999). These data suggest that a CTL response, rather than a humoral response, plays an important role in resolving HCV infection during the acute phase, at least. Mutation of dominant epitopes recognized by CTL appeared in three persistently infected chimpanzees during the acute phase of infection, but most dominant epitopes were sustained in HCV-resolved chimpanzees (Erickson *et al.*, 2001). It appears that once HCV escapes the CTL response during the acute phase, chronic infection may be established.

The molecular mechanism of immunosuppression induced by HCV has not been elucidated. Several lines of evidence suggest that HCV proteins have immunomodulatory function. Hahn and colleagues reported that HCV core protein binds to the gC1q receptor (Kittlesen *et al.*, 2000). T-cell proliferation was decreased by the interaction of HCV core with the gC1q receptor (Kittlesen *et al.*, 2000), which inhibits the phosphorylation of ERK/MEK (Yao *et al.*, 2001). Thus, inhibition of ERK/MEK may lead to inhibition of interleukin (IL)-2 and IL-2R production (Yao *et al.*, 2001). Envelope protein E2 was shown to bind to PKR via the phosphorylation site of E2 that is homologous to that of eIF2 alpha. This sequence is conserved in the E2 proteins of type 1a strains and results in the suppression of eIF2 alpha phosphorylation (Taylor *et al.*, 1999). Furthermore, inhibition of natural killer cell activation, cytokine production and proliferation through cross-linking of hCD81 by E2 protein suggests that HCV can alter innate immunity via inhibition of cytokine production by natural killer cells; this inhibition facilitated the establishment of a persistent infection (Crotta *et al.*, 2002; Tseng & Klimpel, 2002). NS3/4A complex also has the ability to inhibit phosphorylation of IRF-3 (Foy *et al.*, 2003). Viral infection induces the phosphorylation of IRF-3 by viral-activated kinase, leading to the transcription of type I IFN and cytokines (Foy *et al.*, 2003). Expression of the NS3/4A complex or HCV polyprotein inhibits the phosphorylation of IRF and transcription of type I IFN through NS3 protease activity (Foy *et al.*, 2003).

New antiviral therapy against HCV

RNA interference (RNAi) is a recently described phenomena that leads to the post-transcriptional regulation of protein expression (Fire *et al.*, 1998; Paddison & Hannon, 2002). In lower organisms such as *Caenorhabditis elegans*, RNAi is composed of two distinct steps. A double-stranded RNA is processed into small interfering RNA (siRNA) of 21–23 nucleotides by Dicer, an RNAase-III-family nuclease (Zamore *et al.*, 2000). The siRNA is then incorporated into the RNA-induced silencing complex (RISC), which leads to the specific destruction of a target mRNA recognized by the antisense strand of the siRNA (Hammond *et al.*, 2000). Potential therapeutic uses for RNAi have been proposed for HIV (Jacque *et al.*, 2002; Lee *et al.*, 2002; Novina *et al.*, 2002), poliovirus (Gitlin, *et al.*, 2002) and heptitis B virus (McCaffrey *et al.*, 2003).

IFN-alpha/ribavirin combination therapy leads to remission of disease in only 40% of patients with chronic HCV; furthermore, this therapy causes significant side effects (Wedemeyer *et al.*, 1998). Thus, it is very important to develop an alternative therapy for chronic HCV infection. McCaffrey and colleague reported that siRNA specific to NS5B inhibit the translation of NS5B-fused luciferase in mice (McCaffrey *et al.*, 2002), although this experiment did not address the replication step of HCV. Several recent studies demonstrated that RNAi suppresses gene expression and RNA replication of HCV in the replicon system (Kapadia *et al.*, 2003; McCaffrey *et al.*, 2002; Randall *et al.*, 2003; Wilson *et al.*, 2003; Yokota *et al.*, 2003). The application of RNAi to chronic HCV therapy, possibly in conjunction with IFN/ribavirin, holds great promise but needs to be further investigated.

Richardson and colleague reported an interesting approach to reduce the number of HCV-infected cells using a modified pro-apoptotic protein (Hsu *et al.*, 2003a). The Bcl-2-family member Bid becomes pro-apoptotic following caspase-8 activation in the liver (Li *et al.*, 1998; Yin *et al.*, 1999). Adenovirus encoding recombinant Bid, in which the caspase cleavage site was replaced with an HCV NS3 cleavage site, suppressed HCV replication not only in the RNA replicon system *in vitro*, but also in mice implanted with human livers infected with HCV without any cytotoxicity of uninfected human liver cells (Hsu *et al.*, 2003a).

Core protein has also become a target for antiviral therapy. SPP is a presenillin-type protease. The primary accumulation of the primary target of presenillin, beta-amyloid precursor protein, is the cause of Alzheimer's disease. Several compounds developed for presenillin can also inhibit the activity of SPP in addition to $(z\text{-LL})_2$ keton. In order to be effective for SPP, a compound must pass through the plasma membrane as SPP is retained in the ER membrane by ER-retrieval signal (Weihofen *et al.*, 2002). The development of such compounds could prove useful for the treatment of chronic HCV infection.

Conclusions

As described above, several putative cell surface receptors for HCV envelope proteins have been identified by different groups. The report of cell surface expression of wild-type HCV envelope proteins by Bartosh *et al.* (Bartosch, *et al.*, 2003; Pohlmann *et al.*, 2003) was extremely surprising, given that HCV envelope proteins are believed to have ER retention signals (Cocquerel *et al.*, 1999; Cocquerel *et al.*, 2002). Genuine receptor(s) will hopefully be identified in the near future if both envelope proteins expressed on the cell surface sustain their native forms and the ability to interact with their cognate receptor(s). Replicon systems are useful tools for the study of HCV replication and for the development of new drugs for HCV therapy. Huh-7 cells can acquire the ability to allow the HCV genome to replicate, but they are deficient in the assembly and/or release of HCV particles. If the Huh-7 cell line was reconstituted by the introduction of the true HCV receptor, co-receptor and other host factors essential for viral assembly and release of infectious particles, the cell line would provide a breakthrough in HCV study. However, an adaptive mutation of type 1b strain reverted back to authentic amino acid, and most adaptively mutated clones could not establish infection when inoculated into chimpanzees (Bukh *et al.*, 2002). Environmental conditions surrounding the HCV RNA replicon system may hold a unique position in Huh-7 cell line. The establishment of a robust culture system for HCV replication is the most important issue that needs to be resolved in order to better understand the pathogenicity of HCV and develop an effective measure for the treatment of chronic HCV infection.

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Proteasome Activator PA28γ-Dependent Nuclear Retention and Degradation of Hepatitis C Virus Core Protein

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Hepatitis C virus (HCV) core protein plays an important role in the formation of the viral nucleocapsid and a regulatory protein involved in hepatocarcinogenesis. In this study, we have identified proteasome activator PA28γ (11S regulator γ) as an HCV core binding protein by using yeast two-hybrid system. This interaction was demonstrated not only in cell culture but also in the livers of HCV core transgenic mice. These findings are extended to human HCV infection by the observation of this interaction in liver specimens from a patient with chronic HCV infection. Neither the interaction of HCV core protein with other PA28 subtypes nor that of PA28γ with other *Flavivirus* core proteins was detected. Deletion of the PA28γ-binding region from the HCV core protein or knockout of the PA28γ gene led to the export of the HCV core protein from the nucleus to the cytoplasm. Overexpression of PA28γ enhanced the proteolysis of the HCV core protein. Thus, the nuclear retention and stability of the HCV core protein is regulated via a PA28γ-dependent pathway through which HCV pathogenesis may be exerted.

Hepatitis C virus (HCV) is the causative agent in most cases of acute and chronic non-A, non-B hepatitis (16, 51). Over 50% of patients with acute infection evolve into a chronic carrier state (26), and persistent infection frequently results in chronic hepatitis. Chronic HCV infection may lead to the development of cirrhosis and eventually hepatocellular carcinoma (21, 51). HCV belongs to the *Flaviviridae* family, a family that also includes *Japanese encephalitis virus* (JEV) and *Dengue fever virus* (DEN), and possesses a viral genome consisting of a single positive-strand RNA of approximately 9.6 kb and encoding approximately 3,000 amino acids in a single polypeptide (9, 58). HCV proteins are produced as a single polypeptide that is posttranslationally cleaved by host cellular peptidases and viral proteases to yield at least 10 viral proteins (7, 10, 12, 54).

A comparison of the genome structure of HCV with other flaviviruses, as well as the observation of a specific interaction of viral sense RNA with HCV core protein in cells (53, 68), suggests that the HCV core protein forms the nucleocapsid with viral genome RNA. An HCV core protein consisting of the N-terminal 191 amino acids is generated by protein cleavage by host signal peptidase(s) (37, 52). The HCV core protein is further processed into a mature core protein lacking its C-terminal hydrophobic region by either an unknown host protease (52, 65) or by a signal peptide peptidase (36). The matured core protein is retained on the endoplasmic reticulum (ER) either by an interaction with immature core protein on the ER membrane (29) or via E1 envelope protein (32). The C-terminal hydrophobic region between amino acids 174 and 191 is essential for HCV core protein anchoring on the ER membrane and for the signal sequence of E1 protein to translocate into the ER lumen. Core proteins truncated at the C termini are mainly localized in the nucleus and, to lesser extent, in the cytoplasm (8, 55). Further processing of the HCV core protein yields a 16-kDa product whose C terminus is near amino acid 151; this protein translocates into the nucleus (30, 31, 55).

We have reported that hepatic steatosis and hepatocellular carcinoma are induced in transgenic mice expressing the HCV core protein, suggesting that the HCV core protein has an oncogenic activity in liver. These data further suggest that the cellular components responsible for HCV-induced carcinogenesis exist not only in humans but also in mice (39). Thus, the identification of core-binding partners in mammalian cells could potentially clarify the molecular mechanism(s) of HCV-induced hepatocarcinogenesis. Several cytoplasmic and nuclear proteins have been reported to bind the HCV core protein to both induce carcinogenesis and facilitate virion formation. A report has suggested that the HCV core protein may sequester LZIP, a putative tumor suppressor, in the cytoplasm, with a resulting enhancement of carcinogenesis of NIH 3T3 cells (18). The HCV core protein interacts with the C-terminal region of p53 and enhances its transcriptional activity through augmentation of p53 DNA binding affinity (46). A putative cellular RNA helicase, primarily localized in the nucleus and to a lesser extent in the cytoplasm, interacts with the

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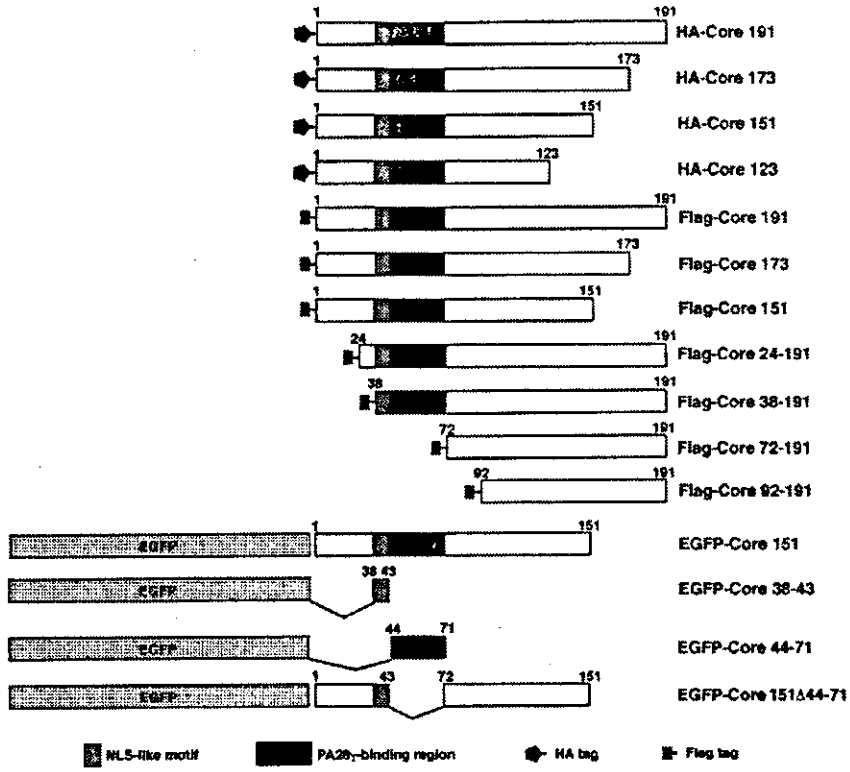

FIG. 1. Expression constructs of HCV core protein used in this study. The genes encoding HA-tagged HCV mutants or Flag-tagged HCV mutants were cloned into pCAG-GS vectors (45) while the genes encoding EGFP-fused proteins were introduced into pEGFP-C3. Other vectors are described in the text or in figure legends.

N-terminal 40 amino acids of the HCV core protein and is colocalized with the HCV core protein in both cellular locations (33, 67). It was recently reported that the HCV core protein directly binds and activates STAT3 by phosphorylation through a JAK-independent pathway; cells overexpressing both HCV core protein and STAT3 exhibited anchorage-independent growth and tumorigenesis (66). These reports suggest that the HCV core protein functions in both the nucleus and cytoplasm.

In this report, we identify proteasome activator PA28γ (11S regulator) as an HCV core binding protein by the yeast two-hybrid system. It is well known that PA28γ enhances the latent proteasome activity of the 20S proteasome and is predominantly localized in the nucleus (48, 62). PA28γ is conserved across the animal kingdom from invertebrates to vertebrates (34, 47), although the biological significance of PA28γ is largely unknown. Here, we demonstrate through several lines of evidence that PA28γ specifically interacts with the HCV core protein and remains in the nucleus, consequently regulating its stability.

MATERIALS AND METHODS

Plasmids. Human PA28γ cDNA was isolated from a human fetal brain library by the advanced yeast two-hybrid technique (MATCHMAKER two-hybrid system 3; Clontech, Palo Alto, Calif.) with amino acids 1 to 173 of the HCV core protein as bait. The gene encoding HCV core protein was amplified from HCV strain J1 (genotype 1b) (2) and cloned into the pGBKT7 vector (pGBK T7HCVCore173). The cDNA of PA28γ was amplified by PCR with *Pfu* turbo DNA polymerase (Stratagene, La Jolla, Calif.) and cloned into pEFFlagpGPK-puro (17), pEGFP-C3, and pDsRed2N1; the sequence was verified by DNA sequencing. The gene encoding PA28γ, with amino acids 82 to 90 deleted, was amplified by splicing the overlapping extension (13, 15) and cloned into pDsRed2N1 (Clontech). Other mutant constructs of the HCV core protein were introduced into pCAG-GS (45) and pEGFP-C3 (Fig. 1). The genes encoding the core proteins of DEN (amino acids 1 to 100) and JEV (amino acids 1 to 105), both lacking the C-terminal hydrophobic regions, were amplified by PCR and cloned into pEGFP-C3. F protein was shown to be synthesized by ribosomal frameshift of the core protein-coding sequence (64). The gene encoding F protein of the −2/+1 frame attached to a Flag tag at N terminus was generated by deletion of one adenine in codon 10 and then introduced into the pEFFlagpGK-puro vector. The gene encoding human Bad or human FKBP attached to a hemagglutinin (HA) tag were isolated from human fetal libraries and introduced into pIRESbleo (Clontech). Mouse anti-Flag (M2), mouse anti-HA (HA.11), and mouse anti-cytochrome *c* oxygenase subunit IV (20E) antibodies were purchased from Sigma (St. Louis, Mo.), Babco (Richmond, Calif.), and Molecular Probes (Eugene, Oreg.), respectively. Horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit antisera were purchased from ICN Pharmaceuticals (Aurora, Ohio). Rabbit antisera against synthetic peptides corresponding to amino acids 70 to 85 of PA28γ, 5 to 19 of PA28α, or 15 to 31 of PA28β were purchased from AFFINITI (Exeter, United Kingdom).

Yeast two-hybrid assay and library screening. The bait vector, pGBK T7HCVCore173, described above, was introduced into *Saccharomyces cerevisiae* strain AH109. The yeast containing pGBKT7HCVCore173 were grown in yeast extract-peptone-dextrose medium and transfected with library plasmids based on pACT2 (Clontech), a vector encoding ampicillin resistance. Clones (2×10^6) from a human fetal brain library were screened (Clontech). The yeast clones encoding pGBKT7-53 and pGADT7-T (Clontech) were used as a positive control while yeast containing pGBKT7 and pGADT7 were considered the negative control. Yeast colonies grown on dropout plates lacking tryptophan, leucine, histidine, and adenine were inoculated on two new dropout plates lacking leucine and tryptophan. One of the two plates was subjected to β-galactosidase assay

according to the method of Duttweiller (11), and the other plate was kept at 4°C as a master plate. One of 85 dropout-plate-positive clones led to dark blue staining on a β-galactosidase assay plate to the same extent as the positive control and was called C1-24. The total DNA was recovered from C1-24 and introduced into *Escherichia coli* strain JM109. The plasmid from C1-24 was recovered from the clones independently grown on the Luria-Bertani plate containing 10 μg of ampicillin/ml. The sequences of the insert were determined by using the Big Dye terminator reaction mixture and an ABI Prism 310 genetic analyzer (Applied Biosystems Japan, Tokyo, Japan). All 3 clones contained the same insert encoding human PA28γ.

Transfection, immunoprecipitation, and immunoblotting. Plasmid vectors were transfected into HeLa or 293T cells by liposome-mediated transfection. Immunoprecipitation and liposome-mediated transfection were reported previously (38). Human and mouse liver samples were washed twice with cold phosphate-buffered saline (PBS) and then homogenized in 10 volumes of 20 mM Tris-HCl (pH 7.4) containing 135 mM NaCl, 1% Triton X-100, 10% glycerol, supplemented with 0.5 μg of Pefabloc (Pentapharm, Munich, Germany)/ml, 1 mM phenylmethylsulfonyl fluoride, 1 μg of soybean trypsin inhibitor/ml, 50 mM NaF, and 5 mM Na_3VO_4 (Tris lysis buffer). Mouse monoclonal antibodies to the HCV core protein (clones 11-4, 11-10, and 11-14) (5) were used for immunoprecipitation and immunoblotting (38).

Laser scanning confocal microscopy. Transfectants were grown on glass slides at 37°C overnight, washed twice with PBS, and fixed with 4% paraformaldehyde for 15 min at room temperature. Enhanced green fluorescent protein (EGFP) fusion protein-expressing cells were examined directly. For samples requiring immunostaining, cells were washed twice with PBS after fixation, permeabilized for 15 min at room temperature with PBS containing 0.5% Triton X-100, and incubated in PBS containing 1% bovine serum albumin (PBS-BSA) in order to block nonspecific binding. Cells were then incubated at room temperature for 30 min in PBS-BSA containing 1 μg of rabbit anti-PA28γ antiserum (AFFINITI)/ml and mouse anti-HA antibody. Cells were washed three times with PBS-BSA and incubated at room temperature for 30 min in PBS-BSA containing 0.5 μg of Alexa Fluor 488-conjugated anti-rabbit immunoglobulin G (IgG) and Alexa Fluor 594-conjugated anti-mouse IgG antisera (Molecular Probes)/ml. After being washed three times with PBS-BSA, all samples were visualized with a Bio-Rad (Tokyo, Japan) confocal laser-scanning microscope.

Mouse embryonic fibroblasts isolated from PA28γ knockout mice. Embryonic fibroblasts from PA28γ knockout mice were prepared as previously described (42). Cells were cultured at 37°C (5% CO_2) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, sodium pyruvate, and nonessential amino acids. Plasmid vectors were transfected into cells after three passages, and the intracellular localization of proteins was examined.

Time-lapse microscopy. HeLa cells or mouse embryonic fibroblasts were seeded on 35-mm-diameter culture dishes, grown overnight to 70% confluence, and transfected with 2.5 μg of plasmid DNA with Lipofectamine 2000 per the manufacturer's instructions (Invitrogen Corp., Carlsbad, Calif.). For immunofluorescent microscopy, cells were incubated at 37°C overnight after transfection, trypsinized, reseeded, and cultivated for 20 h. For time-lapse microscopy, cells were seeded on 35-mm-diameter dishes, grown for 24 h after transfection, and incubated in Dulbecco's modified Eagle's medium containing 10% fetal calf serum at 37°C. Cells were viewed at 37°C (5% CO_2) with an Olympus (Tokyo, Japan) IX71 and Cool SNAP HQ charge-coupled device camera (Roper Scientific JAPAN, Tokyo, Japan). Digital images were analyzed with Metamorph software (Universal Imaging, Downingtown, Pa.).

Effect of MG132 on stability of the HCV core protein. To determine the effect of proteasome inhibitors on the degradation of HCV core proteins in the presence of overexpressed PA28γ, 2×10^5 293T cells were transfected with expression plasmids encoding the HCV core protein and PA28γ by lipofection on 35-mm-diameter plates. The proteasome inhibitor MG132 (Sigma) or solvent, dimethyl sulfoxide, was added as a 10^{-3} volume of medium into wells at 8 h posttransfection. Cells were harvested at 24 h posttransfection and lysed in lysis buffer as described above. Proteins were detected by Western blotting with mouse monoclonal anti-Flag, anti-HA, goat polyclonal anti-actin (Santa Cruz Biotechnology, Santa Cruz, Calif.) or rabbit polyclonal anti-PA28γ.

RESULTS

Isolation of PA28γ cDNA from human libraries. To determine the protein(s) that interact with HCV core protein in mammalian cells, we choose to employ a yeast two-hybrid system with the HCV core protein as bait. Human fetal brain and liver libraries were used for this screening because it is not known whether the target protein is specifically expressed in the liver. Many light blue colonies emerged on dropout plates, but these were eliminated from further screening so that proteins exhibiting strong binding could be examined more fully. Several clones exhibited dark blue color on a dropout plate containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside to an extent similar to that of the positive control containing p53 and large T antigen. No gene has been included which has previously been reported as a core-binding protein in the dark blue colonies, and we selected the darkest one. The total DNA was extracted from this clone and introduced into *E. coli* strain JM109 with the goal of recovering the pACT2 plasmid encoding the candidate core-binding protein. The nucleotide sequence of the DNA insert was determined from three independent colonies. The sequence isolated from the positive clone included the 5′ and 3′ noncoding regions as well as the full coding region of proteasome activator PA28γ; all sequences were in frame. There are two splicing variants of PA28γ in human tissue (3, 23). The isolated cDNA of PA28γ encoded the major isoform that is comprised of 254 amino acids; this isoform demonstrates 100% identity with mouse PA28γ based on amino acid sequence. The isolated pACT2 plasmid containing PA28γ cDNA was introduced into yeast strain AH109 together with either an empty bait plasmid, pGBKT7, or a plasmid encoding the HCV core protein, pGBKT7HCVCore173, in order to confirm that the isolated plasmid encodes an HCV core-binding protein. The yeast clone containing pACT2-PA28γ and pGBKT7HCVCore173 grew on a dropout plate deficient in leucine, tryptophan, histidine, and adenine, but the yeast clone containing pACT2-PA28γ and pGBKT7 did not (data not shown). These data suggest that PA28γ binds to the HCV core protein in yeast. The cDNAs of HCV core protein and its mutants were introduced into several mammalian expression vectors as shown in Fig. 1.

Interaction of the HCV core protein with PA28γ in mammalian cells, livers of HCV core transgenic mice, and a patient with chronic hepatitis C. Because it is generally known that many false-positive clones are identified by using the yeast two-hybrid system, protein-protein interaction and coincidence of intracellular localization between bait and prey proteins should be examined in mammalian cells. When Flag-tagged PA28γ (Flag-PA28γ) was coexpressed in 293T cells with HA-Core191, HA-Core173, HA-Core151, HA-Bad, or HA-FKBP, Flag-PA28γ was coprecipitated with HA-Core191, HA-Core173, and HA-Core151 but not with HA-Bad and HA-FKBP by mouse anti-HA antibody. The interaction of Flag-PA28γ with HA-Bad and HA-FKBP was not observed even though these constructs were expressed at a higher level than the HA-Core proteins (Fig. 2A). To eliminate the possibility of an artificial interaction of the HCV core protein with PA28γ due to overexpression, the association of HCV core proteins with endogenous PA28γ was examined. Endogenous PA28γ was coprecipitated with HCV core proteins in HA-Core-expressing 293T cells but not in nontransfected cell lysates (Fig. 2B).

Hepatic steatosis and hepatocellular carcinoma have been shown to be induced in transgenic mice expressing the HCV core protein; in this system, expression levels of the HCV core

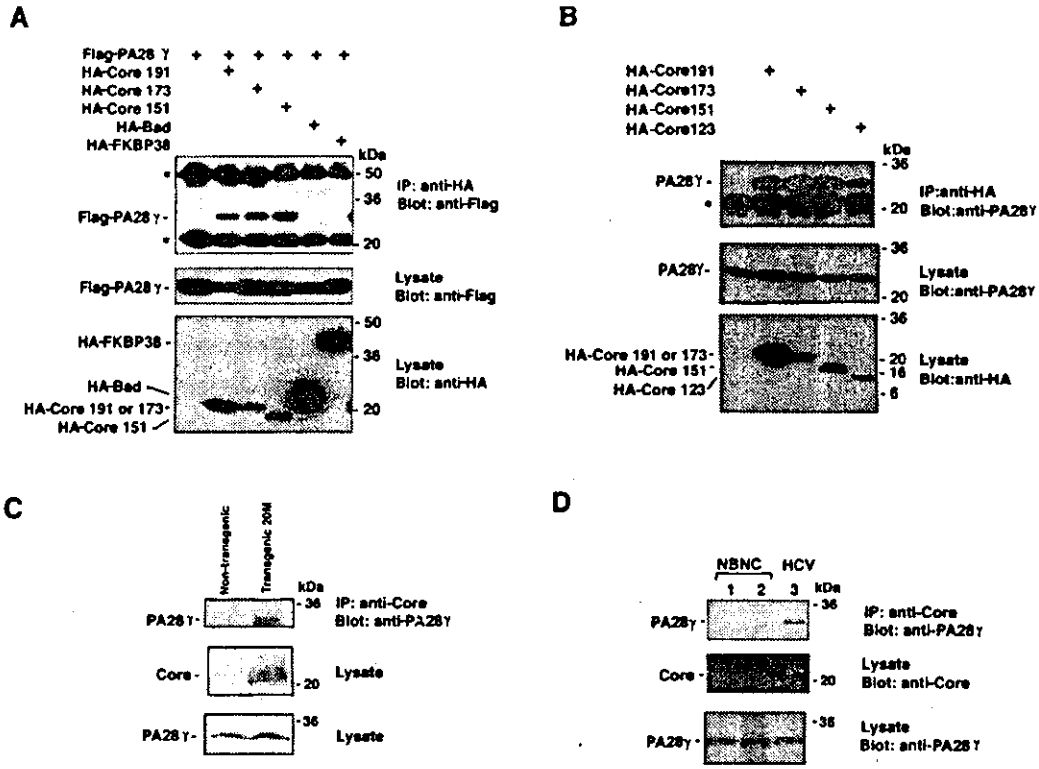

FIG. 2. Interaction of HCV core protein with PA28-γ. (A) Human embryonic kidney 293T cells were transfected with the expression plasmids encoding HCV core proteins and/or PA28-γ. A Flag epitope tag was added to PA28-γ at its amino terminus while an HA epitope tag was added to the HCV core protein and control proteins (Bad and FKBP38). PA28-γ was able to be coimmunoprecipitated with the HCV core protein by anti-HA antibody and was visualized by Western blotting with anti-Flag antibody. HA-Core 151 was expressed by using two times amount of plasmid DNA compared to HA-Core 191 and 173 because coexpression with PA28-γ decreased the amount of HA-Core 151. (B) 293T cells were transfected with the expression plasmids encoding HA-tagged HCV core proteins. Endogenous PA28-γ was coimmunoprecipitated with HCV core proteins and was detected by immunoblotting with anti-PA28-γ antiserum. Asterisks indicate IgG bands. (C) Liver homogenates of HCV core transgenic mice and nontransgenic mice were immunoprecipitated (IP) with anti-core antibody; endogenous PA28-γ was coprecipitated with HCV core protein and was detected by immunoblotting with anti-PA28-γ antibody. (D) Liver homogenates of non-B and non-C hepatitis patients (lanes 1 and 2) and a patient with chronic hepatitis C (lane 3) were immunoprecipitated with anti-core antibody. Endogenous PA28-γ was coimmunoprecipitated with HCV core protein and was detected by immunoblotting with rabbit anti-PA28-γ antiserum.

protein in mouse livers were similar to those in patients with chronic hepatitis C (25, 39). The amino acid sequence of human PA28-γ is identical to that of mouse PA28-γ (22, 23, 44). Liver tissue of HCV core transgenic and nontransgenic mice were homogenized in lysis buffer. Endogenous PA28-γ was coprecipitated with HCV core protein by anti-HCV core antibody in liver lysates of HCV core transgenic mice but not in those of nontransgenic mice (Fig. 2C), indicating that the HCV core protein specifically interacts with PA28-γ in the liver of core transgenic mice. To further confirm the specific interaction of the HCV core protein with endogenous PA28-γ, this interaction was examined in liver specimens from a patient with chronic hepatitis C infection (Fig. 2D). Endogenous PA28-γ was also coprecipitated with HCV core protein in liver lysates from this patient (Fig. 2D, lane 3), but not in patients with non-B and non-C hepatitis (Fig. 2D, lanes 1 and 2), by anti-HCV core antibody. These results indicate that the HCV core protein specifically binds to PA28-γ not only in mammalian cell lines but also in liver tissue.

Intracellular localization of the HCV core protein with PA28α, β, and γ. The nonessential, flexible loop region of PA28, termed the homologue-specific insert region, lies between the N terminus of the protein and the proteasome activation domain. This region does not show any homology with other PA28 isotypes (49). The nuclear localization signal (NLS) of PA28-γ encompasses amino acids 82 to 90 and lies within the homologous specific insert (amino acids 72 to 102); there is no NLS in PA28α and β. PA28-γ is primarily localized to the nucleus in mammalian cells through its NLS motif, but PA28α and β are predominantly found in the cytoplasm (6). Figure 3 shows the intracellular localization of the HCV core protein and endogenous PA28-γ and PA28α. HA-Core191 was predominantly detected in the cytoplasm and to a lesser extent in the nucleus or perinuclear region in HeLa cells. Conversely, HA-Core173 and HA-Core151 were primarily found in the nucleus with less cytoplasmic staining. Endogenous PA28-γ was visualized by indirect immunostaining with polyclonal rabbit anti-PA28-γ antiserum and was predominantly detected in the nucleus of HeLa cells irrespective of the expression of HCV core proteins. HA-Core191 was partially colocalized with PA28-γ in the nucleus. In contrast to these findings, a large proportion of HA-Core151 or 173 was found to be colocalized

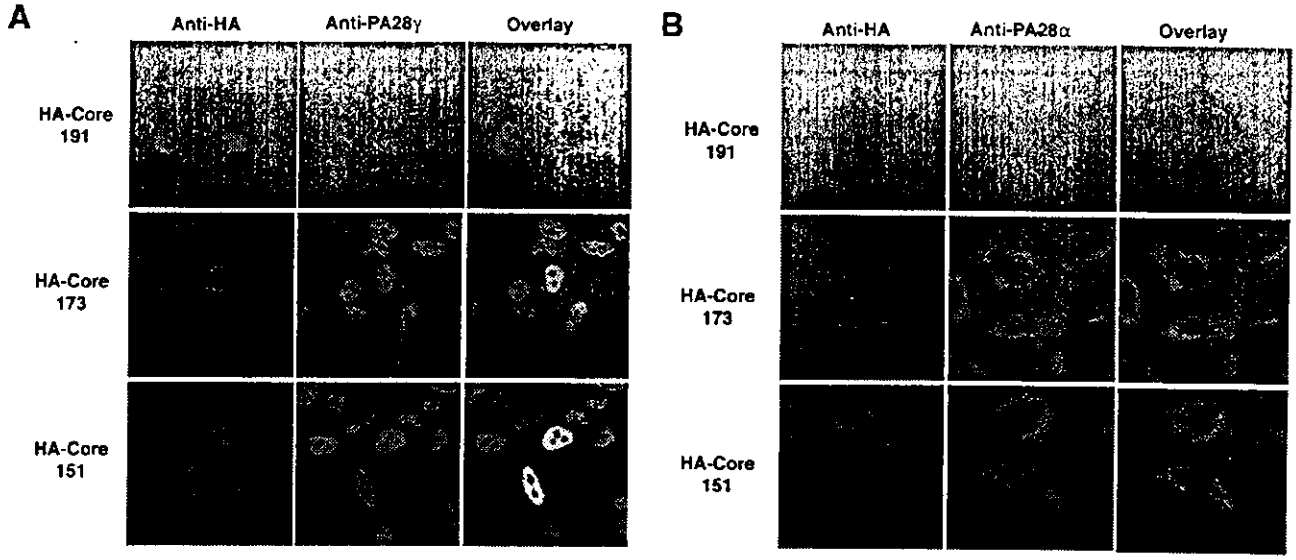

FIG. 3. Intracellular localization of HCV core protein with PA28α and γ. HeLa cells were transfected with plasmids encoding HA-Core proteins and then fixed with paraformaldehyde. The HCV core protein was visualized by indirect immunostaining with anti-HA antibody. Endogenous PA28γ and α were visualized by indirect immunostaining with rabbit anti-PA28γ (A) and anti-PA28α (B) antisera, respectively. All samples were observed with a confocal microscope.

with PA28γ in the nucleus. PA28α and β share 41.3 and 33.6% homology to PA28γ, respectively. A heteroheptamer of PA28α and β binds to the 20S proteasome in the cytoplasm to activate the peptidase activity of this proteasome (1). Endogenous PA28α was predominantly detected in the cytoplasm and, to a lesser extent, in the nucleus. When HA-Core191 was expressed in HeLa cells, it was mainly localized to the cytoplasm, but it did not colocalize with PA28α. When HA-Core151 and 173 were expressed in HeLa cells, endogenous PA28α was not translocated from the cytoplasm to the nucleus, and no colocalization with HCV core proteins was observed. Similar results were also obtained in 293T cells (data not shown). Endogenous PA28α was not able to be coimmunoprecipitated with Flag-HCV Core191 in 293T cells. Endogenous PA28γ, however, was clearly coprecipitated with the core protein (Fig. 4). Endogenous PA28β was not colocalized with HCV core proteins in HeLa cells by indirect immunostaining (data not shown). These data indicate that the HCV core protein interacts with PA28γ but not with PA28α and β.

Intracellular localization of *Flaviviridae* core proteins with PA28γ. The interaction of the HCV core protein with PA28γ was demonstrated by coimmunoprecipitation, and the colocalization of these proteins was examined by immunostaining. It was still unknown, however, whether the HCV core protein interacts with PA28γ under living cell conditions. Since the nuclear localization of PA28γ is dependent on a c-Myc-like NLS, deletion of the NLS in PA28γ should shift its localization into the cytoplasm. When PA28γ was fused to the C terminus of the red fluorescence protein (DsRed) (DsRed-PA28γ) and coexpressed with EGFP-Core151 in HeLa cells (Fig. 1), EGFP-Core151 colocalized with DsRed-PA28γ in the nucleus (Fig. 5A, upper panels). In the presence of DsRed-PA28γ lacking the NLS (DsRed-PA28γΔNLS), however, EGFP-Core151 was predominantly detected in the cytoplasm and was colocalized with DsRed-PA28γΔNLS (Fig. 5A, lower panels). The detection of EGFP-Core151 in the nucleus of cells overexpressing DsRed-PA28γΔNLS was probably due to the interaction of the core protein with endogenous PA28γ in the nucleus. The cytoplasmic localization of EGFP-Core151 was also detected with DsRed-PA28γΔNLS in 293T cells (data not shown). These data indicate that the HCV core protein binds to PA28γ in living cells.

DEN and JEV are both members of the *Flaviviridae* family, which also includes HCV (35, 50). The HCV core protein shares 22 and 30% homology with the DEN and JEV core proteins within the N-terminal 50 amino acids, respectively. Also similar to HCV, the core proteins of DEN and JEV are basic. The EGFP-fused JEV core protein lacking the C-termi-

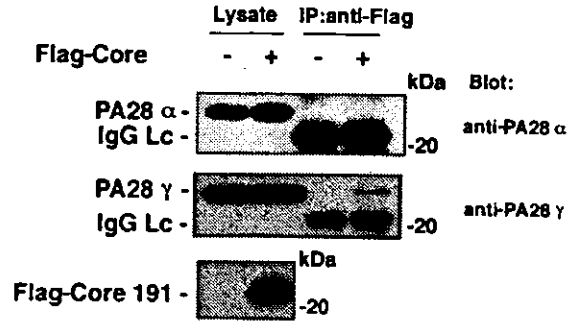

FIG. 4. HCV core protein does not bind to PA28α. After overnight cultivation, 293T cells were transfected with the expression plasmid encoding Flag-HCV Core191. Immunoprecipitation (IP) was performed as described in Materials and Methods. Endogenous PA28α and PA28γ coprecipitated with HCV core protein were stained with anti-PA28α and anti-PA28γ antisera, respectively. Lc, light chain.

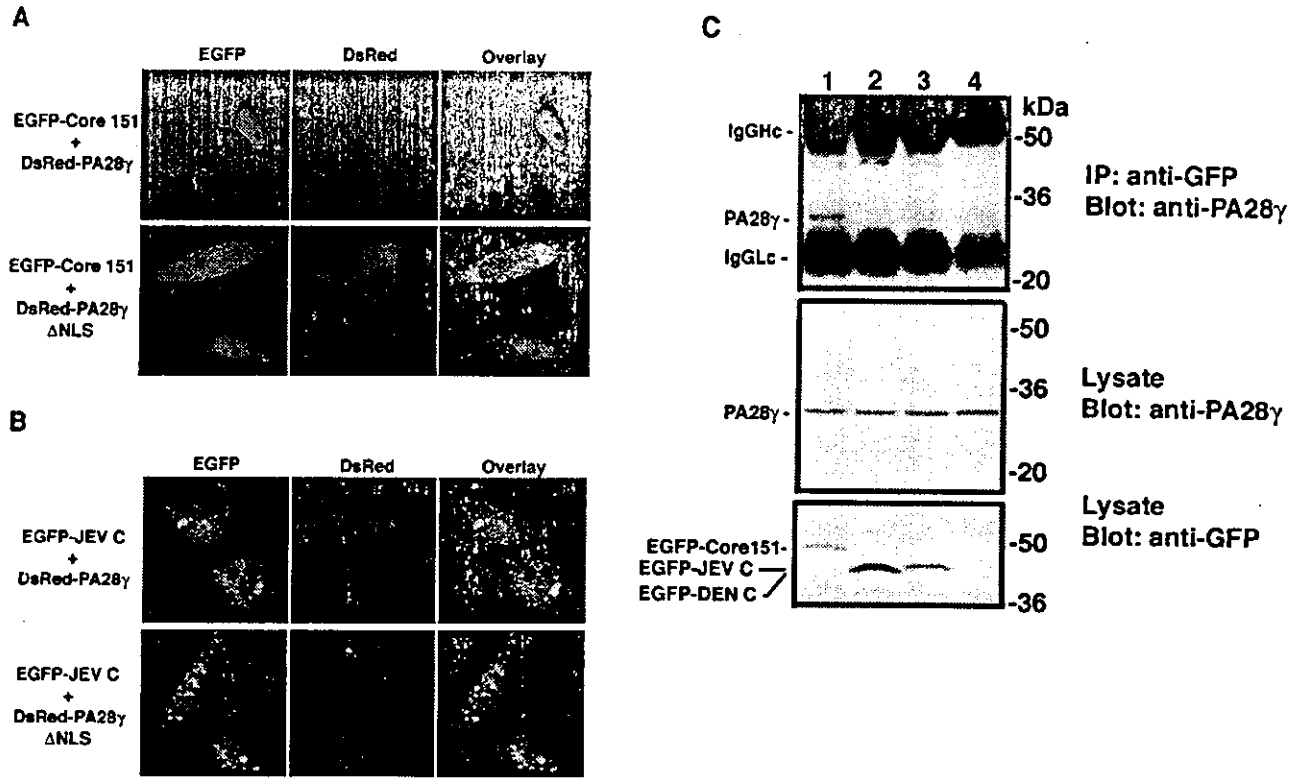

FIG. 5. Interaction of *Flaviviridae* core proteins with PA28γ. HeLa cells were transfected with the expression plasmids encoding DsRed-PA28γ (upper panels) or DsRed-PA28γΔNLS (lower panels) together with EGFP-Core151 (A) or EGFP-JEV C (B). All samples were observed with a confocal microscope. (C) The cells were transfected with the expression plasmid encoding EGFP-Core151 (lane 1), EGFP-DEN C (lane 2), or EGFP-JEV C (lane 3) and then harvested at 36 h posttransfection. EGFP fusion proteins were precipitated with anti-GFP antibody. Endogenous PA28γ was coimmunoprecipitated with anti-GFP antibody and then was visualized by immunoblotting with anti-PA28γ antiserum. The untransfected cells were used as a negative control (lane 4). IP, immunoprecipitation; Hc, heavy chain; Lc, light chain.

nal hydrophobic region (EGFP-JEV C) can be visualized in both the cytoplasm and nucleus (Fig. 5B, upper panels). The intracellular localization of EGFP-JEV C was quite distinct from that of DsRed-PA28γ, and coexpression with DsRed-PA28γΔNLS did not affect the subcellular localization of the protein (Fig. 5B, lower panels). Similar results were obtained by coexpression of the EGFP-fused DEN core protein lacking the C-terminal hydrophobic region (EGFP-DEN C). EGFP-DEN C was not colocalized with DsRed-PA28γ and was not affected by expression of DsRed-PA28γΔNLS (data not shown). Endogenous PA28γ was coprecipitated with EGFP-Core151 by anti-GFP antibody but not with EGFP-DEN C or EGFP-JEV C (Fig. 5C). These data suggest that PA28γ specifically interacts with the HCV core protein but not with DEN and JEV core proteins in living cells.

Mapping of the PA28γ-binding region of the HCV core protein. To determine the region of the HCV core protein responsible for PA28γ binding, the interactions of PA28γ with deletion mutants of the HCV core protein were examined. When Flag-Core mutants (Fig. 1) were expressed in 293T cells, endogenous PA28γ was coimmunoprecipitated with Flag-Core191, Flag-Core24-191, and Flag-Core38-191 by anti-Flag antibody but not with Flag-Core72-191 and Flag-Core92-191; the levels of protein expression were the same for all constructs (Fig. 6A). Conversely, Flag-Core191, Flag-Core24-191, and Flag-Core38-191, but not Flag-Core72-191 and Flag-Core92-191, were coprecipitated with endogenous PA28γ by anti-PA28γ antibody. These results indicate that the N-terminal 37 amino acids of the HCV core protein are not involved in the interaction with PA28γ. Because HA-Core151 was shown to interact with PA28γ (Fig. 2A) and localized to the nucleus (Fig. 3A), we examined the effect of deletion the N-terminal amino acids on the localization of Core-151 in living cells by using EGFP-Core151 (Fig. 5A). EGFP-Core24-151 and EGFP-Core38-151 were localized entirely within the nucleus, and EGFP-Core72-151 and EGFP-Core92-151 were predominantly localized in the cytoplasm (Fig. 6B). These results give rise to the question of whether amino acids 38 to 71 of the HCV core protein might be involved in the interaction with PA28γ and in the nuclear localization of the HCV core protein. To determine the precise region of the HCV core protein responsible for binding with PA28γ, we constructed additional mutant core proteins, EGFP-Core38-43 and EGFP-Core44-71 (Fig. 1). EGFP-Core44-71 was primarily localized to the nucleus, but EGFP-Core38-43 displayed a diffuse cellular staining similar to that of EGFP alone (Fig. 6B). EGFP-Core44-71, but not EGFP-Core38-43, was coprecipitated with endogenous PA28γ by rabbit anti-GFP antiserum in 293T cells (Fig. 6C). These results suggest that a cluster of amino acids from 44 to

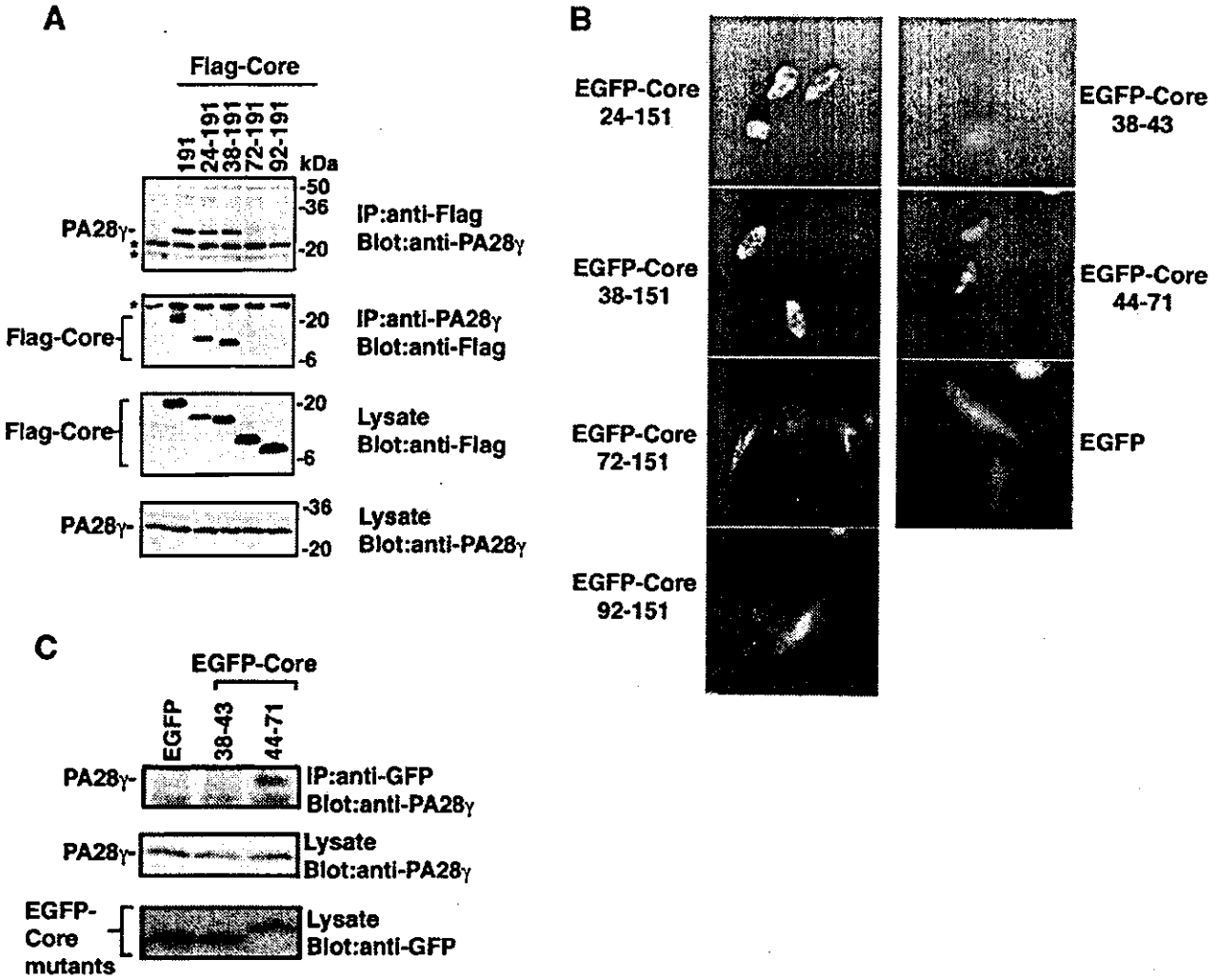

FIG. 6. Mapping of the PA28γ-binding region in HCV core protein. (A) 293T cells were transfected with plasmids encoding Flag-tagged HCV core deletion mutants. Endogenous PA28γ was coimmunoprecipitated with an anti-Flag antibody and was visualized by immunoblotting with anti-PA28γ antiserum. (B) Intracellular localization of EGFP-Core deletion mutants in 293T cells. All samples were observed at 24 h posttransfection. (C) EGFP-tagged HCV core deletion mutants were expressed in 293T cells. Endogenous PA28γ was coimmunoprecipitated with anti-GFP antiserum and then was visualized by immunoblotting with anti-PA28γ antiserum. IP, immunoprecipitation.

71 in the HCV core protein is responsible for both its interaction with PA28γ and its nuclear localization.

Deletion of the PA28γ-binding region or knockout of PA28γ leads to export of the HCV core protein from nucleus to cytoplasm. To determine whether the PA28γ-binding region identified in HCV core protein amino acids 44 to 71 functioned as anNLS, the localization of a deletion mutant lacking amino acids 44 to 71 was determined (Fig. 7A). EGFP-Core151 was detected in the nucleus of HeLa cells and retained there until at least 48 h posttransfection. Conversely, EGFP-Core151Δ44-71 (Fig. 1) was detected in the nucleus at 3 h posttransfection and gradually translocated into the cytoplasm. Most of the EGFP-Core151Δ44-71 was detected in the cytoplasm at 24 h posttransfection. These results indicate that HCV core protein amino acids 44 to 71 have a function in both PA28γ binding and nuclear retention. To further confirm this observation, we examined embryonic fibroblasts derived from PA28γ knockout mice (2) (Fig. 7B). When EGFP-Core151 was expressed in PA28γ$^{+/-}$or PA28γ$^{-/-}$mouse embryonic fibroblasts, EGFP-Core151 was localized to the nucleus at 24 h posttransfection, irrespective of PA28γ expression. EGFP-Core151 was retained in the nucleus of PA28γ$^{+/-}$mouse embryonic fibroblasts until 42 h posttransfection, when cell death was induced (Fig. 7B, left panels). In PA28γ$^{-/-}$fibroblasts, however, EGFP-Core151 was exported to the cytoplasm at 27 h posttransfection and no cell damage was observed until 44 h posttransfection (Fig. 7B, right panels). These data clearly indicate that an interaction with PA28γ is essential for the nuclear retention of the HCV core protein.

Degradation of HCV core protein via PA28γ-dependent pathway. It was previously reported that HCV core proteins truncated at the C termini (HCV Core151 and 173), although

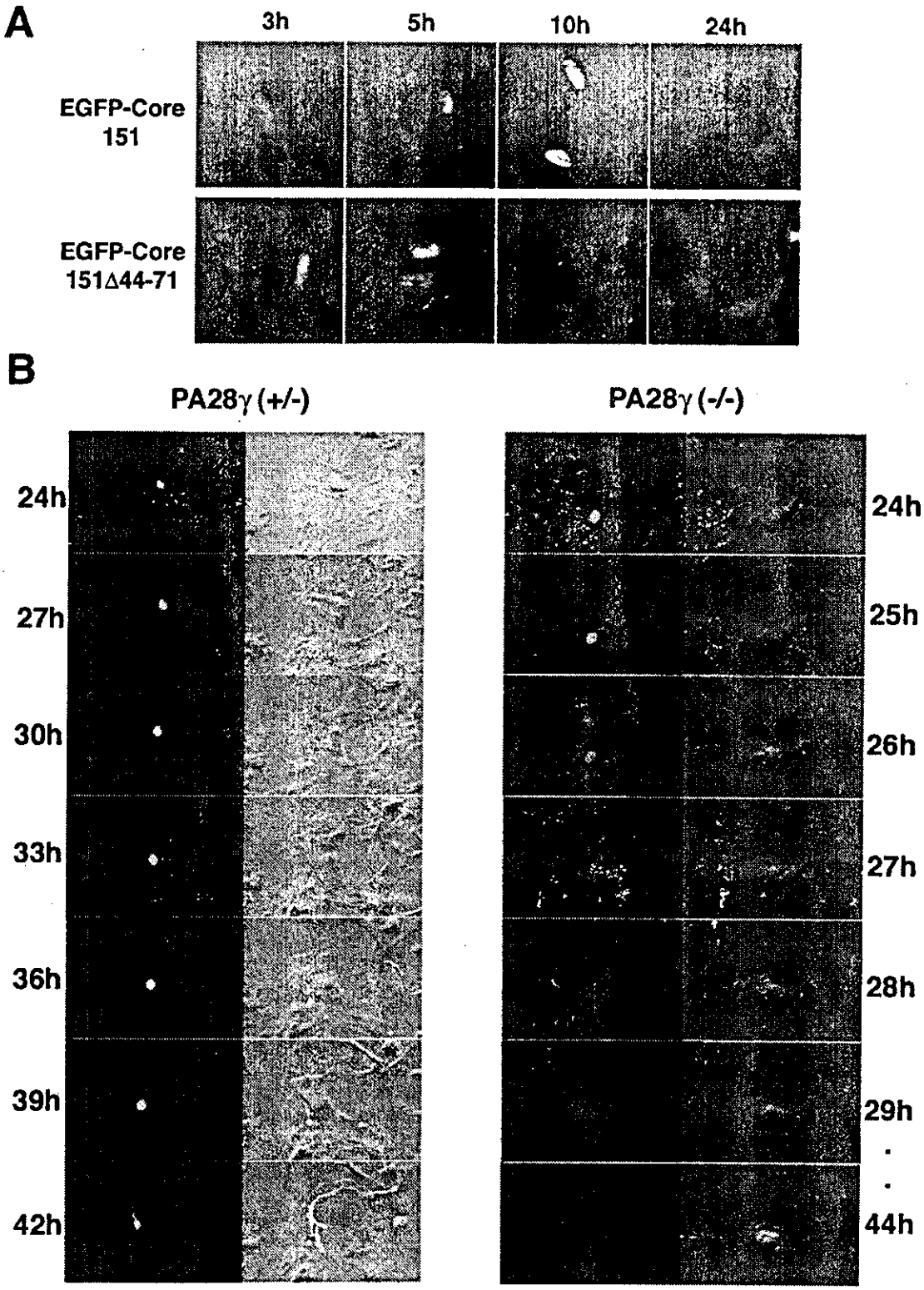

FIG. 7. Intracellular localization of HCV core protein. (A) EGFP-Core151 and EGFP-Core151Δ44-71 were expressed in HeLa cells. The intracellular localization of EGFP-Core151 (upper panels) or EGFP-Core151Δ44-71 (lower panels) was observed at 3, 5, 10, and 24 h posttransfection. (B) Intracellular localization of EGFP-Core 151 in embryonic fibroblasts prepared from a PA28$\gamma^{+/-}$ mouse (left) or from a PA28$\gamma^{-/-}$ mouse (right) was examined from 24 to 42 h posttransfection by time-lapse microscopy.

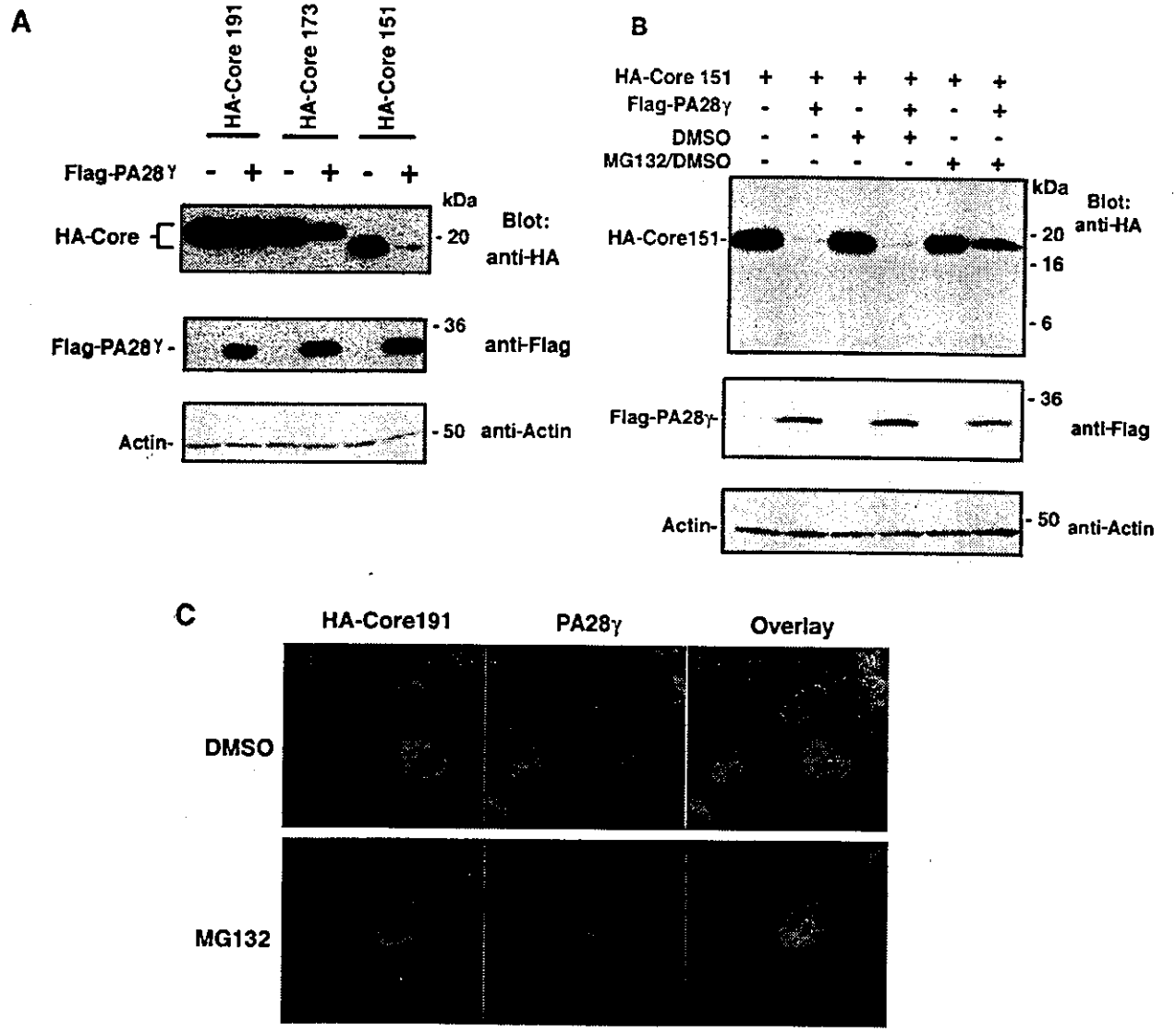

FIG. 8. The proteasome inhibitor MG132 blocks degradation of the HCV core protein. (A) 293T cells were transfected with the expression plasmids encoding HA-Core 191, 173, or 151 together with an empty plasmid or a plasmid encoding Flag-PA28γ. Cell lysates were analyzed by immunoblotting with anti-HA, anti-Flag, or anti-actin antibodies. (B) 293T cells were transfected with an expression plasmid encoding HA-Core 151 with either an empty plasmid or a plasmid encoding Flag-PA28γ. Cells were treated with either MG132 in dimethyl sulfoxide (DMSO) or dimethyl sulfoxide alone as a control where indicated. (C) HeLa cells transfected with plasmid encoding HA-Core191 were treated with 30 μM MG132 at 10 h posttransfection and then fixed with paraformaldehyde at 24 h posttransfection. Endogenous PA28γ and HA-Core were stained with rabbit anti-PA28γ antiserum and mouse anti-HA antibody, respectively. All samples were observed with a confocal microscope. +, present; −, absent.

normally rapidly degraded, were able to be detected after the addition of a proteasome inhibitor (57). To determine the effect of PA28γ expression on the stability of HCV core protein, HA-Core191, HA-Core173, or HA-Core151 was coexpressed with Flag-PA28γ in 293T cells. The amounts of HA-Core173 and HA-Core151 were decreased by overexpression of Flag-PA28γ, but expression levels of HA-Core191 were unchanged (Fig. 8A). Degradation of HA-Core151 by PA28γ overexpression was eliminated by the addition of the proteasome inhibitor MG132 (Fig. 8B), thus suggesting that nucleus-localized HCV core protein undergoes degradation by the proteasome in a PA28γ-dependent manner. To confirm the nuclear localization and degradation of the processed HCV core proteins derived from HA-Core191, MG132 was added to HeLa cells transfected with the plasmid encoding HA-Core191 (Fig. 8C). Treatment with MG132 enhanced the expression of HCV core protein colocalized with endogenous PA28γ in the nucleus of HeLa cells expressing HA-Core191. F protein was generated by the −2/+1 ribosomal frameshift in the gene encoding HCV core protein (64). The expected molecular mass of the F protein of the J1 strain is about 14 kDa. Endogenous PA28γ was coprecipitated by anti-Flag antibody with Flag-