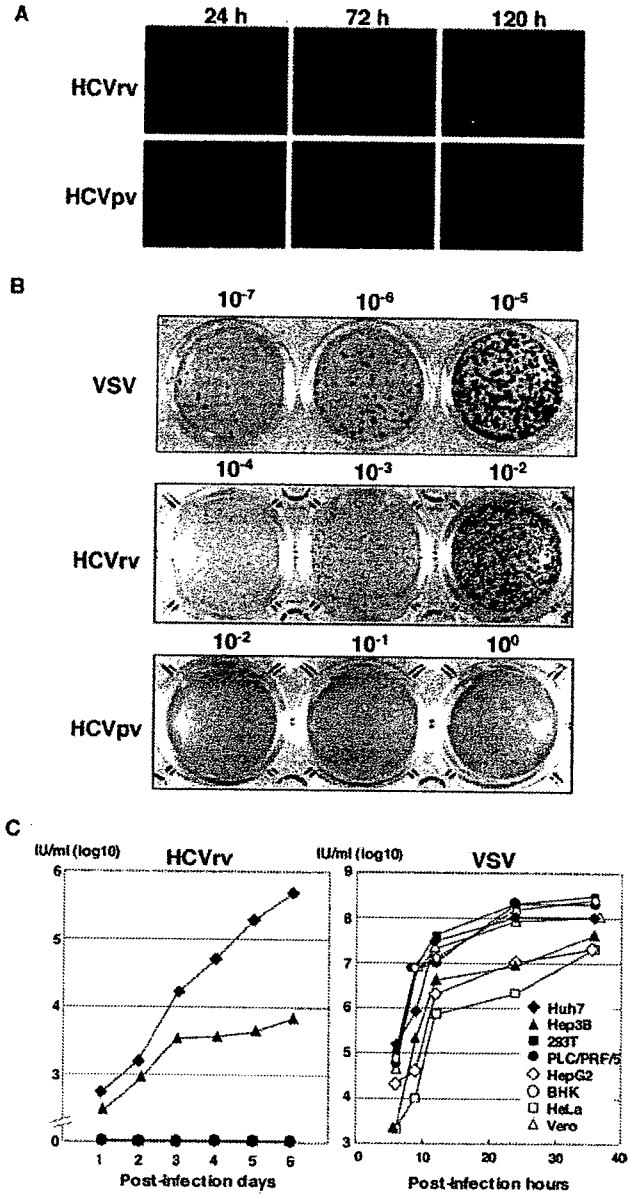

FIG. 4. Propagation of HCVrv. (A) Detection of viral proteins in Huh7 cells infected with HCVpv or HCVrv. Huh7 cells were infected with HCVpv or HCVrv at an MOI of 0.01. Twenty-four, 72, and 120 h after infection, cells were fixed and stained with monoclonal antibody to VSV N protein and Alexa 488-conjugated secondary antibody. Cell nuclei were stained by Hoechst 33258. Pictures were taken using a fluorescence microscope by double exposure of the same fields with filters for Alexa 488 or Hoechst 33258. (B) Focus formation of HCVpv, HCVrv, or VSV in Huh7 cells. Huh7 cells were infected with serial 10-fold dilutions of HCVpv, HCVrv, or VSV and incubated at 30°C for 72 h for HCVpv and HCVrv or 24 h for VSV in a culture medium containing 0.8% methylcellulose. Foci of infected cells were detected by immunohistochemical staining. (C) Kinetics of HCVrv (left) and VSV (right) propagation in various cell lines. HCVrv and VSV generated in Huh7 cells were used to infect cells at an MOI of 0.01. The culture supernatant was collected at the indicated time points and titrated by a focus-formation assay. Infectious titers are expressed in IU/milliliter.

the expression of hCD81 in HepG2 cells (HepCD81) (Fig. 5A and D) rendered them permissive to infection with all of the viruses. Furthermore, Hc were not susceptible to the infection with HCVpv, HCVrv, or HCVpp, despite the expression of hCD81. These results suggest that expression of hCD81 is essential for the infection with HCVpv and HCVrv, as reported for infection with HCVpp and HCVcc, but conditions with a lack of hCD81 are insufficient for the infection with HCVpv, HCVrv, and HCVpp.

Neutralization of HCVpv and HCVrv infection by antibodies to HCV envelope proteins and sera of HCV patients. It has been reported that HCVpp can be neutralized by several well-characterized E2-specific monoclonal and polyclonal antibodies (5, 23, 49). The neutralization activity of anti-E1 (AP21.010) and anti-E2 (AP33) monoclonal antibodies (49) and anti-E1 (R852) and anti-E2 (R646) rabbit polyclonal antibodies raised against the E1 and E2 proteins of the H77 strain on the infection with HCVpv and HCVrv was determined (Fig. 6A). The infections with both HCVpv and HCVrv bearing E1 and E2 proteins of the H77 strain were clearly inhibited by anti-E2 (AP33) antibody or anti-E2 (R646) rabbit serum, consistent with a previous report on the effect of these antibodies on HCVpp infection (49), whereas no neutralization by AP21.010 and R852 antibodies was observed. The infections with HCVpv and HCVrv bearing E1 and E2 proteins of the Con1 strain were also inhibited by AP33 and R646 antibodies (data not shown), suggesting that the infectivity of HCVpv and HCVrv was cross-neutralized by anti-E2 antibody, as reported for HCVpp (49). These results indicate that the E2 protein plays a crucial role in the infectivity of both HCVpv and HCVrv. Although the addition of naive human sera (HD) inhibited infection with VSVpv, infection with HCVpv or HCVrv was clearly enhanced, as reported for HCVpp infection of Huh7 cells (28, 42). To assess the neutralization ability of these antibodies in patients, HCVpv and HCVrv were incubated with a 2% concentration of the sera of chronic HCV patients infected with genotype 1b HCV (Fig. 6B). All of the sera of patients of genotype 1b showed high levels of neutralization activity against infection with HCVpv and HCVrv bearing envelope proteins of genotype 1a, whereas they had no effect on the infectivity of VSVpv, in contrast to the inhibition achieved by the naïve sera. These results indicate that HCV patients elicit high levels of antibodies that are likely to cross-neutralize the infectivity of HCVpv and HCVrv.

Inhibition of HCVpv and HCVrv infection by bafilomycin A_1. Enveloped viruses enter target cells through two different pathways: one is a pH-independent direct fusion at the plasma membrane, and the other is a pH-dependent receptor-mediated endocytosis (58). Previous studies have revealed that both HCVpp and HCVcc were sensitive to the inhibitors of vacuolar acidification, such as ammonium chloride, concanamycin A, or bafilomycin A_1, suggesting that these viruses enter via a pH-dependent endocytosis into target cells (23, 61). To determine the entry pathway of HCVpv and HCVrv, Huh7 cells were pretreated with various concentrations of bafilomycin A_1, and then the cells were inoculated with HCVpv, HCVrv, VSVpv, and MLVpv (Fig. 7). As expected, the treatment did not affect the infection with MLVpv bearing an envelope protein of MLV that enters cells via a pH-independent pathway. In contrast, infection with VSVpv bearing the G protein of VSV,

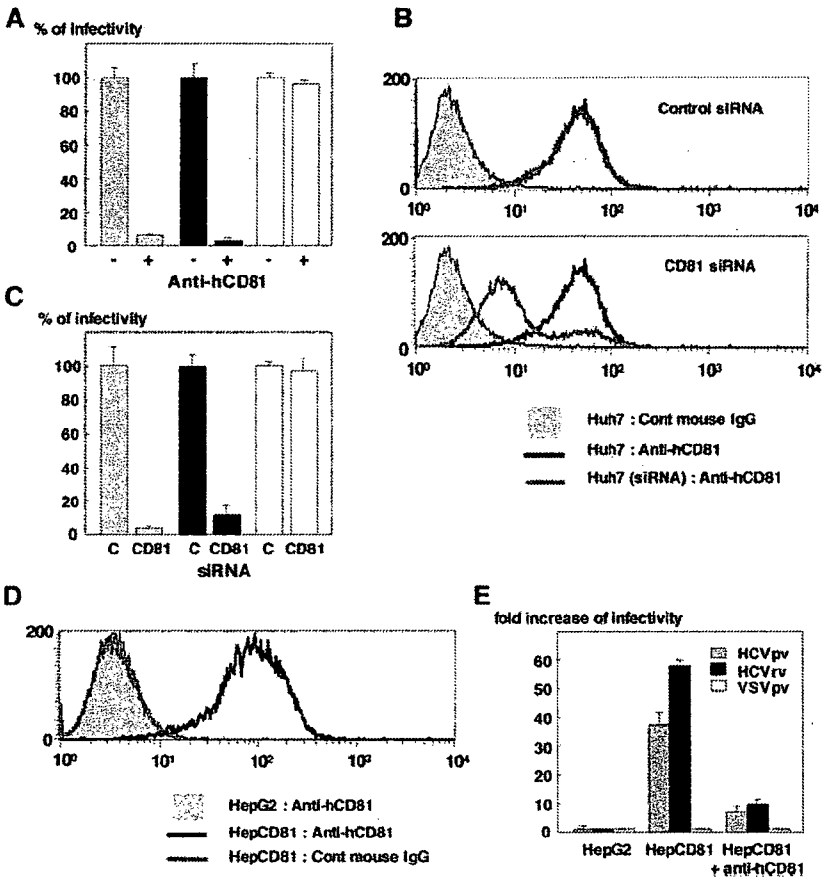

FIG. 5. Involvement of hCD81 in the infection of HCVpv and HCVrv. (A) Effect of anti-hCD81 antibody on the infectivity of HCVpv (gray-filled bars), HCVrv (black-filled bars), or VSVpv (open bars) in Huh7 cells. (B) Cell surface expression of hCD81 on Huh7 cells transfected with siRNA targeted to hCD81 or control siRNA was examined by FACS analysis after staining with anti-hCD81 antibody. (C) Effect of knockdown of hCD81 in Huh7 cells by siRNA targeted to hCD81 on the infection of HCVpv, HCVrv, or VSVpv. (D) Cell surface expression of hCD81 on HepG2 and HepCD81 cells was examined by FACS analysis after staining with anti-hCD81 antibody. (E) Infectivity of HCVpv, HCVrv, or VSVpv to HepG2 or HepCD81 cells and the effect of anti-hCD81 antibody on the infection of the viruses to HepCD81 cells. The results shown are from three independent assays, with the error bars representing the standard deviations.

which enters cells through pH-dependent endocytosis, was inhibited by the treatment with bafilomycin A_1 in a dose-dependent manner. Infection with HCVpv and HCVrv was also clearly inhibited by the treatment with bafilomycin A_1 in a dose-dependent manner, as with VSVpv. This suggests that low pH exposure is essential for the entry of HCVpv and HCVrv.

Effects of ER α-glucosidase inhibitors on HCVrv infection. Previous studies have shown that deoxynojirimycin (DNJ) and Nn-DNJ, a long-alkyl-chain iminosugar derivative of DNJ, inhibit the infection of flaviviruses such as Japanese encephalitis virus (JEV) and dengue virus in a dose-dependent manner (15, 64). Although the effects of glycosylation inhibitors on the folding and assembly of HCV envelope proteins in the N-glycosylation steps and the binding properties of HCV-LP produced in insect cells have been reported (11, 12), glycobiological analyses of HCV envelope proteins involved in virus infectivity have not been reported yet. To determine the effects of the inhibitor of Golgi mannosidase (DMJ) and of ER α-glucosidase (Nn-DNJ) on the infectivity of HCVrv, Huh7 cells were treated with these inhibitors. Treatment of Huh7 cells with Nn-DNJ but not with DMJ reduced the infectivity of HCVrv in a dose-dependent manner, and this reduction was more efficient than that in the infectivity of VSV (Fig. 8A, top). Although immunoblotting and Coomassie staining of the particles revealed that incorporation of the envelope proteins and generation of HCVrv and VSV particles recovered from cells treated with 100 μM of Nn-DNJ were severely impaired by the cytotoxic effects of Nn-DNJ (Fig. 8A, bottom left), treatment with 10 μM of Nn-DNJ selectively reduced the infectivity of HCVrv but not of VSV without any cytotoxic effect (Fig. 8A, top left). In contrast, Huh7 cells treated with more than 0.5 mM of DMJ exhibited a slight reduction of molecular sizes of E1 or VSVG proteins incorporated into the particles (Fig. 8A, bottom right); no effect on the incorporation of the envelope proteins into the viral particles and the infectivity was observed (Fig. 8A, top right). Next, we assessed the effects of the inhibitors on the propagation of the viruses. Focus formation of HCVrv was also inhibited by the treatment with Nn-DNJ but not with DMJ (Fig. 8B). To further confirm the effect of modification of the envelope glycoproteins by ER α-glucosidase on the infectivity of HCV, Huh7.5.1 cells were treated with the

TABLE 1. Infectivity of HCVpv, HCVrv, or HCVpp in various cells

Target cells	Cell surface expression of[a]:		Virus, producer cells, and strain (genotype)[b]								HCVpp virus produced in 293T cells and of strain H77 (genotype 1a)[b]
			HCVpv				HCVrv				
			293T		Huh7		293T		Huh7		
	hCD81	SR-BI	H77 (1a)	Con1 (1b)	H77 (1a)	Con1 (1b)	H77 (1a)	Con1 (1b)	H77 (1a)	Con1 (1b)	
Huh7	++	++	+++	+++	++	++	+++	+++	+++	+++	+++
HepG2	−	++	−	−	−	−	−	−	−	−	−
HepCD81	++	++	++	++	+	+	+++	+++	+++	+++	++
Hep3B	++	+	++	++	+	+	+++	+++	+++	+++	++
PLC/PRF/5	++	+	+	+	−	−	+	+	+	+	−
FLC4	−	++	−	−	−	−	−	−	−	−	−
Hc	++	−	−	−	−	−	−	−	−	−	−
HeLa	+	+	−	−	−	−	−	−	−	−	−
293T	++	+	+	+	−	−	+	+	+	+	−
Vero	−	−	+	+	−	−	+	+	+	+	−
BHK	−	−	−	−	−	−	−	−	−	−	−
CHOK1	−	−	−	−	−	−	−	−	−	−	−
CHOCD81	++	−	−	−	−	−	−	−	−	−	−

[a] Cell surface expression of receptor candidates was examined by FACS analyses with specific antibodies. Mean fluorescence intensity shifts of less than 1, between 1 and 2, and between 2 and 3 are indicated as −, +, and ++, respectively.

[b] Infectious titers higher than 5×10^4 IU/ml, between 5×10^3 and 5×10^4 IU/ml, between 5×10^2 and 5×10^3 IU/ml, and lower than 5×10^2 IU/ml are indicated as +++, ++, +, and −, respectively. The results were derived from at least three independent experiments, and the standard deviations did not exceed 30% of the mean values.

inhibitors, and infectivity of HCVcc was determined (Fig. 8C, top). Treatment with Nn-DNJ clearly inhibited the infection with HCVcc in a dose-dependent manner, as it did the infection with HCVrv. Focus formation of HCVcc was also inhibited by the treatment of Huh7.5.1 cells with Nn-DNJ (Fig. 8C, bottom). These results indicate that modification of the glycans of HCV E1 and E2 proteins in the ER by α-glucosidase rather than that in the Golgi is crucial for the infectivity of both HCVrv and HCVcc.

DISCUSSION

In general, enveloped viruses attach to host target cells and enter into cells through the interaction between viral envelope proteins and cell surface receptors and coreceptors. Due to the lack of a robust cell culture system to support the replication of various HCV genotypes, surrogate systems have been developed to examine the mechanisms of HCV infection. Although in vitro binding assays have identified several candidate receptors for HCV (4), the final determination of a true entry receptor or coreceptor capable of internalizing HCV particles has to be made by an infection assay. Toward this end, pseudotype virus systems based on VSV (27, 39) and retroviruses (5, 23) have been established. Both VSV and retroviruses normally bud from the plasma membrane, and therefore foreign envelope proteins expressed on the cell surface have been believed to incorporate into the pseudotype particles. HCV E1 and E2 proteins form heterodimers that have static ER retention signals in their C-terminal transmembrane region (17) and pulse-chase experiments and endoglycanase treatment of the intracellular forms of the proteins or those incorporated into the HCVpp have revealed that only a small fraction of the HCV envelope proteins are translocated to the plasma membrane and modified to the complex-type glycans (48). In addition, it was demonstrated that recruitment of the foreign envelope proteins by MLV and the lentivirus core protein does not occur at the cell surface but takes place intracellularly in the endosomal pathway (55, 56). Production of pseudotype VSVs bearing unmodified envelope glycoproteins of bunyaviruses has also been reported, in spite of the static retention of the envelope glycoproteins in the intracellular compartment and the lack of translocation into the plasma membrane (46). Therefore, cell surface expression of HCV envelope glycoproteins may not necessarily be a prerequisite for generation of pseudotype particles based on VSV or retroviruses.

Recombinant VSV encoding foreign viral envelope proteins in place of the G protein has been shown to be a powerful tool for the investigation of viral infection and the development of vaccines for diseases caused by infection with viruses such as influenza virus, human immunodeficiency virus, respiratory syncytial virus, human papillomavirus, and filoviruses (20, 31). Although recombinant VSV encoding HCV envelope proteins has been generated as a surrogate model for HCV infection and a vaccine vector (9, 35), recombinant VSV generated in rodent cells possessing the chimeric E1 and/or E2 proteins has been shown to be noninfectious in a human hepatoma cell line that is susceptible to HCVpp infection (9). In this study, we successfully generated infectious recombinant and pseudotype VSVs incorporating unmodified E1 and E2 proteins in hepatic and nonhepatic human cell lines. The previously observed lack of infectivity of the recombinant VSV carrying the chimeric HCV envelope proteins might be attributable to the production of viral particles in rodent (BHK) cells (9), because in this study the HCVrv generated in BHK cells exhibited no infectivity in the target cells in spite of a sufficient amount of incorporation of the HCV envelope proteins. These results suggest that posttranslational modification or host factor(s) specific to human cells might be involved in the endowment of infectivity of recombinant VSVs. Furthermore, HCVrv can be produced in various cell lines upon infection with the G-complemented particles, which are known to exhibit infectivity in several cell lines, in contrast to the pseudotype viruses, infec-

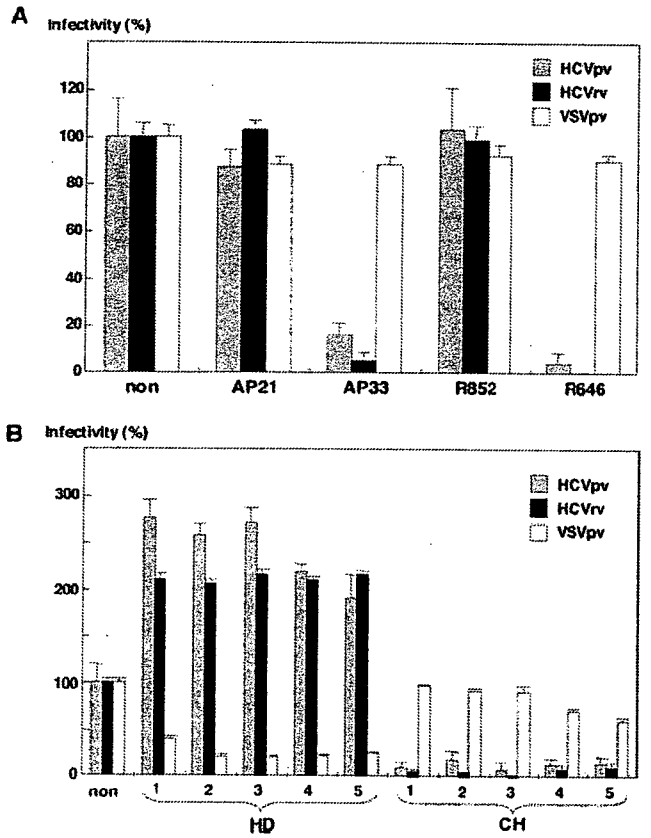

FIG. 6. Neutralization of HCVpv and HCVrv infection by antibodies to HCV envelope proteins and sera of HCV patients. (A) Effect of anti-E1 (AP21.010) and anti-E2 (AP33) monoclonal antibodies and anti-E1 (R852) and anti-E2 (R646) rabbit sera on the infectivity of HCVpv (gray-filled bars), HCVrv (black-filled bars), or VSVpv (open bars) to Huh7 cells. The viruses were preincubated for 1 h at room temperature with the antibodies before infection of Huh7 cells. (B) Effects of human sera from healthy donors and HCV patients on the infection of HCVpv, HCVrv, or VSVpv. The viruses were preincubated for 1 h at room temperature with five different healthy human sera (HD) or chronic HCV patient sera (CH) diluted 1:50 before infection of Huh7 cells.

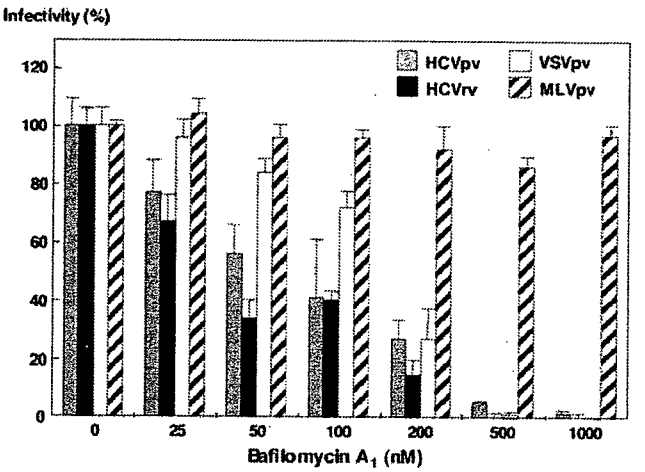

FIG. 7. Inhibition of HCVpv and HCVrv infection by bafilomycin A_1. HCVpv (gray-filled bars), HCVrv (black-filled bars), VSVpv (open bars), or MLVpv (striped bars) were inoculated to Huh7 cells after treatment with various concentrations of bafilomycin A_1. The results shown are from three independent assays, with the error bars representing the standard deviations.

tious particles of which were recovered only in cells exhibiting a high competency of transfection, such as 293T cells. Therefore, generation of HCVrv in various human cells, including nonhepatic cells such as B cells, might be useful for investigating the cell-specific modification and/or factors determining the cell tropism of HCV infection.

Overwhelming evidence that hCD81 facilitates the entry of HCV into Hc via interaction with the E2 protein has been accumulated not only by surrogate models, such as purified E2 proteins, HCV-LP, and HCVpp, but also by authentic HCV particles and HCVcc of genotype 2a (4). In this study, both HCVpv and HCVrv were shown to be infectious in Huh7 cells, and this infectivity was shown to be mediated through the interaction with hCD81. Although overexpression of hCD81 in HepG2 cells which lack endogenous expression of hCD81 renders them susceptible to infection by surrogate viruses, primary human Hc and HeLa cells expressing hCD81 and the rodent CHO cells stably expressing hCD81 (CHOCD81 cells) were resistant to infection by HCVrv and HCVpv (Table 1) (5, 14, 67), suggesting that hCD81 is one of the important factors for HCV entry but is not sufficient for infectivity of HCV in target cells. Recently, it was shown that participation of hCD81 in the infection of HCVpp or HCVcc bearing HCV envelope proteins isolated during chronic HCV infection was reduced, suggesting that the affinity of HCV envelope proteins to hCD81 was reduced and HCV utilizes receptors other than hCD81 (62, 69). HCVrv is useful for studies of the generation of various genotypes of escape variants under pressure of neutralization antibody or antagonist against HCV receptor candidates. Further studies of the functional relevance of hCD81 and other receptor candidates in the entry steps of HCV, such as binding, endocytosis, and membrane fusion, are needed.

Bafilomycin A_1, an H^+-ATPase inhibitor, was shown to reduce the infectivities of both HCVpv and HCVrv in a dose-dependent manner, as it did for the infectivities of both HCVpp and HCVcc (6, 23, 29, 61), suggesting that these viruses require low-pH-induced conformational changes of the envelope proteins upon entry. Furthermore, as with HCVcc (40, 61), preexposure of HCVpv and HCVrv to acidic pH did not reduce their infectivity (data not shown), indicating that additional factors are required for the internalization of the viruses. Recently, entry of HCVpp was shown to depend on the clathrin-mediated endocytosis through the knockdown of clathrin heavy chain by siRNA or chlorpromazine (8, 40), and dominant-negative mutants of Rab5 or Rab7, which are involved in the transport of clathrin-coated vesicles, revealed that entry of HCVpp requires delivery to early but not to late endosomes (40). N-linked glycosylation processing events in the ER are important for the secretion of several enveloped viruses. ER α-glucosidase I and II are involved in the trimming of terminal glucose on the core oligosaccharides, and the resulting monoglucosylated glycoproteins are able to bind to the ER chaperones calnexin (CNX) and/or calreticulin (CRT). ER α-glucosidase inhibitors, DNJ or castanospermine, which block

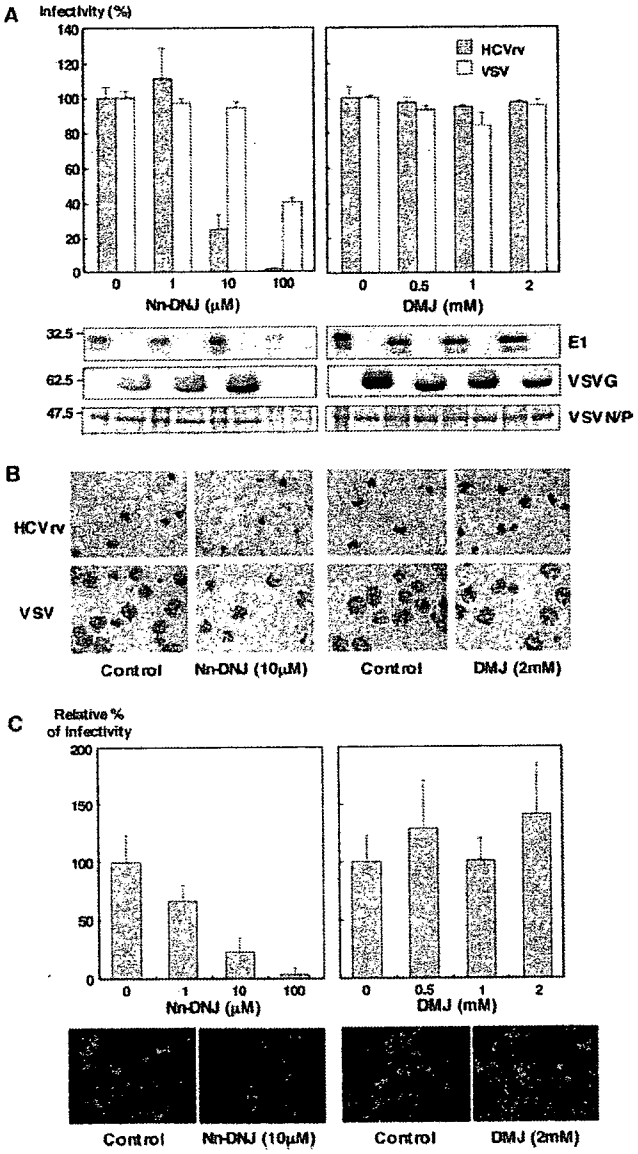

FIG. 8. Effects of ER α-glucosidase inhibitors on the infection with HCVrv and HCVcc. (A) (Top) Production of HCVrv and VSV in the presence of Nn-DNJ (left) or DMJ (right). Huh7 cells infected with HCVrv and VSV at MOIs of 0.1 and 0.01, respectively, were treated with various concentrations of Nn-DNJ or DMJ. Seventy-two hours (HCVrv) or 24 h (VSV) postinfection, culture supernatants were collected and titrated on Huh7 cells by a focus-forming assay. The results shown are from three independent assays, with the error bars representing the standard deviations. (Bottom) Purified viruses generated in Huh7 cells treated with Nn-DNJ or DMJ were analyzed by immunoblotting with anti-E1 (BDI198) and anti-VSVG (ab34774) or Coomassie staining. (B) Focus formation of HCVrv and VSV in the presence of Nn-DNJ or DMJ. Huh7 cells were infected with HCVrv or VSV, treated with Nn-DNJ (10 μM) or DMJ (2 mM) prior to an overlay of culture media containing 0.8% of methylcellulose, and stained with an anti-VSV N antibody after fixation at 72 h (HCVrv) and 24 h (VSV) postinfection. (C) (Top) Production of HCVcc in the presence of Nn-DNJ (left) or DMJ (right). Huh7.5.1 cells infected with HCVcc at an MOI of 0.01 were treated with various concentrations of Nn-DNJ or DMJ. Culture supernatants were collected and titrated by a quantitative core enzyme-linked immunosorbent assay at 96 h postinfection. (Bottom) Immunofluorescence assay of HCVcc infection in the presence of Nn-DNJ or DMJ. Huh7.5.1 cells were infected with HCVcc at an MOI of 0.01, treated with 10 μM of Nn-DNJ or 2 mM of DMJ prior to an overlay of culture media containing 0.8% of methylcellulose, and stained with an anti-NS5A antibody and Alexa 488-conjugated secondary antibody after fixation at 96 h postinfection. Cell nuclei were stained by Hoechst 33258. Pictures were taken using a fluorescence microscope by double exposure of the same fields with filters for Alexa 488 or Hoechst 33258.

the trimming step of N-linked glycosylation, have been shown to prevent the interaction of CNX and/or CRT with the folding glycoproteins, and the production of many enveloped viruses is inhibited by these inhibitors (41). In this study, we found that infection with both HCVrv and HCVcc was inhibited in a dose-dependent manner by treatment with Nn-DNJ, which is an N-alkylated derivative of DNJ exhibiting a stronger effect than DNJ. HCV E1 and E2 proteins were shown to interact with CNX and CRT, and these interactions were inhibited by the treatment with ER α-glucosidase inhibitors (12). One possible function of the HCV p7 protein, the formation of ion channels, has also been shown to be inhibited by the treatment with long-alkyl-chain iminosugar derivatives (50). Recently, it was reported that HCV-LPs produced in the presence of ER α-glucosidase inhibitors incorporated unprocessed, triglucosylated N-glycans and misfolded E1 and E2 proteins and lost their ability to bind hepatoma cell lines (11). Our results demonstrate that the modification of E1 and E2 proteins in the glycosylation steps in the ER is required to confer infectivity to HCVrv and HCVcc. The presence of E1 and E2 proteins on the surrogate viruses and HCVcc possessing high-mannose glycans indicate that these viruses are not released through the *trans*-Golgi network. In the case of West Nile virus, mature particles propagated in mammalian cells possess complex types of carbohydrates, in contrast to those generated in insect cells, which have high-mannose glycans (16). We still do not know the exact nature of modifications of the mature envelope proteins on authentic HCV particles. Further studies of the relationship between the modification of HCV envelope proteins and their infectivity are needed to clarify the life cycle of HCV. The neutralizing activity of antibodies against HCV have been assessed in the past using HCVpv (10, 43), HCVpp (3, 33, 42), and HCVcc (63, 65), as well as by the inhibition of binding of purified E2 protein to hCD81 (24, 53) and of HCV-LP to target cells (59). Sera from patients chronically infected with HCV and experimentally infected chimpanzees were shown to specifically neutralize HCVpp infection (3, 33, 42). In the present study, sera from patients infected with genotype 1b of HCV and anti-E2 monoclonal antibodies exhibited high levels of neutralization activity against infection with both HCVpv and HCVrv bearing HCV envelope proteins of genotypes 1a and 1b. One of the characteristics of HCV infection is the establishment of a persistent infection. Therefore, the high prevalence of neutralizing antibodies to the surrogate viruses and HCVcc suggests that HCV particles exhibiting similar phenotypes to surrogate viruses and HCVcc would be easily eliminated by neutralizing antibodies and thus not be able to participate in the establishment of a persistent infection. Recently, it was reported that HCV escapes from neutralizing antibody and T-cell responses by the continuous generation of escape

variants during chronic infection (51, 62). However, it was demonstrated that viral clearance in acute HCV infection was not correlated with the presence of neutralizing antibodies against HCVpp (33, 42), and 75% of HCVpp bearing HCV envelope proteins of various genotypes are not infectious (29). Therefore, it is reasonable to speculate that HCV particles exhibiting characteristics similar to those of the surrogate viruses are produced in large numbers and act as decoys in HCV patients, eliciting strong neutralizing antibodies against the viruses, and that a small portion of HCV particles exhibiting characteristics different from those of the surrogate viruses may participate in the establishment of persistent infection by escaping from the host immune surveillance system. The authenticity of the surrogate virus systems for the study of HCV infection remains controversial, and further studies are needed to clarify their profiles.

In conclusion, we generated replication-incompetent HCVpv and replication-competent HCVrv possessing HCV envelope proteins as novel surrogate models for the study of HCV. HCVpv and HCVrv were shown to have infection mechanisms similar to those of HCVpp and HCVcc. HCVrv has the following advantages compared to HCVcc: (i) infectious particles bearing HCV envelope proteins of various genotypes are capable of generating in various cell lines or primary cells, in contrast to the strict restriction of generating the infectious HCVcc in the Huh7-derived cell lines; (ii) isolation of escape mutants carrying mutations in the envelope proteins under various pressures may be easily obtained due to the higher replication efficiency than that of HCVcc; and (iii) in vivo investigation of the HCV envelope proteins for entry using humanized mice with human Hc and for immunogenicity for a future vaccine development are possible. Therefore, replication-competent HCVrv established in this study may provide valuable tools not only for understanding the entry mechanisms of HCV in a manner that is cell type and species dependent but also for developing novel therapeutics and vaccines.

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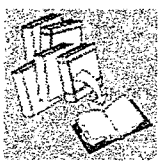
REVIEW



Host factors involved in the replication of hepatitis C virus

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SUMMARY

Hepatitis C virus (HCV) is the major causative agent of blood-borne hepatitis. The majority of HCV-infected individuals develop chronic hepatitis, which eventually progresses to liver cirrhosis, and hepatocellular carcinoma. Although the precise mechanisms of entry, replication, assembly, egress and pathogenesis of HCV are largely unknown, information about viral receptor candidates has accumulated by the development of pseudotype viruses and an *in vitro* replication system of the HCV JFH1 strain. Furthermore, the autonomous RNA replication system based on the artificial viral genome revealed that HCV replicates in the intracellular replication complex composed of viral and host proteins. Recently, an immunosuppressant, cyclosporin A and inhibitors for sphingolipid synthesis and chaperon were reported to inhibit the replication of HCV by counteracting the interplay between host and viral proteins. This review considers the current knowledge of the host proteins that participate in HCV replication and the possibility of developing novel therapeutics intervention for chronic hepatitis C. Copyright © 2007 John Wiley & Sons, Ltd.

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INTRODUCTION

Hepatitis C, which is caused by infection with hepatitis C virus (HCV), is a serious form of chronic hepatitis with steatosis and cirrhosis, and eventually leads to hepatocellular carcinoma [1]. HCV is classified into a member of genus *Hepacivirus* of the family *Flaviviridae* [1]. Epidemiological study reveals that 170 million individuals worldwide are infected with HCV, mostly through blood-borne infection [2]. Introduction of combination therapy with interferon alpha and ribavirin improved therapeutic efficacy, but had no effect on half of the individuals infected with a high viral load of HCV genotype 1 [3,4]. Therefore, effective therapeutic measures are required for the treatment of hepatitis C patients who are not responsive to chemotherapy. An HCV replicon system was established as a representative functional system composed of an antibiotic gene for selection and HCV genomic RNA for autonomous replication in the intracellular compartments around the endoplasmic reticulum (ER) [5]. Studies on HCV replication have used the replicon system, and small chemicals targeted to HCV proteins have been identified [6–10]. On the other hand, a pseudotype viral system based on the vesicular stomatitis virus and retrovirus has been developed to study the receptor determination and the entry mechanism [1]. Recently, an *in vitro* cell culture system for HCV of genotype 2a, which is highly sensitive to interferon therapy [11,12], has been developed [13–15]. However, a robust cell culture system for the HCV 1a and 1b genotypes, which are both the most prevalent genotypes in the world and resistant to interferon therapy, has not yet been successful.

HCV possesses a single positive strand RNA genome encoding a large polyprotein composed of approximately 3000 amino acid residues [1]. The polyprotein is cleaved by the viral proteases

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Abbreviations used

CaM, calmodulin binding domain; Dhh, Desert hedgehog; ER, endoplasmic reticulum; FBD, FK506-binding domain; FKBP, FK506-binding proteins; HCV, hepatitis C virus; Ihh, Indian hedgehog; MSP, major sperm protein; NS, nonstructural; ORPs, oxysterol-binding protein-related proteins; Ptc, Patched; Smo, Smoothened; Shh, Sonic hedgehog; VAMP, vesicle-associated membrane protein.

NS2 and NS3 and by host proteases including signal peptidase and signal peptide peptidase. Viral structural protein, capsid protein (core) and two envelope proteins (E1 and E2) occupy the N-terminal third of the polyprotein, while nonstructural (NS) proteins located in the remaining region. NS3, NS4A, NS4B, NS5A and NS5B are essentially required for autonomous replication in the replicon cells [5]. NS3 possesses the RNA helicase and protease activities [16,17], and NS4A fulfils anchoring NS3 on the intracellular membrane [18]. NS4B is a membrane protein modelling the ER membrane in order to make it suitable for efficient HCV viral replication [19]. NS5A is a phosphoprotein required for HCV replication [20], because adaptive mutations for efficient RNA replication in the HCV replicon were selectively introduced into the NS5A coding region [21]. NS5B is the active subunit of the replication complex known as an RNA-dependent RNA polymerase [22]. Recent reports suggest that several host proteins attend to the formation of the HCV replication complex [9,10,23,24]. In this review, we summarise the physiological and pathological functions of the host proteins that directly or indirectly participate in the replication of HCV.

IMMUNOPHILINS AND HSP90

The peptide bond *cis/trans* isomerases catalyse the conversion between *cis* and *trans* peptide bonds for correct folding of the protein substrate, including peptidyl prolyl *cis/trans* isomerase (PPIase), such as the families of cyclophilins [25], FK506-binding proteins (FKBP) [26,27] and parvulins [28] and the secondary amide peptide bond *cis/trans* isomerase (APIase) [29]. Cyclophilin and FKBP are classified as immunophilins capable of binding to immunosuppressants cyclosporine and FK506, respectively [30]. The family members do not share a homologous domain with each other, based on their amino acid sequences, substrate specificities and inhibitor sensitivities. Recently, cyclophilin B and FKBP8 were shown to interact with NS5B and NS5A, respectively, and to regulate HCV replication [9,10], suggesting that the immunophilins are promising therapies for chronic hepatitis C (Figure 1).

Cyclophilin B

A study of the host gene related to resistance to retrovirus infection revealed that HIV capsid interacts with cyclophilin A [31], which is incorporated into viral particles, but its precise functions in the viral life cycle have not been elucidated yet. HIV particles lacking cyclophilin A exhibited no abnormality in virus packaging, reverse transcriptase activity or capsid stability [32]. However, in macaque cells, cyclophilin A modulates conformation of gag capsid protein to facilitate the interaction with TRIM5alpha, a potent antiretroviral restriction factor and confers resistance to human

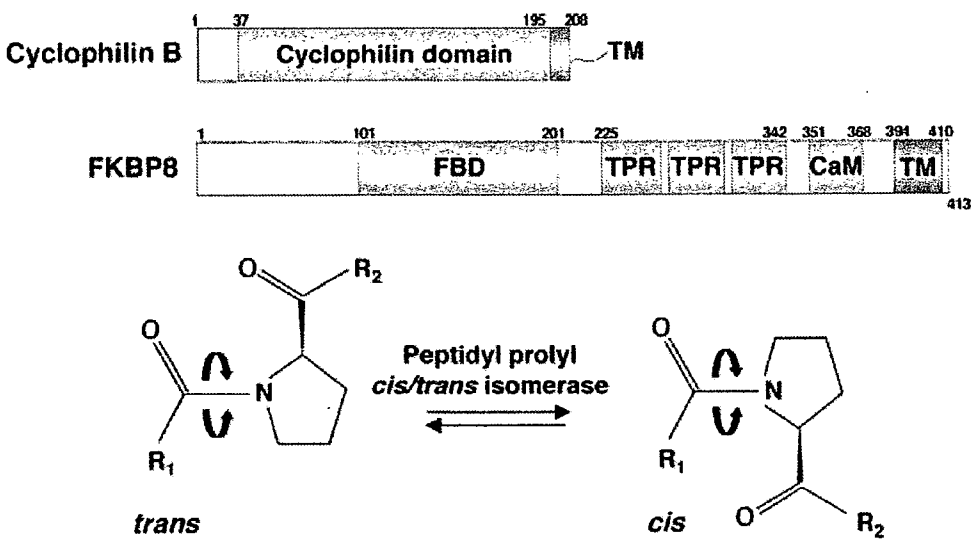

Figure 1. Structures of cyclophilin B and FKBP8. Cyclophilin B possesses a cyclophilin domain and a transmembrane region. FKBP8 has an FK506-binding domain (FBD), three sets of tricopeptide repeats (TPRs), a calmodulin-binding domain (CaM) and a transmembrane region (TM). Both proteins catalyse the conversion between *cis* and *trans* propyl peptide bonds for correct folding of protein substrate

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retrovirus, which participates in the establishment of host range restriction [33,34].

Cyclophilin B, formerly called s-cyclophilin, is identified as a 20 kDa secreted neurotrophic factor for spinal cord cells of chick embryo [35], and it is secreted into human milk and blood [36,37]. Extracellular cyclophilin B enhances the retrotranslocation of prolactin into nucleus [38], is implicated in the presynaptic function by interacting with synaptin I, and impairs the correct folding of prion protein in the presence of cyclosporin A, leading to accumulation in aggresomes [39]. Therefore, cyclophilin B may regulate the correct folding and translocation of host proteins under extracellular and intracellular conditions, although its precise functions are still unknown.

Cyclosporin A and its derivatives capable of inhibiting cyclophilins were shown to inhibit HCV RNA replication and to be effective in the treatment of hepatitis C patients [9,40,41]. Inoue *et al.* [42] reported at the first time that cyclosporin A is effective for the treatment of hepatitis C patients. Cyclosporin derivatives lacking the ability to interact with cyclophilin lost their inhibitory effect on HCV replication [9]. Cyclophilin B was shown to specifically interact with NS5B, the HCV RNA-dependent RNA polymerase, around the ER of the HCV replicon cells and to promote NS5B's association with the viral RNA [9]. Cyclosporin A was shown to disrupt interaction between NS5B and cyclophilin B [9] (Figure 2). Treatment with cyclosporin A and knockdown of cyclophilin B suppressed the replication of HCV, suggesting that cyclophilin B plays an important role in HCV genome replication by enhancing the interaction between NS5B and viral RNA [9].

FKBP8

HCV NS5A is an essential component of the viral replication complex, although NS5A's function has not been clarified yet. We screened the human fetal brain and liver libraries using a yeast two-hybrid system that employs HCV NS5A as bait and identified FKBP8 as an NS5A-binding partner [10] (Figure 2). An immunoprecipitation analysis revealed that NS5A bound to FKBP8 but not to FKBP52 or cyclophilin D, all three of which have homology to each other.

FKBP8 belongs to the FKBP family based on sequence similarity, but lacks the amino acid residues essential for either FK506 binding or PPIase activity [43]. Recent biochemical and enzymological studies indicate that FKBP8 has weak PPIase activity and low affinity to FK506 [44,45], suggest-

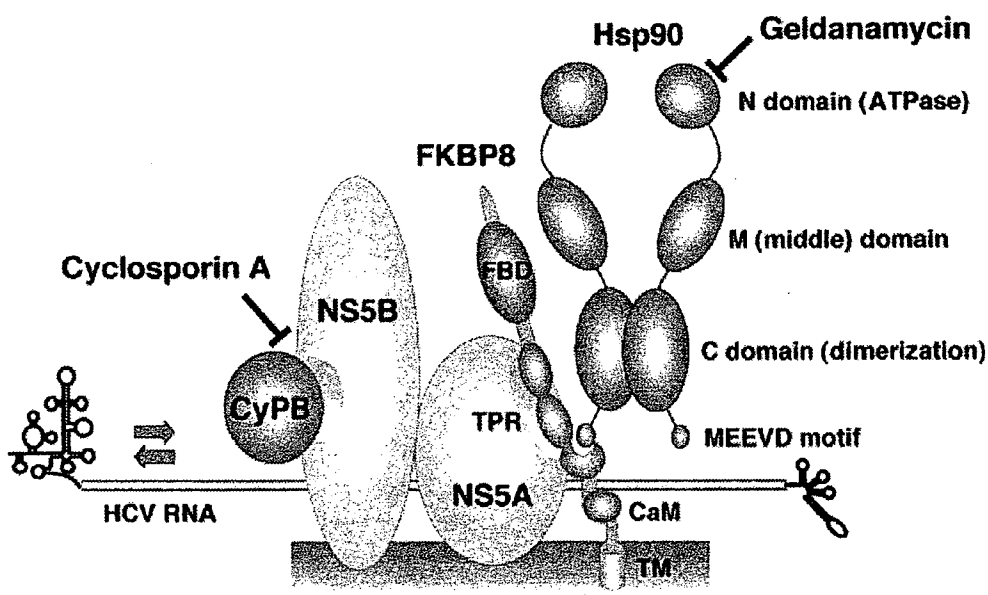

Figure 2. Interaction of HCV NS5A and NS5B proteins with immunophilins and Hsp90. Cyclophilin B interacts with NS5B. FKBP8 interacts with NS5A and Hsp90 through the different regions within TPR domains. Lys^{307} and Arg^{311} of the FKBP8 carboxylate clamp motif are required for binding to the MEEVD motif of Hsp90. Cyclosporin A inhibits interaction between cyclophilin B and NS5B. Geldanamycin is an inhibitor of the ATPase activity of Hsp90

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ing that FK506 is unable to modulate FKBP8 function. Previously, FKBP8 was termed FKBP38 or FKBPr38 (FKBP-related protein 38 kDa) from the deduced molecular weight of 38 kDa based on the fact that the incomplete amino acid sequence was missing the N-terminal part of the authentic FKBP8. The true transcription and translation initiation sites were identified in the upstream of the original start site in the genomic sequences [46]. The FKBP8 splicing variants of 44 and 46 kDa were detected in mouse but not in human, and the 45 kDa of human FKBP8 corresponds to the 44 kDa of murine protein [46].

The physiological function of FKBP8 is largely unknown, but is slightly elucidated from the data of genetically manipulated mice [47]. FKBP8−/− mice exhibit a phenotype similar to that of mutant mice under the excessive activation of the Sonic hedgehog (Shh) protein, a secreted morphogen that regulates the patterning and growth of many tissues in the developing mouse embryo [47]. Human and mouse have three species of hedgehog proteins: Indian hedgehog (Ihh), Desert hedgehog (Dhh) and Shh [48,49]. Ihh and Dhh are predominantly expressed in bone and gonads, respectively, whereas Shh is ubiquitously expressed in many organs such as brain, liver and lungs. Shh is secreted as glycoprotein from the ventral midline of the spinal cord and is involved in the regulation of the genes related to the control of ventral fate in the spinal cord and forebrain [50,51]. Hedgehog protein generally binds to the receptor protein Patched (Ptc) and then inhibits the function of the membrane protein Smoothened (Smo) [52,53]. Smo activates the protein kinase A, which suppresses the transcription factor GLI protein by phosphorylation [54]. Phosphorylated GLI was inactivated by cleavage and acts as a transcriptional repressor against a full length of GLI in hedgehog signalling [54]. Hedgehog protein binds to the receptor Ptc and then inhibits Smo, leading to the accumulation of the full length of the GLI protein [55]. Deficiency in the murine Shh gene or knockouts of the genes required for Shh signal transduction abolished control over morphological formation [51,56]. On the other hand, excessive Shh signalling exhibited the opposite phenotype, including cells that inappropriately adopt ventral identities for dorsal identities [48,57]. FKBP8-deficient mice were reported to exhibit phenotypes similar to those of mice expressing excessive Shh signalling, except that the FKBP8-deficient mice had no abnormalities of the limb pads, bronchial arches or somites [47]. Shh−/− and FKBP8−/− double knockout embryos showed partial rescue of cyclopia and holoprosencephaly, but still showed limb outgrowth defect [47]. These results suggest that Shh signalling in the brain is overlapped with FKBP8-controlled signalling including phosphorylation and protein–protein interaction. Shirane *et al.* [58] suggest that FKBP8 is an inherent phosphatase inhibitor and retains Bcl-2 on mitochondrial membrane to inhibit apoptosis. However, there was no difference between wild-type and FKBP8-deficient mice with respect to apoptosis, suggesting that FKBP8 deficiency does not affect physiological apoptosis. FKBP8 may modulate a phosphatase such as calcineurin to enhance the phosphorylation required for suppression of Shh signalling.

Hsp90

Proteomics analysis reveals that FKBP8 forms a complex with Hsp90 to act as a co-chaperone [10]. Although both NS5A and Hsp90 bound to the TPR domain of FKBP8, interaction between NS5A and FKBP8 did not affect homomultimerisation of FKBP8 or complex formation with Hsp90. The amino acid residues of the carboxylate clump position in the TPR domain of FKBP8 grasp the C-terminal MEEVD motif of Hsp90. Mutations of the residues in the carboxylate clump of FKBP8 suppressed the interaction with Hsp90 but not that with NS5A, suggesting that FKBP8 interacts with NS5A and Hsp90 at different sites within the TPR domain. Knockdown of FKBP8 and treatment with geldanamycin, an ATPase inhibitor of Hsp90, downregulated HCV replication in HCV replicon cells. These data suggest that recruitment of Hsp90 to the replication complex through the interaction between FKBP8 and NS5A is crucial for the replication of HCV (Figure 2). It is also feasible to speculate that NS5A modulates the activity of unidentified phosphatases by the interaction with FKBP8 to facilitate the replication of HCV RNA. Although Hsp90 was shown to be involved in the cleavage between NS2 and NS3 [59], NS2 is not required for the replication of the HCV genome [5].

Hsp90 was suggested to be involved in the enzymatic activity and intracellular localisation of several viral enzymes, including polymerases. Hsp90 was shown to bind to a viral polymerase subunit

of influenza virus to facilitate the replication complex formation and the nuclear localisation of the viral polymerase subunit [60,61]. The DNA polymerase of herpes simplex virus type 1 required the chaperone activity of Hsp90 for the nuclear localisation of the polymerase [62]. Flock house virus utilises Hsp90 to assemble the complex of the RNA-dependent RNA polymerase on the intracellular membrane [63]. Knockdown and treatment with Hsp90 inhibitor revealed that Hsp90 activity is important for the rapid growth of negative strand RNA viruses [64]. Furthermore, Hsp90 was shown to be required for the activity of the hepatitis B reverse transcriptase [65,66]. Hsp90 generally requires the co-chaperone protein to acquire specificity to the substrate client. Therefore, Hsp90 and co-chaperones are crucial molecules required for the efficient replication of a broad range of viruses and are an ideal target for antivirals with broad spectra. Recently, Hsp90 inhibitors were shown to drastically impair the replication of poliovirus without any emergence of escape mutants [67].

Immunophilins and Hsp90 may be involved in HCV replication through the correct folding of the replication complex required for efficient enzymatic activity. In addition, cyclophilin B may also participate in the translocation of NS5B, as seen in the polymerase subunits of influenza virus, to facilitate binding to viral RNA. Elucidation of the HCV replication complex may lead to the development of new therapeutics for chronic hepatitis C.

VESICLE-ASSOCIATED MEMBRANE PROTEIN-ASSOCIATED PROTEINS

VAPs were originally identified as proteins that bind to vesicle-associated membrane protein (VAMP) in the nematode *Aplysia* and were designated as VAMP-associated protein 33 kDa (VAP-33) [68]. After that, one homologue and its splicing variant were identified as VAP-B and -C, respectively [69], and VAP-33 has been renamed VAP-A. Although VAP-A was suggested to be required for delivery of components into the presynaptic membrane of *Aplysia* ganglion [68,70], in mouse organs both VAP-A and -B localise in the intracellular membrane compartments, including ER, but not in the VAMP [68,71]. In addition, VAP-A, -B and -C are ubiquitously expressed in mammalian organs, such as heart, placenta, lung, liver, skeletal muscle and pancreas [72], suggesting that VAP proteins possess have other functions besides neurotransmitter release [69,70,73].

VAP is a type II membrane protein composed of three functional domains: the N-terminal half of the protein, which is highly homologous with the nematode major sperm protein (MSP); the coiled-coil domain and the transmembrane domain. VAP-A shares 60% identity with VAP-B, while VAP-C is the splicing variant of VAP-B that lacks a transmembrane domain [69]. MSP was identified as one of the major proteins of the nematode sperm [74] and forms a microfilament required for amoeboid motility through the push–pull theory. MSPs form a subfilament by homodimerisation through the Ig-like domain and coiled coil around each other to form a filament. Several filaments are further assembled around each other to make a macrofiber [75,76]. The MSP-like domain was identified in several mammalian, avian, arthropod, plant and fungal proteins but not in protist proteins [77].

VAP-interacting proteins share the FFAT motif represented by the consensus amino acid sequence EFFDAxE as determined by a comparison of oxysterol-binding protein-related proteins (ORPs) [78]. However, both VAMP and tubulin are capable of binding to VAP proteins in an FFAT-independent manner [70,79–81]. In yeast, Opi1p is the transcriptional repressor of the INO1 gene, which encodes an inositol-1-phosphate synthase [72,82]. SCS2p is a yeast homologue of VAP and interacts with Opi1p through the FFAT motif to regulate the expression of the INO1 gene [78]. In mammals, ceramide is transported by the cargo protein CERT from ER to Golgi for the synthesis of sphingomyelin [83,84]. VAP-A and -B could anchor CERT via the FFAT motif to uptake ceramide by CERT in ER [85], suggesting that VAPs serve as anchors for the transporter of ceramide in mammalian cells rather than as a component of neurotransmitter release machinery.

VAP-A and -B were reported to be NS5A-binding host proteins by the screening of the human hepatoma cell line library using NS5A as bait in yeast [23,24]. GST pulldown and immunoprecipitation analyses revealed that NS5A and NS5B interact with human VAP-A and that the N-terminal MSP domain and the coiled-coil domain of VAP-A are responsible for the binding to NS5B and NS5A, respectively [24] (Figure 3). Several host kinases were shown to phosphorylate NS5A,

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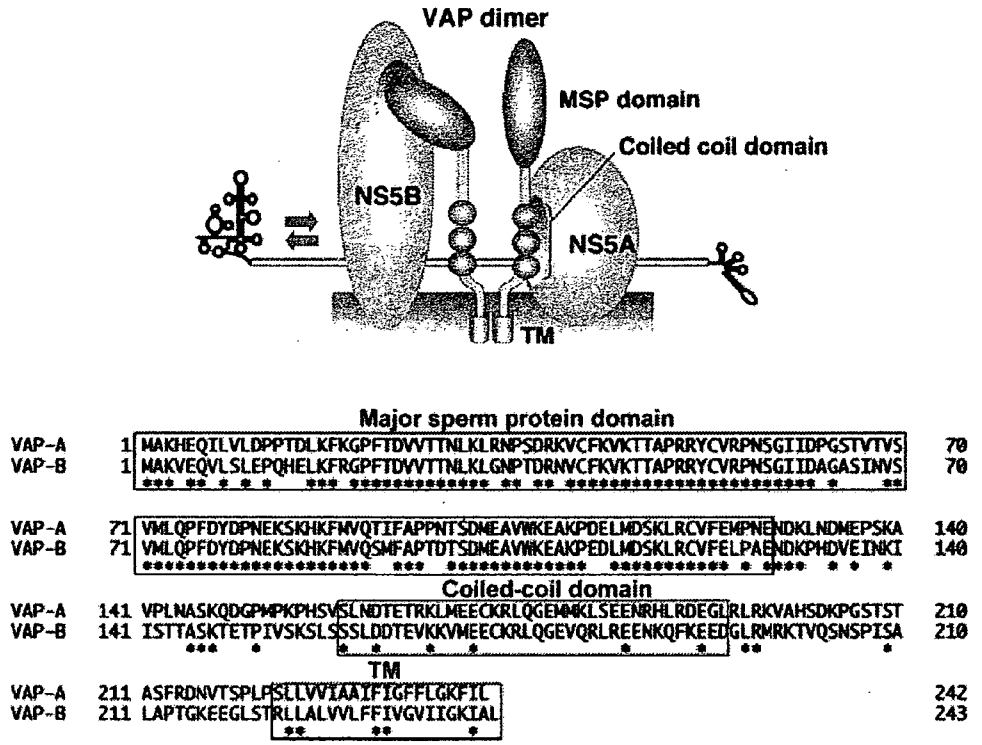

Figure 3. Interaction between HCV NS5A protein and VAPs. VAP-A and VAP-B make homo- and hetero-dimers with each other. The VAP dimer interacts with NS5A and NS5B through the coiled-coil domain and the MSP domain, respectively. VAP-A and VAP-B share 62.9 and 84.9% homology in total and in the MSP domain, respectively

and the hyperphosphorylation of NS5A abrogates the interaction with human VAP-A, which leads to the downregulation of HCV replication [20,86–88]. Adaptive mutation for an efficient replication of HCV RNA in the Huh7 cell line was associated with hypophosphorylation of NS5A, which enhances binding to VAP-A [20]. NS5A of HCV genotype 1a H77 strain was shown to be hyperphosphorylated in both yeast and replicon cells, and no interaction with VAP-A was detected in yeast, suggesting that hyperphosphorylation of NS5A may suppress HCV RNA replication through by counteracting binding to VAP-A [20]. However, we have demonstrated that NS5A of genotype 1a H77 strain is capable of binding not only to VAP-A but also to VAP-B at levels similar to that of genotype 1b in mammalian cells [23].

Several reports suggest that HCV replication takes place on the detergent-resistant membrane fraction [6,89,90]. NS4B is predominantly associated with a lipid-raft-like detergent-resistant fraction, and both NS5A and NS5B are co-localised in the similar fraction in the presence of NS4B [89]. VAP-A was also localised in the detergent-resistant fraction, suggesting that it plays an important role in HCV replication, because the dominant negative mutant of VAP-A suppressed the replication of HCV RNA [89]. VAP-B forms a homodimer and heterodimer with VAP-A, and knockdown of VAP-A or VAP-B led to a substantial suppression of HCV replication [23,91], suggesting that heterodimerisation of VAPs could regulate HCV replication (Figure 3). The host proteins possessing the FFAT motif are related to biosynthesis and translocation of lipid [81], whereas NS5A and NS5B do not have the typical FFAT motif. Although replication of HCV RNA did not affect lipid biosynthesis, lipid components are required to form the HCV replication complex as described below. VAPs might be involved in the transport of lipid components to the HCV replication complex through the interaction with NS5A and NS5B, resulting in the upregulation of HCV replication. VAP-B was shown to interact with Nir2 protein through the FFAT motif and to remodel the ER structure [92]. It can therefore be speculated that VAPs are asso-

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ciated with remodelling of the HCV replication complex in the ER membrane through interaction with Nir2 protein.

HOST PROTEINS MODIFIED BY LIPID AND INVOLVED IN LIPID BIOSYNTHESIS

Lipid components are required for the assembly, budding and replication of several viruses [93–97]. Increases in saturated and monounsaturated fatty acids enhance HCV RNA replication, in contrast to suppression by polyunsaturated fatty acids [98], suggesting that enzymes associated with lipid biosynthesis are also involved in HCV replication. SREBP-1c regulates the transcription of acetyl-CoA carboxylase, fatty acid synthase and stearoyl-CoA desaturase, leading to the production of saturated and monounsaturated fatty acids and triglycerides [99]. Expression of HCV core protein induces the production of lipid droplets composed mainly of triglyceride [100]. Our recent study suggests that SREBP-1c was upregulated in the liver of transgenic mice expressing HCV core protein through the LXRalpha/RXRalpha-dependent pathway, which leads to the development of fatty liver [101]. The upregulation of SREBP-1c in the core transgenic mice was required for expression of PA28gamma, an HCV core-binding host protein involved in the activation of nuclear proteasome activity. Saturated or monounsaturated fatty acid may be utilised for the formation of HCV replication complex with cholesterol and sphingolipid [98]. A lipophilic long-chain compound derived from microbial metabolites, an inhibitor of sphingolipid biosynthesis, was shown to inhibit HCV replication [6]. The HCV replication complex is shown to be localised in the lipid raft including sphingolipid [89,90,102]. Therefore, compounds disrupting sphingolipid biosynthesis may inhibit the replication of HCV through the modification of the lipid raft (Figure 4).

HCV replication was also disrupted with an inhibitor of geranylgeranyl transferase I but not with that of farnesyl transferase [103], suggesting that geranylgeranylation of viral or host protein regulates HCV replication efficiency [103]. Geranylgeranylate is an intermediate of the mevalonate pathway and is attached to various cellular proteins for anchoring to plasma or intracellular membrane [99]. Wang *et al.* [104] reported that geranylgeranylated FBL2 is required for the efficient replication of HCV genomic RNA. FBL2 had been identified as a structural homologue of Skp2, which interacts with Skp1 for S-phase entry and conserves the structural motif of F-box for Skp1 binding [105]. The immunoprecipitation analysis revealed that NS5A interacts with FBL2 [104]. The F-box motif is located in the N-terminus of FBL2, followed by 11 leucine-rich repeats [105]

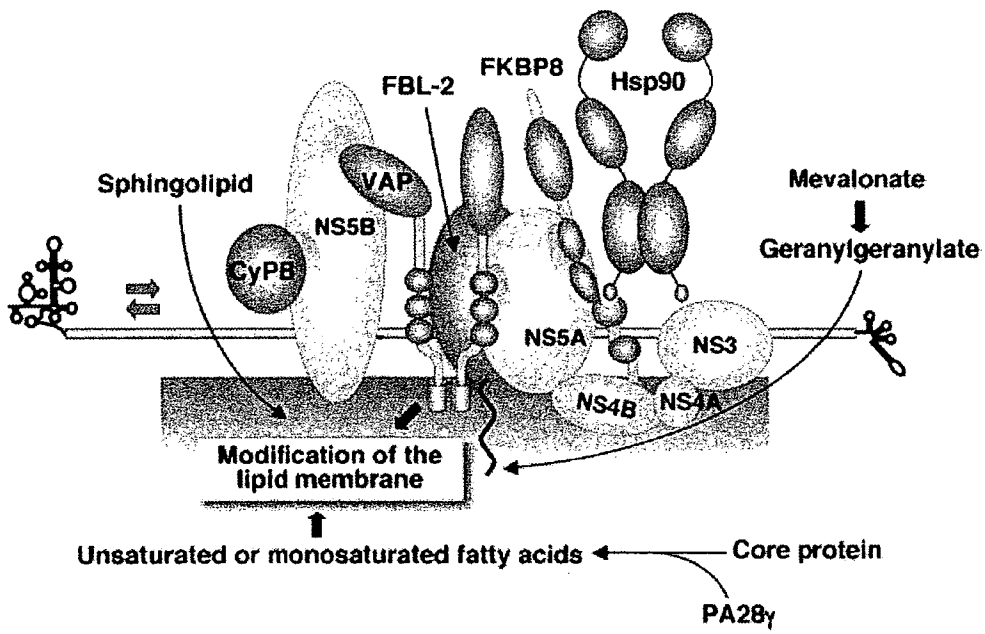

Figure 4. Putative model of HCV replication complex composed of viral and host proteins

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and the CAAX motif (CVIL), which is suggested to be modified by geranylgeranylation [104]. FBL2 lacking the CAAX motif was not modified by geranylgeranylation and lost the interaction with NS5A [104]. An F-box-truncated FBL2 mutant suppressed the replication of HCV as a dominant negative, whereas a mutant in the residues responsible for geranylgeranylation exhibited no suppressive effect [104]. The geranylgeranylated FBL2 is required for the replication of HCV but not for that of West Nile virus [104]. Furthermore, knockdown of FBL2 in the replicon cells induced suppression of HCV replication but not in cells expressing an siRNA-resistant FBL2 [104]. The F-box motif is generally essential for the formation of the ubiquitin ligase complex [105], suggesting that FBL2 regulates the ubiquitination of host or viral proteins through the interaction with NS5A. Another possibility is that FBL2 may retain the viral replication complex by interacting with NS5A (Figure 4).

CONCLUSION

The host machineries of lipid biosynthesis, protein folding and anchoring in the intracellular compartment may cooperate with HCV proteins to facilitate the replication of the viral genome. In addition, translation of the viral genome is also expected to utilise the host proteins to generate viral proteins. Other host factors such as cellular RNA helicase p68 and nucleolin were also reported to be involved in HCV RNA replication [106,107]. The primary concern of chronic hepatitis C is the development of hepatocellular carcinoma through liver steatosis and fibrosis. HCV proteins could potentiate the production of reactive oxygen species, which may activate STAT3 leading to carcinogenesis [101,108–111]. Among HCV proteins, only the core protein was shown to be involved in the induction of carcinogenesis [112–114]. Data on the replication of HCV cooperating with host proteins have been accumulated by using RNA replicon and cell culture systems. Further studies on the host proteins involved in viral replication and carcinogenesis are needed for the development of therapeutic measures for chronic hepatitis C.

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Evaluation systems for anti-HCV drugs ☆

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Abstract

Development of therapeutics for chronic hepatitis C has been hampered by the lack of an efficient cell culture system and a small animal model for the hepatitis C virus (HCV). An RNA replicon system, in which the HCV genome replicates autonomously in cells, and replication competent viruses derived from an HCV genotype 2a JFH1 strain efficiently propagating in Huh7 cells have been developed, and these systems have contributed to the evaluation of anti-HCV drugs targeted to viral and host proteins involved in the replication of HCV. Several compounds counteracting the viral enzymes, such as RNA polymerase and proteases, and host proteins involved in the lipid synthesis and protein folding are reported to have anti-HCV activities based on assessments using these *in vitro* systems. Furthermore, a mouse model transplanted with human liver fragments was shown to be capable of replicating HCV and has been used to evaluate the efficacy of antiviral drugs *in vivo*. In this review, we summarize information regarding systems for studying the HCV life cycle and potential new targets for therapeutic intervention for chronic hepatitis C.

Keywords: Anti-HCV drugs; Protease inhibitor; Nucleotide analogue; Cell-based model system; Animal model

Contents

1. Introduction

Hepatitis C virus (HCV), a member of genus *Hepacivirus* of the family *Flaviviridae*, infects approximately 170 million people worldwide and induces serious chronic hepatitis that results in steatosis, cirrhosis, and ultimately hepatocellular carcinoma.

☆ This review is part of the *Advanced Drug Delivery Reviews* theme issue on "Toward Evidence Based Control of Hepatitis C Virus Infection".

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