

FIG. 5. Construction of a mutant JEV carrying the capsid protein resistant to cleavage by cathepsin L. (A) Predicted RNA secondary structures of the wild-type (WT) and mutant viral genomes. Nucleotides 135 to 152 and bases 10858 to 10875 in the 5' and 3' termini, respectively, connected by 8 nonsense nucleotides (X) alternative to bases 153 to 10857, were applied to the computer program GENETYX-MAC, version 12, to calculate free energies (E). The secondary RNA structures with minimum free energies are illustrated. RNA sequences encoding Asp¹⁵ (P4), Met¹⁶ (P3), and Leu¹⁷ (P2) in the 5' cyclization sequences of the WT JEV, Leu¹⁷ (P2) for WT/CSmt, and Ala¹⁷ (P2) for L17A and L17A/CSmt are boxed. The mutated nucleotides are shown by lowercase letters. (B) Vero cells (5×10^6) were electroporated with 10 μ g of in vitro-transcribed genomic RNA of WT, L17A, WT/CSmt, or L17A/CSmt virus and immunostained with an anti-E antibody at 4 days posttransfection. (C) Expression of capsid proteins in cells infected with WT or L17A/CSmt JEV. Vero cells were inoculated with the JEVs at an MOI of 10 and analyzed by immunoblotting with anticapsid antibody at 1 day postinfection.

was generated (Fig. 5A). However, the electroporation of the mutant RNA did not result in the production of the viral antigen (Fig. 5B) and infectious particles (data not shown). The coding region for Leu¹⁷ slightly overlaps the 5' cyclization sequences, suggesting that the lack of replication of the L17A mutant is caused by the unstable and inappropriate secondary structure of viral RNA (Fig. 5A). To examine this possibility, L17A/CSmt, carrying additional complementary mutations in the 3' UTR, which was predicted to have a stable secondary structure, and WT/CSmt, carrying a mutation only in the 3' UTR as a control, were generated (Fig. 5A). Upon electroporation of the genomic RNAs into Vero cells, RNA of L17A/CSmt but not of WT/CSmt exhibited replication (Fig. 5B). As we expected, the C2 protein was not detected in Vero cells infected with L17A/CSmt (Fig. 5C). These results further confirm that RNA-RNA base pairing mediated by the two com-

plementary cyclization sequences in the capsid coding region and 3' UTR is required for replication of JEV.

Involvement of capsid protein cleavage on the cell type-specific replication of JEV. To examine the biological function of the C2 protein, the growth kinetics of the mutant L17A/CSmt was examined in several cell lines. As shown in Fig. 6A, L17A/CSmt was comparably replicated in Vero, C6/36, and PK15 cells compared with wild-type JEV, whereas growth of L17A/CSmt was 3.3- to 6.1-fold lower and 10.8- to 11.8-fold lower than that of wild-type JEV in N18 and RAW264.7 cells, respectively. In addition, L17A/CSmt exhibited reduced synthesis of the viral protein in N18 cells but not in Vero cells (Fig. 6B), suggesting that impairment of L17A/CSmt replication in N18 cells might be attributable to the reduction of viral protein synthesis. To further confirm the involvement of capsid protein cleavage in the cell type-specific restriction of L17A/CSmt rep-

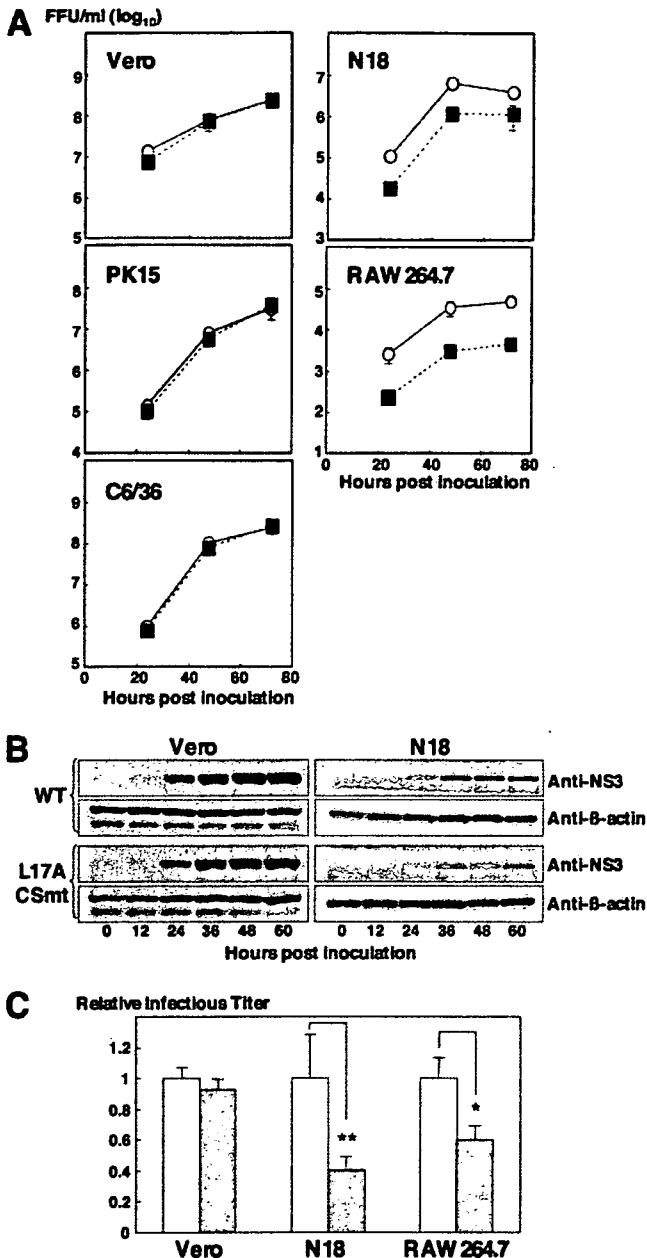


FIG. 6. Growth kinetics of L17A/CSmt in various cell lines. (A) The wild-type (WT; open circles) and mutant L17A/CSmt (solid squares) JEVs were inoculated into Vero, C6/36, PK15, N18, and RAW264.7 cells at an MOI of 10. After the indicated times, the infective titers in the culture supernatants on Vero cells were determined. (B) Viral protein synthesis in Vero and N18 cells infected with the WT or L17/CSmt virus. The NS3 and β-actin proteins were detected by immunoblotting with anti-JEV NS3 and anti-β-actin MAbs, respectively. (C) The WT JEV was inoculated into Vero, N18, and RAW264.7 cells at an MOI of 10 and incubated in the presence of DMSO (white bars) or 1 μM Z-FY-DMK (gray bars). At 24 h after inoculation, the infectious titers in the culture supernatants on Vero cells were determined. Asterisks showed significant differences by *t* test (**, *P* < 0.01; *, *P* < 0.05).

lication, we examine the effect of the cathepsin L inhibitor on JEV replication. The cathepsin L inhibitor suppressed the growth of the wild-type virus in N18 and RAW264.7 cells, but not in Vero cells (Fig. 6C). Furthermore, the wild-type virus

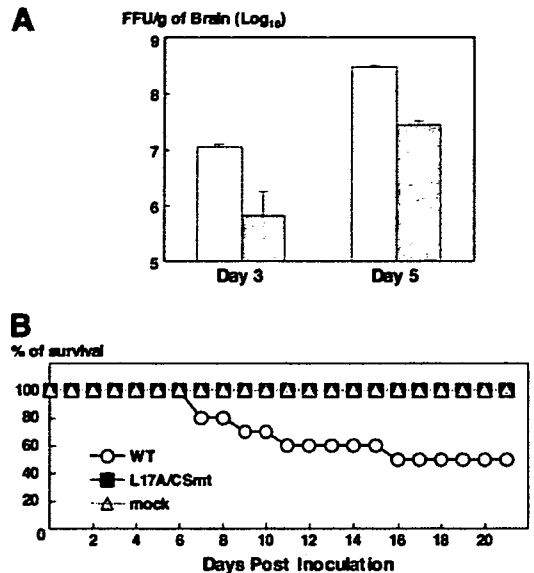


FIG. 7. Neurovirulence and neuroinvasiveness of L17A/CSmt in mice. (A) Growth of the wild-type (WT; white bars) and mutant L17A/CSmt (gray bars) virus in mouse brain. One hundred FFU of each virus were intracerebrally injected into 3-week-old ICR mice, and the progeny viruses in the brain at 3 or 5 days after inoculation on Vero cells were determined. (B) Neuroinvasiveness of the WT and mutant JEVs to mice. Ten ICR mice were intraperitoneally inoculated with 1×10^6 FFU of each virus, and the survival rates of the mice were determined for 21 days.

replicated equally in Vero/siNC and Vero/siCTSL cells (data not shown). These results suggest that generation of the C2 protein is required for the efficient replication of JEV in murine macrophage and neural cells.

Neurovirulence and neuroinvasiveness of L17A/CSmt in mice. To compare the levels of neurovirulence of the wild-type and mutant viruses, we determined the LD₅₀ values by intracerebral inoculation of the viruses in 3-week-old ICR mice. The LD₅₀ value of L17A/CSmt (12.3 FFU) was approximately five times higher than that of the wild-type JEV (2.7 FFU). Although no significant difference in symptoms was observed between mice inoculated with 100 FFU of the wild-type and the mutant viruses, L17A/CSmt required longer periods than the wild-type JEV to kill mice (wild type versus L17A/CSmt: 6.8 ± 0.9 versus 8.4 ± 1.4 days postinoculation). To examine the growth kinetics of the viruses in the mouse brain, 100 FFU of each virus were intracerebrally injected and the progeny viruses in the brain were determined. The growth of L17A/CSmt was 16.3 and 11.0 times lower than that of the wild-type virus at 3 and 5 days after inoculation, respectively (Fig. 7A). Next, to compare the levels of neuroinvasiveness of the wild-type and mutant viruses, ICR mice were intraperitoneally inoculated with 1×10^6 FFU of each virus. All of the 10 mice inoculated with L17A/CSmt survived, whereas one-half of the mice inoculated with the wild-type JEV died by 10.0 days postinoculation on average (Fig. 7B). These results indicated that the L17A/CSmt mutant resistant to the cleavage by cathepsin L exhibits impaired neurovirulence and neuroinvasiveness in mice.

DISCUSSION

Posttranslational modifications, including proteolysis, glycosylation, and phosphorylation, play a key role in regulating the functions of various proteins. Flavivirus proteins are translated as a single large precursor polyprotein, and proteolysis by host and viral proteases, such as signal peptidase, NS2B/3, and furin, is crucial for viral propagation (24). In this study, we demonstrate that some fraction of the mature JEV capsid proteins (C1) are further processed into a small form of capsid protein (C2) by cathepsin L, a papain-like cysteine protease. Furthermore, the C2 protein was shown to play a role in the replication of JEV in neural and macrophage cells and pathogenicity in mice. It is well established that cathepsins, a large group of lysosomal proteases, are involved in the bulk degradation of proteins in the lysosome. On the other hand, limited proteolysis by cathepsins has also been shown to convert a hormone (12), a neurotransmitter (51), and transactivators (15, 16, 33) from inactive precursors to the active forms and facilitate entry of several viruses (7, 13, 41).

It has been shown that the JEV C2 protein can be generated by the cleavage of the amino-terminal 18 amino acids from the C1 capsid protein by cathepsin L. However, the amino-terminal part of the cleavage product was not detected even though a FLAG tag was added (Fig. 2B). Therefore, the fate of the N-terminal 18 residues is currently unknown. The C2 protein was detected only in the cells, not in the viral particles, in contrast to the C1 protein, which was detected in both. The amino-terminal 32 amino acids and carboxyl-terminal 26 amino acids of the capsid protein of Kunjin virus (KUN), an Australian subtype of WNV, are essential for binding to the genomic RNA (20). The amino-terminal region of the capsid protein is well conserved between JEV and WNV. Therefore, it is possible that the JEV C2 protein is not incorporated into viral particles due to lack of the amino-terminal region of the capsid protein, required for binding to the viral RNA. Three-dimensional structural analyses revealed that the DEN and KUN capsid proteins contain four α -helices and form a homodimer and a homotetramer (11, 27), and the amino-terminal 20 amino acids of the DEN capsid protein were shown to be flexible and not resolvable by nuclear magnetic resonance assay (27). In addition, a deletion mutant of the capsid protein of KUN lacking the amino-terminal 22 amino acids was used to determine the crystal structure (11). Therefore, the amino-terminal region of the flaviviral capsid proteins might not be involved in the self-assembly of the capsid proteins.

The capsid proteins in the fraction that are degraded rather than secreted as virions are likely to come in contact with cathepsin L in the acidic compartments such as the lysosome. Furthermore, subcellular fractionation indicated that the C2 protein had also migrated into the nucleus after processing. Our previous studies have shown that nuclear localization of the capsid protein and binding with the host nucleolar protein B23 are important for JEV replication (32, 46). These data suggest that the JEV capsid protein is translocated from the cytoplasm to the nucleus through the acidic compartment. Although the trafficking mechanisms of the capsid protein remain unknown, the C2 protein is able to migrate into the nucleus through the nuclear localization signal and B23-binding domain at Gly⁴² and Pro⁴³ (32, 46). The C2 protein of a

mutant JEV in which Gly⁴² and Pro⁴³ were replaced with alanines (32) was impaired in nuclear localization, and the mutant capsid protein missing the amino-terminal amino acids was detected in the nucleus, especially in the nucleolus, when it was expressed by plasmid transfection (data not shown). On the other hand, it has been reported that cathepsin L or a cathepsin L-like protease is expressed in the nucleus and cleaves some host proteins, such as CDP/Cux (16), RB, and SP-1 (15, 33). Therefore, the JEV capsid protein might be alternatively processed in the nucleus by the proteases. In the case of WNV, the export of the capsid protein from the nucleus was facilitated in a Jab1-binding manner (34), and the Jab1-binding motif (Pro-Gly-Gly-Pro; residues 5 to 8) was also conserved in the JEV capsid protein. Therefore, the C2 protein lacking the Jab1-binding motif due to cleavage with cathepsin L might be able to escape from Jab1-dependent nuclear export and accumulate in the nucleus.

It has been established that the primary determinants of the specificity for cathepsin L are the S2 subsite (as shown in other papain-like proteases) and the hydrophobic residues at the P2 position of the substrates (37, 38). In addition, basic residues show a preference for the P1 position of substrates (38). These properties are in good agreement with our results that the cleavage site of the JEV capsid protein by cathepsin L is between Lys¹⁸ and Arg¹⁹ and that Leu¹⁷ at the P2 site was crucial for the cleavage. The residues P4 to P1' are well conserved among mosquito-borne flaviviruses except for YFV, and the amino acid changes of the YFV capsid protein occur only within hydrophobic (Leu to Val at the P2 site) and basic (Lys to Arg at the P1 site) residues, respectively (Fig. 4C). Therefore, the capsid protein of YFV may also be cleaved by cathepsin L.

On the other hand, it has been reported that a small capsid protein of DEN2 was generated by leaky scanning (9). Due to the lack of a Kozak consensus sequence around the first start codon in many mosquito-borne flaviviruses, including DEN2, the smaller capsid protein of DEN2 is translated from the second or third AUG codon (9). In this context, two independent mechanisms of leaky scanning and processing by cathepsin L might be involved in the production of the small capsid protein. If both mechanisms were involved in the processing of the capsid protein of DEN, the C2 products that were generated by leaky scanning that started at residue Met¹⁵ and then were processed by cathepsin L at Arg¹⁸ should be present. This hypothesis is supported by the detection of the slowly migrating C2 proteins of DEN2 and -4 by SDS-PAGE due to treatment with E64d (Fig. 4E). The fast-migrating forms of the C2 proteins of DEN2 and -4 may be generated by cleavage by cathepsin L, while the slowly migrating forms detected in the presence of the inhibitor may be generated by leaky scanning. In contrast, the JEV genome possesses the ideal Kozak consensus sequence around the first AUG codon (9), and thus leaky scanning should not be involved in the production of the C2 protein.

Generation of the L17A capsid mutant in combination with the changes in the CSmt region (L17A/CSmt mutant) was necessary to ensure that altering the Leu¹⁷ codon did not also affect the 3' cyclization sequence essential for viral replication. The RNA-RNA interaction between the 5' cyclization sequences, in which the conserved amino acids required for ca-

thepsin L cleavage are partially encoded, and the 3' cyclization sequences was predicted for the flaviviruses (19), and the importance of the interaction for replication has been demonstrated in many flaviviruses (1, 10, 14, 19, 25). In this study we further confirmed the crucial role of the interaction of both the 5' and 3' ends of the viral RNA for JEV replication. Replication is a prerequisite for the viral life cycle; therefore, the capacity for the processing of the capsid protein, which is partially encoded in the 5' cyclization sequences, by cathepsin L should be acquired during the viral adaptation to the hosts.

The growth kinetics of the L17A/CSmt JEV was reduced in RAW264.7 and N18 cells, but not in Vero, PK15, and C6/36 cells. It is noteworthy that the neural cells and the cells of monocyte/macrophage lineage are known to support JEV replication *in vivo* (22, 30, 50). The present study could not completely exclude the possibility that the complementary mutations in the cyclization sequences and/or the structure of the mutant capsid protein may be responsible for the reduced replication of the mutant virus in specific cells in culture or *in vivo*. It has been previously reported that DEN RNA with complementary mutations in the cyclization sequences recovered its direct interaction and self-primed RNA synthesis to the same level as seen in the wild-type RNA in a cell-free system (52, 53), whereas similar mutations significantly delayed RNA replication of the KUN replicon (19). Suppression of viral replication in N18 and RAW264.7 cells by treatment with the cathepsin L inhibitor further supports the possibility that the cleavage of capsid protein rather than RNA alteration in the cyclization sequences plays a crucial role in viral replication. Generation of the C2 protein is not a prerequisite for the cell-specific replication of JEV, because the processing of the capsid protein by cathepsin L was observed in all of the cells examined. However, we do not know the reason why the cell lines that showed the lowest production of the C2 protein exhibited the lowest viral production and the largest difference in growth of wild-type and L17A/CSmt viruses at the moment. Interaction of the C2 protein with a host factor(s) may be required for efficient replication of JEV in neural and macrophage cells, in which virus replicates at a low level, whereas the C2 protein may be unnecessary for replication in highly replication-competent cells, such as Vero, C6/36, and PK15 cells. The importance of the small capsid protein for viral replication has been shown in a study of DEN2, but a cell tropism for viral replication has not been reported (9).

Consistent with the data obtained *in vitro*, the L17A/CSmt mutant exhibited slow growth in the mouse brain. In addition, the limited growth of the mutant JEV in RAW264.7 and N18 cells may be a reflection of its reduced neuroinvasiveness. The symptoms of mice intracerebrally inoculated with the L17A/CSmt mutant were indistinguishable from those inoculated with the wild type, although disease induction required more time and a larger amount of virus than that due to inoculation with the wild type. These results suggest that the C2 protein is involved in viral replication *in vivo* but does not directly participate in virulence. This is in clear contrast to the mutant JEV defective in the nuclear localization of the capsid protein, which exhibited neurovirulence comparable to that of the wild type in spite of severe impairment of growth in the brain (32).

The present study demonstrated that cleavage of the capsid protein by cathepsin L and the resulting C2 protein missing the

amino-terminal 18 amino acids plays a role in JEV replication in the nerve and macrophage cell lines, suggesting that the capsid protein has additional functions other than nucleocapsid formation. The limited genomic information of flaviviruses may constrain the multiassignment strategies of the viral proteins during the evolutionary adaptation of the viruses to their hosts.

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Replication-Competent Recombinant Vesicular Stomatitis Virus Encoding Hepatitis C Virus Envelope Proteins[∇]

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Although *in vitro* replication of the hepatitis C virus (HCV) JFH1 clone of genotype 2a (HCVcc) has been developed, a robust cell culture system for the 1a and 1b genotypes, which are the most prevalent viruses in the world and resistant to interferon therapy, has not yet been established. As a surrogate virus system, pseudotype viruses transiently bearing HCV envelope proteins based on the vesicular stomatitis virus (VSV) and retrovirus have been developed. Here, we have developed a replication-competent recombinant VSV with a genome encoding unmodified HCV E1 and E2 proteins in place of the VSV envelope protein (HCVrv) in human cell lines. HCVrv and a pseudotype VSV bearing the unmodified HCV envelope proteins (HCVpv) generated in 293T or Huh7 cells exhibited high infectivity in Huh7 cells. Generation of infectious HCVrv was limited in some cell lines examined. Furthermore, HCVrv but not HCVpv was able to propagate and form foci in Huh7 cells. The infection of Huh7 cells with HCVpv and HCVrv was neutralized by anti-hCD81 and anti-E2 antibodies and by sera from chronic HCV patients. The infectivity of HCVrv was inhibited by an endoplasmic reticulum α -glucosidase inhibitor, *N*-(*n*-nonyl) deoxynojirimycin (Nn-DNJ), but not by a Golgi mannosidase inhibitor, deoxymannojirimycin. Focus formation of HCVrv in Huh7 cells was impaired by Nn-DNJ treatment. These results indicate that the HCVrv developed in this study can be used to study HCV envelope proteins with respect to not only the biological functions in the entry process but also their maturation step.

Hepatitis C virus (HCV) is the major causative agent of blood-borne chronic non-A, non-B hepatitis, infecting at least 3% of the world's population. The majority of HCV-infected individuals develop chronic hepatitis that eventually progresses to liver cirrhosis and hepatocellular carcinoma (36). HCV is an enveloped single-stranded plus-sense RNA virus belonging to the genus *Hepacivirus* in the *Flaviviridae* family, which also includes members of the genus *Flavivirus*, such as yellow fever virus, dengue virus, and West Nile virus, and of the genus *Pestivirus*, such as bovine viral diarrhea virus and classical swine fever virus. The genome of HCV encodes a polyprotein of approximately 3,000 amino acids, which is subsequently processed into at least 10 viral proteins. The HCV envelope glycoproteins E1 and E2 are cleaved from the polyprotein by host signal peptidases and play a crucial role in the initiation of infection through interaction with cell surface receptor(s) in the HCV life cycle (17, 38).

A number of cellular components have been shown to participate in HCV adsorption and/or internalization, including

human CD81 (hCD81) (52), low-density lipoprotein receptor (LDLr) (1), human scavenger receptor class B type I (SR-BI) (57), dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN), liver/lymph node-specific intercellular adhesion molecule-3-grabbing nonintegrin (L-SIGN or DC-SIGNR) (21, 34), glycosaminoglycans (2), and a tight junction component, claudin-1 (18). Recently, an *in vitro* cell culture system was developed for HCV of the genotype 2a JFH1 strain (HCVcc) isolated from a fulminant HCV patient (32, 63, 68). However, a robust cell culture system for HCV of the 1a and 1b genotypes, the most prevalent genotypes in the world, has not yet been successfully developed, except for the cell culture system of H77 or H77-S strain (1a genotype) (26, 65). Furthermore, it is currently not possible to obtain a sufficient amount of HCV particles for biological and physicochemical studies due to the low viral load in the sera of hepatitis C patients and the low yield of HCV particles in cell culture. Thus, the relative contribution of these receptor candidates in HCV attachment and entry remains unclear (44).

As surrogate systems for the investigation of HCV infection mechanisms, HCV-like particles (HCV-LP) produced in insect or mammalian cells by recombinant baculovirus vectors have been developed (7, 37). Although the binding of HCV-LP to the target cells has been well characterized, HCV-LP are not suitable for the analysis of the HCV entry steps due to the absence of a clear distinction between binding and internalization. On the other hand, both murine leukemia virus (MLV)- and human immunodeficiency virus-based pseudotype retrovi-

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ral particles (HCVpp) bearing unmodified E1 and E2 proteins (5, 23) are capable of infecting human hepatoma cells, including Huh7 cells, and this infection can be inhibited by treatment with anti-hCD81 antibody and the soluble hCD81 protein or by a knockdown of hCD81 expression by small interfering RNAs (siRNAs) (67). Furthermore, the ectopic expression of hCD81 confers permissiveness to infection with HCVpp in normally nonpermissive HepG2 cells lacking expression of hCD81. These data suggest that expression of hCD81 is crucial for HCVpp infection (4). However, expression of this candidate receptor molecule is not sufficient to render nonhepatic cells permissive for HCVpp infection (6, 23). Indeed, it is also interesting to note that although neutralizing antibodies to HCVpp have been detected in the sera from persistently infected humans and chimpanzees (3, 23, 33, 66), these antibodies do not appear to play a significant role in the outcome of acute HCV infection (42). Therefore, further investigation is needed to assess the authenticity of the HCVpp as a surrogate system for HCV infection.

We and others have previously reported the generation of vesicular stomatitis virus (VSV)-based pseudotype viruses bearing chimeric or unmodified HCV E1 and E2 glycoproteins (HCVpv) in nonhepatic cell lines (27, 39, 60). Although HCVpv infected several cell lines, including human hepatoma cell lines (27, 39, 60), recombinant VSV bearing chimeric HCV E1 and E2 glycoproteins in place of VSV glycoprotein (G) was not infectious (9). This discrepancy in the cell tropism might be attributable to the differences in the constructs and strains of HCV envelope proteins or in the systems and cells in which the viruses were generated.

Human hepatocytes (Hc) are believed to be a main target for HCV replication, and it is reasonable to speculate that hepatocyte-specific host factors regulate the entry, replication, and assembly of HCV. Although HCVpp is an excellent system for examining the entry mechanisms of HCV, the system requires a high level of transfection of the expression plasmids, and thus production of HCVpp is limited to 293T cells due to their high transfectability. Furthermore, HCVpp are replication-defective and do not produce progeny virus in infected Hc, and thus reinfection with progeny viruses cannot be assessed. In this study, we generated replication-competent recombinant VSVs encoding the unmodified HCV E1 and E2 polyproteins of genotypes 1a and 1b in place of the G protein (HCVrv) in human cell lines. HCVrv was able to infect human hepatoma cell lines through an hCD81-dependent pathway and to form foci in Huh7 cells. Treatment with an ER α -glucosidase inhibitor was shown to inhibit not only infection but also focus formation of HCVrv, suggesting that modifications of envelope glycoproteins in the endoplasmic reticulum (ER) are required for infection with HCVrv.

MATERIALS AND METHODS

Plasmids and cells. The cDNAs encoding the C-terminal 60 amino acids of the core to the last residue of p7 protein (c60-p7; nucleotides 735 to 2746) of the H77 (provided by Bukh) and Con1 (provided by Bartenschlager) (residues atg + 521 to 2773 bp) strains were generated by PCR amplification. All PCR products were cloned into pCAGGS/MCS-PM, carrying the puromycin gene for the establishment of the cell lines derived from pCAGGS (45) and designated pCAGc60-p7. The plasmid used for construction of HCVrv was pVSV Δ G-P/M2.6, which has additional transcription units with two multiple cloning sites (MCS) located between the P and M genes (MCS-2) and the M and L genes (MCS-1). The

c60-p7 gene was subcloned into pBluescript SK(+) from pCAGc60-p7 by digestion with EcoRI and EcoRV and designated pBSc60-p7. To construct pVSV Δ G-c60-p7, the c60-p7 gene was excised from pBSc60-p7 with KpnI and XbaI and ligated into the KpnI and NheI sites of MCS-2 of pVSV Δ G-P/M2.6. The cDNA of hCD81 was amplified by PCR from Huh7 cells and cloned into the BamHI and XbaI sites of the pcDNA3.1 plasmid, resulting in pHCD81. The hepatic (Huh7, HepG2, Hep3B, and PLC/PRF/5) and nonhepatic (293T, HeLa, Vero, BHK, and CHOK1) cell lines were obtained from the American Type Culture Collection (Rockville, MD). The FLC4 cell line was established as described previously (37). The Huh7.5.1 cell line was kindly provided by F. Chisari. All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS). Human primary Hc were purchased from the Applied Cell Biology Research Institute (Kirkland, WA) and maintained using a CS-C serum-free medium kit (Applied Cell Biology Research Institute). To establish stable HepG2 or CHOK1 cell lines expressing hCD81, cells were transfected with pHCD81 by the *TransIT-LT1* (Mirus, Madison, WI) reagent, selected with DMEM containing 10% FBS and 2 mg/ml (HepG2) or 3.5 mg/ml (CHOK1) of G418 (PAA Laboratories GmbH, Linz, Austria), and sorted twice by FACSCalibur (Becton Dickinson, San Jose, CA) after staining with anti-hCD81 monoclonal antibody (JS-81; BD Biosciences Pharmingen, Mountain View, CA) to obtain high-expressing clones. Anti-E1 (BDI198; Biondesign International, Saco, ME) and anti-E2 (AP33) (13, 49) monoclonal antibodies or anti-VSVG polyclonal antibody (ab34774; Abcam Inc., Cambridge, MA) was used for detection of E1 and E2 of the H77 strain or VSVG by immunoblotting, respectively.

Reverse genetics of VSV. Recombinant VSVs were generated as described previously (25, 30). Briefly, BHK cells were grown to 90% confluence on 35-mm tissue culture plates. The cells were infected with a recombinant vaccinia virus encoding T7 RNA polymerase (vTF7-3) (19) at a multiplicity of infection (MOI) of 5. After incubation at room temperature for 1 h, the cells were transfected with 3 μ g of pBS-N, 5 μ g of pBS-P, 1 μ g of pBS-L, 8 μ g of pBS-G, and 5 μ g of p Δ G-c60-p7 plasmids using a cationic liposome reagent (54). After 4 h, the supernatants were replaced with 10% FBS DMEM, and cells were incubated at 37°C for 48 h. The supernatants were then filtered through a 0.22- μ m-pore-size filter (Millex-GS; Millipore) to remove vaccinia virus and were applied to BHK cells that had been transfected with pCAGVSVG (39) 24 h previously. Recovery of the virus was assessed by examining the cells for the cytopathic effects that are typical of a VSV infection after 24 to 36 h. Stocks of *G-complemented viruses, i.e., VSV Δ G virus or recombinant viruses transiently bearing VSVG protein on the virion surface, were grown from single plaques on BHK cells transfected with pCAGVSVG and then stored at -80°C. The infectious titers of the recovered viruses were determined by a plaque assay.

Production and characterization of HCVpv, HCVrv, or HCVpp. The construction of HCVpv and HCVrv is summarized in Fig. 1. To generate HCVpv in 293T or Huh7 cells transiently expressing E1 and E2 proteins, cells were transfected with pCAGc60-p7 (H77 or Con1 strain) using *TransIT-LT1* (Mirus). After 24 h of incubation at 37°C, cells were infected at an MOI of 5 with the VSV Δ G-GFP/G, in which the G envelope gene was replaced with the green fluorescent protein (GFP) gene and which was pseudotyped with the VSV G glycoprotein (39). The virus was adsorbed for 2 h at 37°C and then extensively washed four times with DMEM. After 24 h of incubation at 37°C, the culture supernatants were collected, centrifuged to remove cell debris, and stored at -80°C. HCVpp were produced as previously described from 293T cells cotransfected with an MLV Gag-Pol packaging construct, an MLV-based transfer vector encoding GFP, and the HCV envelope protein expression constructs (5). To generate HCVrv in various mammalian cell lines, cells were infected with the VSVG-complemented VSV Δ G-c60-p7 at an MOI of 5 for 2 h at 37°C and then extensively washed four times with DMEM. After 48 h of incubation at 30°C, the culture supernatants were collected and stored at -80°C. The culture supernatants were pelleted through a 20% (wt/vol) sucrose cushion at 25,000 rpm for 2 h by using an SW28 rotor (Beckman Coulter, Tokyo, Japan). The pellets were resuspended in phosphate-buffered saline (PBS), mixed with 33% (wt/wt) cesium chloride, and centrifuged at 50,000 rpm for 48 h at 4°C by using an SW55Ti rotor (Beckman Coulter). After centrifugation, 12 fractions (0.5 ml each) were collected from the top and pelleted through a 20% (wt/vol) sucrose cushion by centrifugation at 50,000 rpm for 1 h at 4°C using an SW55Ti rotor. The pellets were resuspended in PBS and analyzed by immunoblotting to detect the incorporation of E1 or E2 proteins with anti-E1 (BDI198) or anti-E2 (AP33) monoclonal antibody, respectively. VSV N, P, and M were detected by anti-VSV polyclonal antibody, which was prepared by immunization of goats with purified VSV Δ G. To determine the infectivities of HCVpv and HCVpp, infected cells were identified as GFP-positive cells under fluorescence microscopy or using FACSCalibur and expressed as infectious units (IU)/milliliter. The infectious

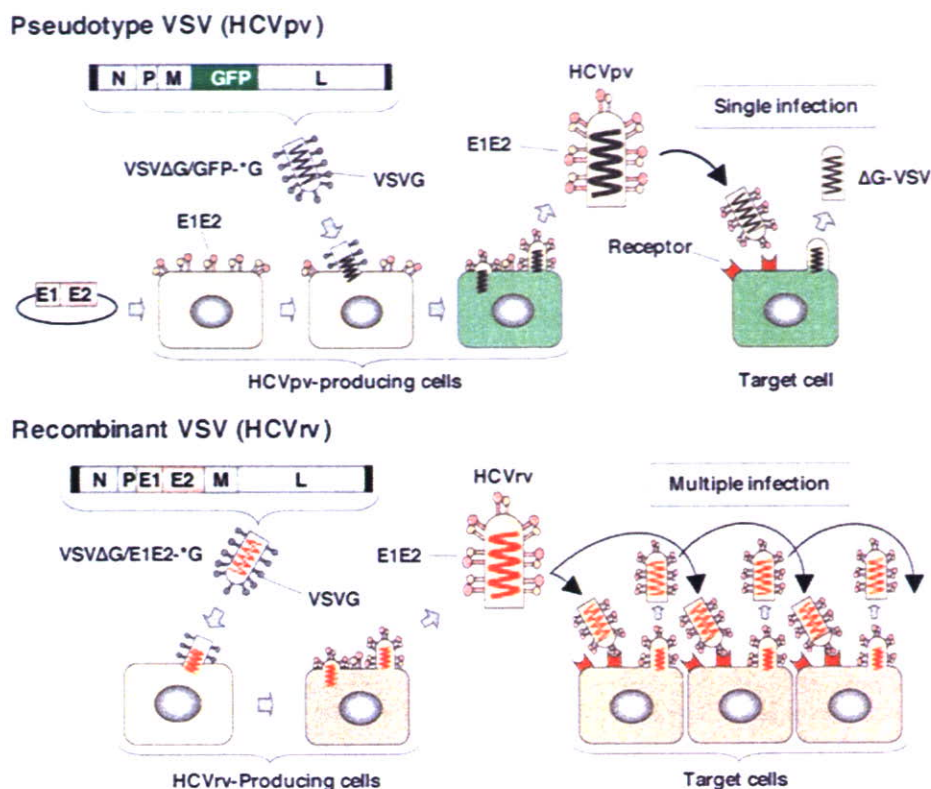


FIG. 1. Schematic representation of the production of HCVpv and HCVrv. Pseudotype VSV (HCVpv): producer cells (Huh7 or 293T) were transfected with an expression plasmid encoding the HCV E1 and E2 genes and then infected with a VSVG-complemented pseudotype virus (VSVΔG/GFP-*G). The HCVpv released from the producer cells infected target cells but was not able to produce infectious progeny virus. Recombinant VSV (HCVrv): various mammalian producer cells were inoculated with a VSVG-complemented recombinant virus (VSVΔG/E1E2-*G) encoding the HCV E1 and E2 genes instead of VSVG. HCVrv was capable of undergoing a fully productive infection generating infectious progeny virus that could be passaged into naive cells.

titers of HCVrv were determined by a focus-forming assay as described below. To examine the effects of oligosaccharide modification of the E1 or E2 envelope proteins on the infectivity of the HCVpv and HCVrv, the cell lysates and the purified virions were digested with endoglycosidase H (Endo H) or peptide-N-glycosidase F (PNGase F) (Boehringer Mannheim, Mannheim, Germany), following a protocol provided by the manufacturer, and analyzed by immunoblotting. Pseudotype VSVs bearing VSVG (VSVpv) and MLV RD114 envelope protein (MLVpv) were produced in 293T cells transfected with pCAGVSVG and pFBASALF (provided by Miyazawa), respectively, and used as controls.

Immunofluorescence and focus-forming assay. The cells infected with HCVpv, HCVrv, VSV, or HCVcc were cultured at 37°C with 0.8% methylcellulose in 10% FBS DMEM for the indicated periods and fixed with 4% paraformaldehyde solution for 1 h. Cells were washed once with PBS, treated with 0.5% Triton X-100 for 20 min for permeabilization, and then incubated with mouse monoclonal antibody to VSV N (10G4) (HCVpv, HCVrv, and VSV) or rabbit polyclonal antibody to NSSA (22) (HCVcc) for 1 h. Then, the cells were visualized by staining with Alexa 488-conjugated anti-mouse immunoglobulin G (IgG) or anti-rabbit IgG (Molecular Probes, Eugene, OR) for the immunofluorescence assay. The nuclei were counterstained with Hoechst 33258 (Molecular Probes). For the focus-forming assay, cells were treated with secondary antibodies and stained by using a VECTASTAIN Elite ABC anti-mouse IgG kit with a VIP substrate (Vector Laboratories, Burlingame, CA), following a protocol provided by the manufacturer. The infectious titers of the viruses were expressed as focus-forming units.

Inhibition of HCVpv or HCVrv infection by treatment with antibodies against hCD81, E1 and E2, HCV patient sera, and siRNA. To determine the involvement of hCD81 in infection, Huh7 or HepCD81 cells were pretreated with 5 μg/ml of anti-hCD81 for 1 h at 37°C and inoculated with HCVpv or HCVrv. In addition, Huh7 cells on six-well plates were transfected with 80 nM of siRNAs targeted to hCD81 by using Nucleofector II (Amaxa GmbH, Cologne, Germany) according

to the manufacturer's protocol. The hCD81 siRNAs (sc-35030) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). At 24 h posttransfection, cells were trypsinized, seeded at 8×10^3 cells/well into 96-well plates, and cultured for 48 h at 37°C. HCVpv or HCVrv was inoculated into the target cells, and infectivity was determined at 24 h postinfection. To characterize the infection with HCVpv and HCVrv, viruses were preincubated with 20 μg/ml of anti-E1 (AP21.010) (13) or anti-E2 (AP33) monoclonal antibodies, 1:50 diluted anti-E1 (R852) or anti-E2 (R646) polyclonal rabbit sera, and sera from chronic HCV patients or healthy donors for 1 h at 37°C and then inoculated into Huh7 cells. Informed consent was obtained from the patients and the donors. After 1 h of adsorption at 37°C, the cells were washed three times with DMEM containing 10% FBS, and infectivity was determined after 24 h of incubation at 37°C.

Effects of chemicals on HCVpv, HCVrv, and HCVcc infection. To determine the entry pathways of the viruses, Huh7 cells were preincubated with various concentrations of bafilomycin A₁ (Sigma) for 1 h at 37°C followed by infection with HCVpv, HCVrv, VSVpv, or MLVpv. The residual infectivity was determined as described above. *N*-(*n*-Nonyl) deoxyjirimycin (Nn-DNJ) and 1-deoxymannojirimycin hydrochloride (DMJ) were purchased from Toronto Research Chemicals Inc. (Downsview, ON, Canada). Nn-DNJ and DMJ were dissolved in ethanol and PBS, respectively, and diluted with medium before use. HCVcc was generated as previously described (47). Huh7 cells were inoculated with the viruses for 2 h at 37°C, replaced with medium containing either Nn-DNJ or DMJ, and cultured for 24 h (VSV), 72 h (HCVrv), or 96 h (HCVcc). The effects of Nn-DNJ or DMJ on the incorporation of the envelope proteins and generation of infectious particles were analyzed by immunoblotting and Coomassie staining. For the focus-forming assay, 0.8% methylcellulose in 10% FBS DMEM containing the reagents was overlaid on the cells. The infectious titers of VSV and HCVrv were determined by a focus-forming assay as described below. The infectious titers of HCVcc were evaluated by a quantitative core enzyme-linked immunosorbent assay as described previously (37).

RESULTS

Production and characterization of HCVrv. HCVrv was recovered from plasmids using established methods for the recovery of recombinant VSV in BHK cells. To ensure that infectious virus was produced, the recoveries were performed in cells transiently expressing VSV G protein. To determine if the HCV envelope proteins could mediate infection, the G-pseudotyped viruses were used to infect either Huh7 or 293T cells, and then the supernatants were titrated on Huh7 cells. VSV lacking an envelope protein (VSVΔG) was used as a negative control. Infectivities of HCVrv generated in either 293T cells or Huh7 cells were dependent on the combination of incubation temperature and period (data not shown). The highest infectivity was constantly recovered in either cell line when cultured at 30°C for 48 h rather than when cultured at 37°C. Thus, HCVrv was prepared at 30°C for the remaining experiments. To determine whether the cell line used affected the generation of HCVrv, the infectivity in Huh7 cells and incorporation of HCV proteins into particles of HCVrv generated in various cell lines were examined (Fig. 2). HCVrv generated in Huh7 and 293T cells exhibited the highest infectivity in Huh7 cells, followed by Hep3B, PLC/PRF/5, and HepG2 cells. Significant infectivity was not observed for virus produced in HeLa, Vero, or BHK cells. Incorporation of the E1 and E2 proteins varied among the particles produced in the different cell lines. Although incorporation of E1 and E2 proteins into the particles was high in HCVrv generated in HepG2, BHK, and Huh7 cells, HCVrv produced in BHK cells exhibited the lowest infectivity to Huh7 cells. On the other hand, incorporation of the E1 and E2 proteins into HCVrv particles generated in 293T and Hep3B cells was low, whereas these viruses exhibited substantial infectivity to Huh7 cells. These results indicate that there is no clear correlation between the quantity of incorporation of HCV envelope proteins and the infectivity of HCVrv in Huh7 cells, although the producer cell type is important.

Characterization of HCVrv and HCVpv. To examine the properties of the HCV envelope proteins incorporated into the recombinant and pseudotype VSV particles, E1 and E2 proteins of the H77 strain (genotype 1a) expressed in 293T cells and incorporated into the viral particles were examined by immunoblotting with anti-E1 (BDI198) and anti-E2 (AP33) monoclonal antibodies (Fig. 3A). The E1 and E2 proteins of the cell lysates and virions of HCVpv or HCVrv were sensitive to both Endo H and PNGase F, suggesting that both HCVrv and HCVpv possess E1 and E2 proteins with high-mannose glycans, as reported for the E1 and E2 proteins of HCVpp (48). Next, to examine the infectivity of HCVrv and HCVpv to the target cells, viruses bearing HCV envelope proteins of genotypes 1a (H77 strain) and 1b (Con1 strain) were generated in 293T or Huh7 cells and inoculated into Huh7 cells. The infectivities in Huh7 cells of HCVrv carrying E1 and E2 proteins of the H77 or Con1 strains were 10- to 20-fold higher ($\sim 1 \times 10^6$ IU/ml) than those of HCVpv ($\sim 1 \times 10^5$ IU/ml) (Fig. 3B). HCVpv generated in 293T cells exhibited higher infectivity than that generated in Huh7 cells. No difference in the infectivities of HCVpv and HCVrv between the 1a and 1b genotypes was observed. To determine the relationship between the incorporation of E1 and E2 proteins into HCVrv

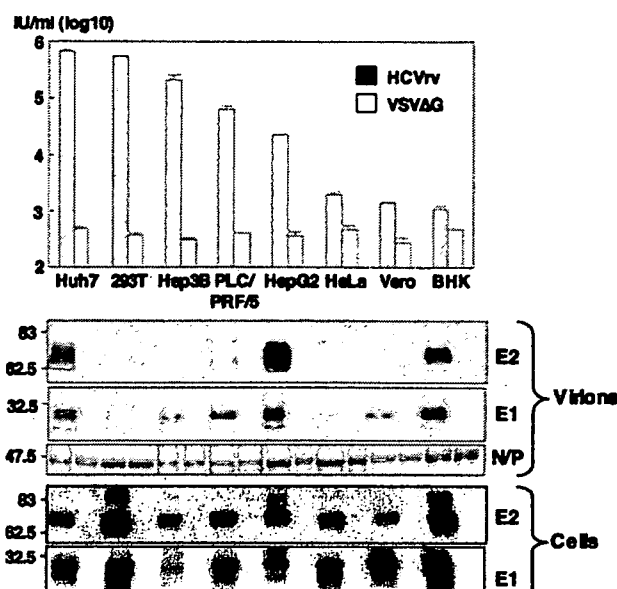


FIG. 2. Production and characterization of HCVrv. (Top) The infectivity of HCVrv of the H77 strain produced in the indicated cell lines at 30°C for 48 h was determined in Huh7 cells at 37°C for 24 h postinfection by counting VSV N-positive cells. The results shown are from three independent assays, with the error bars representing the standard deviations. (Bottom) Expression and incorporation of the HCV E1 and E2 proteins in cells and purified viral particles. HCV E1 or E2 proteins were detected by immunoblotting with anti-E1 or anti-E2 monoclonal antibodies. An envelope-less pseudotype virus, VSVΔG, was used as a negative control.

particles and their infectivities, the culture supernatants of 293T cells infected with HCVrv (H77 strain) were subjected to CsCl equilibrium gradient centrifugation, and each fraction was analyzed by immunoblotting and titration of infectivity in Huh7 cells (Fig. 3C). Immunoblot analyses revealed that incorporation of E1 and E2 proteins into HCVrv particles was detected in fractions 4 to 8 (Fig. 3C, top). These fractions exhibited the highest infectious titers (5×10^5 to 1×10^6 IU/ml), corresponding to buoyant densities of 1.2 to 1.3 g/ml (Fig. 3C, bottom).

Propagation of HCVrv. To examine the propagation of HCVrv in the target cells, Huh7 cells were infected with HCVrv at an MOI of 0.01 and incubated for up to 120 h. As a negative control, HCVpv was employed (Fig. 4A). A visible cytopathic effect was observed in Huh7 cells infected with HCVrv but not with HCVpv after 48 h incubation (data not shown). Immunofluorescence staining of Huh7 cells infected with HCVrv with antibody against VSV N revealed that VSV N protein was present from 24 h postinfection and had infected all cells at 120 h postinfection. In contrast, VSV N protein staining was decreased in cells infected with HCVpv at 120 h postinoculation. Focus formation of HCVrv in Huh7 cells was visualized by immunostaining under a methylcellulose overlay (Fig. 4B). Although the focus sizes of HCVrv were smaller than those of wild-type VSV, focus formation of HCVrv was clearly detected in a dose-dependent manner. In contrast, no focus formation was detected in cells infected with HCVpv. These results indicate that HCVrv is replication competent in

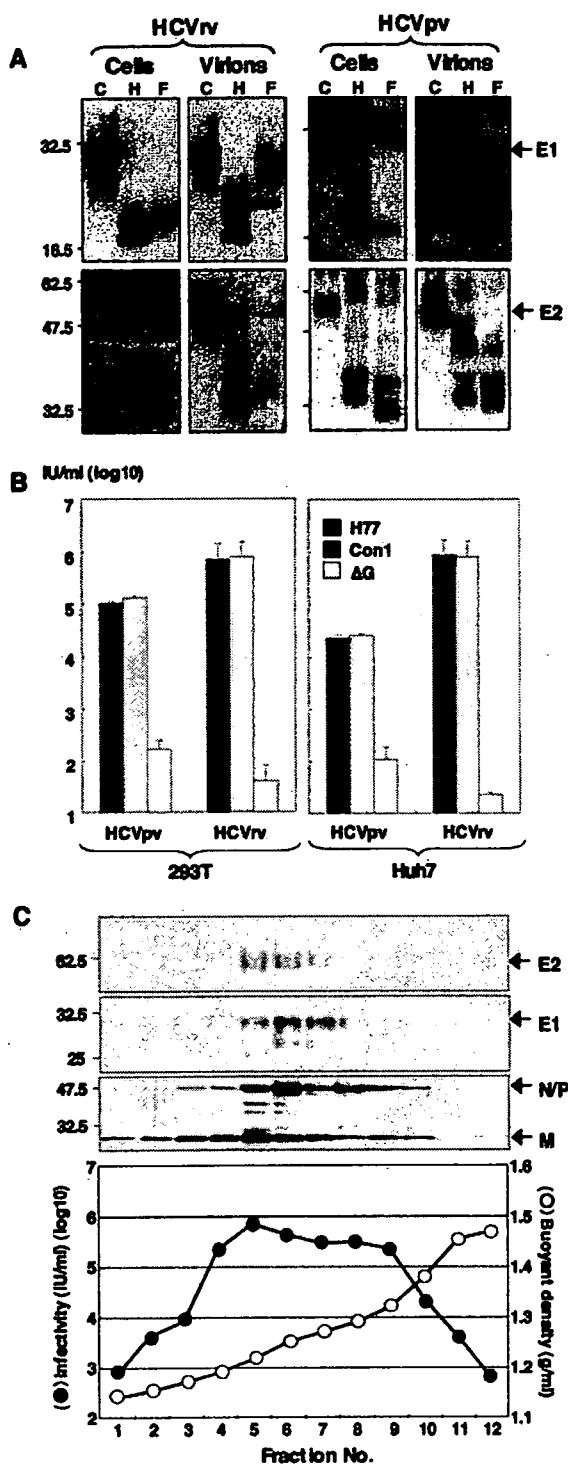


FIG. 3. Characterization of HCVrv and HCVpv. (A) The E1 and E2 proteins of the H77 strain expressed in 293T cells and incorporated into the particles of HCVrv and HCVpv were either untreated (C) or treated with endoglycosidase H (H) or peptide-*N*-glycosidase F (F). Following fractionation on sodium dodecyl sulfate-polyacrylamide gel gels, the glycoproteins were detected by immunoblotting with anti-E1 (BDI198) and anti-E2 (AP33) monoclonal antibodies. (B) The infectivities of HCVrv and HCVpv bearing HCV envelope proteins of genotypes 1a (H77 strain) and 1b (Con1 strain) generated

Huh7 cells. To further determine the cell tropism for virus propagation, HCVrv was generated in various cell lines, and replication was assessed during incubation for up to 6 days (Fig. 4C, left). The growth kinetics of the wild-type VSV revealed an efficient replication of VSV in all the cell lines examined (Fig. 4C, right). Huh7 cells exhibited the highest susceptibility to propagation of HCVrv, followed by Hep3B cells, and no propagation was detected in the other cell lines. These results indicate that various human cell lines are capable of producing HCVrv that is infectious to Huh7 cells and that Huh7 cells are highly permissive to the propagation of HCVrv.

Involvement of hCD81 in the infection with HCVpv and HCVrv. Among the candidates for entry receptor of HCV, hCD81 was shown to be most essential for the infection with HCVpp (5, 23) and HCVcc (27, 56, 60). The infection of Huh7 cells with HCVpv and HCVrv was inhibited by anti-hCD81 antibody, whereas no inhibition of VSVpv infection was observed (Fig. 5A). Treatment with siRNA targeted to hCD81 induced a reduction of hCD81 expression on the surface of Huh7 cells (Fig. 5B), and the susceptibility of hCD81-knock-down cells to infection with HCVpv and HCVrv, but not to that with VSVpv, was clearly reduced (Fig. 5C). To further determine the involvement of hCD81 in the infectivity of HCVpv and HCVrv, hCD81-negative HepG2 cells stably expressing hCD81 (HepCD81) were established, and fluorescence-activated cell sorter (FACS) analysis revealed that expression of hCD81 on the cell surface was higher than that of Huh7 cells (Fig. 5D). Although HCVpv and HCVrv are not infectious in HepG2 cells, HepCD81 cells were permissive to both HCVpv and HCVrv infection, and pretreatment with the anti-hCD81 antibody inhibited the infection of HepCD81 cells with HCVpv and HCVrv (Fig. 5E). These results indicate that hCD81 plays a crucial role in infection with HCVpv and HCVrv, as it has been reported to play in infection with HCVpp and HCVcc.

Infectivity of HCVpv and HCVrv in various cell lines. To further examine the cell tropism of the viruses, HCVpv and HCVrv of the H77 and Con1 strains generated in 293T or Huh7 cells and HCVpp of the H77 strain generated in 293T cells were inoculated into various cell lines and primary Hc (Table 1). As expected, the control VSVΔG exhibited no infectivity in any of the cells examined (data not shown). The HCVpv and HCVrv derived from both genotypes were highly infectious in Huh7 cells, followed by HepCD81 and Hep3B cells, and weakly infectious in PLC/PRF/5, 293T, and Vero cells. No infectivity was detected in the other cell lines examined. The cell tropisms of the HCVpp were similar to those of HCVpv and HCVrv. Although the ectopic expression of hCD81 in Chinese hamster ovary cells (CHOCD81) did not confer susceptibility to HCVpv, HCVrv, or HCVpp infection,

in 293T or Huh7 cells were determined with Huh7 cells. The envelopeless VSV (ΔG) was used as a control. (C) (Top) CsCl gradient sedimentation of HCVrv produced in 293T cells. The supernatant was fractionated from the top of the gradient and analyzed by immunoblotting with anti-E2, anti-E1, and anti-VSV antibodies. (Bottom) The infectivity (filled circles) of each fraction was determined after the removal of CsCl with column purification. Fraction densities (open circles) are expressed in grams/milliliter.

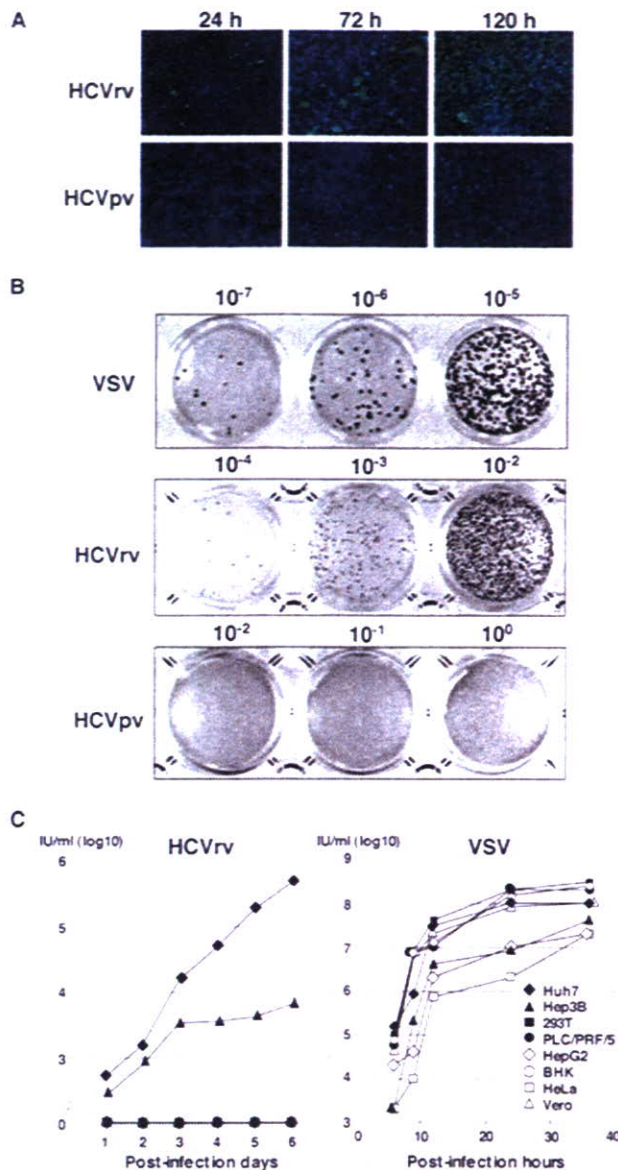


FIG. 4. Propagation of HCVrv. (A) Detection of viral proteins in Huh7 cells infected with HCVpv or HCVrv. Huh7 cells were infected with HCVpv or HCVrv at an MOI of 0.01. Twenty-four, 72, and 120 h after infection, cells were fixed and stained with monoclonal antibody to VSV N protein and Alexa 488-conjugated 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 naïve 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 A₁. 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 A₁, suggesting that these viruses enter via a pH-dependent endocytosis into target cells (25, 61). To determine the entry pathway of HCVpv and HCVrv, Huh7 cells were pretreated with various concentrations of bafilomycin A₁, 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,

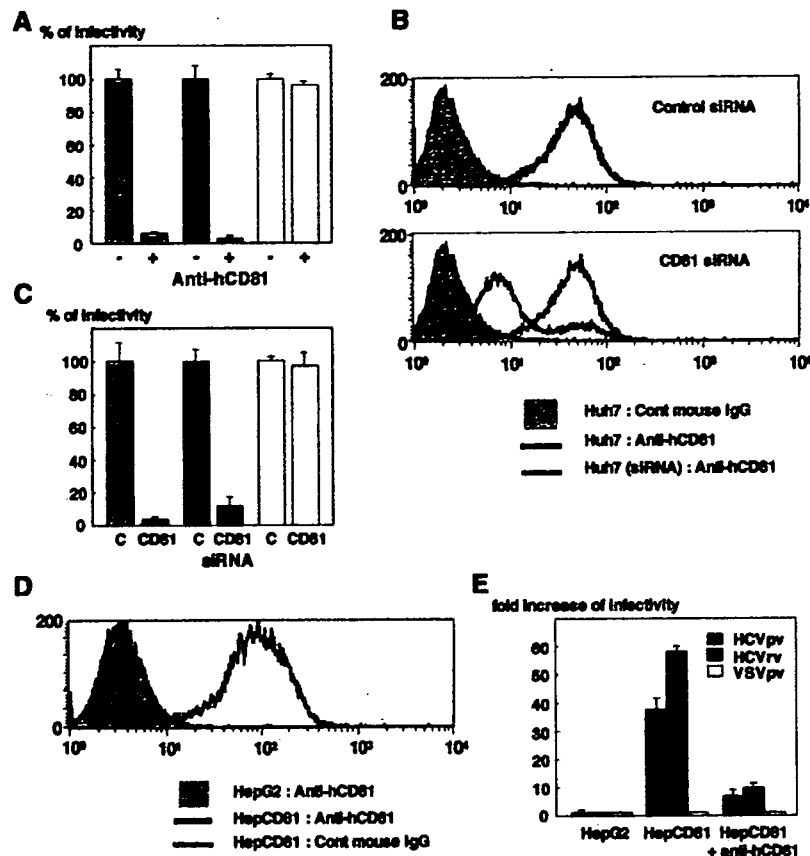


FIG. 5. Involvement of hCD81 in the infection of HCVpv and HCVrv. (A) Effect of anti-hCD81 antibody on the infectivity of HCVpv (gray-filled bars), HCVrv (black-filled bars), or VSVpv (open bars) in Huh7 cells. (B) Cell surface expression of hCD81 on Huh7 cells transfected with siRNA targeted to hCD81 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 A₁ in a dose-dependent manner. Infection with HCVpv and HCVrv was also clearly inhibited by the treatment with bafilomycin A₁ 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), glyco-biological 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 μ M of Nn-DNJ were severely impaired by the cytotoxic effects of Nn-DNJ (Fig. 8A, bottom left), treatment with 10 μ M 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-

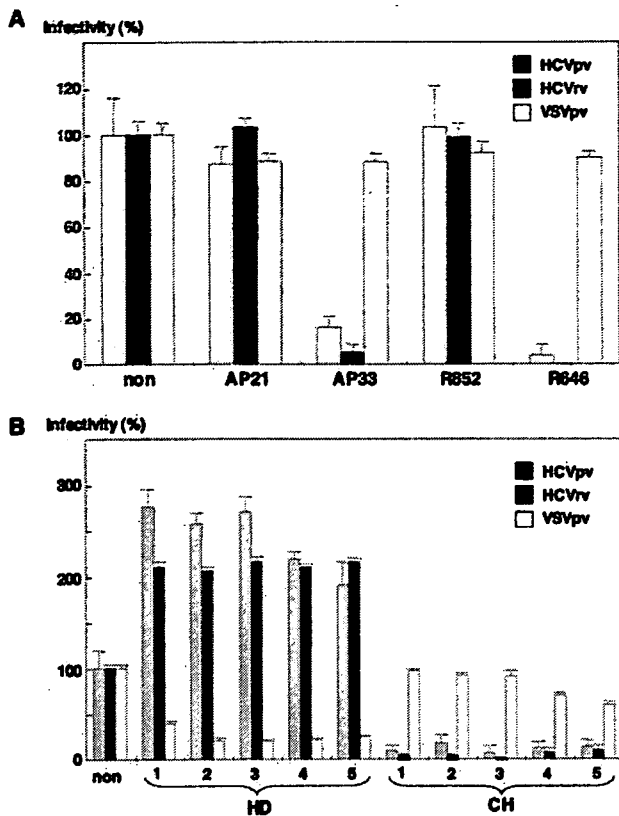


FIG. 6. Neutralization of HCVpv and HCVrv infection by antibodies to HCV envelope proteins and sera of HCV patients. (A) Effect of anti-E1 (AP21.010) and anti-E2 (AP33) monoclonal antibodies and anti-E1 (R852) and anti-E2 (R646) rabbit sera on the infectivity of HCVpv (gray-filled bars), HCVrv (black-filled 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.

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 (CHOC81 cells) were

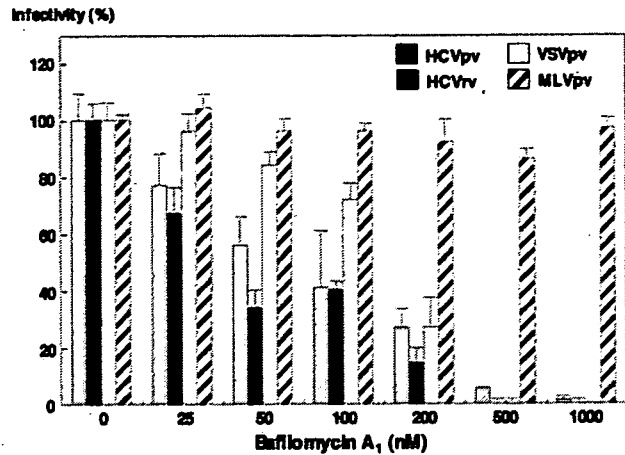


FIG. 7. Inhibition of HCVpv and HCVrv infection by bafilomycin A₁. HCVpv (gray-filled bars), HCVrv (black-filled bars), VSVpv (open bars), or MLVpv (striped bars) were inoculated to Huh7 cells after treatment with various concentrations of bafilomycin A₁. The results shown are from three independent assays, with the error bars representing the standard deviations.

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 A₁, 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

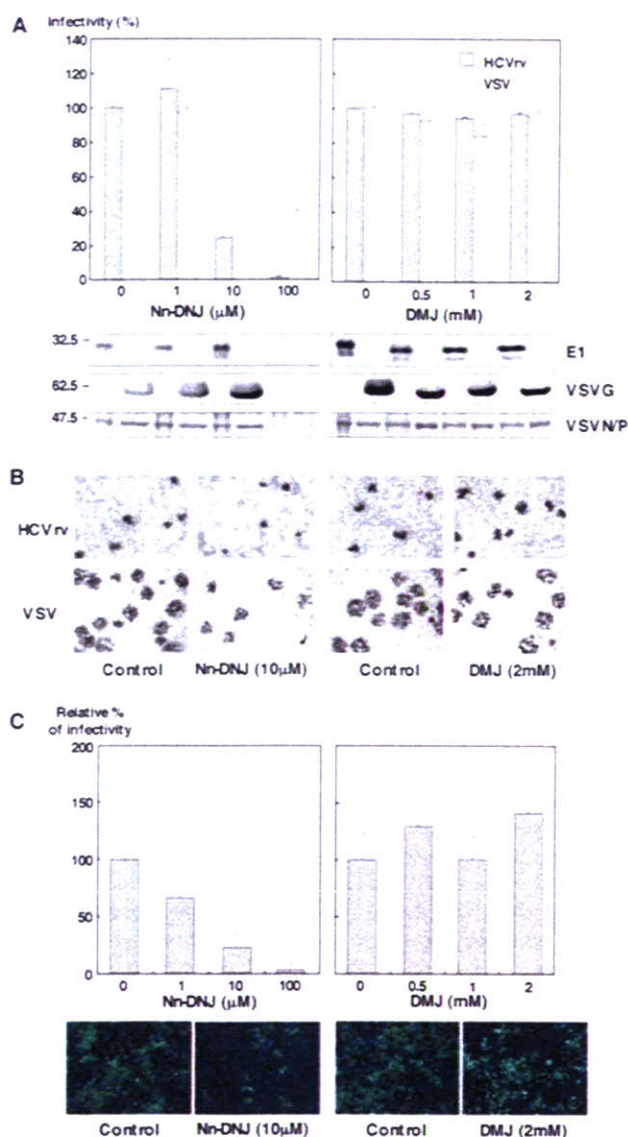


FIG. 8. Effects of ER α -glucosidase inhibitors on the infection with HCVrv and HCVcc. (A) (Top) Production of HCVrv and VSV in the presence of Nn-DNJ (left) or DMJ (right). Huh7 cells infected with HCVrv and VSV at MOIs of 0.1 and 0.01, respectively, were treated with various concentrations of Nn-DNJ 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 μ M) 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

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, triglycosylated 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

an MOI of 0.01, treated with 10 μ M 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.

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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Enhanced TLR-mediated NF-IL6-dependent gene expression by Trib1 deficiency

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Toll-like receptors (TLRs) recognize a variety of microbial components and mediate downstream signal transduction pathways that culminate in the activation of nuclear factor κ B (NF- κ B) and mitogen-activated protein (MAP) kinases. Trib1 is reportedly involved in the regulation of NF- κ B and MAP kinases, as well as gene expression *in vitro*. To clarify the physiological function of Trib1 in TLR-mediated responses, we generated Trib1-deficient mice by gene targeting. Microarray analysis showed that Trib1-deficient macrophages exhibited a dysregulated expression pattern of lipopolysaccharide-inducible genes, whereas TLR-mediated activation of MAP kinases and NF- κ B was normal. Trib1 was found to associate with NF-IL6 (also known as CCAAT/enhancer-binding protein β). NF-IL6-deficient cells showed opposite phenotypes to those in Trib1-deficient cells in terms of TLR-mediated responses. Moreover, overexpression of Trib1 inhibited NF-IL6-dependent gene expression by down-regulating NF-IL6 protein expression. In contrast, Trib1-deficient cells exhibited augmented NF-IL6 DNA-binding activities with increased amounts of NF-IL6 proteins. These results demonstrate that Trib1 is a negative regulator of NF-IL6 protein expression and modulates NF-IL6-dependent gene expression in TLR-mediated signaling.

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Abbreviations used: 24p3, lipocalin-2; BLP, bacterial lipoprotein; C/EBP, CCAAT/enhancer-binding protein; Jnk, c-Jun N-terminal kinase; MALP-2, macrophage-activating lipopeptide-2; MAP, mitogen-activated protein; mPGES, prostaglandin E synthase; TLR, Toll-like receptor.

Innate immunity is promptly activated after the invasion of microbes through recognition of pathogen-associated molecular patterns by pattern-recognition receptors, including Toll-like receptors (TLRs) (1). The recognition of microbial components by TLRs effectively stimulates host immune responses such as proinflammatory cytokine production, cellular proliferation, and up-regulation of co-stimulatory molecules, accompanied by the activation of NF- κ B and mitogen-activated protein (MAP) kinases (2, 3). Although the inhibitory protein I κ B family members sequester NF- κ B in the cytoplasm of unstimulated cells, TLR-dependent I κ B phosphorylation by the I κ B kinase complex and degradation by the ubiquitin-proteasome pathway permit translocation of NF- κ B to the nucleus (4). MAP kinases such as c-Jun N-terminal kinase (Jnk) and p38 are also rapidly phosphorylated

and activated by upstream kinases in response to TLR stimulation (5). Moreover, TLR-mediated activity of NF- κ B and MAP kinases is shown to be regulated at multiple steps regarding the strength and the duration of the activation (6).

Recent extensive experiments have identified a variety of modulators that have positive and negative effects on the activation of NF- κ B and MAP kinases, including a family of serine/threonine kinase-like proteins called Trib (7). Trib consists of three family members: Trib1 (also known as c8fw, GIG2, or SKIP1), Trib2 (also known as c5fw), and Trib3 (also known as NIPK, SINK, or SKIP3) (7–12). Trib3 has been shown to interact with the p65 subunit of NF- κ B and to inhibit NF- κ B-dependent gene expression *in vitro* (11). In terms of MAP kinases, Trib1, Trib2, and Trib3 reportedly bind to Jnk and p38, and affect the activity of MAP kinases and IL-8 production in response to PMA or

The online version of this article contains supplemental material.

TLR ligands/IL-1 (12). However, whether Trib family members regulate TLR-mediated signaling pathways under physiological conditions is still unknown.

In this study, we generated Trib1-deficient mice by gene targeting and analyzed TLR-mediated responses. Although the activation of NF- κ B and MAP kinases in response to LPS was comparable between wild-type and Trib1-deficient cells, microarray analysis revealed that a subset of LPS-inducible genes was dysregulated in Trib1-deficient cells. Subsequent yeast two-hybrid analysis identified the CCAAT/enhancer-binding protein (C/EBP) family member NF-IL6 (also known as C/EBP β) as a binding partner of Trib1, and phenotypes found in NF-IL6-deficient cells were opposite to those observed in Trib1-deficient cells. Moreover, overexpression of Trib1 inhibited NF-IL6-mediated gene expression and reduced amounts of NF-IL6 proteins. Inversely, NF-IL6 DNA-binding activity and LPS-inducible NF-IL6-target gene expression were up-regulated in Trib1-deficient cells, in which amounts of NF-IL6 proteins were increased. These results demonstrate that Trib1 plays an important role in NF-IL6-dependent gene expression in the TLR-mediated signaling pathways.

RESULTS

Comprehensive gene expression analysis in Trib1-deficient macrophages

To assess the physiological function of Trib1 in TLR-mediated immune responses, we performed a microarray analysis to compare gene expression profiles between wild-type and Trib1-deficient macrophages in response to LPS (Fig. 1 A and Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20070183/DC1>). Out of 45,102 transcripts, we first defined the genes induced more than twofold after LPS stimulation in wild-type cells as "LPS-inducible genes" and identified 790 of them (Table S1). We next compared the LPS-inducible genes in wild-type and Trib1-deficient macrophages after LPS stimulation and found 59, 703, and 28 genes as up-regulated, similarly expressed, and down-regulated in Trib1-deficient cells, respectively (Table S1).

Among the up-regulated genes, several were subsequently tested by Northern blotting to confirm the accuracy. LPS-induced expression of prostaglandin E synthase (mPGES), lipocalin-2 (24p3), arginase type II, and plasminogen activator inhibitor type II, which were highly up-regulated in the microarray analysis (Table S1), was indeed enhanced in Trib1-deficient macrophages (Fig. 1 B). Furthermore, in contrast to proinflammatory cytokines such as TNF- α and IL-6, which were similarly expressed between wild-type and Trib1-deficient cells in response not only to LPS but also to other TLR ligands, IL-12 p40 was down-regulated in Trib1-deficient cells compared with wild-type cells (Fig. 1 C; Fig. S2, A–C, available at <http://www.jem.org/cgi/content/full/jem.20070183/DC1>; and Table S1). Thus, the comprehensive microarray analysis revealed that a subset of LPS-inducible genes is dysregulated in Trib1-deficient cells.

Previous *in vitro* studies demonstrate that human Trib family members modulate activation of MAP kinases and

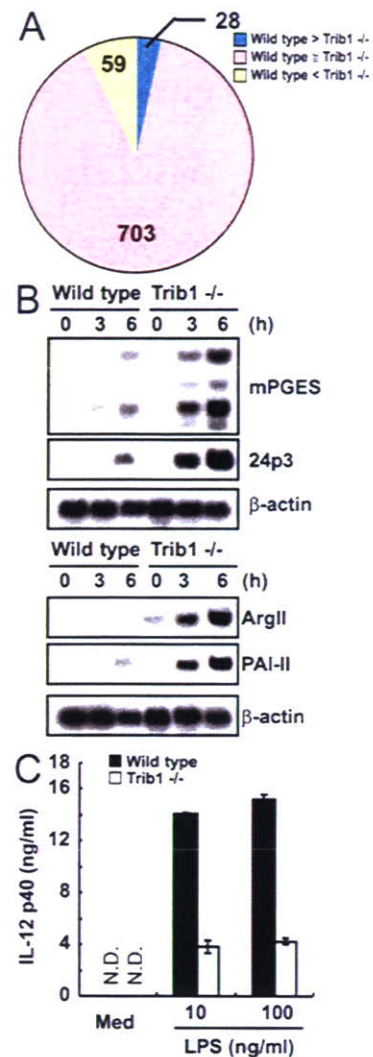


Figure 1. Dysregulation of a subset of LPS-inducible genes in Trib1-deficient cells. (A) Summary of DNA chip microarray analysis. 790 LPS-inducible genes were divided into up-regulated (yellow), similarly expressed (pink), and down-regulated (blue) groups, with the indicated amounts of each. (B) Peritoneal macrophages from wild-type or Trib1-deficient mice were stimulated with 10 ng/ml LPS for the indicated periods. Total RNA (10 μ g) was extracted and subjected to Northern blot analysis for the expression of the indicated probes. (C) Peritoneal macrophages from wild-type and Trib1-deficient mice were cultured with the indicated concentrations of LPS in the presence of 30 ng/ml IFN- γ for 24 h. Concentrations of IL-12 p40 in the culture supernatants were measured by ELISA. Indicated values are means \pm SD of triplicates. Data are representative of three (B) or two (C) independent experiments. N.D., not detected.

NF- κ B (7–12). Both wild-type and Trib1-deficient cells showed similar levels and time courses of phosphorylation of p38, Jnk and extracellular signal-regulated kinase, and I κ B α degradation (Fig. S2 D), indicating that the dysregulated

expression of LPS-inducible genes in Trib1-deficient cells might be independent of activation of NF- κ B and MAP kinases.

Interaction of Trib1 with NF-IL6

To explore signaling aspects of Trib1 deficiency other than NF- κ B and MAP kinases, we performed a yeast-two-hybrid screen with the full length of human Trib1 as bait to identify a binding partner of Trib1 and identified several clones as being positive. Sequence analysis subsequently revealed that three clones encoded the N-terminal portion of a member of the C/EBP NF-IL6 (unpublished data). We initially tested the interaction of Trib1 and NF-IL6 in yeasts. AH109 cells were transformed with a plasmid encoding the full length of Trib1 together with a plasmid encoding the N-terminal portion of NF-IL6 obtained by the screening (Fig. 2 A). We next examined the interaction in mammalian cells using immunoprecipitation experiments. HEK293 cells were transiently transfected with a plasmid encoding the full length of mouse Trib1 together with a plasmid encoding the full length of mouse NF-IL6. Myc-tagged NF-IL6 was coimmunoprecipitated

with Flag-Trib1 (Fig. 2 B), showing the interaction of Trib1 and NF-IL6 in mammalian cells.

TLR-mediated immune responses in NF-IL6-deficient macrophages

An in vitro study showing the interaction of Trib1 and NF-IL6 prompted us to examine the TLR-mediated immune responses in NF-IL6-deficient cells, because LPS-induced expression of mPGES is shown to depend on NF-IL6 (13). We initially analyzed the expression pattern of genes affected by the loss of Trib1 in NF-IL6-deficient macrophages by Northern blotting. LPS-induced expression of 24p3, plasminogen activator inhibitor type II, and arginase type II, as well as mPGES, was profoundly defective in NF-IL6-deficient cells (Fig. 2 C). We next tested IL-12 p40 production by ELISA. As previously reported, IL-12 p40 production by LPS stimulation was increased in a dose-dependent fashion in NF-IL6-deficient cells compared with control cells (Fig. 2 D) (14). In addition, the production in response to bacterial lipoprotein (BLP), macrophage-activating lipopeptide-2 (MALP-2), or CpG DNA was also augmented in

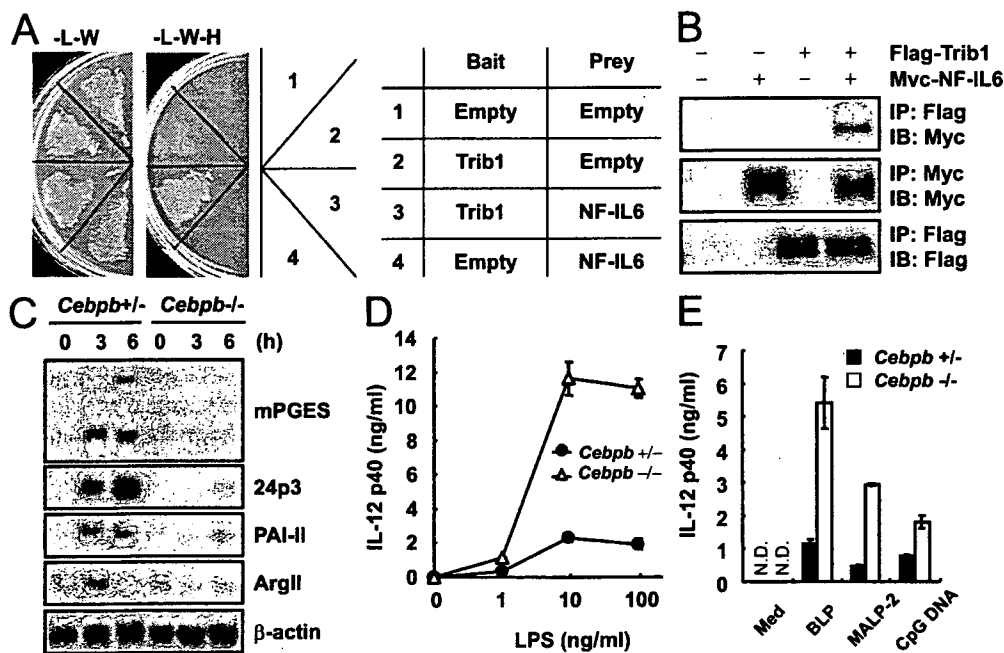


Figure 2. Association of Trib1 with NF-IL6 and TLR-mediated responses in NF-IL6-deficient macrophages. (A) Plasmids expressing human Trib1 fused to the GAL4 DNA-binding domain or an empty vector were cotransfected with a plasmid expressing NF-IL6 fused to GAL4 transactivation domain or an empty vector. Interactions were detected by the ability of cells to grow on medium lacking tryptophan, leucine, and histidine (-L-W-H). The growth of cells on a plate lacking tryptophan and leucine (-L-W) is indicative of the efficiency of the transfection. (B) Lysates of HEK293 cells transiently cotransfected with 2 μ g of Flag-tagged Trib1 and/or 2 μ g Myc-tagged NF-IL6 expression vectors were immunoprecipitated with the indicated antibodies. (C) Peritoneal macrophages from wild-type or NF-IL6-deficient mice were stimulated with 10 ng/ml LPS for the indicated periods. Total RNA (10 μ g) was extracted and subjected to Northern blot analysis for expression of the indicated probes. (D and E) Peritoneal macrophages from wild-type and NF-IL6-deficient mice were cultured with the indicated concentrations of LPS (D) or with 100 ng/ml BLP, 30 ng/ml MALP-2, or 1 μ M, CpG DNA (E) in the presence of 30 ng/ml IFN- γ for 24 h. Concentrations of IL-12 p40 in the culture supernatants were measured by ELISA. Indicated values are means \pm SD of triplicates. Data are representative of three (B) and two (C-E) separate experiments. N.D., not detected.