

## Processing of Capsid Protein by Cathepsin L Plays a Crucial Role in Replication of Japanese Encephalitis Virus in Neural and Macrophage Cells<sup>†</sup>

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The flavivirus capsid protein not only is a component of nucleocapsids but also plays a role in viral replication. In this study, we found a small capsid protein in cells infected with Japanese encephalitis virus (JEV) but not in the viral particles. The small capsid protein was shown to be generated by processing with host cysteine protease cathepsin L. An *in vitro* cleavage assay revealed that cathepsin L cleaves the capsid protein between amino acid residues Lys<sup>18</sup> and Arg<sup>19</sup>, which are well conserved among the mosquito-borne flaviviruses. A mutant JEV resistant to the cleavage of the capsid protein by cathepsin L was generated from an infectious cDNA clone of JEV by introducing a substitution in the cleavage site. The mutant JEV exhibited growth kinetics similar to those of the wild-type JEV in monkey (Vero), mosquito (C6/36), and porcine (PK15) cell lines, whereas replication of the mutant JEV in mouse macrophage (RAW264.7) and neuroblastoma (N18) cells was impaired. Furthermore, the neurovirulence and neuroinvasiveness of the mutant JEV to mice were lower than those of the wild-type JEV. These results suggest that the processing of the JEV capsid protein by cathepsin L plays a crucial role in the replication of JEV in neural and macrophage cells, which leads to the pathogenesis of JEV infection.

The genus *Flavivirus* within the family *Flaviviridae* comprises over 70 viruses, many of which are predominantly arthropod-borne viruses, such as Japanese encephalitis virus (JEV), West Nile virus (WNV), Murray Valley encephalitis virus (MVE), dengue virus (DENV), yellow fever virus (YFV), and tick-borne encephalitis virus (TBEV). They frequently cause significant morbidity and mortality in mammals and birds (5). JEV is distributed in the south and southeast regions of Asia and is kept in a zoonotic transmission cycle between pigs or birds and mosquitoes (5, 42, 45). JEV spreads to dead-end hosts, including humans, through the bite of JEV-infected mosquitoes and causes infection of the central nervous system with a high mortality rate (5, 45). JEV has a single-stranded positive-strand RNA genome of approximately 11 kb, which is capped at the 5' end but lacks a 3' polyadenine tail (24). The ability of the flaviviral genomic RNA to cyclize is crucial for viral replication (1, 14). Among mosquito-borne flaviviruses, two complementary cyclization sequences, mapped in the capsid protein-coding region and 3' untranslated region (UTR), mediated the cyclization by RNA-RNA base pairing, together with a second pair of complementary sequences, named 5' and 3' upstream AUG regions (1, 10, 14, 19, 25). The genomic RNA includes a single large open reading frame, and a polyprotein translated at the endoplasmic reticulum (ER) membrane is cleaved co- and posttranslationally by host and

viral proteases to yield three structural proteins, the capsid, precursor membrane (prM), and envelope (E) proteins, and at least seven nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (24).

Although the capsid protein has very little amino acid homology among flaviviruses—for example, the homologies of the capsid protein of JEV to those of WNV, DEN type 2 (DEN2), and TBEV were only 67%, 33%, and 25%, respectively—the structural properties, such as the hydrophobicity profile, abundance of basic amino acid residues, and secondary and tertiary structures, are well conserved (11, 18, 27). The flavivirus capsid protein commonly contains two hydrophobic sequences in the center and the carboxyl terminus. The latter serves as a signal sequence of prM. The signal/anchor sequence is cleaved off by the viral protease NS2B/3, and this cleavage is required for the subsequent liberation of the amino terminus of prM by the host signal peptidase (26, 43, 49). The mature capsid protein may be associated with the ER membrane through the central hydrophobic region (23, 29). Because the capsid protein has RNA-binding ability via the basic amino acid clusters at its amino and carboxyl termini, it is believed to bind to the genomic RNA to form a nucleocapsid (20). Unlike other envelope viruses, the nucleocapsid structures are rarely found in cells infected with flaviviruses (48), although the nucleocapsid of TBEV can assemble *in vitro* (21). Therefore, viral assembly is thought to be a coordinated process between the membrane-associated capsid protein and two envelope glycoproteins, prM and E, in the ER membrane.

In conflict with their roles as structural proteins, the capsid proteins of some flaviviruses are localized not only in the cytoplasm but also in the nuclei of the infected cells (4, 28, 32, 44, 46–48). We previously reported that the JEV capsid protein

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has also been detected in both the nucleoli and cytoplasm and that the mutant virus defective in the nuclear localization of capsid protein exhibited impaired viral growth in mammalian cells and neuroinvasiveness in mice (32). Furthermore, we have also reported that the nuclear and cytoplasmic localizations of the JEV capsid protein are dependent on binding to the host nucleolar protein B23 (46). It has been reported that, in addition to the JEV capsid protein, the WNV and DEN capsid proteins bind to several host proteins, such as Jab1, a component of the COP9 signalosome complex (34), the chaperone protein HSP70 (35), and the heterogenous nuclear ribonucleoprotein K (8), to regulate these functions. Recently, Clyde and Harris have shown that the small capsid protein isoform translated from the second AUG codon of the DEN genome by leaky scanning is important for viral replication (9). In this context, these properties of the flaviviral capsid proteins raised the possibility that they play some roles in viral growth as "nonstructural" proteins.

In this study, we detected a small capsid protein in JEV-infected cells, but not in the released viral particles. The small capsid protein has been shown to be generated by host protease cathepsin L. Cathepsin L was capable of cleaving the capsid protein between amino acid residues Lys<sup>18</sup> and Arg<sup>19</sup>. Furthermore, we have generated a mutant JEV carrying a capsid protein resistant to cleavage by cathepsin L. The characterization of this mutant JEV indicated that cleavage of the capsid protein by cathepsin L plays important roles in viral replication in mouse neuroblastoma and macrophage cells and in the pathogenesis of encephalitis *in vivo*. These results suggest a novel mechanism for JEV to adapt host cells by the processing of the capsid protein.

#### MATERIALS AND METHODS

**Cells.** The mammalian cell lines Vero (monkey kidney), 293T (human kidney), PK15 (pig kidney), RAW264.7 (mouse macrophage), and N18 (mouse neuroblastoma) were maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Mosquito cell line C6/36 (*Aedes albopictus*) was grown in Eagle's minimal essential medium supplemented with 10% FBS. Vero cell lines Vero/siNC and Vero/siCTSL, stably expressing the hairpin small interfering RNAs (siRNA) for the nonsense sequence and cathepsin L, respectively, were established by transfection with plasmids pSilencer/NC and pSilencer/CTSL (see below), respectively, and selected with DMEM containing 10% FBS and 50 µg/ml hygromycin B (Sigma, St. Louis, MO).

**Plasmids.** The cDNA for the capsid protein of JEV AT31 (amino acid residues 2 to 105) was amplified from pMWATG1 (54) by PCR using Ex-Jaq (Takara, Shiga, Japan) and cloned between the FLAG and hemagglutinin (HA) tags in pcDNA3.1FLAGHA (36). From this plasmid, the capsid cDNAs with or without FLAG and/or HA tags were amplified by PCR and subcloned into a mammalian expression vector pCAGPM (31) and designated pCAG/FLAG-JEC-HA, pCAG/FLAG-JEC, pCAG/JEC-HA, and pCAG/JEC. By the same procedure, the plasmids encoding FLAG- and HA-tagged DEN2 and DEN4 capsid proteins, pCAG/FLAG-DEN2C-HA and pCAG/FLAG-DEN4C-HA, were generated from the plasmids encoding the capsid proteins of DEN2 and DEN4, respectively (the kind gifts from F. Hasebe and M. Tadano, respectively). For mutational analyses of the amino acid residues from 14 to 23 (based on the JEV capsid protein sequence), a series of point mutants of the FLAG- and HA-tagged JEV capsid proteins were synthesized by PCR-based mutagenesis (17). All of the mutant genes, as well as the wild-type gene, were cloned into pCAGPM. The JEV capsid gene was cloned into pcDNA 3.1/myc-His (Invitrogen, Carlsbad, CA), and the cDNA encoding the JEV capsid protein fused with myc and His tags was amplified and cloned into bacterial expression vector pET32a (Merck Novagen, Darmstadt, Germany). The resulting plasmid was designated pET32/JECmycHis. The cDNAs of human cathepsins B and L were amplified from 293T cells by reverse transcription-PCR and cloned into pcDNA 3.1/myc-His. An

enzymatically inactive mutation of cathepsin L in which Cys<sup>136</sup> was replaced with Ala was generated by PCR-based mutagenesis. Expression vector pSilencer/CTSL for a hairpin siRNA for African green monkey cathepsin L was generated by annealing with synthesized nucleotides (sense, GAT CCG GCG ATG CAC AAC AGA TTA TTC AAG AGA TAA TCT GTT GTG CAT CGC CTT TTT TGG AAA; antisense, AGC TTT TCC AAA AAA GGC GAT GCA CAA CAG ATT ATC TCT TGA ATA AAT TGT TGT GCA TCC CCG) and insertion into the BamHI and HindIII sites of pSilencer 2.1 U6 hygro (Ambion Inc., Austin, TX). pSilencer/NC, encoding an siRNA with no homology to mammalian genes, was used as a negative control. pMWATL17A carrying replacements of cytosine at nucleotide 144 and thymine at nucleotide 145 with guanine and cytosine, respectively, in pMWATG1, an infectious cDNA clone of JEV, was constructed by PCR-based mutagenesis which results in the replacement of Leu<sup>17</sup> in the capsid protein with Ala (see Fig. 5A). In addition, adenine-to-guanine and guanine-to-cytosine mutations were introduced into pMWATG1 and pMWATL17A at nucleotides 10865 and 10866 of the JEV gene, respectively. The resulting plasmids were named pMWAT/CSmt and pMWATL17A/CSmt, respectively.

**Viruses.** The wild-type and L17A/CSmt JEVs were generated from plasmids pMWATG1 and pMWATL17A/CSmt, respectively, by a method described previously (54). The infectivity of the viruses was determined by an immunostaining focus assay as described previously (32) and expressed in focus-forming units (FFU). The JEV particles were purified from the supernatant of the infected Vero cells as described previously with some modifications (32). Briefly, the virions were clarified by centrifugation at 6,000 × g for 30 min and precipitated with 10% polyethylene glycol (molecular mass, approximately 6,000 kDa). The precipitates were collected by centrifugation at 10,000 × g for 45 min and centrifuged at 147,000 × g for 20 h on a 20 to 60% sucrose gradient. The fractions ranging from 1.16 to 1.19 g/ml in gravity were used as the purified virion.

**Antibodies.** Anti-JEV capsid protein rabbit polyclonal antibody (PAb) was prepared as described previously (32). Monoclonal antibodies (MAbs) to JEV E (10B4) and NS3 proteins (34A1) were generous gifts from E. Konishi and K. Yasui, respectively. Anti-FLAG tag (M2) and anti-β-actin MAbs were purchased from Sigma. Anti-HA (HA11) and anti-myc tag (9E10) MAbs were purchased from Covance (Richmond, CA). An antinucleolin MAh (MS-3) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PA28-α and anti-cathepsin L rabbit PABs were purchased from Affinity Bioreagents (Golden, CO) and Merck Calbiochem (Darmstadt, Germany), respectively.

**Infection, transfection, immunoblotting, and cell fractionation.** A monolayer of Vero or N18 cells was infected at multiplicities of infection (MOI) of 5 and 10 with the wild-type and L17A/CSmt JEVs. Plasmids were transfected by *TransIT* LT-1 (Mirus, Madison, WI) and Lipofectamine 2000 (Invitrogen) for Vero and 293T cells, respectively, according to the manufacturers' instructions. At 24 h after inoculation or transfection, cells were lysed on ice by Triton lysis buffer (20 mM Tris-HCl [pH 7.4], 135 mM NaCl, 1% Triton X-100, 10% glycerol) supplemented with a protease inhibitor cocktail (Biovision, Mountain View, CA) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting as previously described (36, 46). JEV-infected cells were fractionated using a Nuclear/Cytosol Fractionation kit (Biovision).

**Inhibition of capsid protein processing.** E64d and CA074Me were purchased from the Peptide Institute (Osaka, Japan). Z-Phe-Tyr-(*tert*-butyl)-diazomethyl ketone (DMK) (Z-FY-DMK), Z-Val-Ala-Asp-fluoromethyl ketone (FMK) (Z-VAD-FMK), PD150606, and bafilomycin A1 were purchased from Merck Calbiochem. Chloroquine and ammonium chloride were obtained from Sigma and Nacalai Tesque (Kyoto, Japan), respectively. Chloroquine and ammonium chloride were dissolved in distilled water, and bafilomycin A1 was dissolved in ethanol. The other reagents were dissolved in dimethyl sulfoxide (DMSO). At 24 h after inoculation or transfection, cells were incubated with the culture medium containing each reagent or solvent for 8 h at 37°C and examined by immunoblotting. To determine the effects of CA074Me or FY-DMK on the cleavage of the capsid protein, cells transfected with pCAG/FLAG-JEC-HA were treated with the inhibitor for 8 h at 37°C. The ratios of the densities of the slower- and faster-migrating capsid proteins (C1 and C2, respectively) detected by immunoblotting were calculated by Multi Gauge software (Fujifilm, Tokyo, Japan). The relative cleavage values were determined as the C2 to C1 ratio in the presence of inhibitor/the C2 to C1 ratio in the absence of inhibitor. The inhibitory effects of CA074Me or Z-FY-DMK to cathepsins B and L were determined as described previously (7, 13) with some modifications. Briefly, Vero cells (2 × 10<sup>5</sup>) were treated with CA074Me or Z-FY-DMK for 4 h at 37°C and lysed with 25 µl of acidic lysis buffer consisting of 100 mM sodium acetate (pH 5.0), 1 mM EDTA, 0.5% Triton X-100, 2 mM AEBFS [4-(2-aminoethyl)benzenesulfonfyl fluoride] (Merck Calbiochem), 5 µg/ml aprotinin (Nacalai Tesque), 100 µM bestatin (Sigma), and 15 µM pepstatin (Peptide Institute). Insoluble materials



were sedimented in a microcentrifuge at 4°C. Ten microliters of each lysate was mixed with 90  $\mu$ l of reaction buffer (100 mM sodium acetate [pH 5.0], 1 mM EDTA, 4 mM dithiothreitol, 2 mM AEBSEF, 5  $\mu$ g/ml aprotinin, 100  $\mu$ M bestatin, 15  $\mu$ M pepstatin). The resulting samples were mixed with 100  $\mu$ l of cathepsin B-specific (100  $\mu$ M Z-Arg-Arg-MCA [4-methylcoumaryl-7-amide; Peptide Institute], 0.1% Brij 35) (3) or cathepsin L-specific (100  $\mu$ M [Z-Phe-Arg]<sub>2</sub>-R110 [Molecular Probes, Eugene, OR], 0.1% Brij 35) (2) substrate solutions in a black 96-well plate (Corning, Corning, NY). After incubation for 30 min at room temperature, fluorescence was measured using a fluorescence multiwell plate reader (CytoFluor 4000 LX1; Applied Biosystems, Foster City, CA) with an excitation of 360 nm and an emission of 460 nm for cathepsin B and with an excitation of 485 nm and an emission of 460 nm for cathepsin L. The relative cleavage value in the absence of each inhibitor was defined as 1.

**In vitro processing of the JEV capsid protein.** The JEV capsid protein fused with thioredoxin and myc-His tags in the N and C termini, respectively, was purified using TALON metal affinity resin (Clontech, Mountain View, CA) from the lysate of *Escherichia coli* transformed by pET32/JECmycHis. The purified protein was dialyzed with acidic dialysis buffer (50 mM sodium acetate [pH 5.5], 1 mM EDTA) for 24 h at 4°C. The recombinant JEV capsid protein (35  $\mu$ g [1 nmol]/100  $\mu$ l) was incubated with 0.01 units (170 ng) of human cathepsin L (Merck Calbiochem) for 2 h at room temperature. According to the manufacturer's instructions, one unit is defined as an amount of the enzyme capable of hydrolyzing 1.0  $\mu$ mol of Z-Phe-Arg-AMC (7-amino-4-methylcoumarin) per minute at 37°C. The resulting samples were subjected to SDS-PAGE and Western blotting using anti-myc Mab. The N-terminal peptide sequences of the cleaved capsid proteins were determined by the Edman degradation method at the APRO Life Science Institute (Tokushima, Japan).

**Computer analyses of the flavivirus capsid genes.** The amino acid sequences of the flavivirus capsid proteins were aligned with the software package GENETYX-MAC, version 12 (GENETYX, Tokyo, Japan). The GenBank accession numbers of the analyzed sequences are as follows: JEV AT31 strain, AB196923; MVE 1-51 strain, AF161266; WNV IS-98 STD1 strain, AF481864; DEN1 Singapore S275/90 strain, M87512; DEN2 New Guinea C strain, M29095; DEN3 H87 strain, M93130; DEN4 814669 strain, AF326573; YFV 17D strain, X03700. Nucleotides 135 to 152 and bases 10858 to 10875 in the 5' and 3' termini, respectively, connected by 8 X nucleotides alternative to bases 153 to 10857, of the wild-type and mutant JEV genomes were applied to GENETYX-MAC to predict RNA secondary structures with minimum free energy.

**Growth kinetics of JEVs in vitro.** Vero, C6/36, PK15, N18, RAW264.7, Vero/siNC, and Vero/siCTSL cells in 24-well plates ( $2 \times 10^5$ ) were infected with the wild-type or L17A/Csmt virus at an MOI of 5 for 1 h, washed three times with a medium to remove unbound viruses, and incubated with a medium supplemented with 5% FBS for a total duration of 72 h. To examine the effect of the cathepsin L inhibitor on virus growth, DMSO or 1  $\mu$ M Z-FY-DMK was added to the culture medium over the incubation period (24 h). The culture supernatants were used for titration of infectious virus.

**Mouse experiments.** The pathogenicity of JEV to mice was determined as described previously (32). Briefly, 3-week-old female ICR mice were purchased from CLEA Japan (Osaka, Japan) and kept in special pathogen-free environments. Groups of 10 mice were intracerebrally inoculated with 30  $\mu$ l of 10-fold-diluted solutions of wild-type or L17A/Csmt virus. The virus-diluting solution (DMEM) was administered to two mice as a control. The mice were observed for 2 weeks after inoculation to determine survival rates. The value of the 50% lethal dose ( $LD_{50}$ ) of each virus was determined by the method by Reed and Muench (39). To examine viral growth in the brain, 100 FFU of the viruses were intracerebrally administered to the mice. At 3 and 5 days after inoculation, the mice were euthanized, and the brains were collected. The infectious titers in the homogenates of the brains were determined in Vero cells as described above. Groups of 10 mice were inoculated intraperitoneally with  $1 \times 10^6$  FFU (100  $\mu$ l) of the viruses. The mice were observed for 3 weeks after inoculation to determine survival rates.

## RESULTS

**JEV-infected cells contained a small capsid protein.** Western blotting analyses of Vero cells infected with JEV revealed capsid proteins of 14 and 12 kDa, which were designated C1 and C2, respectively, in contrast to the purified viral particles, in which only C1 was detected (Fig. 1A), indicating that C1 is a mature capsid protein missing a signal sequence of the prM protein. The C2 protein was also detected in the other cell lines

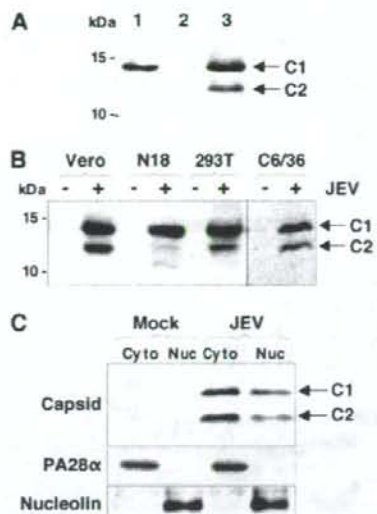


FIG. 1. Detection of C2 protein in cells infected with JEV. (A) Detection of the capsid proteins from the purified viral particles and cells infected with JEV. Lane 1, purified JEV particles produced in Vero cells; lanes 2 and 3, mock- and JEV-infected Vero cells, respectively. Arrows indicate a mature capsid protein (C1) and a further-processed capsid protein (C2). (B) Detection of the capsid protein from various cell lines infected with JEV. (C) Detection of the C1 and C2 proteins in the cytoplasmic (Cyto) and nuclear (Nuc) fractions of Vero cells infected with JEV. PA28- $\alpha$  and nucleolin are control proteins of the cytoplasmic and nuclear fractions, respectively.

examined, and a further processed capsid protein was detected in N18 cells infected with JEV (Fig. 1B). It was shown that the JEV capsid protein is localized in the nuclei as well as in the cytoplasm of the infected cells (32). The C1 and C2 proteins were also detected in both the cytoplasmic and nuclear fractions (Fig. 1C). These results indicate that two forms of the capsid proteins, C1 and C2, are generated in cells infected with JEV, and the larger capsid (C1) is selectively incorporated into the viral particles.

**The C2 protein lacks the amino terminus.** To determine which terminus is missing in the C2 protein, expression plasmids encoding a series of capsid proteins with or without amino-terminal FLAG and carboxyl-terminal HA tags (F-JEC-H, F-JEC, JEC-H, and JEC) were generated (Fig. 2A). Both the C1 and C2 isoforms were detected in Vero cells transfected with each of the expression plasmids by immunoblotting with anti-JEV capsid PAb (Fig. 2B). The size of the C2 proteins in cells transfected with JEC was similar to that of F-JEC, which has the amino-terminal FLAG tag, whereas larger products were detected in the cells transfected with F-JEC-H and JEC-H, which have the carboxyl-terminal HA tag. Consistent with this observation, anti-HA antibody recognized both isoforms in cells expressing F-JEC-H and JEC-H, whereas anti-FLAG antibody detected only C1 in cells expressing F-JEC-H and F-JEC. These results indicate that the C2 protein lacks the amino-terminal region of the JEV capsid protein.

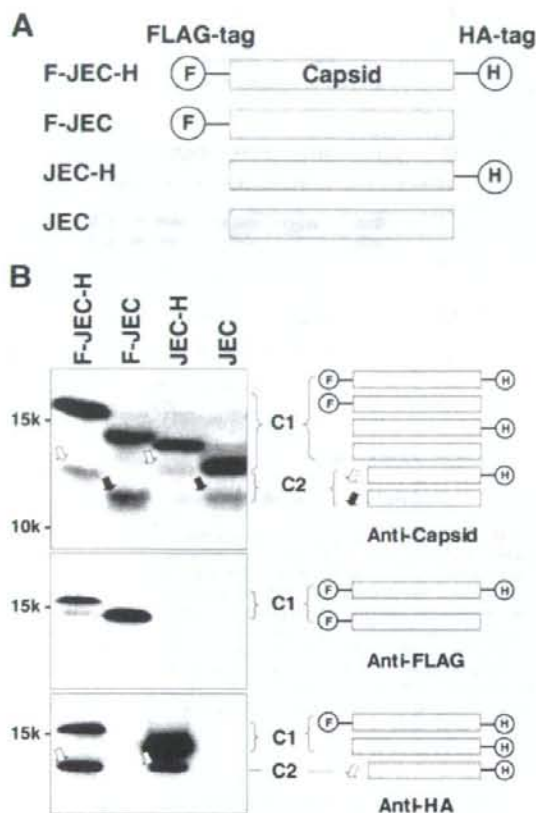


FIG. 2. The C2 protein lacks the amino terminus. (A) Series of the capsid protein constructs with or without FLAG and HA tags in the amino and carboxyl termini, respectively. (B) Expression of a series of the capsid proteins in Vero cells. The cell lysates expressing F-JEC-H, F-JEC, JEC-H, and JEC were examined by immunoblotting using anti-capsid, anti-FLAG, and anti-HA antibodies. The molecules detected by the immunoblotting are indicated on the right. White and black arrows indicate the C2 proteins with and without HA tags in the carboxyl terminus, respectively.

**The JEV capsid protein is processed by cathepsin L.** The C2 protein missing the amino-terminal region of the JEV capsid protein may be generated through cleavage by a host cell protease(s) or translation from the second start codon by leaky scanning, as reported in the case of DEN2 (9). To assess these possibilities, cells expressing F-JEC-H were treated with various protease inhibitors. C2 production was completely abrogated by treatment with broad-spectrum cysteine protease inhibitor E64d at the concentration of 50  $\mu$ M, along with an increase in C1 expression (Fig. 3A), indicating that the JEV C2 protein was generated via cleavage of the C1 protein by a cysteine protease(s) but not leaky scanning. To identify the cysteine protease responsible for the processing of the JEV capsid protein, specific inhibitors for individual cysteine proteases were examined in cells expressing F-JEC-H. The inhib-

itors for cathepsins B and L, CA074Me (10  $\mu$ M) (6) and Z-FY-DMK (10  $\mu$ M) (40), impaired the processing, while an inhibitor of caspases, Z-VAD-FMK (20  $\mu$ M), and an inhibitor of calpains, PD150606 (20  $\mu$ M), exhibited no effect (Fig. 3A). Cathepsins B and L are known to be present in the late endosome and lysosome. The treatments with inhibitors of these acidic compartments, ammonium chloride (10 mM), chloroquine (50  $\mu$ M), and bafilomycin A1 (100 nM), also blocked the processing of the capsid protein (Fig. 3B). To determine whether cathepsin B or L is a dominant protease for cleavage of the JEV capsid protein, the dose dependency of the effects of cathepsin inhibitors CA074Me and Z-FY-DMK on the cleavage of F-JEC-H was examined. The processing of the JEV capsid protein was inhibited in a manner that correlated closely with the inactivation of cathepsin L rather than that of cathepsin B (Fig. 3C). Furthermore, overexpression of cathepsin L, but not cathepsin B and inactive cathepsin L (C138A), resulted in an increase of C2 production in 293T cells (Fig. 3D). In addition, production of C2 from F-JEC-H was significantly decreased in two independent clones of Vero cells stably expressing siRNA for cathepsin L (Fig. 3E). These results indicate that cathepsin L is responsible for the processing of the JEV capsid protein to generate the C2 protein.

**Identification of the site of the cleavage of the JEV capsid protein by cathepsin L.** To determine the site of the cleavage of the JEV capsid protein by cathepsin L, a recombinant capsid protein possessing amino-terminal thioredoxin, His, and S tags and carboxyl-terminal myc and His tags was prepared (Fig. 4A). The *in vitro* incubation of the purified capsid protein with cathepsin L at room temperature for 60 min generated two major cleaved products, detectable by anti-myc antibody (Fig. 4B). The amino-terminal amino acid sequencing revealed that the mass of cleaved product 1 contained two peptides beginning with the residues Ser-Asp-Lys-Ile-Ile (a minor peptide) and Arg-Gln-His-Met-Asp (a major peptide), corresponding to a region of the thioredoxin and S tags, respectively (Fig. 4A and B). On the other hand, cleaved product 2 contained a single peptide beginning with Arg-Gly-Leu-Pro-Arg, corresponding to amino acid residues 19 to 23 of the JEV capsid protein. This result indicates that the JEV capsid protein is cleaved between Lys<sup>18</sup> and Arg<sup>19</sup> by cathepsin L *in vitro* (Fig. 4C). To further confirm the cleavage of the capsid protein in mammalian cells, a series of F-JEC-H proteins with alanine substitutions in each residue around the cleavage site (Ile<sup>14</sup> to Arg<sup>23</sup>) was expressed in Vero cells (Fig. 4D). As indicated in the reports that a hydrophobic amino acid residue at position P2 is responsible for the substrate specificity of cathepsin L (37, 38), the replacement of Leu<sup>17</sup> (P2) with alanine was crucial for capsid protein processing. In addition, although the single replacements at the cleavage site of Lys<sup>18</sup> (P1) and Arg<sup>19</sup> (P1') with alanine had no effect on cleavage, the double substitution of acidic amino acids (Lys<sup>18</sup> to Glu and Arg<sup>19</sup> to Asp) resulted in impairment of C2 production (Fig. 4D). These results indicate that the JEV capsid protein is cleaved between Lys<sup>18</sup> and Arg<sup>19</sup> by cathepsin L *in vitro* and *in vivo*.

**Production of the C2 proteins of DENs.** The P4 to P1' region of the cathepsin L cleavage site is conserved among many mosquito-borne flaviviruses, including MVE, WNV, and DENs (Fig. 4C), and the 5'-complementary cyclization sequences are over-



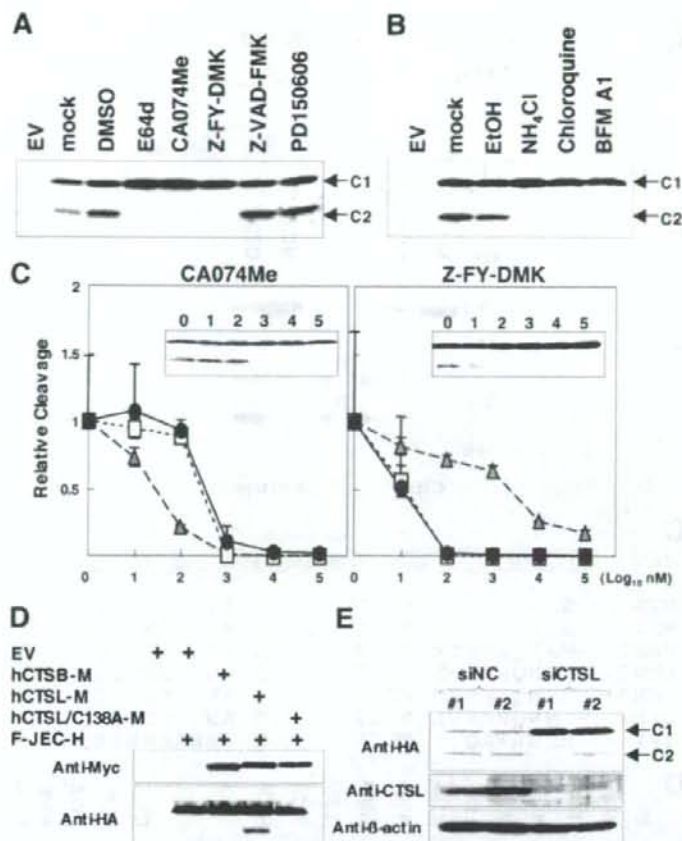


FIG. 3. JEV capsid protein is processed by cathepsin L. (A) Effects of cysteine protease inhibitors on the processing of the JEV capsid protein. Vero cells expressing F-JEC-H were treated with 50  $\mu$ M E64d, 10  $\mu$ M CA074Me, 10  $\mu$ M Z-FY-DMK, 20  $\mu$ M Z-VAD-FMK, or 20  $\mu$ M PD150606 for 8 h at 37°C and examined by immunoblotting using an anti-HA antibody. EV, empty vector. (B) Effects of anti-acidic compartment reagents on the processing of the JEV capsid protein. Vero cells expressing F-JEC-H were treated with 10 mM ammonium chloride, 50  $\mu$ M chloroquine, or 100 nM bafilomycin A1 (BFM A1) for 8 h at 37°C and examined by immunoblotting using an anti-HA antibody. EtOH, ethanol. (C) Dose-dependent effects of two cathepsin inhibitors, CA074Me and Z-FY-DMK, on F-JEC-H processing. Vero cells expressing F-JEC-H were treated with CA074Me or Z-FY-DMK at the indicated concentrations for 8 h at 37°C and examined by immunoblotting using an anti-HA antibody. The relative cleavage values for the capsid protein (solid circles) were calculated as the intensity of C2 compared to that of C1 in three independent experiments. A representative image of the immunoblotting is indicated in each graph panel. The relative levels of cleavage of the substrates specific to cathepsin B (gray triangles) and cathepsin L (open squares) were determined as described in Materials and Methods. The value for the control sample without treatment of each inhibitor was taken as 1. (D) Effects of the overexpression of cathepsins on the processing of the JEV capsid protein. 293T cells were cotransfected with plasmids encoding myc-tagged human cathepsin B (hCTSB-M), cathepsin L (hCTSL-M), or inactive cathepsin L (hCTSL/C138A-M) with F-JEC-H. Immunoblot analysis was carried out using the antibodies shown at the left. (E) Processing of F-JEC-H in Vero cells stably expressing hairpin siRNA corresponding to the negative control (siNC) or cathepsin L (siCTSL). Immunoblot analysis was carried out using the antibodies shown at the left.

lapped through the P4 to P2 sites (1, 19) (Fig. 5A). The C2 proteins were also detected in cells expressing the capsid proteins of DEN2 and DEN4 (Fig. 4E). To determine whether the C2 proteins of DEN are generated in the same manner as the C2 proteins of JEV, we examined the effect of the cysteine protease inhibitor E64d on the productions of the DEN C2 proteins. When cells were treated with E64d at a concentration of 50  $\mu$ M, the C2 protein was diminished in cells expressing the capsid protein of JEV, but not in those expressing DEN2 and

DEN4. However, it should be noted that treatment with the inhibitor induced a slight delay in migration of the C2 proteins of DENs. These results suggest that cysteine proteases do not play a major role in the production of the C2 proteins of DENs but play some roles in their processing.

**Construction of a mutant JEV carrying the capsid protein resistant to cleavage by cathepsin L.** To assess the biological significance of the cleavage of the JEV capsid protein by cathepsin L, a mutant JEV with Leu<sup>17</sup> replaced by Ala (L17A)

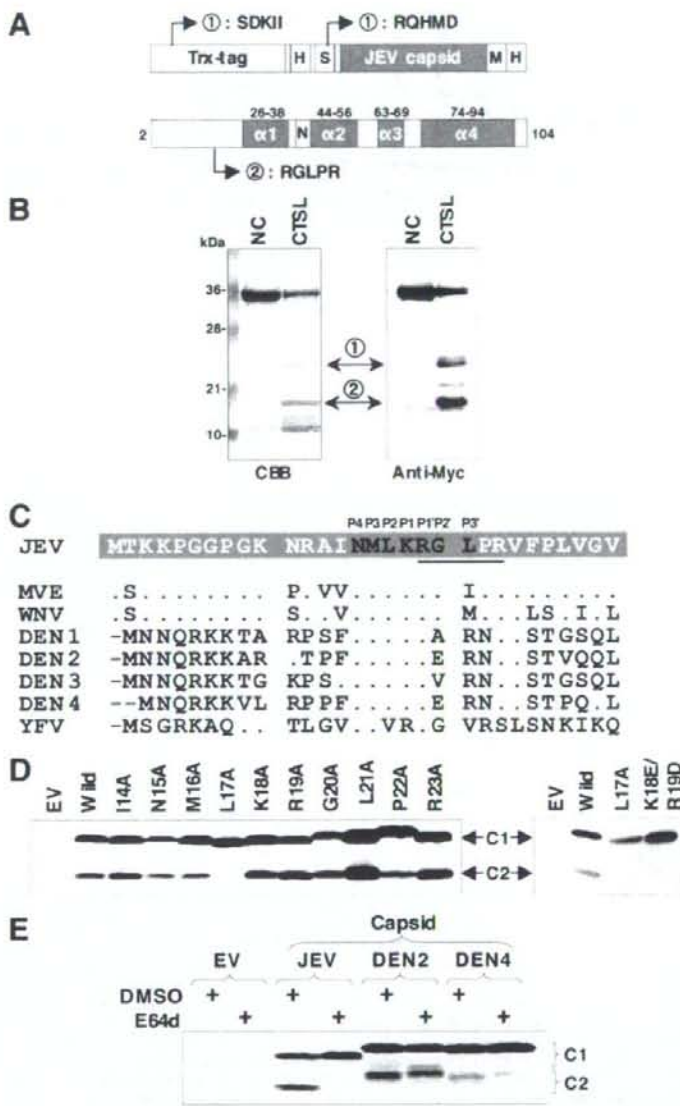


FIG. 4. Identification of the site of cleavage of JEV capsid protein by cathepsin L. (A) Schematic diagram of the recombinant JEV capsid protein. The His, S, and myc tags are indicated as H, S, and M, respectively. Four  $\alpha$ -helices ( $\alpha 1$  to  $\alpha 4$ ) of the JEV capsid protein were predicted by Ma et al. (27). The nuclear localization signal (N) was mapped to residues Gly<sup>42</sup> and Pro<sup>43</sup> (32). Products 1 and 2 of in vitro cleavage by cathepsin L began at the indicated positions. Trx, thioredoxin. (B) The purified capsid protein (33  $\mu$ g [1 nmol]/100  $\mu$ l) was treated with 0.01 units of recombinant human cathepsin L (CTSL) at room temperature for 60 min and analyzed by Coomassie brilliant blue (CBB) staining and immunoblotting using an anti-myc antibody after SDS-PAGE. The amino-terminal amino acid sequences of cleavage products 1 and 2 were determined by the Edman degradation method. (C) Alignment of the amino-terminal amino acid sequences of the mosquito-borne flaviviral capsid proteins. Positions P4 to P3' of the site of cleavage of the JEV capsid protein by cathepsin L are shown at the top of the sequences. The amino-terminal amino acid sequences of cleavage product 2 generated by cathepsin L in vitro are underlined. Identical and deleted residues compared with the JEV capsid protein are indicated as dots and bars, respectively. (D) Identification of crucial residues for capsid protein processing by cathepsin L in vivo. A series of the mutant constructs derived from F-JEC-H were expressed in Vero cells and analyzed by immunoblotting using an anti-HA antibody. (E) Effect of a cysteine protease inhibitor E64d on the processing of the DEN capsid proteins. Vero cells expressing the FLAG- and HA-tagged capsid proteins of JEV, DEN2, and DEN4 were treated with DMSO or 50  $\mu$ M E64d for 8 h at 37°C and examined by immunoblotting using an anti-HA antibody.

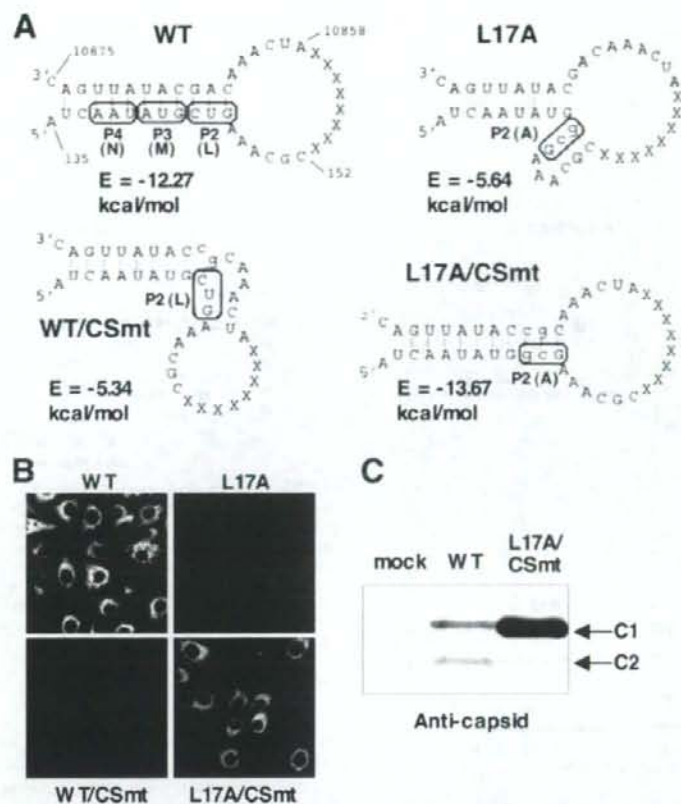


FIG. 5. Construction of a mutant JEV carrying the capsid protein resistant to cleavage by cathepsin L. (A) Predicted RNA secondary structures of the wild-type (WT) and mutant viral genomes. Nucleotides 135 to 152 and bases 10858 to 10875 in the 5' and 3' termini, respectively, connected by 8 nonsense 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 Asp<sup>15</sup> (P4), Met<sup>16</sup> (P3), and Leu<sup>17</sup> (P2) in the 5' cyclization sequences of the WT JEV, Leu<sup>17</sup> (P2) for WT/CSmt, and Ala<sup>17</sup> (P2) for L17A and L17A/CSmt are boxed. The mutated nucleotides are shown by lowercase letters. (B) Vero cells ( $5 \times 10^6$ ) were electroporated with 10  $\mu$ 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 Leu<sup>17</sup> 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-



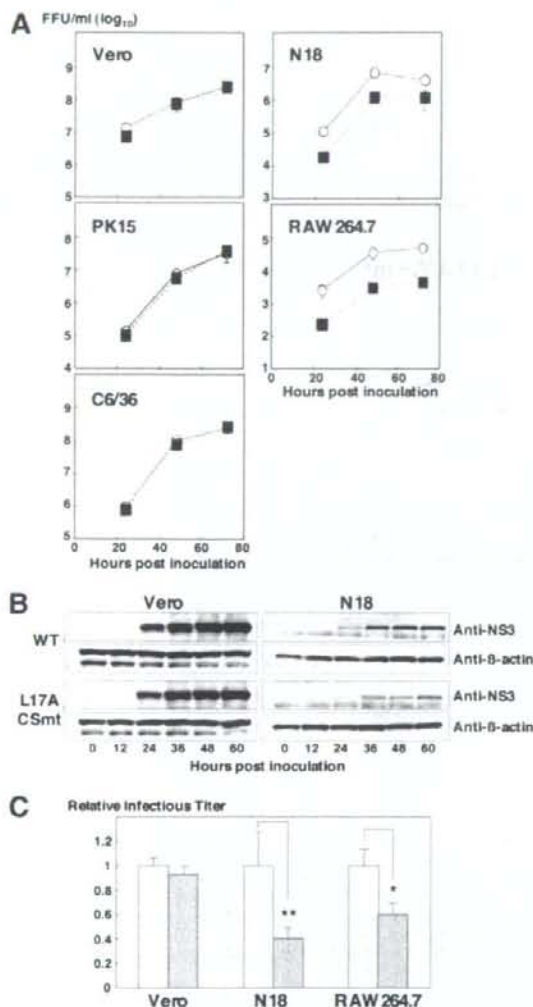


FIG. 6. Growth kinetics of L17A/CSmt in various cell lines. (A) The wild-type (WT; open circles) and mutant L17A/CSmt (solid squares) JEVs were inoculated into Vero, C6/36, PK15, N18, and RAW264.7 cells at an MOI of 10. After the indicated times, the infective titers in the culture supernatants on Vero cells were determined. (B) Viral protein synthesis in Vero and N18 cells infected with the WT or L17A/CSmt virus. The NS3 and  $\beta$ -actin proteins were detected by immunoblotting with anti-JEV NS3 and anti- $\beta$ -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  $\mu$ M 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

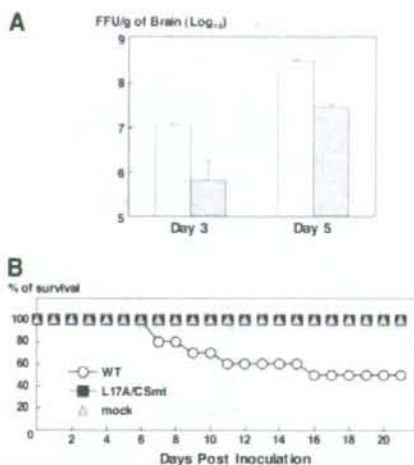


FIG. 7. Neurovirulence and neuroinvasiveness of L17A/CSmt in mice. (A) Growth of the wild-type (WT; white bars) and mutant L17A/CSmt (gray bars) virus in mouse brain. One hundred FFU of each virus were intracerebrally injected into 3-week-old ICR mice, and the progeny viruses in the brain at 3 or 5 days after inoculation on Vero cells were determined. (B) Neuroinvasiveness of the WT and mutant JEVs to mice. Ten ICR mice were intraperitoneally inoculated with  $1 \times 10^6$  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 LD<sub>50</sub> values by intracerebral inoculation of the viruses in 3-week-old ICR mice. The LD<sub>50</sub> 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:  $6.8 \pm 0.9$  versus  $8.4 \pm 1.4$  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  $1 \times 10^6$  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  $\alpha$ -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 Gly<sup>42</sup> and Pro<sup>43</sup> (32, 46). The C2 protein of a

mutant JEV in which Gly<sup>42</sup> and Pro<sup>43</sup> 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 Lys<sup>18</sup> and Arg<sup>19</sup> and that Leu<sup>17</sup> 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 Met<sup>15</sup> and then were processed by cathepsin L at Arg<sup>18</sup> 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 Leu<sup>17</sup> 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<sup>∇</sup>

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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  $\alpha$ -glucosidase inhibitor, *N*-(*n*-nonyl) deoxyjirimycin (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  $\alpha$ -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 $\Delta$ 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 $\Delta$ 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 $\Delta$ 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 pHC81. 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 pHC81 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 (BD1198; Biodesign 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  $\mu$ g of pBS-N, 5  $\mu$ g of pBS-P, 1  $\mu$ g of pBS-L, 8  $\mu$ g of pBS-G, and 5  $\mu$ g of p $\Delta$ 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- $\mu$ 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 $\Delta$ 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 $\Delta$ 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 $\Delta$ 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 (BD1198) 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 $\Delta$ 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



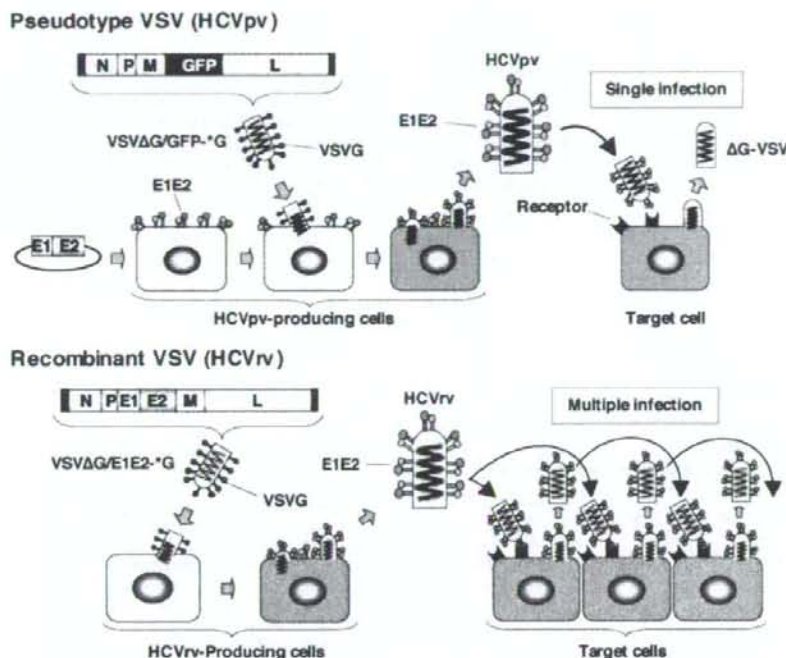


FIG. 1. Schematic representation of the production of HCVpv and HCVrv. Pseudotype VSV (HCVpv): producer cells (Huh7 or 293T) were transfected with an expression plasmid encoding the HCV E1 and E2 genes and then infected with a VSVG-complemented pseudotype virus (VSVΔG/GFP-ΔG). The HCVpv released from 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-ΔG) 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 30°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 NS5A (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  $8 \times 10^3$  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 A<sub>1</sub> (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) deoxyinosin (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 titered 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

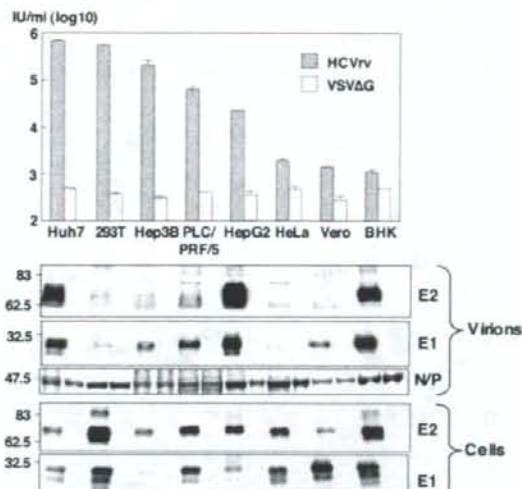


FIG. 2. Production and characterization of HCVrv. (Top) The infectivity of HCVrv of the H77 strain produced in the indicated cell lines at 30°C for 48 h was determined in Huh7 cells at 37°C for 24 h postinfection by counting VSV N-positive cells. The results shown are from three independent assays, with 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 ( $5 \times 10^5$  to  $1 \times 10^6$  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

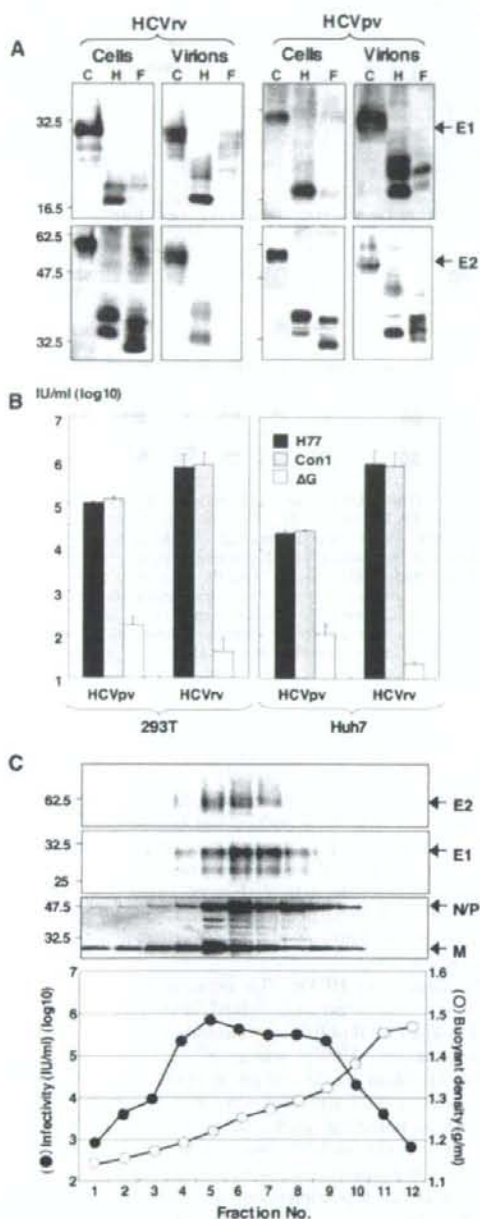


FIG. 3. Characterization of HCVrv and HCVpv. (A) The E1 and E2 proteins of the H77 strain expressed in 293T cells and incorporated into the particles of HCVrv and HCVpv were either untreated (C) or treated with endoglycosidase H (H) or peptide-N-glycosidase F (F). Following fractionation on sodium dodecyl sulfate-polyacrylamide gel, the glycoproteins were detected by immunoblotting with anti-E1 (BD1198) 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-knockdown 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.



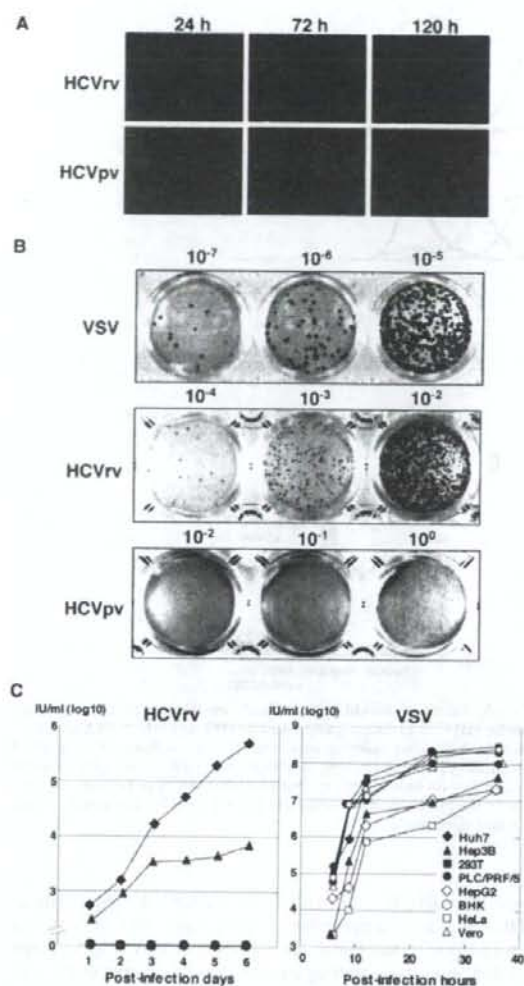


FIG. 4. Propagation of HCVrv. (A) Detection of viral proteins in Huh7 cells infected with HCVpv or HCVrv. Huh7 cells were infected with HCVpv or HCVrv at an MOI of 0.01. Twenty-four, 72, and 120 h after infection, cells were fixed and stained with monoclonal antibody to VSV N protein and Alexa 488-conjugated 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 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 naive 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<sub>1</sub>.** 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<sub>1</sub>, 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<sub>1</sub>, 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,

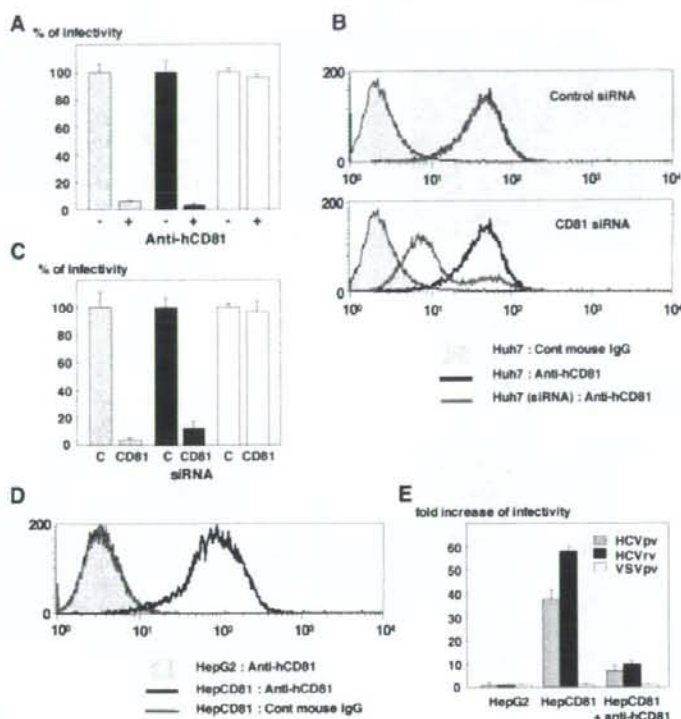


FIG. 5. Involvement of hCD81 in the infection of HCVpv and HCVrv. (A) Effect of anti-hCD81 antibody on the infectivity of HCVpv (gray-filled bars), HCVrv (black-filled bars), or VSVpv (open bars) in Huh7 cells. (B) Cell surface expression of hCD81 on Huh7 cells transfected with siRNA targeted to hCD81 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  $\alpha$ -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), glycobiochemical 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  $\alpha$ -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  $\mu$ M of Nn-DNJ were severely impaired by the cytotoxic effects of Nn-DNJ (Fig. 8A, bottom left), treatment with 10  $\mu$ 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  $\alpha$ -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 <sup>a</sup> :		Virus, producer cells, and strain (genotype) <sup>b</sup>								HCVpp virus produced in 293T cells and of strain H77 (genotype 1a) <sup>c</sup>
			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	-	-	-	-	-	-	-	-	-	-	-
CHOC81	++	-	-	-	-	-	-	-	-	-	-

<sup>a</sup> 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.

<sup>b</sup> Infectious titers higher than  $5 \times 10^4$  IU/ml, between  $5 \times 10^3$  and  $5 \times 10^4$  IU/ml, between  $5 \times 10^2$  and  $5 \times 10^3$  IU/ml, and lower than  $5 \times 10^2$  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  $\alpha$ -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-

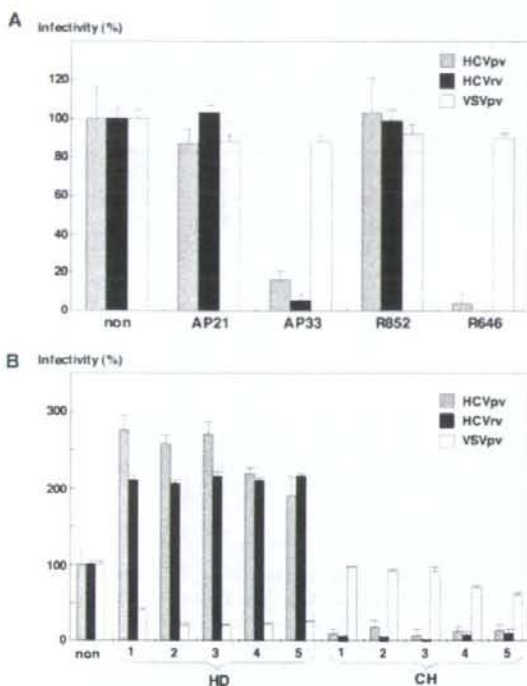


FIG. 6. Neutralization of HCVpv and HCVrv infection by antibodies to HCV envelope proteins and sera of HCV patients. (A) Effect of anti-E1 (AP21.010) and anti-E2 (AP33) monoclonal antibodies and anti-E1 (R852) and anti-E2 (R646) rabbit sera on the infectivity of HCVpv (gray-filled bars), HCVrv (black-filled 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 (CHOCD81 cells) were

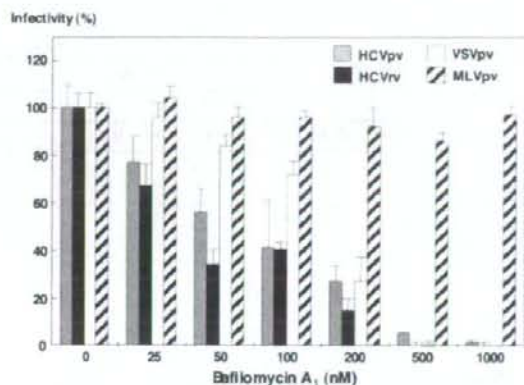


FIG. 7. Inhibition of HCVpv and HCVrv infection by baflomycin  $A_1$ . HCVpv (gray-filled bars), HCVrv (black-filled bars), VSVpv (open bars), or MLVpv (striped bars) were inoculated to Huh7 cells after treatment with various concentrations of baflomycin  $A_1$ . The results shown are from three independent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

Baflomycin  $A_1$ , 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  $\alpha$ -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  $\alpha$ -glucosidase inhibitors, DNJ or castanospermine, which block