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^{18} and Arg^{19}. 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-*Taq* (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^{138} 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 ATC TGT TGT GCA TCG CCG) and insertion into the BamHI and HindIII sites of p*Silencer* 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. pMWAT/L17A 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^{17} in the capsid protein with Ala (see Fig. 5A). In addition, adenine-to-guanine and guanine-to-cytosine mutations were introduced into pMWATG1 and pMWAT/L17A at nucleotides 10865 and 10866 of the JEV gene, respectively. The resulting plasmids were named pMWAT/CSmt and pMWAT/L17ACSmt, respectively.

Viruses. The wild-type and L17A/CSmt JEVs were generated from plasmids pMWATG1 and pMWAT/L17ACSmt, 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 MAb (MS-3) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PA28-alpha 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 *Trans*IT 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 (2×10^5) 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 AEBSF [4-(2-aminoethyl)benzenesulfonyl 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 μl of reaction buffer (100 mM sodium acetate [pH 5.0], 1 mM EDTA, 4 mM dithiothreitol, 2 mM AEBSF, 5 μg/ml aprotinin, 100 μM bestatin, 15 μM pepstatin). The resulting samples were mixed with 100 μl of cathepsin B-specific (100 μM Z-Arg-Arg-MCA [4-methylcoumaryl-7-amide; Peptide Institute], 0.1% Brij 35) (3) or cathepsin L-specific (100 μM $[Z\text{-Phe-Arg}]_2$-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 (33 μg [1 nmol]/100 μl) 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 μmol 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 (2×10^5) 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 μM 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 μl 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 Müench (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 1×10^6 FFU (100 μl) 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

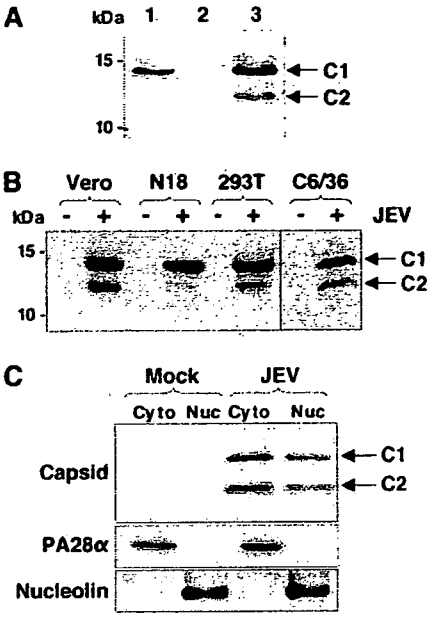


FIG. 1. Detection of C2 protein in cells infected with JEV. (A) Detection of the capsid proteins from the purified viral particles and cells infected with JEV. Lane 1, purified JEV particles produced in Vero cells; lanes 2 and 3, mock- and JEV-infected Vero cells, respectively. Arrows indicate a mature 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-α 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.

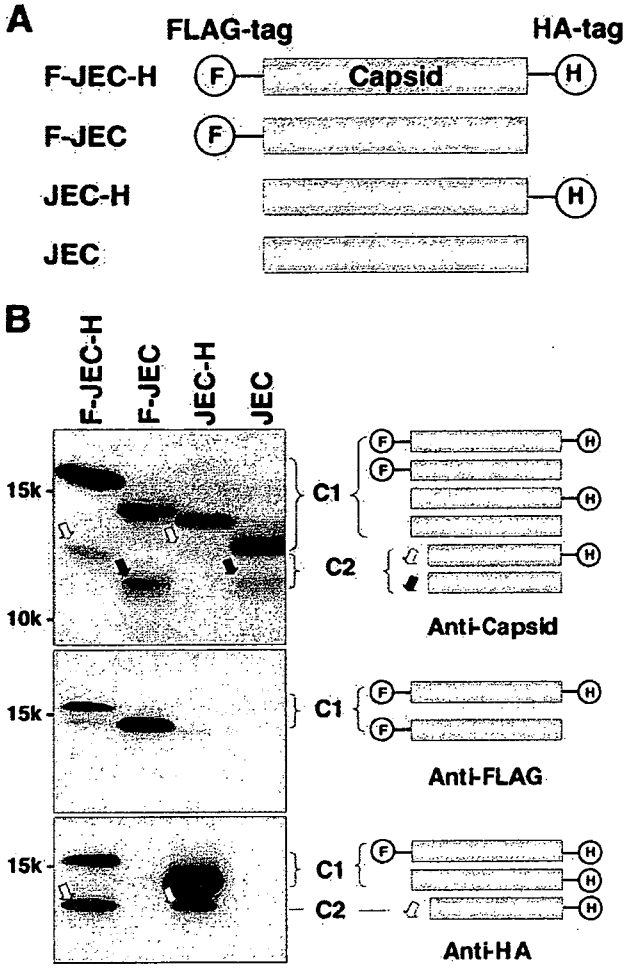


FIG. 2. The C2 protein lacks the amino terminus. (A) Series of the capsid protein constructs with or without FLAG and HA tags in the amino and carboxyl termini, respectively. (B) Expression of a series of the capsid proteins in Vero cells. The cell lysates expressing F-JEC-H, F-JEC, JEC-H, and JEC were 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 μM, 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 inhibitors for cathepsins B and L, CA074Me (10 μM) (6) and Z-FY-DMK (10 μM) (40), impaired the processing, while an inhibitor of caspases, Z-VAD-FMK (20 μM), and an inhibitor of calpains, PD150606 (20 μM), 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 μM), 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^{18} and Arg^{19} 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^{14} to Arg^{23}) 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^{17} (P2) with alanine was crucial for capsid protein processing. In addition, although the single replacements at the cleavage site of Lys^{18} (P1) and Arg^{19} (P1') with alanine had no effect on cleavage, the double substitution of acidic amino acids (Lys^{18} to Glu and Arg^{19} to Asp) resulted in impairment of C2 production (Fig. 4D). These results indicate that the JEV capsid protein is cleaved between Lys^{18} and Arg^{19} 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-

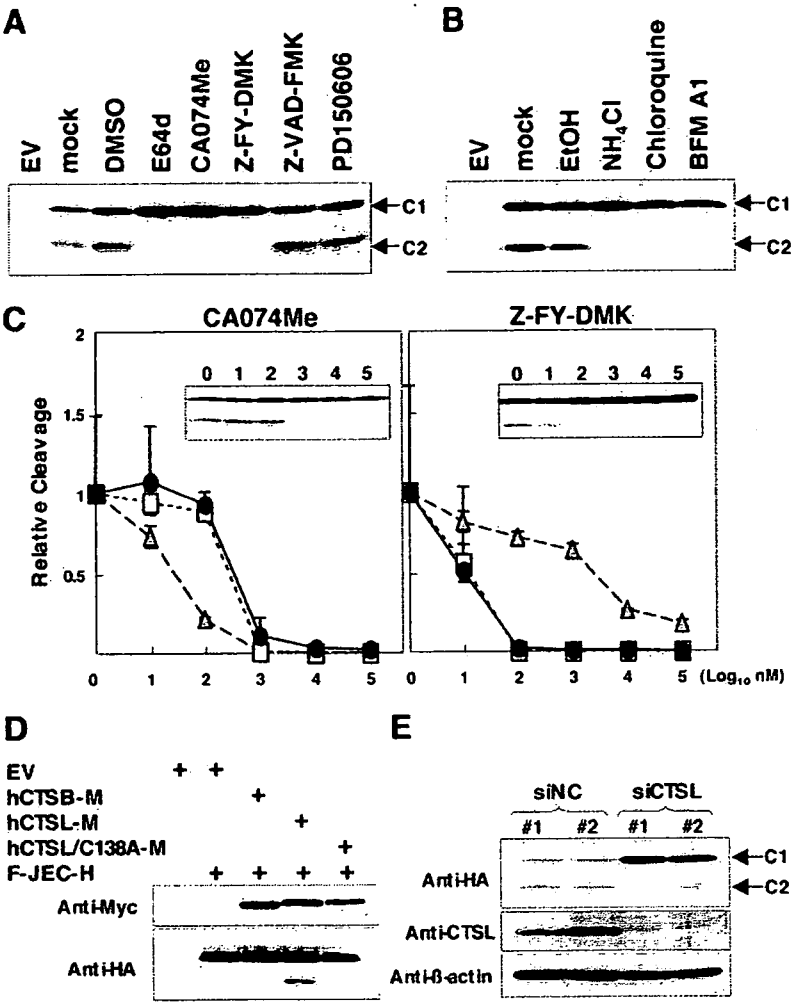


FIG. 3. JEV capsid protein is processed by cathepsin L. (A) Effects of cysteine protease inhibitors on the processing of the JEV capsid protein. Vero cells expressing F-JEC-H were treated with 50 μM E64d, 10 μM CA074Me, 10 μM Z-FY-DMK, 20 μM Z-VAD-FMK, or 20 μM PD150606 for 8 h at 37°C and examined by 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 μM 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 μM, 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^{17} replaced by Ala (L17A)

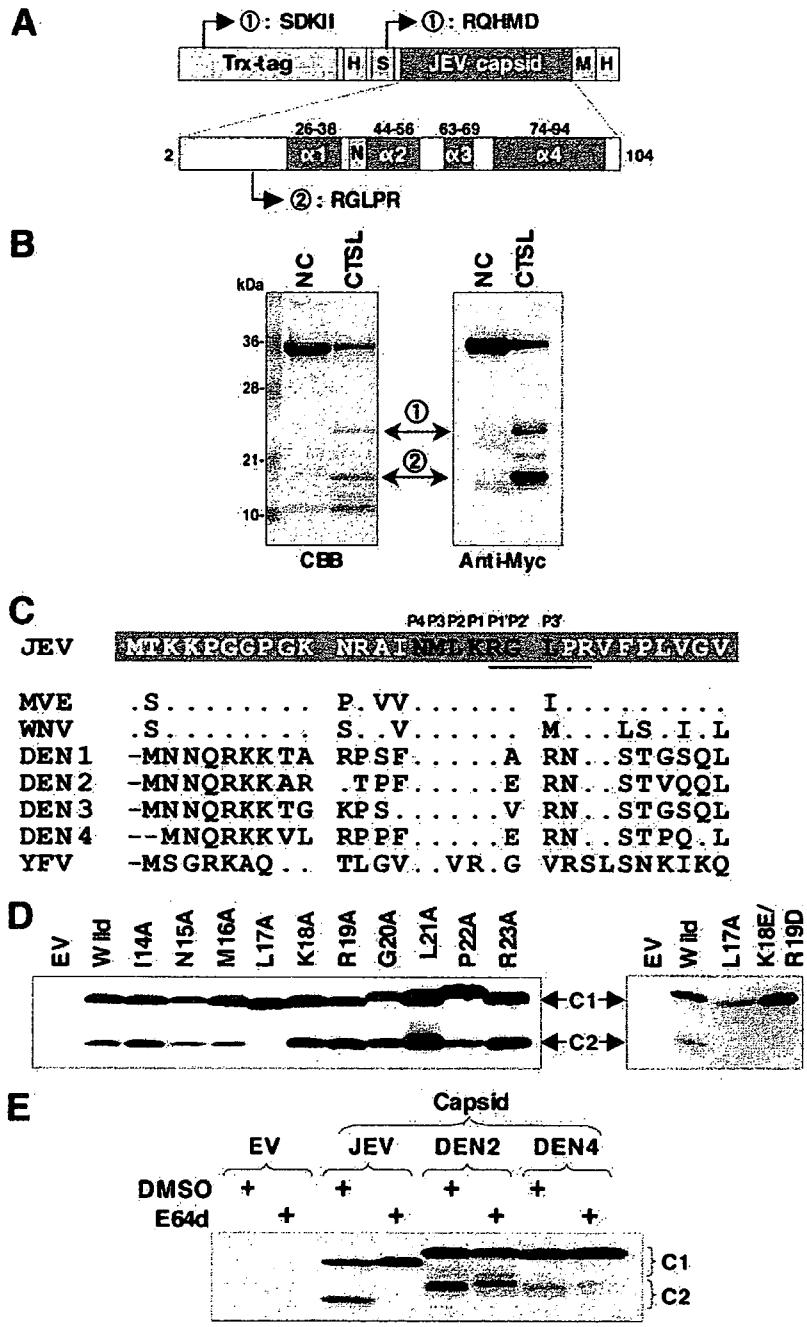


FIG. 4. Identification of the site of cleavage of JEV capsid protein by cathepsin L. (A) Schematic diagram of the recombinant JEV capsid protein. The His, S, and myc tags are indicated as H, S, and M, respectively. Four α-helices (α1 to 4) of the JEV capsid protein were predicted by Ma et al. (27). The nuclear localization signal (N) was mapped to residues Gly^{42} and Pro^{43} (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 μg [1 nmol]/100 μl) 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 μM E64d for 8 h at 37°C and examined by immunoblotting using an anti-HA antibody.

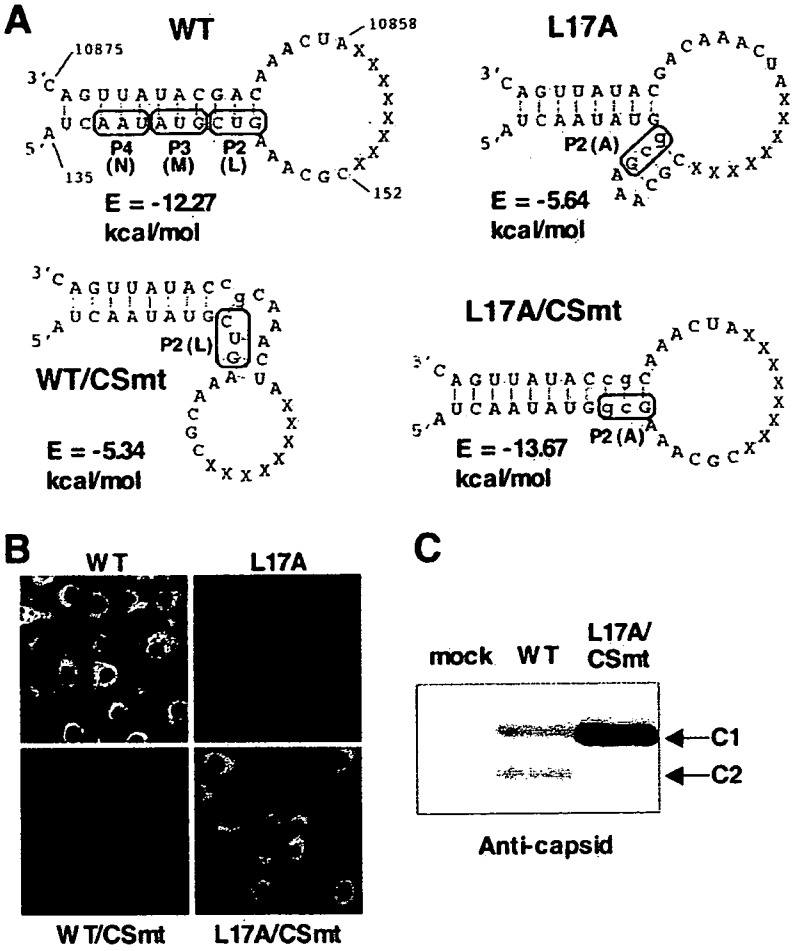


FIG. 5. Construction of a mutant JEV carrying the capsid protein resistant to cleavage by cathepsin L. (A) Predicted RNA secondary structures of the wild-type (WT) and mutant viral genomes. Nucleotides 135 to 152 and bases 10858 to 10875 in the 5′ and 3′ termini, respectively, connected by 8 nonsense 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^{15} (P4), Met^{16} (P3), and Leu^{17} (P2) in the 5′ cyclization sequences of the WT JEV, Leu^{17} (P2) for WT/CSmt, and Ala^{17} (P2) for L17A and L17A/CSmt are boxed. The mutated nucleotides are shown by lowercase letters. (B) Vero cells (5×10^6) were electroporated with 10 μg 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^{17} 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 complementary cyclization sequences in the capsid coding region and 3′ UTR is required for replication of JEV.

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

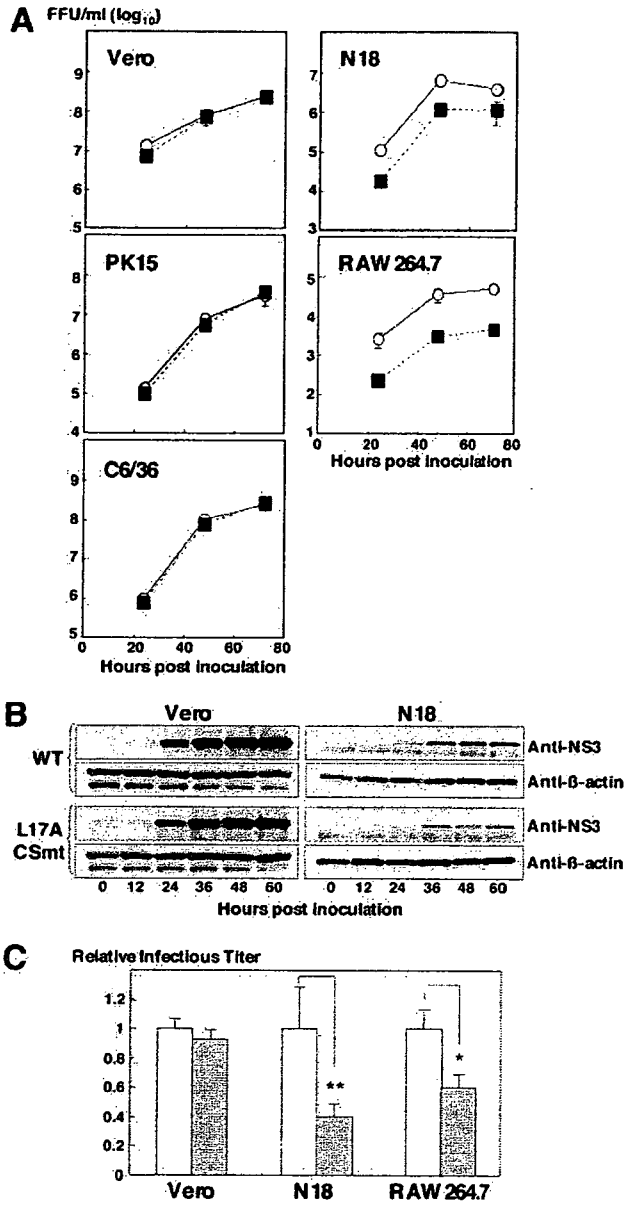


FIG. 6. Growth kinetics of L17A/CSmt in various cell lines. (A) The wild-type (WT; open circles) and mutant L17A/CSmt (solid squares) JEVs were inoculated into Vero, C6/36, PK15, N18, and RAW264.7 cells at an MOI of 10. After the indicated times, the infective titers in the culture supernatants on Vero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

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

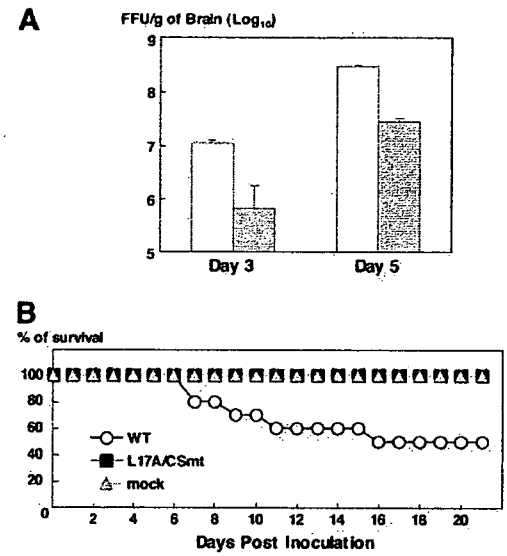


FIG. 7. Neurovirulence and neuroinvasiveness of L17A/CSmt in mice. (A) Growth of the wild-type (WT; white bars) and mutant L17A/CSmt (gray bars) virus in mouse brain. One hundred FFU of each virus were intracerebrally injected into 3-week-old ICR mice, and the progeny viruses in the brain at 3 or 5 days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

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

Neurovirulence and neuroinvasiveness of L17A/CSmt in mice. To compare the levels of neurovirulence of the wild-type and mutant viruses, we determined the LD_{50} values by intracerebral inoculation of the viruses in 3-week-old ICR mice. The LD_{50} value of L17A/CSmt (12.3 FFU) was approximately five times higher than that of the wild-type JEV (2.7 FFU). Although no significant difference in symptoms was observed between mice inoculated with 100 FFU of the wild-type and the mutant viruses, L17A/CSmt required longer periods than the wild-type JEV to kill mice (wild type versus L17A/CSmt: 6.8 ± 0.9 versus 8.4 ± 1.4 days postinoculation). To examine the growth kinetics of the viruses in the mouse brain, 100 FFU of each virus were intracerebrally injected and the progeny viruses in the brain were determined. The growth of L17A/CSmt was 16.3 and 11.0 times lower than that of the wild-type virus at 3 and 5 days after inoculation, respectively (Fig. 7A). Next, to compare the levels of neuroinvasiveness of the wild-type and mutant viruses, ICR mice were intraperitoneally inoculated with 1×10^6 FFU of each virus. All of the 10 mice inoculated with L17A/CSmt survived, whereas one-half of the mice inoculated with the wild-type JEV died by 10.0 days postinoculation on average (Fig. 7B). These results indicated that the L17A/CSmt mutant resistant to the cleavage by cathepsin L exhibits impaired neurovirulence and neuroinvasiveness in mice.

DISCUSSION

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

It has been shown that the JEV C2 protein can be generated by the cleavage of the amino-terminal 18 amino acids from the C1 capsid protein by cathepsin L. However, the amino-terminal part of the cleavage product was not detected even though a FLAG tag was added (Fig. 2B). Therefore, the fate of the N-termimal 18 residues is currently unknown. The C2 protein was detected only in the cells, not in the viral particles, in contrast to the C1 protein, which was detected in both. The amino-terminal 32 amino acids and carboxyl-terminal 26 amino acids of the capsid protein of Kunjin virus (KUN), an Australian subtype of WNV, are essential for binding to the genomic RNA (20). The amino-terminal region of the capsid protein is well conserved between JEV and WNV. Therefore, it is possible that the JEV C2 protein is not incorporated into viral particles due to lack of the amino-terminal region of the capsid protein, required for binding to the viral RNA. Three-dimensional structural analyses revealed that the DEN and KUN capsid proteins contain four α-helixes 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^{42} and Pro^{43} (32, 46). The C2 protein of a mutant JEV in which Gly^{42} and Pro^{43} 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^{18} and Arg^{19} and that Leu^{17} 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^{15} and then were processed by cathepsin L at Arg^{18} 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^{17} 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 evolutional adaptation of the viruses to their hosts.

ACKNOWLEDGMENTS

We thank H. Murase for her secretarial work. We also thank T. Wakita for providing the JEV infectious clone plasmids, E. Konishi and K. Yasui for the gifts of the anti-E and NS3 antibodies, respectively, and F. Hasebe and M. Tadano for the plasmids encoding the DEN2 and DEN4 capsid proteins, respectively.

This research was supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare; the Ministry of Education, Culture, Sports, Science, and Technology; the 21st Century Center of Excellence Program; the Foundation for Biomedical Research and Innovation; and the Zoonoses Control Project of the Ministry of Agriculture, Forestry and Fisheries of Japan.

REFERENCES

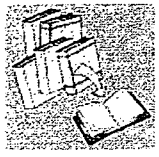
1. **Alvarez, D. E., M. F. Lodeiro, S. J. Luduena, L. I. Pietrasanta, and A. V. Gamarnik.** 2005. Long-range RNA-RNA interactions circularize the dengue virus genome. J. Virol. 79:6631–6643.
2. **Assfalg-Machleidt, I., G. Rothe, S. Klingel, R. Banati, W. F. Mangel, G. Valet, and W. Machleidt.** 1992. Membrane permeable fluorogenic rhodamine substrates for selective determination of cathepsin L. Biol. Chem. Hoppe-Seyler. 373:433–440.
3. **Barrett, A. J., and H. Kirschke.** 1981. Cathepsin B, cathepsin H, and cathepsin L. Methods Enzymol. 80:535–661.
4. **Bulich, R., and J. G. Aaskov.** 1992. Nuclear localization of dengue 2 virus core protein detected with monoclonal antibodies. J. Gen. Virol. 73:2999–3003.
5. **Burke, D. S., and T. P. Monath.** 2001. Flaviviruses, p. 1043–1125. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 4th ed., vol. 1. Lippincott Williams & Wilkins, Philadelphia, PA.
6. **Buttle, D. J., M. Murata, C. G. Knight, and A. J. Barrett.** 1992. CA074 methyl ester: a proinhibitor for intracellular cathepsin B. Arch. Biochem. Biophys. 299:377–380.
7. **Chandran, K., N. J. Sullivan, U. Felbor, S. P. Whelan, and J. M. Cunningham.** 2005. Endosomal proteolysis of the Ebola virus glycoprotein is necessary for infection. Science 308:1643–1645.
8. **Chang, C. J., H. W. Luh, S. H. Wang, H. J. Lin, S. C. Lee, and S. T. Hu.** 2001. The heterogeneous nuclear ribonucleoprotein K (hnRNP K) interacts with dengue virus core protein. DNA Cell Biol. 20:569–577.
9. **Clyde, K., and E. Harris.** 2006. RNA secondary structure in the coding region of dengue virus type 2 directs translation start codon selection and is required for viral replication. J. Virol. 80:2170–2182.
10. **Corver, J., E. Lenches, K. Smith, R. A. Robison, T. Sando, E. G. Strauss, and J. H. Strauss.** 2003. Fine mapping of a *cis*-acting sequence element in yellow fever virus RNA that is required for RNA replication and cyclization. J. Virol. 77:2265–2270.
11. **Dokland, T., M. Walsh, J. M. Mackenzie, A. A. Khromykh, K. H. Ee, and S. Wang.** 2004. West nile virus core protein; tetramer structure and ribbon formation. Structure 12:1157–1163.
12. **Dunn, A. D., H. E. Crutchfield, and J. T. Dunn.** 1991. Thyroglobulin processing by thyroidal proteases. Major sites of cleavage by cathepsins B, D, and L. J. Biol. Chem. 266:20198–20204.
13. **Ebert, D. H., J. Deussing, C. Peters, and T. S. Dermody.** 2002. Cathepsin L and cathepsin B mediate reovirus disassembly in murine fibroblast cells. J. Biol. Chem. 277:24609–24617.
14. **Filomatori, C. V., M. F. Lodeiro, D. E. Alvarez, M. M. Samsa, L. Pietrasanta, and A. V. Gamarnik.** 2006. A 5′ RNA element promotes dengue virus RNA synthesis on a circular genome. Genes Dev. 20:2238–2249.
15. **Fu, Y. H., T. Nishinaka, K. Yokoyama, and R. Chiu.** 1998. A retinoblastoma susceptibility gene product, RB, targeting protease is regulated through the cell cycle. FEBS Lett. 421:89–93.
16. **Goulet, B., A. Baruch, N. S. Moon, M. Poirier, L. L. Sansregret, A. Erickson, M. Bogyo, and A. Nepveu.** 2004. A cathepsin L isoform that is devoid of a signal peptide localizes to the nucleus in S phase and processes the CDP/Cux transcription factor. Mol. Cell 14:207–219.
17. **Higuchi, R., B. Krummel, and R. K. Saiki.** 1988. A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. Nucleic Acids Res. 16:7351–7367.

18. Jones, C. T., L. Ma, J. W. Burgner, T. D. Groesch, C. B. Post, and R. J. Kuhn. 2003. Flavivirus capsid is a dimeric alpha-helical protein. J. Virol. 77:7143–7149.
19. Khromykh, A. A., H. Meka, K. J. Guyatt, and E. G. Westaway. 2001. Essential role of cyclization sequences in flavivirus RNA replication. J. Virol. 75:6719–6728.
20. Khromykh, A. A., and E. G. Westaway. 1996. RNA binding properties of core protein of the flavivirus Kunjin. Arch. Virol. 141:685–699.
21. Kiermayr, S., R. M. Kofler, C. W. Mandl, P. Messner, and F. X. Heinz. 2004. Isolation of capsid protein dimers from the tick-borne encephalitis flavivirus and in vitro assembly of capsid-like particles. J. Virol. 78:8078–8084.
22. Kimura-Kuroda, J., M. Ichikawa, A. Ogata, K. Nagashima, and K. Yasui. 1993. Specific tropism of Japanese encephalitis virus for developing neurons in primary rat brain culture. Arch. Virol. 130:477–484.
23. Kofler, R. M., F. X. Heinz, and C. W. Mandl. 2002. Capsid protein C of tick-borne encephalitis virus tolerates large internal deletions and is a favorable target for attenuation of virulence. J. Virol. 76:3534–3543.
24. Lindenbach, B. D., and C. M. Rice. 2001. *Flaviviridae*: the viruses and their replication, p. 991–1041. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.). Fields virology, 4th ed., vol. 1. Lippincott Williams & Wilkins, Philadelphia, PA.
25. Lo, M. K., M. Tilgner, K. A. Bernard, and P. Y. Shi. 2003. Functional analysis of mosquito-borne flavivirus conserved sequence elements within 3′ untranslated region of West Nile virus by use of a reporting replicon that differentiates between viral translation and RNA replication. J. Virol. 77:10004–10014.
26. Lobigs, M., and E. Lee. 2004. Inefficient signalase cleavage promotes efficient nucleocapsid incorporation into budding flavivirus membranes. J. Virol. 78: 178–186.
27. Ma, L., C. T. Jones, T. D. Groesch, R. J. Kuhn, and C. B. Post. 2004. Solution structure of dengue virus capsid protein reveals another fold. Proc. Natl. Acad. Sci. USA 101:3414–3419.
28. Makino, Y., M. Tadano, T. Anzai, S. P. Ma, S. Yasuda, and T. Fukunaga. 1989. Detection of dengue 4 virus core protein in the nucleus. II. Antibody against dengue 4 core protein produced by a recombinant baculovirus reacts with the antigen in the nucleus. J. Gen. Virol. 70:1417–1425.
29. Markoff, L., B. Falgout, and A. Chang. 1997. A conserved internal hydrophobic domain mediates the stable membrane integration of the dengue virus capsid protein. Virology 233:105–117.
30. Mathur, A., M. Bharadwaj, R. Kulshreshtha, S. Rawat, A. Jain, and U. C. Chaturvedi. 1988. Immunopathological study of spleen during Japanese encephalitis virus infection in mice. Br. J. Exp. Pathol. 69:423–432.
31. Matsuo, E., H. Tani, C. Lim, Y. Komoda, T. Okamoto, H. Miyamoto, K. Moriishi, S. Yagi, A. H. Patel, T. Miyamura, and Y. Matsuura. 2006. Characterization of HCV-like particles produced in a human hepatoma cell line by a recombinant baculovirus. Biochem. Biophys. Res. Commun. 340:200–208.
32. Mori, Y., T. Okabayashi, T. Yamashita, Z. Zhao, T. Wakita, K. Yasui, F. Hasebe, M. Tadano, E. Konishi, K. Moriishi, and Y. Matsuura. 2005. Nuclear localization of Japanese encephalitis virus core protein enhances viral replication. J. Virol. 79:3448–3458.
33. Nishinaka, T., Y. H. Fu, L. I. Chen, K. Yokoyama, and R. Chiu. 1997. A unique cathepsin-like protease isolated from CV-1 cells is involved in rapid degradation of retinoblastoma susceptibility gene product, RB, and transcription factor SP1. Biochim. Biophys. Acta 1351:274–286.
34. Oh, W., M. R. Yang, E. W. Lee, K. M. Park, S. Pyo, J. S. Yang, H. W. Lee, and J. Song. 2006. Jab1 mediates cytoplasmic localization and degradation of West Nile virus capsid protein. J. Biol. Chem. 281:30166–30174.
35. Oh, W. K., and J. Song. 2006. Hsp70 functions as a negative regulator of West Nile virus capsid protein through direct interaction. Biochem. Biophys. Res. Commun. 347:994–1000.
36. Okamoto, K., K. Moriishi, T. Miyamura, and Y. Matsuura. 2004. Intramembrane proteolysis and endoplasmic reticulum retention of hepatitis C virus core protein. J. Virol. 78:6370–6380.
37. Portaro, F. C., A. B. Santos, M. H. Cezari, M. A. Juliano, L. Juliano, and E. Carmona. 2000. Probing the specificity of cysteine proteinases at subsites remote from the active site: analysis of P4, P3, P2′ and P3′ variations in extended substrates. Biochem. J. 347:123–129.
38. Puzer, L., S. S. Cotrin, M. F. Alves, T. Egborge, M. S. Araujo, M. A. Juliano, L. Juliano, D. Bromme, and A. K. Carmona. 2004. Comparative substrate specificity analysis of recombinant human cathepsin V and cathepsin L. Arch. Biochem. Biophys. 430:274–283.
39. Reed, L. J., and H. Müench. 1938. A simple method of estimationg fifty per cent endpoints. Am. J. Hyg. 27:493.
40. Shaw, E., S. Mohanty, A. Colic, V. Stoka, and V. Turk. 1993. The affinity-labelling of cathepsin S with peptidyl diazomethyl ketones. Comparison with the inhibition of cathepsin L and calpain. FEBS Lett. 334:340–342.
41. Simmons, G., D. N. Gosalia, A. J. Rennekamp, J. D. Reeves, S. L. Diamond, and P. Bates. 2005. Inhibitors of cathepsin L prevent severe acute respiratory syndrome coronavirus entry. Proc. Natl. Acad. Sci. USA 102:11876–11881.
42. Solomon, T., H. Ni, D. W. Beasley, M. Ekkelenkamp, M. J. Cardosa, and A. D. Barrett. 2003. Origin and evolution of Japanese encephalitis virus in southeast Asia. J. Virol. 77:3091–3098.
43. Stocks, C. E., and M. Lobigs. 1998. Signal peptidase cleavage at the flavivirus C-prM junction: dependence on the viral NS2B-3 protease for efficient processing requires determinants in C, the signal peptide, and prM. J. Virol. 72:2141–2149.
44. Tadano, M., Y. Makino, T. Fukunaga, Y. Okuno, and K. Fukai. 1989. Detection of dengue 4 virus core protein in the nucleus. I. A monoclonal antibody to dengue 4 virus reacts with the antigen in the nucleus and cytoplasm. J. Gen. Virol. 70:1409–1415.
45. Tsai, T. F. 2000. New initiatives for the control of Japanese encephalitis by vaccination: minutes of a WHO/CVI meeting, Bangkok, Thailand, 13–15 October 1998. Vaccine 18:1–25.
46. Tsuda, Y., Y. Mori, T. Abe, T. Yamashita, T. Okamoto, T. Ichimura, K. Moriishi, and Y. Matsuura. 2006. Nucleolar protein b23 interacts with Japanese encephalitis virus core protein and participates in viral replication. Microbiol. Immunol. 50:225–234.
47. Wang, S. H., W. J. Syu, K. J. Huang, H. Y. Lei, C. W. Yao, C. C. King, and S. T. Hu. 2002. Intracellular localization and determination of a nuclear localization signal of the core protein of dengue virus. J. Gen. Virol. 83: 3093–3102.
48. Westaway, E. G., A. A. Khromykh, M. T. Kenney, J. M. Mackenzie, and M. K. Jones. 1997. Proteins C and NS4B of the flavivirus Kunjin translocate independently into the nucleus. Virology 234:31–41.
49. Yamshchikov, V. F., and R. W. Compans. 1994. Processing of the intracellular form of the west Nile virus capsid protein by the viral NS2B-NS3 protease: an in vitro study. J. Virol. 68:5765–5771.
50. Yang, K. D., W. T. Yeh, R. F. Chen, H. L. Chuon, H. P. Tsai, C. W. Yao, and M. F. Shaio. 2004. A model to study neurotropism and persistency of Japanese encephalitis virus infection in human neuroblastoma cells and leukocytes. J. Gen. Virol. 85:635–642.
51. Yasothornsrikul, S., D. Greenbaum, K. F. Medzihradszky, T. Toneff, R. Bundey, R. Miller, B. Schilling, I. Petermann, J. Dehnert, A. Logvinova, P. Goldsmith, J. M. Neveu, W. S. Lane, B. Gibson, T. Reinheckel, C. Peters, M. Bogyo, and V. Hook. 2003. Cathepsin L in secretory vesicles functions as a prohormone-processing enzyme for production of the enkephalin peptide neurotransmitter. Proc. Natl. Acad. Sci. USA 100:9590–9595.
52. You, S., B. Falgout, L. Markoff, and R. Padmanabhan. 2001. In vitro RNA synthesis from exogenous dengue viral RNA templates requires long range interactions between 5′- and 3′-terminal regions that influence RNA structure. J. Biol. Chem. 276:15581–15591.
53. You, S., and R. Padmanabhan. 1999. A novel in vitro replication system for dengue virus. Initiation of RNA synthesis at the 3′-end of exogenous viral RNA templates requires 5′- and 3′-terminal complementary sequence motifs of the viral RNA. J. Biol. Chem. 274:33714–33722.
54. Zhao, Z., T. Date, Y. Li, T. Kato, M. Miyamoto, K. Yasui, and T. Wakita. 2005. Characterization of the E-138 (Glu/Lys) mutation in Japanese encephalitis virus by using a stable, full-length, infectious cDNA clone. J. Gen. Virol. 86:2209–2220.

WILEY InterScience® DISCOVER SOMETHING GREAT
Reviews in Medical Virology

Rev. Med. Virol. 2007; **17**: 343–354.
Published online 11 June 2007 in Wiley InterScience (www.interscience.wiley.com)
DOI: 10.1002/rmv.542

REVIEW



Host factors involved in the replication of hepatitis C virus

Kohji Moriishi and Yoshiharu Matsuura*

Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan

SUMMARY

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

Received: 22 March 2007; Accepted: 10 April 2007

INTRODUCTION

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

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

*Corresponding author: Y. Matsuura, DVM, PhD, Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan.
E-mail: matsuura@biken.osaka-u.ac.jp

Abbreviations used

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

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

IMMUNOPHILINS AND HSP90

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

Cyclophilin B

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

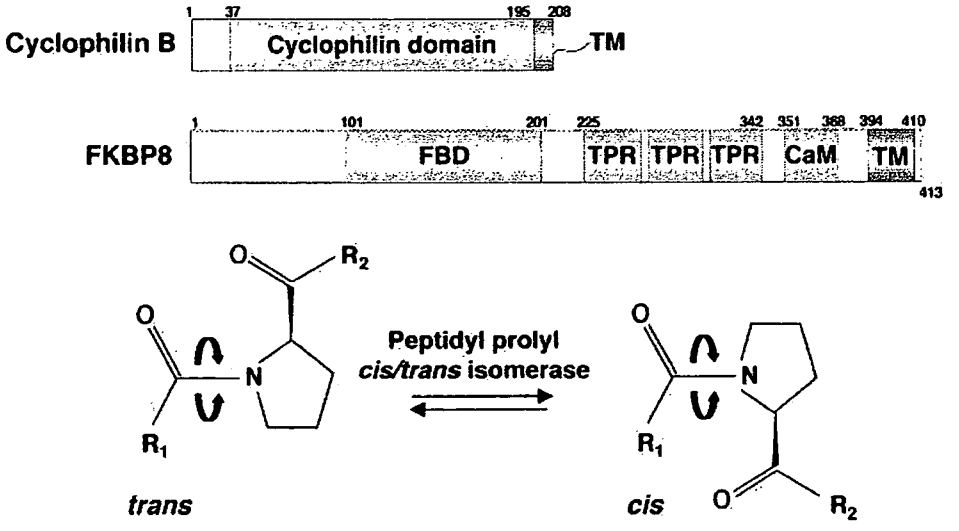


Figure 1. Structures of cyclophilin B and FKBP8. Cyclophilin B possesses a cyclophilin domain and a transmembrane region. FKBP8 has an FK506-binding domain (FBD), three sets of tricopeptide repeats (TPRs), a calmodulin-binding domain (CaM) and a transmembrane region (TM). Both proteins catalyse the conversion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

DOI: 10.1002/rmv

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

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

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

FKBP8

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

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

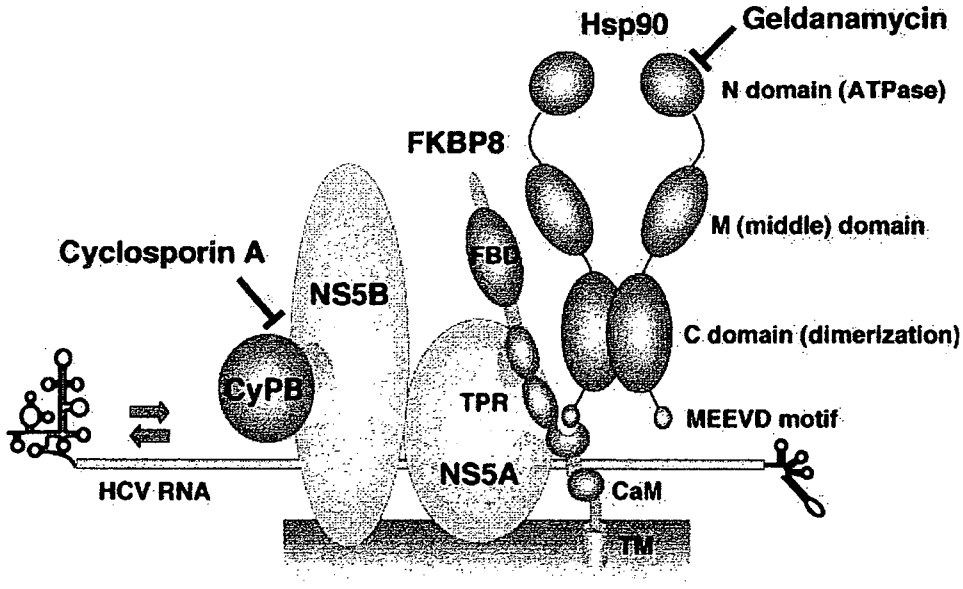


Figure 2. Interaction of HCV NS5A and NS5B proteins with immunophilins and Hsp90. Cyclophilin B interacts with NS5B. FKBP8 interacts with NS5A and Hsp90 through the different regions within TPR domains. Lys^{307} and Arg^{311} of the FKBP8 carboxylate clamp motif are required for binding to the MEEVD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

Rev. Med. Virol. 2007; 17: 343–354.
DOI: 10.1002/rmv

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

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

Hsp90

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

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

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

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

VESICLE-ASSOCIATED MEMBRANE PROTEIN-ASSOCIATED PROTEINS

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

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

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

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

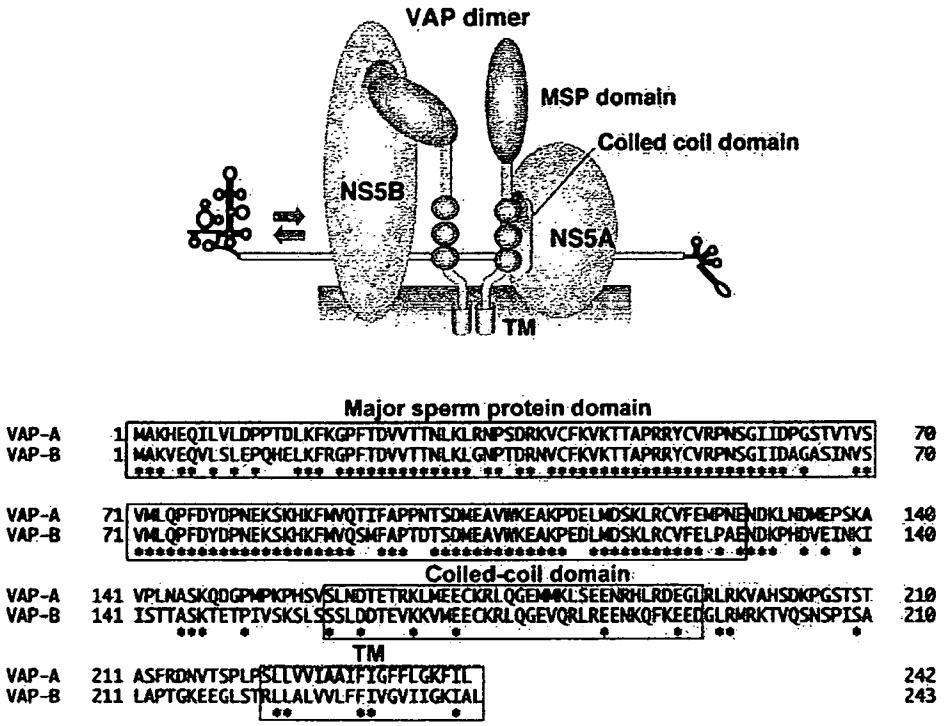


Figure 3. Interaction between HCV NS5A protein and VAPs. VAP-A and VAP-B make homo- and hetero-dimers with each other. The VAP dimer interacts with NS5A and NS5B through the coiled-coil domain and the MSP domain, respectively. VAP-A and VAP-B share 62.9 and 84.9% homology in total and in the MSP domain,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

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

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

DOI: 10.1002/rmv

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

HOST PROTEINS MODIFIED BY LIPID AND INVOLVED IN LIPID BIOSYNTHESIS

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

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

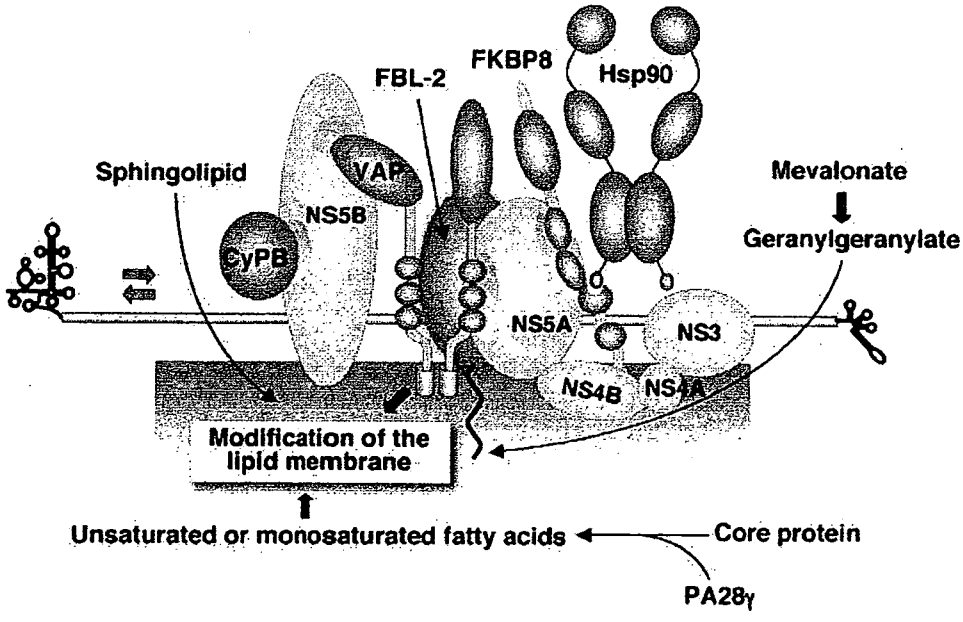


Figure 4. Putative model of HCV replication complex composed of viral and host proteins

Rev. Med. Virol. 2007; **17**: 343–354.
DOI: 10.1002/rmv

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

CONCLUSION

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

ACKNOWLEDGMENTS

We gratefully thank H. Murase for her secretarial work. This work was supported partly by grants-in-aid from the Ministry of Health, Labor, and Welfare; the Ministry of Education, Culture, Sports, Science, and Technology; the 21st Century Center of Excellence Program and the Foundation for Biomedical Research and Innovation, Japan.

REFERENCES

1. Moriishi K, Matsuura Y. Mechanisms of hepatitis C virus infection. *Antivir Chem Chemother* 2003; **14**: 285–297.
2. Wasley A, Alter MJ. Epidemiology of hepatitis C: geographic differences and temporal trends. *Semin Liver Dis* 2000; **20**: 1–16.
3. Strader DB, Wright T, Thomas DL, Seeff LB. Diagnosis, management, and treatment of hepatitis C. *Hepatology* 2004; **39**: 1147–1171.
4. Manns MP, McHutchison JG, Gordon SC, *et al.* Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958–965.
5. Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 1999; **285**: 110–113.
6. Sakamoto H, Okamoto K, Aoki M, *et al.* Host sphingolipid biosynthesis as a target for hepatitis C virus therapy. *Nat Chem Biol* 2005; **1**: 333–337.
7. Ni ZJ, Wagman AS. Progress and development of small molecule HCV antivirals. *Curr Opin Drug Discov Devel* 2004; **7**: 446–459.
8. Lin C, Lin K, Luong YP, *et al.* In vitro resistance studies of hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061: structural analysis indicates different resistance mechanisms. *J Biol Chem* 2004; **279**: 17508–17514.
9. Watashi K, Ishii N, Hijikata M, *et al.* Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. *Mol Cell* 2005; **19**: 111–122.
10. Okamoto T, Nishimura Y, Ichimura T, *et al.* Hepatitis C virus RNA replication is regulated by FKBP8 and Hsp90. *EMBO J* 2006; **25**: 5015–5025.
11. Hadziyannis SJ, Sette H Jr, Morgan TR, *et al.* Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann Intern Med* 2004; **140**: 346–355.
12. Fried MW, Shiffman ML, Reddy KR, *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; **347**: 975–982.
13. Zhong J, Gastaminza P, Cheng G, *et al.* Robust hepatitis C virus infection in vitro. *Proc Natl Acad Sci USA* 2005; **102**: 9294–9299.
14. Wakita T, Pietschmann T, Kato T, *et al.* Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 2005; **11**: 791–796.

15. Lindenbach BD, Evans MJ, Syder AJ, *et al.* Complete replication of hepatitis C virus in cell culture. *Science* 2005; **309**: 623–626.
16. Tomei L, Failla C, Santolini E, De Francesco R, La Monica N. NS3 is a serine protease required for processing of hepatitis C virus polyprotein. *J Virol* 1993; **67**: 4017–4026.
17. Grakoui A, McCourt DW, Wychowski C, Feinstone SM, Rice CM. Characterization of the hepatitis C virus-encoded serine proteinase: determination of proteinase-dependent polyprotein cleavage sites. *J Virol* 1993; **67**: 2832–2843.
18. Hamill P, Jean F. Enzymatic characterization of membrane-associated hepatitis C virus NS3-4A heterocomplex serine protease activity expressed in human cells. *Biochemistry* 2005; **44**: 6586–6596.
19. Egger D, Wolk B, Gosert R, *et al.* Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J Virol* 2002; **76**: 5974–5984.
20. Evans MJ, Rice CM, Goff SP. Phosphorylation of hepatitis C virus nonstructural protein 5A modulates its protein interactions and viral RNA replication. *Proc Natl Acad Sci USA* 2004; **101**: 13038–13043.
21. Blight KJ, Kolykhalov AA, Rice CM. Efficient initiation of HCV RNA replication in cell culture. *Science* 2000; **290**: 1972–1974.
22. Behrens SE, Tomei L, De Francesco R. Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus. *EMBO J* 1996; **15**: 12–22.
23. Hamamoto I, Nishimura Y, Okamoto T, *et al.* Human VAP-B is involved in hepatitis C virus replication through interaction with NS5A and NS5B. *J Virol* 2005; **79**: 13473–13482.
24. Tu H, Gao L, Shi ST, *et al.* Hepatitis C virus RNA polymerase and NS5A complex with a SNARE-like protein. *Virology* 1999; **263**: 30–41.
25. Fischer G, Wittmann-Liebold B, Lang K, Kiefhaber T, Schmid FX. Cyclophilin and peptidyl-prolyl cis-trans isomerase are probably identical proteins. *Nature* 1989; **337**: 476–478.
26. Siekierka JJ, Hung SH, Poe M, Lin CS, Sigal NH. A cytosolic binding protein for the immunosuppressant FK506 has peptidyl-prolyl isomerase activity but is distinct from cyclophilin. *Nature* 1989; **341**: 755–757.
27. Siekierka JJ, Staruch MJ, Hung SH, Sigal NH. FK-506, a potent novel immunosuppressive agent, binds to a cytosolic protein which is distinct from the cyclosporin A-binding protein, cyclophilin. *J Immunol* 1989; **143**: 1580–1583.
28. Rahfeld JU, Schierhorn A, Mann K, Fischer G. A novel peptidyl-prolyl cis/trans isomerase from Escherichia coli. *FEBS Lett* 1994; **343**: 65–69.
29. Schiene-Fischer C, Habazettl J, Schmid FX, Fischer G. The hsp70 chaperone DnaK is a secondary amide peptide bond cis-trans isomerase. *Nat Struct Biol* 2002; **9**: 419–424.
30. Liu J, Farmer JD Jr, Lane WS, Friedman J, Weissman I, Schreiber SL. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* 1991; **66**: 807–815.
31. Luban J, Bossolt KL, Franke EK, Kalpana GV, Goff SP. Human immunodeficiency virus type 1 Gag protein binds to cyclophilins A and B. *Cell* 1993; **73**: 1067–1078.
32. Wiegers K, Rutter G, Schubert U, Grattinger M, Krausslich HG. Cyclophilin A incorporation is not required for human immunodeficiency virus type 1 particle maturation and does not destabilize the mature capsid. *Virology* 1999; **257**: 261–274.
33. Stremlau M, Owens CM, Perron MJ, Kiessling M, Autissier P, Sodroski J. The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. *Nature* 2004; **427**: 848–853.
34. Sayah DM, Sokolskaja E, Berthoux L, Luban J. Cyclophilin A retrotransposition into TRIM5 explains owl monkey resistance to HIV-1. *Nature* 2004; **430**: 569–573.
35. Caroni P, Rothenfluh A, McGlynn E, Schneider C. S-cyclophilin. New member of the cyclophilin family associated with the secretory pathway. *J Biol Chem* 1991; **266**: 10739–10742.
36. Spik G, Haendler B, Delmas O, *et al.* A novel secreted cyclophilin-like protein (SCYLP). *J Biol Chem* 1991; **266**: 10735–10738.
37. Allain F, Denys A, Spik G. Characterization of surface binding sites for cyclophilin B on a human tumor T-cell line. *J Biol Chem* 1994; **269**: 16537–16540.
38. Rycyzyn MA, Reilly SC, O'Malley K, Clevenger CV. Role of cyclophilin B in prolactin signal transduction and nuclear retrotranslocation. *Mol Endocrinol* 2000; **14**: 1175–1186.
39. Cohen E, Taraboulos A. Scrapie-like prion protein accumulates in aggresomes of cyclosporin A-treated cells. *EMBO J* 2003; **22**: 404–417.
40. Ishii N, Watashi K, Hishiki T, *et al.* Diverse effects of cyclosporine on hepatitis C virus strain replication. *J Virol* 2006; **80**: 4510–4520.
41. Watashi K, Hijikata M, Hosaka M, Yamaji M, Shimotohno K. Cyclosporin A suppresses replication of hepatitis C virus genome in cultured hepatocytes. *Hepatology* 2003; **38**: 1282–1288.
42. Inoue K, Sekiyama K, Yamada M, Watanabe T, Yasuda H, Yoshiba M. Combined interferon alpha2b and cyclosporin A in the treatment of chronic hepatitis C: controlled trial. *J Gastroenterol* 2003; **38**: 567–572.
43. Lam E, Martin M, Wiederrecht G. Isolation of a cDNA encoding a novel human FK506-binding

protein homolog containing leucine zipper and tetratricopeptide repeat motifs. *Gene* 1995; **160**: 297–302.
44. Edlich F, Weiwad M, Erdmann F, *et al.* Bcl-2 regulator FKBP38 is activated by Ca2+/calmodulin. *EMBO J* 2005; **24**: 2688–2699.
45. Edlich F, Weiwad M, Wildemann D, *et al.* The specific FKBP38 inhibitor N-(N',N'-dimethylcarboxamidomethyl)cycloheximide has potent neuroprotective and neurotrophic properties in brain ischemia. *J Biol Chem* 2006; **281**: 14961–14970.
46. Nielsen JV, Mitchelmore C, Pedersen KM, Kjaerulff KM, Finsen B, Jensen NA. Fkbp8: novel isoforms, genomic organization, and characterization of a forebrain promoter in transgenic mice. *Genomics* 2004; **83**: 181–192.
47. Bulgakov OV, Eggenschwiler JT, Hong DH, Anderson KV, Li T. FKBP8 is a negative regulator of mouse sonic hedgehog signaling in neural tissues. *Development* 2004; **131**: 2149–2159.
48. Echelard Y, Epstein DJ, St-Jacques B, *et al.* Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* 1993; **75**: 1417–1430.
49. Chang DT, Lopez A, von Kessler DP, *et al.* Products, genetic linkage and limb patterning activity of a murine hedgehog gene. *Development* 1994; **120**: 3339–3353.
50. Briscoe J, Ericson J. Specification of neuronal fates in the ventral neural tube. *Curr Opin Neurobiol* 2001; **11**: 43–49.
51. Chiang C, Litingtung Y, Lee E, *et al.* Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* 1996; **383**: 407–413.
52. Chen Y, Struhl G. Dual roles for patched in sequestering and transducing Hedgehog. *Cell* 1996; **87**: 553–563.
53. Marigo V, Davey RA, Zuo Y, Cunningham JM, Tabin CJ. Biochemical evidence that patched is the Hedgehog receptor. *Nature* 1996; **384**: 176–179.
54. Price MA, Kalderon D. Proteolysis of cubitus interruptus in Drosophila requires phosphorylation by protein kinase A. *Development* 1999; **126**: 4331–4339.
55. Ohlmeyer JT, Kalderon D. Hedgehog stimulates maturation of Cubitus interruptus into a labile transcriptional activator. *Nature* 1998; **396**: 749–753.
56. Wijgerde M, McMahon JA, Rule M, McMahon AP. A direct requirement for Hedgehog signaling for normal specification of all ventral progenitor domains in the presumptive mammalian spinal cord. *Genes Dev* 2002; **16**: 2849–2864.
57. Goodrich LV, Milenkovic L, Higgins KM, Scott MP. Altered neural cell fates and medulloblastoma in mouse patched mutants. *Science* 1997; **277**: 1109–1113.
58. Shirane M, Nakayama KI. Inherent calcineurin inhibitor FKBP38 targets Bcl-2 to mitochondria and inhibits apoptosis. *Nat Cell Biol* 2003; **5**: 28–37.
59. Waxman L, Whitney M, Pollok BA, Kuo LC, Darke PL. Host cell factor requirement for hepatitis C virus enzyme maturation. *Proc Natl Acad Sci USA* 2001; **98**: 13931–13935.
60. Naito T, Momose F, Kawaguchi A, Nagata K. Involvement of Hsp90 in assembly and nuclear import of influenza virus RNA polymerase subunits. *J Virol* 2007; **81**: 1339–1349.
61. Momose F, Naito T, Yano K, Sugimoto S, Morikawa Y, Nagata K. Identification of Hsp90 as a stimulatory host factor involved in influenza virus RNA synthesis. *J Biol Chem* 2002; **277**: 45306–45314.
62. Burch AD, Weller SK. Herpes simplex virus type 1 DNA polymerase requires the mammalian chaperone hsp90 for proper localization to the nucleus. *J Virol* 2005; **79**: 10740–10749.
63. Kampmueller KM, Miller DJ. The cellular chaperone heat shock protein 90 facilitates Flock House virus RNA replication in Drosophila cells. *J Virol* 2005; **79**: 6827–6837.
64. Connor JH, McKenzie MO, Parks GD, Lyles DS. Antiviral activity and RNA polymerase degradation following Hsp90 inhibition in a range of negative strand viruses. *Virology* 2007; **362**: 109–117.
65. Hu J, Toft DO, Seeger C. Hepadnavirus assembly and reverse transcription require a multi-component chaperone complex which is incorporated into nucleocapsids. *EMBO J* 1997; **16**: 59–68.
66. Hu J, Seeger C. Hsp90 is required for the activity of a hepatitis B virus reverse transcriptase. *Proc Natl Acad Sci USA* 1996; **93**: 1060–1064.
67. Geller R, Vignuzzi M, Andino R, Frydman J. Evolutionary constraints on chaperone-mediated folding provide an antiviral approach refractory to development of drug resistance. *Genes Dev* 2007; **21**: 195–205.
68. Skehel PA, Martin KC, Kandel ER, Bartsch D. A VAMP-binding protein from Aplysia required for neurotransmitter release. *Science* 1995; **269**: 1580–1583.
69. Nishimura Y, Hayashi M, Inada H, Tanaka T. Molecular cloning and characterization of mammalian homologues of vesicle-associated membrane protein-associated (VAMP-associated) proteins. *Biochem Biophys Res Commun* 1999; **254**: 21–26.
70. Skehel PA, Fabian-Fine R, Kandel ER. Mouse VAP33 is associated with the endoplasmic reticulum and microtubules. *Proc Natl Acad Sci USA* 2000; **97**: 1101–1106.
71. Soussan L, Burakov D, Daniels MP, *et al.* ERG30, a VAP-33-related protein, functions in protein trans-