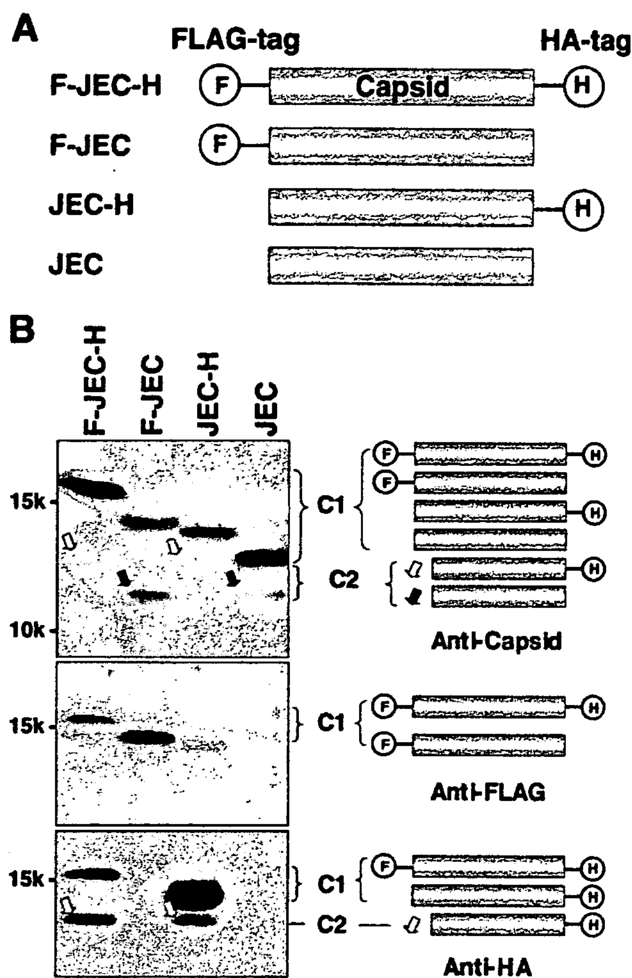

FIG. 2. The C2 protein lacks the amino terminus. (A) Series of the capsid protein constructs with or without FLAG and HA tags in the amino and carboxyl termini, respectively. (B) Expression of a series of the capsid proteins in Vero cells. The cell lysates expressing F-JEC-H, F-JEC, JEC-H, and JEC were 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-

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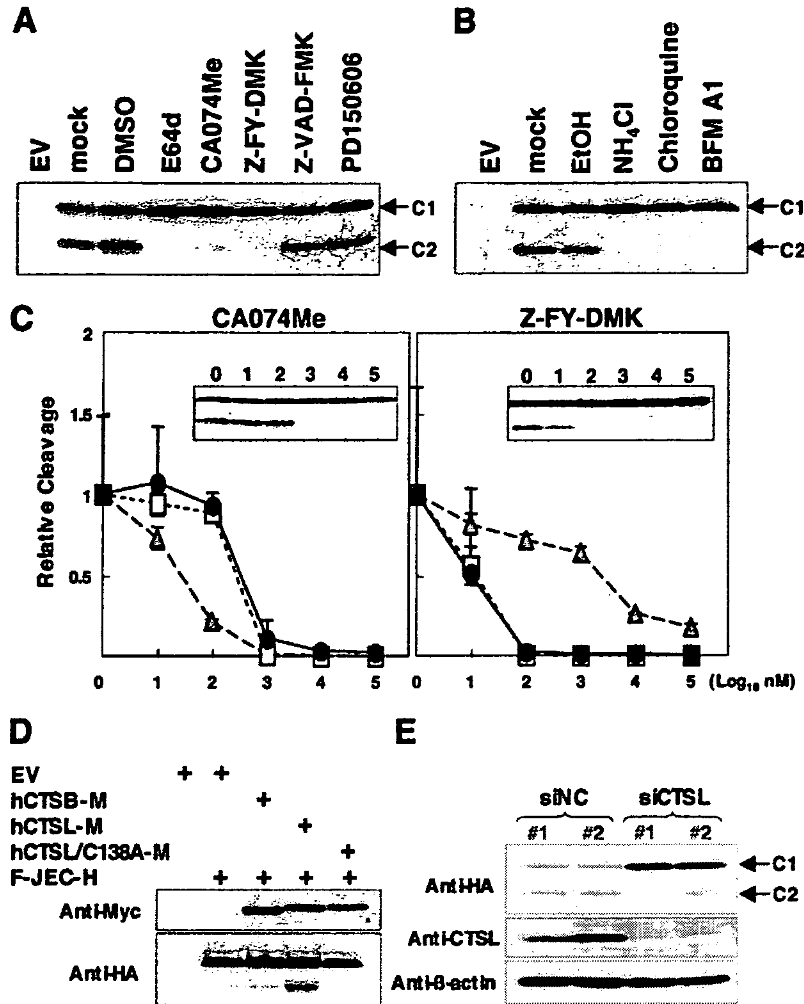

FIG. 3. JEV capsid protein is processed by cathepsin L. (A) Effects of cysteine protease inhibitors on the processing of the JEV capsid protein. Vero cells expressing F-JEC-H were treated with 50 μM E64d, 10 μM CA074Me, 10 μM Z-FY-DMK, 20 μM Z-VAD-FMK, or 20 μM PD150606 for 8 h at 37°C and examined by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

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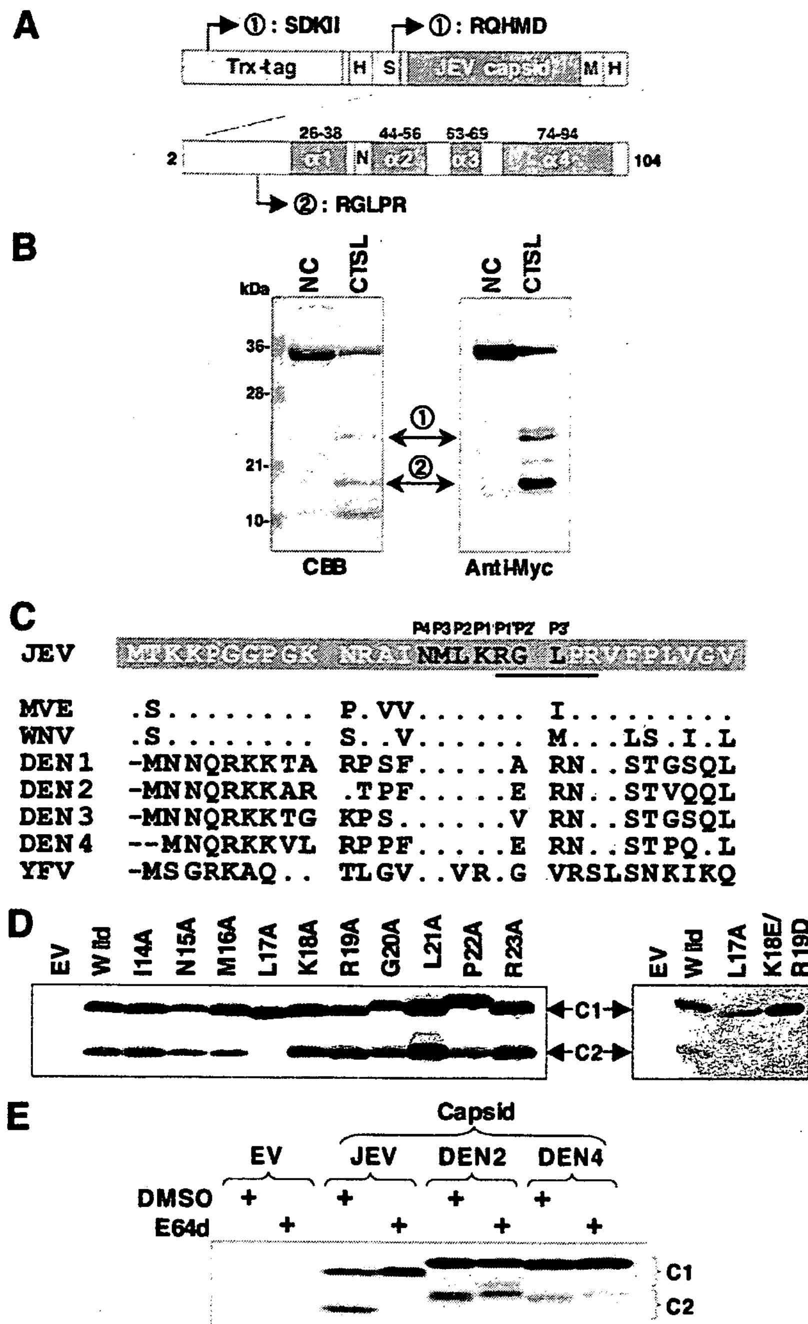

FIG. 4. Identification of the site of cleavage of JEV capsid protein by cathepsin L. (A) Schematic diagram of the recombinant JEV capsid protein. The His, S, and myc tags are indicated as H, S, and M, respectively. Four α-helices (α1 to 4) of the JEV capsid protein were predicted by Ma et al. (27). The 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.

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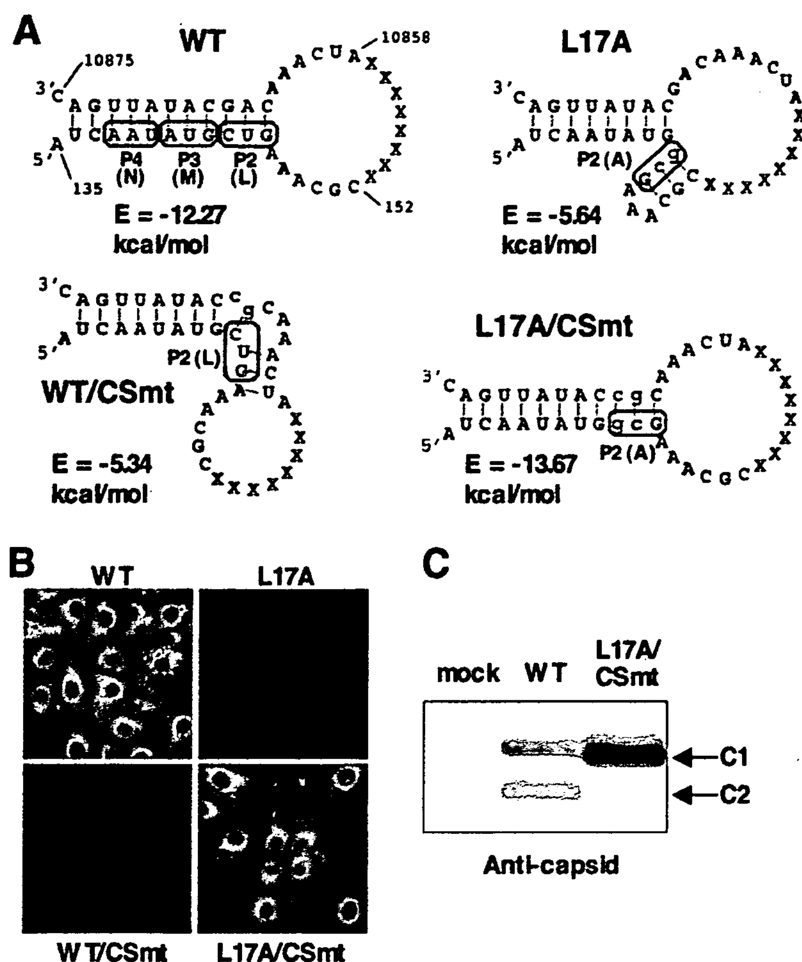

FIG. 5. Construction of a mutant JEV carrying the capsid protein resistant to cleavage by cathepsin L. (A) Predicted RNA secondary structures of the wild-type (WT) and mutant viral genomes. Nucleotides 135 to 152 and bases 10858 to 10875 in the 5′ and 3′ termini, respectively, connected by 8 nonsense 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-

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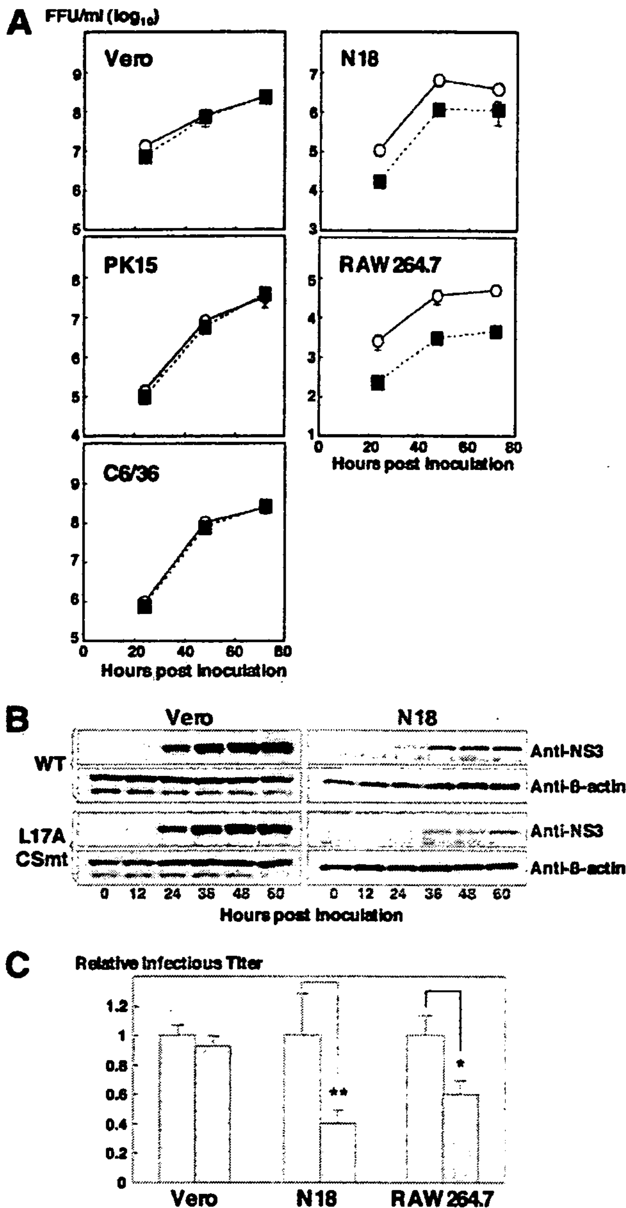

FIG. 6. Growth kinetics of L17A/CSmt in various cell lines. (A) The wild-type (WT; open circles) and mutant L17A/CSmt (solid squares) JEVs were inoculated into Vero, C6/36, PK15, N18, and RAW264.7 cells at an MOI of 10. After the indicated times, the infective titers in the culture supernatants on Vero 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

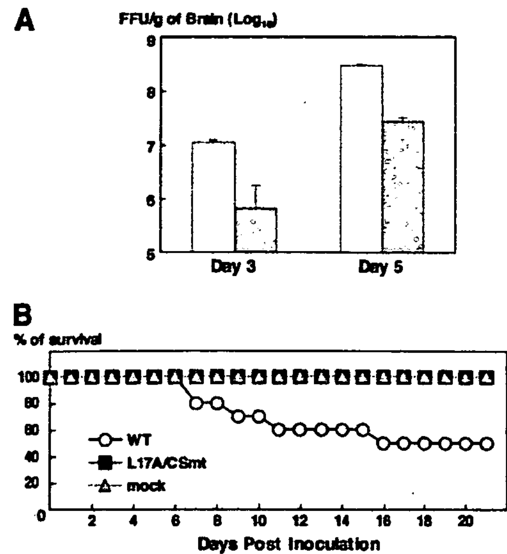

FIG. 7. Neurovirulence and neuroinvasiveness of L17A/CSmt in mice. (A) Growth of the wild-type (WT; white bars) and mutant L17A/CSmt (gray bars) virus in mouse brain. One hundred FFU of each virus were intracerebrally injected into 3-week-old ICR mice, and the progeny viruses in the brain at 3 or 5 days 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.

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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-

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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.

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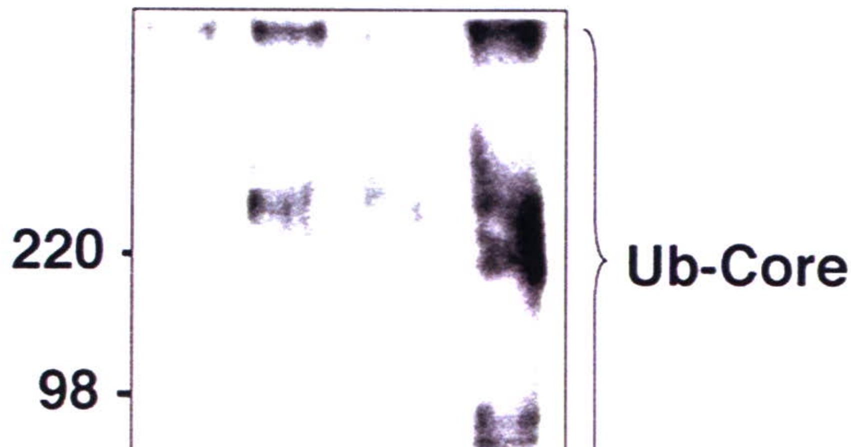

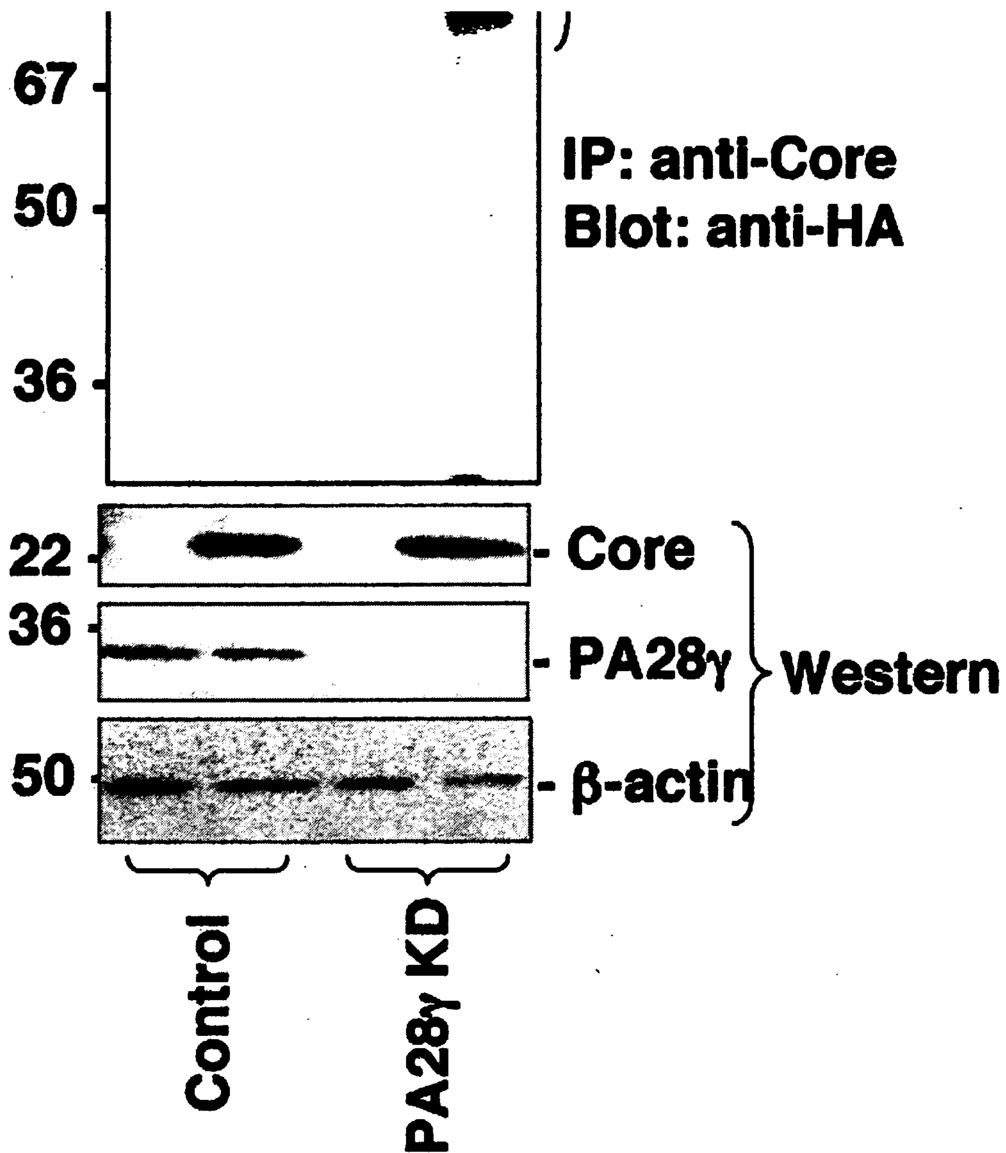

Fig. 6. Effect of PA28g knockdown on the ubiquitination of HCV core protein. HCV core protein was expressed with HA-tagged ubiquitin (HA-Ub) in the parental (Control) or PA28g-knockdown (PA28g KD) human hepatoma (FLC4) cells. The proteasome inhibitor MG132 was added to the culture supernatant at 24 h posttransfection to a final concentration of 10 mM. Cells were harvested at 36 h posttransfection, and cell lysates were subjected to immunoprecipitation (IP) with anti-core antibody and immunoblotting with anti-HA antibody (*Upper*). HCV core protein, PA28g, and b-actin in the cell lysate were detected by immunoblotting (*Lower*).

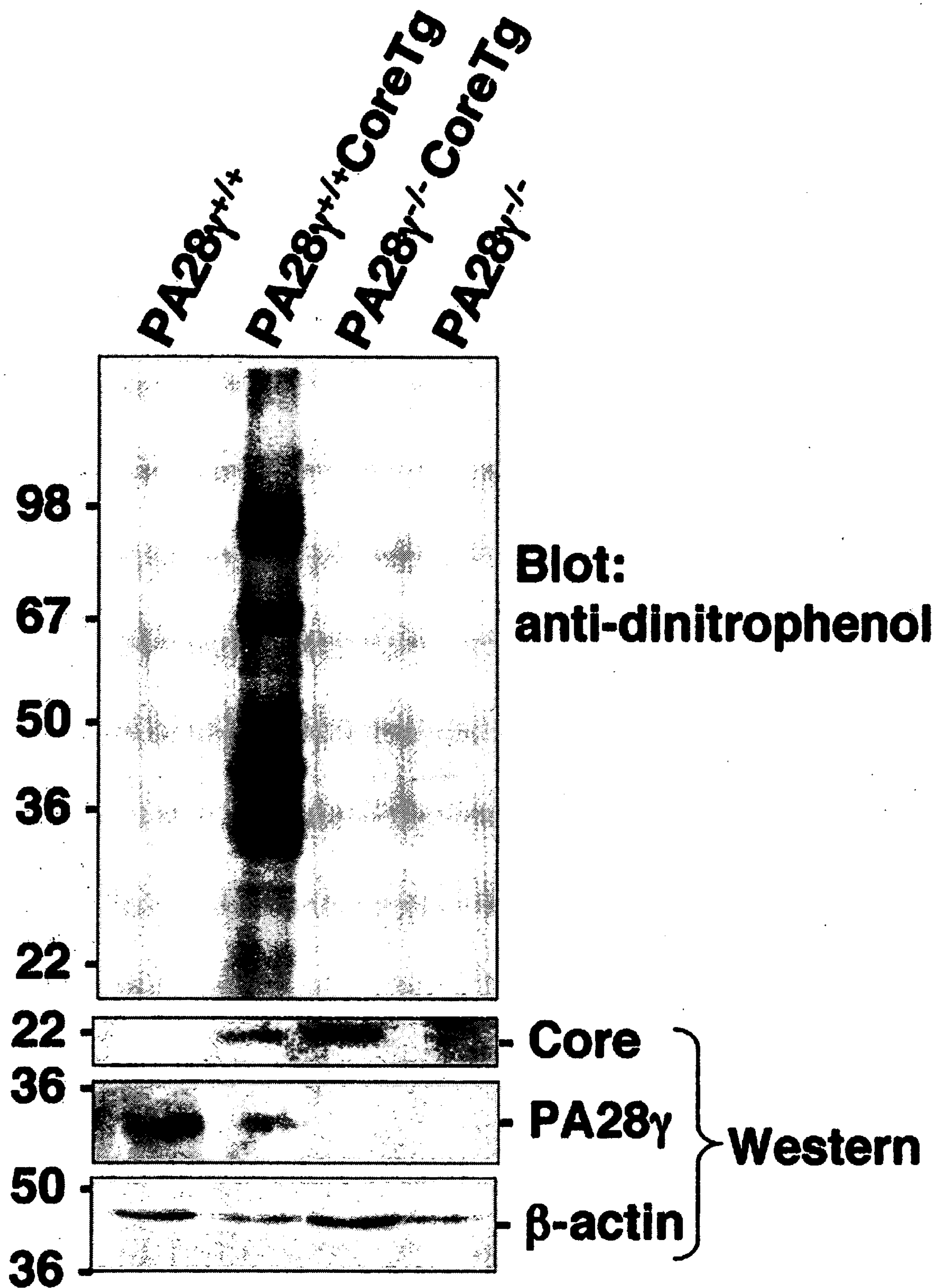

Fig. 7. Knockout of PA28g gene decreases the protein carbonylation induced by HCV core protein. Liver lysates were prepared from 6-month-old mice and subjected to OxyBlot assay. Each sample (20 mg of protein) was applied to a lane after the derivatization reaction (*Top*). HCV core protein, PA28g, and b-actin in the liver lysate were detected by immunoblotting (*Middle* and *Bottom*).

SI Methods and Materials

Histology and Immunohistochemistry. Formalin-fixed tissues were embedded in paraffin according to the standard procedures. Sections were stained with hematoxylin/eosin. To visualize lipids, frozen sections were stained with oil red O. For immunohistochemistry, sections of formalin-fixed tissues were treated with 3% (vol/vol) H_2O_2, washed twice with PBS, blocked with PBS containing 5% BSA, and incubated overnight with rabbit antibody to hepatitis C virus (HCV) core protein, followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG antibody (ICN, Aurora, OH) as a second antibody. Immunoreactive antigen was visualized with 3,3'-diaminobenzidine substrate. The percentage of the area occupied by oil red O-stained lipid droplets was calculated using Image-Pro software (MediaCybernetics, Silver Spring, MD). The area of the lipid droplets was examined in three different fields of every five randomly selected sections, and the areas were calculated by averaging 10 animals per genotype.

Real-Time PCR. RNA was prepared from the mouse livers with the use of TRIzol LS (Invitrogen, Carlsbad, CA). The first-strand cDNAs were synthesized with a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech, Franklin Lakes, NJ). The amount of each cDNA was estimated by using Platinum SYBR Green qPCR super Mix UDG (Invitrogen) according to the manufacturer's protocol. The fluorescent signal was measured by using ABI prism 7000 (Applied Biosystems, Tokyo, Japan). The genes encoding mouse sterol regulatory element-binding proteins (SREBP)-1a, SREBP-1c, SREBP-2, stearoyl-CoA desaturase, acetyl-CoA carboxylase, fatty acid synthase, hydroxymethylglutaryl (HMG)-CoA synthase, HMG-CoA reductase, and hypoxanthine phosphoribosyltransferase were amplified with the primer pairs CACAGCGGTTTTGAACGAC and CTGGCTCCTCTTTGATCCCA, ACGGAGCCATGGATTGCACATTTG and TACATCTTTAAAGCAGCGGGTGCCGATGGT, ACCATTCTCCAGCAGTTCCGT and CCTCTCACAGTGACAGAAGGAGTT, TTCCCTCCTGCAAGCTCTAC and CGCAAGAAGGTGCTAACGAAC, GACAAACGAGTCTGGCTACT and TGATGAGTGACTGCCGAAAC, CTCCAAGACTGACTCGGCTACT and AGCTGGGAGCACATCTCGAA, GGTTGGAGTGTTCTCTTACGG and CTCTGACCAGATACCACGTTC, TATGCCCATCCCTGTTGGAG and CACGTGGAGTTTCTGTAGACGA, and CCAGCAAGCTTGCAACCTTAACCA and GTAATGATCAGTCAACGGGGGAC, respectively. The sense and antisense primers were located in different exons to avoid false-positive amplification from contaminated genomic DNA. Each PCR product was confirmed as a single band of the correct size by agarose gel electrophoresis (data not shown).

Detection of Proteins Modified by Reactive Oxygen Species. Carbonyl groups in proteins were detected by using an OxyBlot kit (Chemicon International, Temecula, CA) according to the manufacturer's instructions. The carbonyl groups in the side

chains of amino acid residues included in the protein are a hallmark of the oxidation status of proteins, and they are reacted with 2,4-dinitophenylhydrazine, resulting in the derivatization to 2,4-dinitrophenylhydrazone. The derivatized proteins are subjected to SDS/PAGE and Western blotting using the antibody to dinitrophenyl moiety.

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