NF-IL6–deficient cells (Fig. 2 E). Together, compared with Trib1-deficient cells, converse phenotypes in terms of TLR-mediated immune responses are observed in NF-IL6–deficient cells.

Inhibition of NF-IL6 by Trib1 overexpression

To test whether Trib1 down-regulates NF-IL6–dependent activation, HEK293 cells were transfected with an NF-IL6–dependent luciferase reporter plasmid together with NF-IL6 and various amounts of Trib1 expression vectors (Fig. 3 A). NF-IL6–mediated luciferase activity was diminished by co-expression of Trib1 in a dose-dependent manner. Moreover, RAW264.7 macrophage cells overexpressing Trib1 exhibited reduced expression of mPGES and 24p3 in response to LPS (Fig. S3 A, available at http://www.jem.org/cgi/content/full/jem.20070183/DC1). We next tested NF-IL6 DNA-binding activity by EMSA and observed less NF-IL6 DNA-binding activity in HEK293 cells coexpressing NF-IL6 and Trib1 than in ones transfected with the NF-IL6 vector alone (Fig. 3 B), presumably accounting for the down-regulation of the NF-IL6–dependent gene expression by Trib1. We then examined the effect of Trib1 on the amounts of NF-IL6 proteins by Western blotting. Although the diminution of NF-IL6 by Trib1 was marginal when excess amounts of NF-IL6 were expressed, we found that the transient expression of lower levels of NF-IL6, together with Trib1, resulted in a reduction of NF-IL6 in HEK293 cells (Fig. 3 C). Also, endogenous levels of NF-IL6 proteins in RAW264.7 cells overexpressing Trib1 were markedly less than those in control cells (Fig. 3 D). These results demonstrated that overproduction of Trib1 might negatively regulate NF-IL6 activity in vitro.

Up-regulation of NF-IL6 in Trib1-deficient cells

We next attempted to check the in vivo status of NF-IL6 in Trib1-deficient cells by comparing the NF-IL6 DNA-binding activity in Trib1-deficient macrophages with that in wild-type cells by EMSA. Although LPS-induced NF-κB–DNA complex formation in Trib1-deficient cells was similarly observed, Trib1-deficient cells exhibited elevated levels of C/EBP–DNA complex formation compared with wild-type cells (Fig. 4 A). We further examined whether the C/EBP–DNA complex in Trib1-deficient cells contained NF-IL6 by supershift assay. Addition of anti–NF-IL6 antibody into the C/EBP–DNA complex yielded more supershifted bands in Trib1-deficient cells than in wild-type cells (Fig. 4 B). In addition, the C/EBP–DNA complex was not shifted by the addition of anti-C/EBPδ (also known as NF-IL6β) antibody (Fig. S4 A, available at http://www.jem.org/cgi/content/full/jem.20070183/DC1), suggesting that NF-IL6 DNA-binding activity is augmented in Trib1-deficient cells. We then examined the amounts of NF-IL6 proteins by Western blotting (Fig. 4 C). Compared with wild-type cells, Trib1-deficient cells showed increased levels of NF-IL6 proteins. Finally, we examined NF-IL6 mRNA levels by Northern blotting and observed enhanced expression of NF-IL6 mRNA in Trib1-deficient cells (Fig. 4 D), which is consistent with the autocrine induction of

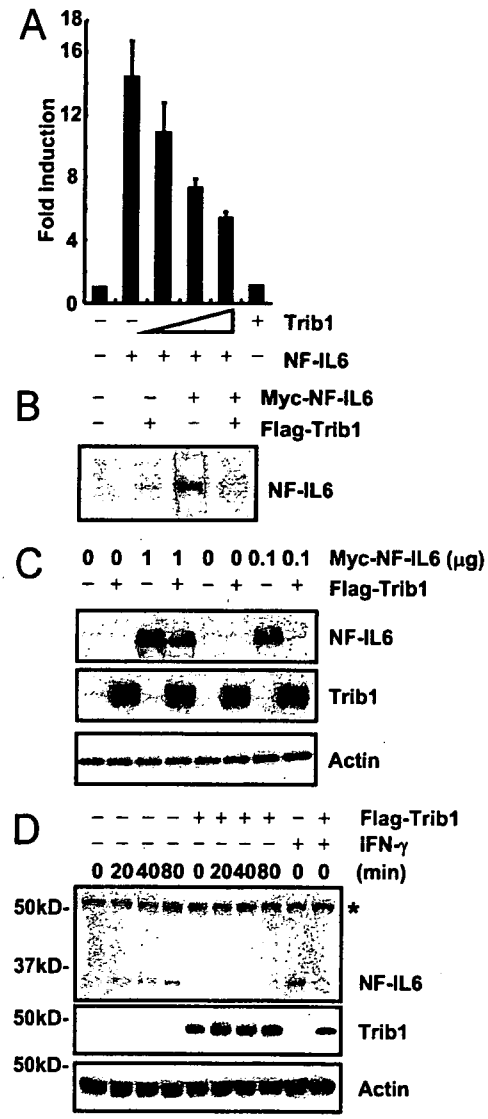

Figure 3. Inhibition of NF-IL6 activity by Trib1 overexpression. (A) HEK293 cells were transfected with an NF-IL6–dependent luciferase reporter together with either Trib1 and/or NF-IL6 expression plasmids. Luciferase activities were expressed as the fold increase over the background shown by lysates prepared from mock-transfected cells. Indicated values are means ± SD of triplicates. (B) HEK293 cells were transfected with 0.1 μg NF-IL6 expression vector together with 4 μg Trib1 expression plasmids. Nuclear extracts were prepared, and C/EBP DNA-binding activity was determined by EMSA using a probe containing the NF-IL6 binding sequence from the mouse 24p3 gene. (C) Lysates of HEK293 cells transiently cotransfected with 2 μg of Flag-tagged Trib1 alone or the indicated amounts of Myc-tagged NF-IL6 expression vectors were immunoblotted with anti-Myc or -Flag for detection of NF-IL6 or Trib1, respectively. (E) RAW 264.7 cells stably transfected with either an empty vector or Flag-Trib1 were stimulated with 10 ng/ml LPS for the indicated periods. The cell lysates were immunoblotted with the indicated antibodies. A protein that cross-reacts with the antibody is indicated (*). Data are representative of three (A and C) and two (B and D), separate experiments.

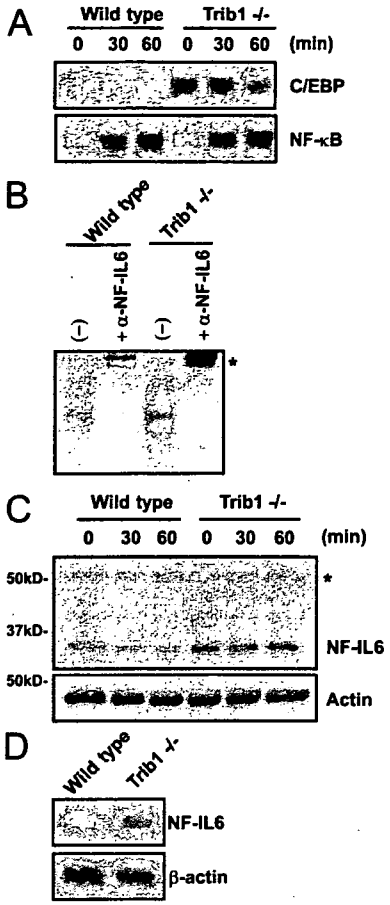

Figure 4. Up-regulation of NF-IL6 activity in Trib1-deficient cells. (A) Peritoneal macrophages from wild-type or Trib1-deficient mice were stimulated with 10 ng/ml LPS for the indicated periods. Nuclear extracts were prepared, and C/EBP DNA-binding activity was determined by EMSA using a C/EBP consensus probe. (B) Nuclear extracts of wild-type and Trib1-deficient unstimulated macrophages were preincubated with anti-NF-IL6, followed by EMSA to determine the C/EBP DNA-binding activity. Supershifted bands are indicated (*). (C) Peritoneal macrophages from wild-type or Trib1-deficient mice were stimulated with 10 ng/ml LPS for the indicated periods and lysed. The cell lysates were immunoblotted with the indicated antibodies. A protein that cross-reacts with the antibody is indicated (*). (D) Total RNA (10 μg) from unstimulated peritoneal macrophages from wild-type or NF-IL6–deficient mice was extracted and subjected to Northern blot analysis for expression of the indicated probes. Data are representative of two (A and B) and three (C and D) separate experiments.

NF-IL6 mRNA in a previous study (15). Thus, Trib1 may negatively control amounts of NF-IL6 proteins, thereby affecting TLR-mediated NF-IL6–dependent gene induction.

DISCUSSION

In this study, we demonstrate by microarray analysis and biochemical studies that Trib1 is associated with NF-IL6 and negates NF-IL6–dependent gene expression by reducing the amounts of NF-IL6 proteins in the context of TLR-mediated responses. Especially regarding IL-12 p40, although the microarray data showed an almost twofold reduction of the mRNA in Trib1-deficient cells (Table S1), the production was three to four times lower than that in wild-type cells (Fig. 1 C), suggesting translational control of IL-12 p40 by Trib1 in addition to the transcriptional regulation. Moreover, the transcription of the IL-12 p40 gene itself may be affected by not only the amount of NF-IL6 proteins but also the phosphorylation or the isoforms such as liver-enriched activator protein and liver-enriched inhibitory protein (16–18). The molecular mechanisms of how Trib1 deficiency affects IL-12 p40 production on the transcriptional or translational levels through NF-IL6 regulation need to be carefully studied in the future.

The name Trib is originally derived from the *Drosophila* mutant strain *tribbles*, in which the *Drosophila* tribbles protein negatively regulates the level of *Drosophila* C/EBP *slbo* protein and C/EBP-dependent developmental responses such as border cell migration in larvae (19–22). It is also of interest that Trib1-deficient female mice and *Drosophila* in adulthood are both infertile (unpublished data) (18). In mammals, other Trib family members such as Trib2 and Trib3 have recently been shown to be involved in C/EBP-dependent responses (23, 24). Mice transferred with bone marrow cells, in which Trib2 is retrovirally overexpressed, display acute myelogenous leukemia–like disease with reduced activities and amounts of C/EBPα (23). In addition, ectopic expression of Trib3 inhibits C/EBP-homologous protein–induced ER stress–mediated apoptosis (24). Thus, the function of tribbles to inhibit C/EBP activities by controlling the amounts appears to be conserved throughout evolution.

Given the up-regulation of the mRNA in Trib1-deficient cells (Fig. 4 D), the reduction of NF-IL6 in Trib1-overexpressing cells (Fig. 3 C), the auto-regulation of NF-IL6 by itself (15), and the degradation of C/EBPα by Trib2 (23) and slbo by tribbles (22), the loss of Trib1 might primarily result in impaired degradation of NF-IL6 and, subsequently, in excessive accumulation of NF-IL6 via the autoregulation in Trib1-deficient cells.

In this study, we focused on the involvement of Trib1 in TLR-mediated NF-IL6–dependent gene expression. However, given that the levels of NF-IL6 proteins were increased in Trib1-deficient cells, it is reasonable to propose that other non–TLR-related NF-IL6–dependent responses might be enhanced in Trib1-deficient mice. Moreover, Trib3 is also shown to be involved in insulin-mediated Akt/PKB activation in the liver by mechanisms apparently unrelated to C/EBP, suggesting that Trib family members possibly function in a C/EBP-independent fashion (25–27). Future studies using mice lacking other Trib family members, as well as Trib1, may help to unravel the nature of mammalian tribbles in wider points of view.

MATERIALS AND METHODS

Generation of Trib1-deficient mice. A genomic DNA containing the *Trib1* gene was isolated from the 129/SV mouse genomic library and characterized by restriction enzyme mapping and sequencing analysis. The gene encoding mouse Trib1 consists of three exons. The targeting vector was constructed by replacing a 0.4-kb fragment encoding the second exon of the

Trib1 gene with a neomycin resistance gene cassette (*neo*) (Fig. S1 A). The targeting vector was transfected into embryonic stem cells (E14.1). G418 and gancyclovir doubly resistant colonies were selected and screened by PCR and Southern blot analysis (Fig. S1 B). Homologous recombinants were microinjected into C57BL/6 female mice, and heterozygous F1 progenies were intercrossed to obtain *Trib1*$^{+/-}$ mice. We interbred the heterozygous mice to produce offspring carrying a null mutation of the gene encoding Trib1. Trib1-deficient mice were born at the expected Mendelian ratio and showed a slight growth retardation with reduced body weight until 2–3 wk after birth (unpublished data). Trib1-deficient that mice survived for >6 wk were analyzed in this study. To confirm the disruption of the gene encoding Trib1, we analyzed total RNA from wild-type and Trib1-deficient peritoneal macrophages by Northern blotting and found no transcripts for Trib1 in Trib1-deficient cells (Fig. S1 C). All animal experiments were conducted with the approval of the Animal Research Committee of the Research Institute for Microbial Diseases at Osaka University.

Reagents, cells, and mice. LPS (a TLR4 ligand) from *Salmonella minnesota* Re 595 and anti-Flag were purchased from Sigma-Aldrich. BLP (TLR1/TLR2), MALP-2 (TLR2/TLR6), and CpG oligodeoxynucleotides (TLR9) were prepared as previously described (28). Antiphosphorylated extracellular signal-regulated kinase, Jnk, and p38 antibodies were purchased from Cell Signaling. Anti–NF-IL6 (C/EBPβ), C/EBPδ, actin, IκBα, and Myc-probe were obtained from Santa Cruz Biotechnology, Inc. NF-IL6–deficient mice were as previously described (29). Epitope-tagged Trib1 fragments were generated by PCR using cDNA from LPS-stimulated mouse peritoneal macrophages as the template and cloned into pcDNA3 expression vectors, according to the manufacturer's instructions (Invitrogen).

Measurement of proinflammatory cytokine concentrations. Peritoneal macrophages were collected from peritoneal cavities 96 h after thioglycollate injection and cultured in 96-well plates (10^5 cells per well) with the indicated concentrations of the indicated ligands for 24 h, as shown in the figures. Concentrations of TNF-α, IL-6, and IL-12 p40 in the culture supernatant were measured by ELISA, according to manufacturer's instructions (TNF-α and IL-12 p40, Genzyme; IL-6, R&D Systems).

Luciferase reporter assay. The NF-IL6–dependent reporter plasmids were constructed by inserting the promoter regions (−1200 to +53) of the mouse 24p3 gene amplified by PCR into the pGL3 reporter plasmid. The reporter plasmids were transiently cotransfected into HEK293 with the control *Renilla* luciferase expression vectors using a reagent (Lipofectamine 2000; Invitrogen). Luciferase activities of total cell lysates were measured using the Dual-Luciferase Reporter Assay System (Promega), as previously described (28).

Yeast two-hybrid analysis. Yeast two-hybrid screening was performed as described for the Matchmaker two-hybrid system 3 (CLONTECH Laboratories, Inc.). For construction of the bait plasmid, the full length of human Trib1 was cloned in frame into the GAL4 DNA-binding domain of pGBKT7. Yeast strain AH109 was transformed with the bait plasmid plus the human lung Matchmaker cDNA library. After screening of 10^6 clones, positive clones were picked, and the pACT2 library plasmids were recovered from individual clones and expanded in *Escherichia coli*. The insert cDNA was sequenced and characterized with the BLAST program (National Center for Biotechnology Information).

Microarray analysis. Peritoneal macrophages from wild-type or Trib1-deficient mice were left untreated or were treated for 4 h with 10 ng/ml LPS in the presence of 30 ng/ml IFN-γ. The cDNA was synthesized and hybridized to Murine Genome 430 2.0 microarray chips (Affymetrix), according to the manufacturer's instructions. Hybridized chips were stained and washed and were scanned with a scanner (GeneArray; Affymetrix). Microarray Suite software (version 5.0; Affymetrix) was used for data analysis. Microarray data have been deposited in the Gene Expression Omnibus under accession no. GSE8788.

Western blot analysis and immunoprecipitation. Peritoneal macrophages were stimulated with the indicated ligands for the indicated periods, as shown in the figures. The cells were lysed in a lysis buffer (1% Nonidet P-40, 150 mM NaCl, 20 mM Tris-Cl [pH 7.5], 5 mM EDTA) and a protease inhibitor cocktail (Roche). The cell lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. For immunoprecipitation, cell lysates were precleared with protein G–sepharose (GE Healthcare) for 2 h and incubated with protein G–sepharose containing 1 μg of the antibodies indicated in the figures for 12 h, with rotation at 4°C. The immunoprecipitants were washed four times with lysis buffer, eluted by boiling with Laemmli sample buffer, and subjected to Western blot analysis using the indicated antibodies, as previously described (28).

EMSA and supershift assay. 2 × 10^6 peritoneal macrophages were stimulated with the indicated stimulants for the indicated periods, as shown in the figures. 2 × 10^6 HEK293 cells were transfected with 0.1 μg Myc–NF-IL6 and/or 4 μg Flag-Trib1 expression vectors. Nuclear extracts were purified from cells and incubated with a probe containing a consensus C/EBP DNA-binding sequence (5′-TGCAGATTGCGCAATCTGCA-3′; Fig. 4, A and B) or mouse 24p3 NF-IL6 binding sequence (sense, 5′-CTTCCTGTTGCTCAACCTTGCA -3′; antisense, 5′-TGCAAGGTTGAGCAACAGGAAG -3′; Fig. 3 B), electrophoresed, and visualized by autoradiography, as previously described (28, 30). When the supershift assay was performed, nuclear extracts were mixed with the supershift-grade antibodies indicated in the figures before the incubation with the probes for 1 h on ice.

Online supplemental material. Fig. S1 showed our strategy for the targeted disruption of the mouse *Trib1* gene. Fig. S2 showed the status of proinflammatory cytokine production in response to various TLR ligands and LPS-induced activation of MAP kinases and IκB degradation. Fig. S3 showed decreased expression of NF-IL6–dependent gene in Trib1-overexpressing cells. Fig. S4 showed that the C/EBP-DNA complex in Trib1-deficient cells contained NF-IL6, but not C/EBPδ. Table S1 provides a complete list of the LPS-inducible genes studied. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20070183/DC1.

We thank M. Hashimoto for excellent secretarial assistance, and N. Okita, N. Iwami, N. Fukuda, and M. Morita for technical assistance.

This study was supported by the Special Coordination Funds, the Ministry of Education, Culture, Sports, Science and Technology, research fellowships from the Japan Society for the Promotion of Science for Young Scientists, the Uehara Memorial Foundation, the Naito Foundation, the Institute of Physical and Chemical Research Junior Research Associate program, and the National Institutes of Health (grant AI070167).

The authors have no conflicting financial interests.

Submitted: 24 January 2007
Accepted: 26 July 2007

REFERENCES

1. Akira, S., S. Uematsu, and O. Takeuchi. 2006. Pathogen recognition and innate immunity. *Cell.* 124:783–801.
2. Beutler, B. 2004. Inferences, questions and possibilities in Toll-like receptor signalling. *Nature.* 430:257–263.
3. Kopp, E., and R. Medzhitov. 2003. Recognition of microbial infection by Toll-like receptors. *Curr. Opin. Immunol.* 15:396–401.
4. Hayden, M.S., and S. Ghosh. 2004. Signaling to NF-κB. *Genes Dev.* 18:2195–2224.
5. Zhang, Y.L., and C. Dong. 2005. MAP kinases in immune responses. *Cell. Mol. Immunol.* 2:20–27.
6. Miggin, S.M., and L.A. O'Neill. 2006. New insights into the regulation of TLR signaling. *J. Leukoc. Biol.* 80:220–226.
7. Hegedus, Z., A. Czibula, and E. Kiss-Toth. 2007. Tribbles: A family of kinase-like proteins with potent signalling regulatory function. *Cell. Signal.* 19:238–250.
8. Kiss-Toth, E., S.M. Bagstaff, H.Y. Sung, V. Jozsa, C. Dempsey, J.C. Caunt, K.M. Oxley, D.H. Wyllie, T. Polgar, M. Harte et al. 2004.

Human tribbles, a protein family controlling mitogen-activated protein kinase cascades. *J. Biol. Chem.* 279:42703–42708.
9. Wilkin, F., N. Suarez-Huerta, B. Robaye, J. Peetermans, F. Libert, J.E. Dumont, and C. Maenhaut. 1997. Characterization of a phosphoprotein whose mRNA is regulated by the mitogenic pathways in dog thyroid cells *Eur. Eur. J. Biochem.* 248:660–668.
10. Mayumi-Matsuda, K., S. Kojima, H. Suzuki, and T. Sakata. 1999. Identification of a novel kinase-like gene induced during neuronal cell death. *Biochem. Biophys. Res. Commun.* 258:260–264.
11. Wu, M., L.G. Xu, Z. Zhai, and H.B. Shu. 2003. SINK is a p65-interacting negative regulator of NF-κB-dependent transcription. *J. Biol. Chem.* 278:27072–27079.
12. Kiss-Toth, E., D.H. Wyllie, K. Holland, L. Marsden, V. Jozsa, K.M. Oxley, T. Polgar, E.E. Qwarnstrom, and S.K. Dower. 2006. Functional mapping and identification of novel regulators for the Toll/Interleukin-1 signalling network by transcription expression cloning. *Cell. Signal.* 18:202–214.
13. Uematsu, S., M. Matsumoto, K. Takeda, and S. Akira. 2002. Lipopolysaccharide-dependent prostaglandin E(2) production is regulated by the glutathione-dependent prostaglandin E(2) synthase gene induced by the Toll-like receptor 4/MyD88/NF-IL6 pathway. *J. Immunol.* 168:5811–5816.
14. Gorgoni, B., D. Maritano, P. Marthyn, M. Righi, and V. Poli. 2002. C/EBPβ gene inactivation causes both impaired and enhanced gene expression and inverse regulation of IL-12 p40 and p35 mRNAs in macrophages. *J. Immunol.* 168:4055–4062.
15. Ramji, D.P., and P. Foka. 2002. CCAAT/enhancer-binding proteins: structure, function and regulation. *Biochem. J.* 365:561–575.
16. Plevy, S.E., J.H. Gemberling, S. Hsu, A.J. Dorner, and S.T. Smale. 1997. Multiple control elements mediate activation of the murine and human interleukin 12 p40 promoters: evidence of functional synergy between C/EBP and Rel proteins. *Mol. Cell. Biol.* 17:4572–4588.
17. Zhu, C., K. Gagnidze, J.H. Gemberling, and S.E. Plevy. 2001. Characterization of an activation protein-1-binding site in the murine interleukin-12 p40 promoter. Demonstration of novel functional elements by a reductionist approach. *J. Biol. Chem.* 276:18519–18528.
18. Bradley, M.N., L. Zhou, and S.T. Smale. 2003. C/EBPβ regulation in lipopolysaccharide-stimulated macrophages. *Mol. Cell. Biol.* 23:4841–4858.
19. Seher, T.C., and M. Leptin. 2000. Tribbles, a cell-cycle brake that coordinates proliferation and morphogenesis during *Drosophila* gastrulation. *Curr. Biol.* 10:623–629.
20. Mata, J., S. Curado, A. Ephrussi, and P. Rorth. 2000. Tribbles coordinates mitosis and morphogenesis in *Drosophila* by regulating string/CDC25 proteolysis. *Cell.* 101:511–522.
21. Grosshans, J., and E. Wieschaus. 2000. A genetic link between morphogenesis and cell division during formation of the ventral furrow in *Drosophila*. *Cell.* 101:523–531.
22. Rorth, P., K. Szabo, and G. Texido. 2000. The level of C/EBP protein is critical for cell migration during *Drosophila* oogenesis and is tightly controlled by regulated degradation. *Mol. Cell.* 6:23–30.
23. Keeshan, K., Y. He, B.J. Wouters, O. Shestova, L. Xu, H. Sai, C.G. Rodriguez, I. Maillard, J.W. Tobias, P. Valk, et al. 2006. Tribbles homolog 2 inactivates C/EBPα and causes acute myelogenous leukemia. *Cancer Cell.* 10:401–411.
24. Ohoka, N., S. Yoshii, T. Hattori, K. Onozaki, and H. Hayashi. 2005. TRB3, a novel ER stress-inducible gene, is induced via ATF4-CHOP pathway and is involved in cell death. *EMBO J.* 24:1243–1255.
25. Du, K., S. Herzig, R.N. Kulkarni, and M. Montminy. 2003. TRB3: a tribbles homolog that inhibits Akt/PKB activation by insulin in liver. *Science.* 300:1574–1577.
26. Koo, S.H., H. Satoh, S. Herzig, C.H. Lee, S. Hedrick, R. Kulkarni, R.M. Evans, J. Olefsky, and M. Montminy. 2004. PGC-1 promotes insulin resistance in liver through PPAR-alpha-dependent induction of TRB-3. *Nat. Med.* 10:530–534.
27. Qi, L., J.E. Heredia, J.Y. Altarejos, R. Screaton, N. Goebel, S. Niessen, I.X. Macleod, C.W. Liew, R.N. Kulkarni, J. Bain, et al. 2006. TRB3 links the E3 ubiquitin ligase COP1 to lipid metabolism. *Science.* 312:1763–1766.
28. Yamamoto, M., T. Okamoto, K. Takeda, S. Sato, H. Sanjo, S. Uematsu, T. Saitoh, N. Yamamoto, H. Sakurai, K.J. Ishii, et al. 2006. Key function for the Ubc13 E2 ubiquitin-conjugating enzyme in immune receptor signaling. *Nat. Immunol.* 7:962–970.
29. Tanaka, T., S. Akira, K. Yoshida, M. Umemoto, Y. Yoneda, N. Shirafuji, H. Fujiwara, S. Suematsu, N. Yoshida, and T. Kishimoto. 1995. Targeted disruption of the NF-IL6 gene discloses its essential role in bacteria killing and tumor cytotoxicity by macrophages. *Cell.* 80:353–361.
30. Shen, F., Z. Hu, J. Goswami, and S.L. Gaffen. 2006. Identification of common transcriptional regulatory elements in interleukin-17 target genes. *J. Biol. Chem.* 281:24138–24148.

Reviews in Medical Virology

Rev. Med. Virol. (in press).
Published online 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

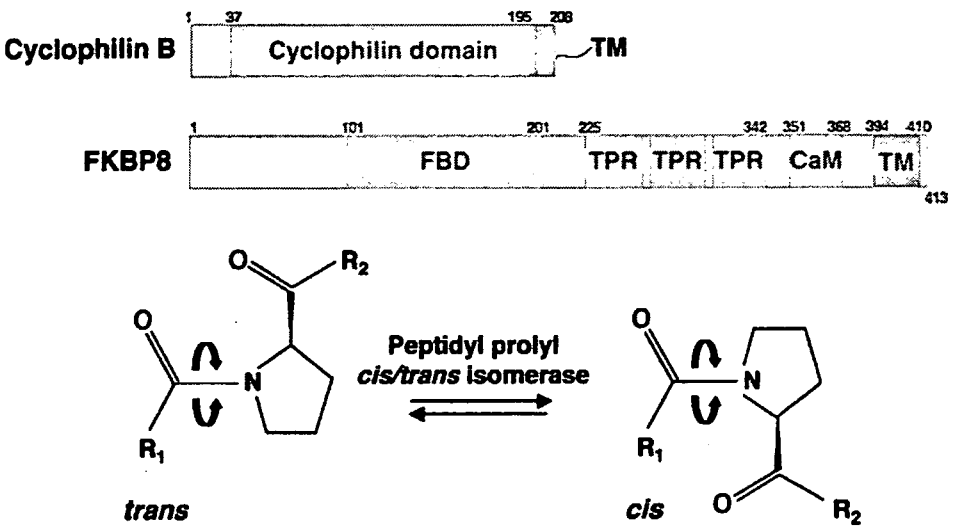

Figure 1. Structures of cyclophilin B and FKBP8. Cyclophilin B possesses a cyclophilin domain and a transmembrane region. FKBP8 has an FK506-binding domain (FBD), three sets of tricopeptide repeats (TPRs), a calmodulin-binding domain (CaM) and a transmembrane region (TM). Both proteins catalyse the conversion 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-

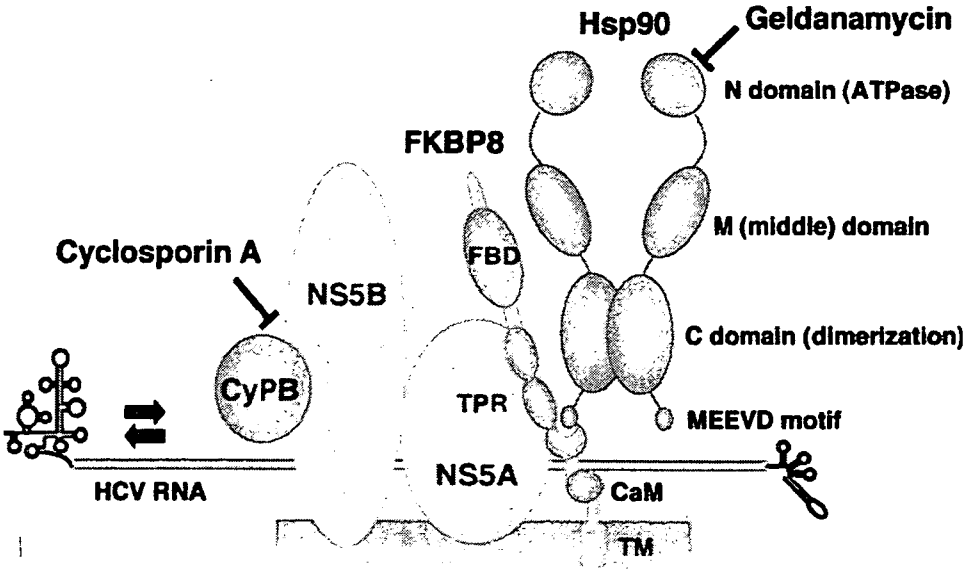

Figure 2. Interaction of HCV NS5A and NS5B proteins with immunophilins and Hsp90. Cyclophilin B interacts with NS5B. FKBP8 interacts with NS5A and Hsp90 through the different regions within TPR domains. Lys^{307} and Arg^{311} of the FKBP8 carboxylate clamp motif are required for binding to the MEEVD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

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,

DOI: 10.1002/rmv

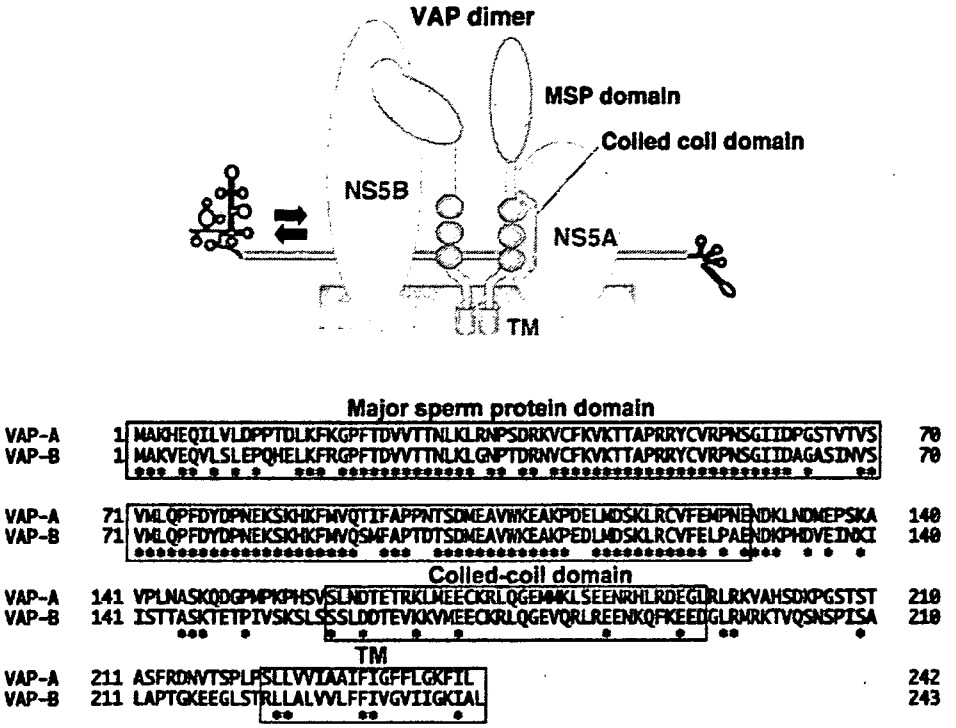

Figure 3. Interaction between HCV NS5A protein and VAPs. VAP-A and VAP-B make homo- and hetero-dimers with each other. The VAP dimer interacts with NS5A and NS5B through the coiled-coil domain and the MSP domain, respectively. VAP-A and VAP-B share 62.9 and 84.9% homology in total and in the MSP domain,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

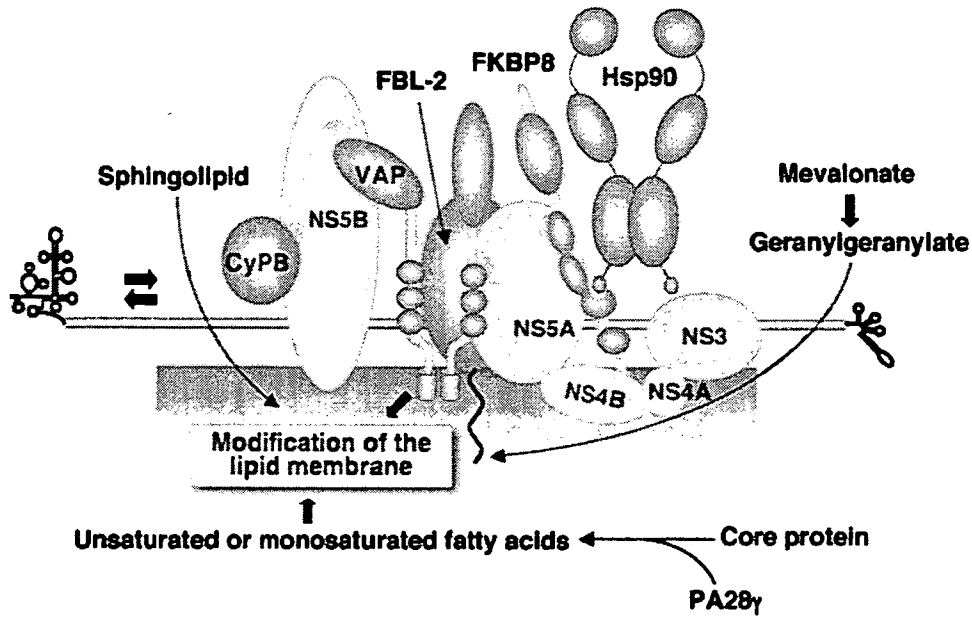

Figure 4. Putative model of HCV replication complex composed of viral and host proteins

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-

port mediated by COPI vesicles. *J Cell Biol* 1999; **146**: 301–311.
72. Kagiwada S, Hosaka K, Murata M, Nikawa J, Takatsuki A. The Saccharomyces cerevisiae SCS2 gene product, a homolog of a synaptobrevin-associated protein, is an integral membrane protein of the endoplasmic reticulum and is required for inositol metabolism. *J Bacteriol* 1998; **180**: 1700–1708.
73. Weir ML, Klip A, Trimble WS. Identification of a human homologue of the vesicle-associated membrane protein (VAMP)-associated protein of 33 kDa (VAP-33): a broadly expressed protein that binds to VAMP. *Biochem J* 1998; **333**(Pt 2): 247–251.
74. Nelson GA, Ward S. Amoeboid motility and actin in Ascaris lumbricoides sperm. *Exp Cell Res* 1981; **131**: 149–160.
75. Italiano JE Jr, Stewart M, Roberts TM. How the assembly dynamics of the nematode major sperm protein generate amoeboid cell motility. *Int Rev Cytol* 2001; **202**: 1–34.
76. Roberts TM, Stewart M. Acting like actin. The dynamics of the nematode major sperm protein (msp) cytoskeleton indicate a push-pull mechanism for amoeboid cell motility. *J Cell Biol* 2000; **149**: 7–12.
77. Tarr DE, Scott AL. MSP domain proteins. *Trends Parasitol* 2005; **21**: 224–231.
78. Loewen CJ, Roy A, Levine TP. A conserved ER targeting motif in three families of lipid binding proteins and in Opi1p binds VAP. *EMBO J* 2003; **22**: 2025–2035.
79. Pennetta G, Hiesinger PR, Fabian-Fine R, Meinertzhagen IA, Bellen HJ. Drosophila VAP-33A directs bouton formation at neuromuscular junctions in a dosage-dependent manner. *Neuron* 2002; **35**: 291–306.
80. Weir ML, Xie H, Klip A, Trimble WS. VAP-A binds promiscuously to both v- and tSNAREs. *Biochem Biophys Res Commun* 2001; **286**: 616–621.
81. Kaiser SE, Brickner JH, Reilein AR, Fenn TD, Walter P, Brunger AT. Structural basis of FFAT motif-mediated ER targeting. *Structure* 2005; **13**: 1035–1045.
82. Brickner JH, Walter P. Gene recruitment of the activated INO1 locus to the nuclear membrane. *PLoS Biol* 2004; **2**: e342.
83. Funakoshi T, Yasuda S, Fukasawa M, Nishijima M, Hanada K. Reconstitution of ATP- and cytosol-dependent transport of de novo synthesized ceramide to the site of sphingomyelin synthesis in semi-intact cells. *J Biol Chem* 2000; **275**: 29938–29945.
84. Fukasawa M, Nishijima M, Hanada K. Genetic evidence for ATP-dependent endoplasmic reticulum-to-Golgi apparatus trafficking of ceramide for sphingomyelin synthesis in Chinese hamster ovary cells. *J Cell Biol* 1999; **144**: 673–685.
85. Kawano M, Kumagai K, Nishijima M, Hanada K. Efficient trafficking of ceramide from the endoplasmic reticulum to the Golgi apparatus requires a VAMP-associated protein-interacting FFAT motif of CERT. *J Biol Chem* 2006; **281**: 30279–30288.
86. Quintavalle M, Sambucini S, Summa E, *et al.* Hepatitis C virus NS5A is a direct substrate of CKI-alpha, a cellular kinase identified by inhibitor affinity chromatography using specific NS5A hyperphosphorylation inhibitors. *J Biol Chem* 2007; **282**: 5536–5544.
87. Neddermann P, Quintavalle M, Di Pietro C, *et al.* Reduction of hepatitis C virus NS5A hyperphosphorylation by selective inhibition of cellular kinases activates viral RNA replication in cell culture. *J Virol* 2004; **78**: 13306–13314.
88. Burckstummer T, Kriegs M, Lupberger J, Pauli EK, Schmittel S, Hildt E. Raf-1 kinase associates with Hepatitis C virus NS5A and regulates viral replication. *FEBS Lett* 2006; **580**: 575–580.
89. Gao L, Aizaki H, He JW, Lai MM. Interactions between viral nonstructural proteins and host protein hVAP-33 mediate the formation of hepatitis C virus RNA replication complex on lipid raft. *J Virol* 2004; **78**: 3480–3488.
90. Shi ST, Lee KJ, Aizaki H, Hwang SB, Lai MM. Hepatitis C virus RNA replication occurs on a detergent-resistant membrane that cofractionates with caveolin-2. *J Virol* 2003; **77**: 4160–4168.
91. Zhang J, Yamada O, Sakamoto T, *et al.* Down-regulation of viral replication by adenoviral-mediated expression of siRNA against cellular cofactors for hepatitis C virus. *Virology* 2004; **320**: 135–143.
92. Amarilio R, Ramachandran S, Sabanay H, Lev S. Differential regulation of endoplasmic reticulum structure through VAP-Nir protein interaction. *J Biol Chem* 2005; **280**: 5934–5944.
93. Giese SI, Woerz I, Homann S, Tibroni N, Geyer M, Fackler OT. Specific and distinct determinants mediate membrane binding and lipid raft incorporation of HIV-1(SF2) Nef. *Virology* 2006; **355**: 175–191.
94. Brugger B, Glass B, Haberkant P, Leibrecht I, Wieland FT, Krausslich HG. The HIV lipidome: a raft with an unusual composition. *Proc Natl Acad Sci USA* 2006; **103**: 2641–2646.
95. Mannova P, Fang R, Wang H, *et al.* Modification of host lipid raft proteome upon hepatitis C virus replication. *Mol Cell Proteomics* 2006; **5**: 2319–2325.
96. Oomens AG, Bevis KP, Wertz GW. The cytoplasmic tail of the human respiratory syncytial virus F protein plays critical roles in cellular localization of the F protein and infectious progeny production. *J Virol* 2006; **80**: 10465–10477.
97. Chen BJ, Takeda M, Lamb RA. Influenza virus hemagglutinin (H3 subtype) requires palmitoylation of its cytoplasmic tail for assembly: M1 pro-

teins of two subtypes differ in their ability to support assembly. *J Virol* 2005; **79**: 13673–13684.
98. Kapadia SB, Chisari FV. Hepatitis C virus RNA replication is regulated by host geranylgeranylation and fatty acids. *Proc Natl Acad Sci USA* 2005; **102**: 2561–2566.
99. Horton JD, Goldstein JL, Brown MS. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest* 2002; **109**: 1125–1131.
100. Barba G, Harper F, Harada T, *et al.* Hepatitis C virus core protein shows a cytoplasmic localization and associates to cellular lipid storage droplets. *Proc Natl Acad Sci USA* 1997; **94**: 1200–1205.
101. Moriishi K, Mochizuki R, Moriya K, *et al.* Critical role of PA28gamma in hepatitis C virus-associated steatogenesis and hepatocarcinogenesis. *Proc Natl Acad Sci USA* 2007; **104**: 1661–1666.
102. Aizaki H, Lee KJ, Sung VM, Ishiko H, Lai MM. Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts. *Virology* 2004; **324**: 450–461.
103. Ye J, Wang C, Sumpter R Jr, Brown MS, Goldstein JL, Gale M Jr. Disruption of hepatitis C virus RNA replication through inhibition of host protein geranylgeranylation. *Proc Natl Acad Sci USA* 2003; **100**: 15865–15870.
104. Wang C, Gale M Jr, Keller BC, *et al.* Identification of FBL2 as a geranylgeranylated cellular protein required for hepatitis C virus RNA replication. *Mol Cell* 2005; **18**: 425–434.
105. Ilyin GP, Rialland M, Glaise D, Guguen-Guillouzo C. Identification of a novel Skp2-like mammalian protein containing F-box and leucine-rich repeats. *FEBS Lett* 1999; **459**: 75–79.
106. Shimakami T, Honda M, Kusakawa T, *et al.* Effect of hepatitis C virus (HCV) NS5B-nucleolin interaction on HCV replication with HCV subgenomic replicon. *J Virol* 2006; **80**: 3332–3340.
107. Goh PY, Tan YJ, Lim SP, *et al.* Cellular RNA helicase p68 relocalization and interaction with the hepatitis C virus (HCV) NS5B protein and the potential role of p68 in HCV RNA replication. *J Virol* 2004; **78**: 5288–5298.
108. Machida K, Cheng KT, Lai CK, Jeng KS, Sung VM, Lai MM. Hepatitis C virus triggers mitochondrial permeability transition with production of reactive oxygen species, leading to DNA damage and STAT3 activation. *J Virol* 2006; **80**: 7199–7207.
109. Kawamura H, Govindarajan S, Aswad F, *et al.* HCV core expression in hepatocytes protects against autoimmune liver injury and promotes liver regeneration in mice. *Hepatology* 2006; **44**: 936–944.
110. Yoshida T, Hanada T, Tokuhisa T, *et al.* Activation of STAT3 by the hepatitis C virus core protein leads to cellular transformation. *J Exp Med* 2002; **196**: 641–653.
111. Ogata H, Kobayashi T, Chinen T, *et al.* Deletion of the SOCS3 gene in liver parenchymal cells promotes hepatitis-induced hepatocarcinogenesis. *Gastroenterology* 2006; **131**: 179–193.
112. Moriya K, Fujie H, Shintani Y, *et al.* The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat Med* 1998; **4**: 1065–1067.
113. Lerat H, Honda M, Beard MR, *et al.* Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. *Gastroenterology* 2002; **122**: 352–365.
114. Koike K, Moriya K, Ishibashi K, *et al.* Sialadenitis histologically resembling Sjogren syndrome in mice transgenic for hepatitis C virus envelope genes. *Proc Natl Acad Sci USA* 1997; **94**: 233–236.

JOURNAL OF VIROLOGY, Aug. 2007, p. 8373 Vol. 81, No. 16
0022-538X/07/$08.00+0 doi:10.1128/JVI.01325-07

SPOTLIGHT

Articles of Significant Interest Selected from This Issue by the Editors

Novel Processing of Japanese Encephalitis Virus Capsid Protein

The Japanese encephalitis virus (JEV) capsid protein is generated from a precursor polyprotein by processing with host and viral proteases. Mori et al. (p. 8477–8487) show that further processing of the capsid protein by cathepsin L is involved in the replication of JEV in neural and macrophage cell lines. This work suggests that JEV uses a novel mechanism to infect host cells via cathepsin-mediated processing of the capsid protein.

Nodavirus RNA Polymerase Synthesis Requires Cellular Chaperones

Genome translation is an essential early step in the life cycle of positive-sense RNA viruses and often involves manipulation of the cellular translation apparatus to benefit the invading virus. Castorena et al. (p. 8412–8420) demonstrate that heat shock protein 90, an abundant cellular chaperone implicated in the replication of numerous viruses, facilitates the efficient synthesis of Flock House virus RNA polymerase. This work highlights the diverse mechanisms whereby viruses exploit cellular machinery to accomplish specific tasks to achieve their replication.

Rotavirus-Induced Alteration of Occludin Expression

Rotavirus can affect the intestinal barrier by diminishing the integrity of tight junctions (TJs). Beau et al. (p. 8579–8586) demonstrate that rhesus monkey rotavirus (RRV) alters the expression of occludin, an integral TJ protein, in enterocyte-like Caco-2 cells. The disappearance of occludin from the TJ plane, decrease in the nonphosphorylated form of occludin, and diminished levels of occludin mRNA are antagonized by inhibitors of protein kinase A (PKA). This work suggests that rotavirus uses PKA-dependent mechanisms to alter TJ function, which might in turn contribute to its pathogenicity or dissemination.

Human Immunodeficiency Virus Escape from Potent Neutralizing Antibodies

A recent clinical trial evaluated neutralizing monoclonal antibodies (MAbs) 2F5 and 4E10 targeting the membrane-proximal external region (MPER) of human immunodeficiency virus (HIV) gp41 together with the carbohydrate-specific MAb 2G12. The results demonstrated that once resistance to 2G12 had evolved, viral replication resumed despite MPER antibody treatment without evidence of escape mutations to these MAbs. Manrique et al. (p. 8793–8808), using cell-culture-based systems, now show that resistance to the MPER MAbs is difficult to achieve and can lead to selection of variants with impaired infectivity. This vulnerability of the virus to interference with the MPER supports the importance of this target in vaccine design.

Superior Smallpox Vaccine Candidates with Integrated Interleukin-15

A smallpox vaccine suitable for contemporary populations with greater numbers of immunodeficient individuals is a priority. Perera et al. (p. 8774–8783) report that the integration of the pleotropic cytokine interleukin-15 (IL-15), which is essential for both innate and adaptive immune responses, into the genome of either a Wyeth vaccine strain derived from the Dryvax vaccine or a nonreplicative modified vaccinia virus Ankara strain results in vaccine candidates with superior immunogenicity, durable efficacy, and safety. These IL-15-integrated derivatives hold promise as more efficacious and safe alternatives to the Dryvax vaccine.

Ancestral Center-of-Tree Human Immunodeficiency Virus Type 1 Proteins Are Functional and Immunogenic

The extensive diversity found in human immunodeficiency virus type 1 (HIV-1) vexingly challenges vaccine development. Rolland et al. (p. 8507–8514) developed a phylogenetics-informed algorithm to reconstruct ancestral HIV-1 sequences, called center-of-tree (COT). COT sequences are designed to minimize genetic distances between the antigen and circulating isolates. Computationally derived COT proteins corresponding to HIV-1 subtype B Gag, Tat, and Nef were generated and shown to retain the functionality of the extant proteins and elicited antigen-specific cellular immune responses in mice. This work establishes a new tool for rational vaccine design.

JOURNAL OF VIROLOGY, Aug. 2007, p. 8477–8487 Vol. 81, No. 16
0022-538X/07/$08.00+0 doi:10.1128/JVI.00477-07

Processing of Capsid Protein by Cathepsin L Plays a Crucial Role in Replication of Japanese Encephalitis Virus in Neural and Macrophage Cells[▽]

Yoshio Mori, Tetsuo Yamashita, Yoshinori Tanaka, Yoshimi Tsuda,† Takayuki Abe, Kohji Moriishi, and Yoshiharu Matsuura*

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

Received 6 March 2007/Accepted 25 May 2007

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

The genus *Flavivirus* within the family *Flaviviridae* comprises over 70 viruses, many of which are predominantly arthropod-borne viruses, such as Japanese encephalitis virus (JEV), West Nile virus (WNV), Murray Valley encephalitis virus (MVE), dengue virus (DEN), yellow fever virus (YFV), and tick-borne encephalitis virus (TBEV). They frequently cause significant morbidity and mortality in mammals and birds (5). JEV is distributed in the south and southeast regions of Asia and is kept in a zoonotic transmission cycle between pigs or birds and mosquitoes (5, 42, 45). JEV spreads to dead-end hosts, including humans, through the bite of JEV-infected mosquitoes and causes infection of the central nervous system with a high mortality rate (5, 45). JEV has a single-stranded positive-strand RNA genome of approximately 11 kb, which is capped at the 5′ end but lacks a 3′ polyadenine tail (24). The ability of the flaviviral genomic RNA to cyclize is crucial for viral replication (1, 14). Among mosquito-borne flaviviruses, two complementary cyclization sequences, mapped in the capsid protein-coding region and 3′ untranslated region (UTR), mediated the cyclization by RNA-RNA base pairing, together with a second pair of complementary sequences, named 5′ and 3′ upstream AUG regions (1, 10, 14, 19, 25). The genomic RNA includes a single large open reading frame, and a polyprotein translated at the endoplasmic reticulum (ER) membrane is cleaved co- and posttranslationally by host and viral proteases to yield three structural proteins, the capsid, precursor membrane (prM), and envelope (E) proteins, and at least seven nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (24).

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

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

* Corresponding author. Mailing address: Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565-0871, Japan. Phone: 81-6-6879-8340. Fax: 81-6-6879-8269. E-mail: matsuura@biken.osaka-u.ac.jp.
† Present address: Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan.
▽ Published ahead of print on 6 June 2007.

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-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 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.

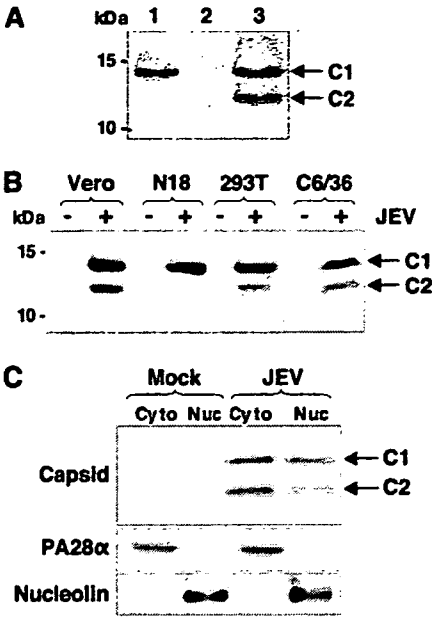

FIG. 1. Detection of C2 protein in cells infected with JEV. (A) Detection of the capsid proteins from the purified viral particles and cells infected with JEV. Lane 1, purified JEV particles produced in Vero cells; lanes 2 and 3, mock- and JEV-infected Vero cells, respectively. Arrows indicate a mature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

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.