

FIG. 6. Effect of Hsp90 inhibitor on the induction of the UPR in HCV replicon cells. (A) Huh7 and Huh9-13 replicon cells were transfected with a reporter plasmid, pGRP78-luc, and an internal-control plasmid, pRL-TK. The transfected cells were treated with IFN- α (+IFN) from 6 to 36 h posttransfection or left untreated and then further incubated for 6 h in the presence or absence of 1 μ M DMAG. The resulting cells were harvested and subjected to a dual-luciferase assay. The firefly luciferase activity is indicated as the RLU (top) after standardization with that of *Renilla* luciferase. The enhancement of promoter activity by treatment with DMAG is presented as the increase (bottom). (B) Huh7 cells, Huh9-13 cells, and Huh9-13 cells cured by IFN- α treatment (Cured) were cultured for 6 h in the presence or absence of 1 μ M DMAG, and the amount of GRP78 mRNA was measured by real-time PCR. The value of the mRNA was normalized with the amount of GAPDH mRNA (upper left), and the transcriptional enhancement by treatment with DMAG is presented as the increase (lower left). The expression levels of GRP78 and β -actin in the cells were determined by immunoblotting (upper right) and are presented as the increase (lower right). (C) The amounts of GADD153 mRNA in Huh7 cells, Huh9-13 cells, and the cured cells cultured for 6 h in the presence or absence of 1 μ M DMAG were measured by real-time PCR. The values of the mRNA were normalized with the amount of GAPDH mRNA (top), and the transcriptional enhancement by treatment with DMAG is presented as the increase (bottom). The error bars indicate standard deviations. The asterisks indicate significant differences ($P < 0.01$) versus the control value. The data shown are representative of three independent experiments.

ity, while small vesicles show fast movement (68), and FM and EM have revealed that NS5A is colocalized with hB-ind1, as well as FKBP8 (45), in the membranous webs. hB-ind1 was first identified as a regulator of Rac1 that activates JNK and NF- κ B (11). Rac1 is a member of the Rho GTPase family and plays

crucial roles in cytoskeletal dynamics, membrane ruffling, and gene transcription through the effectors of the Rho GTPase family members. IQGAP1 and PAK1 are Rac1 effectors that bind to Rac proteins and are also involved in the replication of HCV (5, 7, 19, 31, 50). The tetratricopeptide repeat domain of immunophilin family members, such as FKBP8, has been shown to interact with Hsp90 (12, 45) and the GR-Hsp90 complex that leads to association with dynein for retrograde transport, along with microtubules (12). Hsp90 has been shown to play an important role in the interaction of transcriptase with genomic RNA of hepatitis B virus (27) and the nuclear transportation of the polymerase of influenza virus (40). Flock house virus also recruits Hsp90 in the polymerase synthesis in the early step of infection (9). Hsp90 may be involved in the regulation of the movement and arrangement of the HCV replication complexes through interaction with Rac1, hB-ind1, and FKBP8. Further investigation is needed to clarify the role of the Hsp90 chaperone system in the life cycle of HCV.

The surrounding membranes, including the membranous web, may protect the viral replication complex and RNA genome against digestion by the host proteases and nucleases (69). The replication complex is composed of viral nonstructural proteins and host proteins, including chaperone and co-chaperone proteins. HCV NS5A has been shown to interact with various host proteins, including cochaperones, such as FKBP8 and hB-ind1, and to recruit a chaperone, Hsp90, into the replication complex through interaction with these cochaperones. Recruitment of the chaperone complex into the replication complex is crucial for the correct folding of newly synthesized viral proteins to maintain the efficient replication of the viral genome. HCV replication has been shown to be improved by the adaptive mutations suppressing the phosphorylation status of NS5A in the replicon cells (3). Although suppression of the hyperphosphorylation of NS5A by treatment with kinase inhibitors improves the replication of the replicons that have no adaptive mutations (42), several kinase inhibitors have been shown to suppress the replication of the HCV replicon carrying the adaptive mutations (29), and phosphorylation of NS5A by casein kinase II was shown to improve virus production but not HCV RNA replication (57). Hsp90 is capable of directly modulating the activities of several kinases (37, 53, 54), and thus, it might be feasible that cochaperones, including hB-ind1 and FKBP8, participate in the propagation of HCV by regulating the phosphorylation status of NS5A in cooperation with Hsp90.

The host chaperone system regulates the quality of client proteins, and impairment of the chaperone activity induces accumulation of misfolded proteins and affects the natural cellular function and viability (20, 21, 33). In this study, DMAG treatment induced a higher level of UPR in HCV replicon cells than in parental and cured cells, indicating that the Hsp90 chaperone system participates in the maintenance of correct folding of the viral and host proteins in the replication complex in the membranous web and in the circumvention of the UPR induced by HCV replication. Treatment with geldanamycin or its derivatives has been shown to inhibit GRP94, which is the Hsp90 paralog located in the ER (10), and to disrupt the ER chaperone pathway, leading to the induction of ER-associated protein degradation, transcriptional attenuation, and eventually induction of apoptosis (34). ER chaperones, such as

GRP94, may also participate in the correct folding of the viral and host proteins in the replication complex for efficient replication of the HCV genome.

Geldanamycin and its derivatives have been reported to remarkably inhibit poliovirus replication *in vivo* without any emergence of drug-resistant escape mutants (22), suggesting that an inhibitor of the chaperone system may be a promising candidate for the treatment of viral infectious diseases with low risk of the emergence of drug-resistant viruses. In addition, Hsp90 inhibitors exhibit anticancer activities through the suppression of various cell signals essential for cancer growth and the enhancement of radiation sensitivity (2, 8, 13). In conclusion, our data indicate that hB-ind1 is included within the HCV replication complex and regulates HCV RNA replication through its own cochaperone activity. Hsp90 and cochaperones, including hB-ind1 and FKBP8, which are required for efficient HCV replication, should be ideal targets for the treatment of chronic hepatitis C with a low frequency of emergence of drug-resistant breakthrough viruses.

ACKNOWLEDGMENTS

We thank H. Murase for her secretarial work. We also thank R. Bartenschlager, T. Wakita, and F. V. Chisari for providing the plasmids and cell lines.

This work was supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare; the Ministry of Education, Culture, Sports, Science, and Technology; the Global Center of Excellence Program; the Foundation for Biomedical Research and Innovation; and the Naito Foundation.

REFERENCES

- Abe, T., Y. Kaname, I. Hamamoto, Y. Tsuda, X. Wen, S. Taguwa, K. Morishi, O. Takeuchi, T. Kawai, T. Kanto, N. Hayashi, S. Akira, and Y. Matsuura. 2007. Hepatitis C virus nonstructural protein 5A modulates the toll-like receptor-MyD88-dependent signaling pathway in macrophage cell lines. *J. Virol.* **81**:8953–8966.
- Bisht, K. S., C. M. Bradbury, D. Mattson, A. Kaushal, A. Sowers, S. Markovina, K. L. Ortiz, L. K. Sieck, J. S. Isaacs, M. W. Brechbiel, J. B. Mitchell, L. M. Neckers, and D. Gius. 2003. Geldanamycin and 17-allylamino-17-demethoxygeldanamycin potentiate the *in vitro* and *in vivo* radiation response of cervical tumor cells via the heat shock protein 90-mediated intracellular signaling and cytotoxicity. *Cancer Res.* **63**:8984–8995.
- Blight, K. J., A. A. Kolykhalov, and C. M. Rice. 2000. Efficient initiation of HCV RNA replication in cell culture. *Science* **290**:1972–1974.
- Bohen, S. P., A. Kralli, and K. R. Yamamoto. 1995. Hold 'em and fold 'em: chaperones and signal transduction. *Science* **268**:1303–1304.
- Bost, A. G., D. Venable, L. Liu, and B. A. Heinz. 2003. Cytoskeletal requirements for hepatitis C virus (HCV) RNA synthesis in the HCV replicon cell culture system. *J. Virol.* **77**:4401–4408.
- Brown, G., H. W. Rixon, J. Steel, T. P. McDonald, A. R. Pitt, S. Graham, and R. J. Sugrue. 2005. Evidence for an association between heat shock protein 70 and the respiratory syncytial virus polymerase complex within lipid-raft membranes during virus infection. *Virology* **338**:69–80.
- Bryan, B. A., D. Li, X. Wu, and M. Liu. 2005. The Rho family of small GTPases: crucial regulators of skeletal myogenesis. *Cell Mol. Life Sci.* **62**:1547–1555.
- Calderwood, S. K., M. A. Khaleque, D. B. Sawyer, and D. R. Ciocca. 2006. Heat shock proteins in cancer: chaperones of tumorigenesis. *Trends Biochem. Sci.* **31**:164–172.
- Castorena, K. M., S. A. Weeks, K. A. Stapleford, A. M. Cadwallader, and D. J. Miller. 2007. A functional heat shock protein 90 chaperone is essential for efficient flock house virus RNA polymerase synthesis in *Drosophila* cells. *J. Virol.* **81**:8412–8420.
- Chavany, C., E. Mimnaugh, P. Miller, R. Bitton, P. Nguyen, J. Trepel, L. Whitesell, R. Schnur, J. Moyer, and L. Neckers. 1996. p185erbB2 binds to GRP94 *in vivo*. Dissociation of the p185erbB2/GRP94 heterocomplex by benzoquinone ansamycins precedes depletion of p185erbB2. *J. Biol. Chem.* **271**:4974–4977.
- Courilleau, D., E. Chastre, M. Sabbah, G. Redeuilh, A. Affi, and J. Mester. 2000. B-ind1, a novel mediator of Rac1 signaling cloned from sodium butyrate-treated fibroblasts. *J. Biol. Chem.* **275**:17344–17348.
- Davies, T. H., Y. M. Ning, and E. R. Sanchez. 2002. A new first step in activation of steroid receptors: hormone-induced switching of FKBP51 and FKBP52 immunophilins. *J. Biol. Chem.* **277**:4597–4600.
- Didelot, C., D. Lanneau, M. Brunet, A. L. Joly, A. De Thonel, G. Chiossis, and C. Garrido. 2007. Anti-cancer therapeutic approaches based on intracellular and extracellular heat shock proteins. *Curr. Med. Chem.* **14**:2839–2847.
- Egger, D., B. Wolk, R. Gosert, L. Bianchi, H. E. Blum, D. Moradpour, and K. Bienz. 2002. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J. Virol.* **76**:5974–5984.
- Evans, M. J., C. M. Rice, and S. P. Goff. 2004. Genetic interactions between hepatitis C virus replicons. *J. Virol.* **78**:12085–12089.
- Freeman, B. C., S. J. Felts, D. O. Toft, and K. R. Yamamoto. 2000. The p23 molecular chaperones act at a late step in intracellular receptor action to differentially affect ligand efficacies. *Genes Dev.* **14**:422–434.
- Freeman, B. C., and K. R. Yamamoto. 2002. Disassembly of transcriptional regulatory complexes by molecular chaperones. *Science* **296**:2232–2235.
- Frydman, J., and J. Hohfeld. 1997. Chaperones get in touch: the Hip-Hop connection. *Trends Biochem. Sci.* **22**:87–92.
- Fukata, M., M. Nakagawa, and K. Kaibuchi. 2003. Roles of Rho-family GTPases in cell polarisation and directional migration. *Curr. Opin. Cell Biol.* **15**:590–597.
- Garrido, C., M. Brunet, C. Didelot, Y. Zermati, E. Schmitt, and G. Kroemer. 2006. Heat shock proteins 27 and 70: anti-apoptotic proteins with tumorigenic properties. *Cell Cycle* **5**:2592–2601.
- Garrido, C., S. Gurbuxani, L. Ravagnan, and G. Kroemer. 2001. Heat shock proteins: endogenous modulators of apoptotic cell death. *Biochem. Biophys. Res. Commun.* **286**:433–442.
- Geller, R., M. Vignuzzi, R. Andino, and J. Frydman. 2007. Evolutionary constraints on chaperone-mediated folding provide an antiviral approach refractory to development of drug resistance. *Genes Dev.* **21**:195–205.
- Gosert, R., D. Egger, V. Lohmann, R. Bartenschlager, H. E. Blum, K. Bienz, and D. Moradpour. 2003. Identification of the hepatitis C virus RNA replication complex in Huh-7 cells harboring subgenomic replicons. *J. Virol.* **77**:5487–5492.
- Grakoui, A., D. W. McCourt, C. Wychowski, S. M. Feinstone, and C. M. Rice. 1993. Characterization of the hepatitis C virus-encoded serine proteinase: determination of proteinase-dependent polyprotein cleavage sites. *J. Virol.* **67**:2832–2843.
- Hamamoto, I., Y. Nishimura, T. Okamoto, H. Aizaki, M. Liu, Y. Mori, T. Abe, T. Suzuki, M. M. Lai, T. Miyamura, K. Morishi, and Y. Matsuura. 2005. Human VAP-B is involved in hepatitis C virus replication through interaction with NS5A and NS5B. *J. Virol.* **79**:13473–13482.
- Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77**:51–59.
- Hu, J., D. Flores, D. Toft, X. Wang, and D. Nguyen. 2004. Requirement of heat shock protein 90 for human hepatitis B virus reverse transcriptase function. *J. Virol.* **78**:13122–13131.
- Huang, D. C., S. Cory, and A. Strasser. 1997. Bcl-2, Bcl-XL and adenovirus protein E1B19kD are functionally equivalent in their ability to inhibit cell death. *Oncogene* **14**:405–414.
- Huang, Y., K. Staschke, R. De Francesco, and S. L. Tan. 2007. Phosphorylation of hepatitis C virus NS5A nonstructural protein: a new paradigm for phosphorylation-dependent viral RNA replication? *Virology* **364**:1–9.
- Hutchison, K. A., L. F. Stancato, J. K. Owens-Grillo, J. L. Johnson, P. Krishna, D. O. Toft, and W. B. Pratt. 1995. The 23-kDa acidic protein in reticulocyte lysate is the weakly bound component of the hsp foldosome that is required for assembly of the glucocorticoid receptor into a functional heterocomplex with hsp90. *J. Biol. Chem.* **270**:18841–18847.
- Ishida, H., K. Li, M. Yi, and S. M. Lemon. 2007. p21-activated kinase 1 is activated through the mammalian target of rapamycin/p70 S6 kinase pathway and regulates the replication of hepatitis C virus in human hepatoma cells. *J. Biol. Chem.* **282**:11836–11848.
- Kampmuller, K. M., and D. J. Miller. 2005. The cellular chaperone heat shock protein 90 facilitates Flock House virus RNA replication in *Drosophila* cells. *J. Virol.* **79**:6827–6837.
- Kim, H. P., D. Morse, and A. M. Choi. 2006. Heat-shock proteins: new keys to the development of cytoprotective therapies. *Exp. Opin. Ther. Targets* **10**:759–769.
- Lai, E., T. Teodoro, and A. Volchuk. 2007. Endoplasmic reticulum stress: signaling the unfolded protein response. *Physiology* **22**:193–201.
- Lohmann, V., F. Korner, J. Koch, U. Herian, L. Theilmann, and R. Bartenschlager. 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **285**:110–113.
- McLauchlan, J., M. K. Lemberg, G. Hope, and B. Martoglio. 2002. Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. *EMBO J.* **21**:3980–3988.
- Miyata, Y., and I. Yahara. 1992. The 90-kDa heat shock protein, HSP90, binds and protects casein kinase II from self-aggregation and enhances its kinase activity. *J. Biol. Chem.* **267**:7042–7047.
- Momose, F., T. Naito, K. Yano, S. Sugimoto, Y. Morikawa, and K. Nagata.

2002. Identification of Hsp90 as a stimulatory host factor involved in influenza virus RNA synthesis. *J. Biol. Chem.* 277:45306–45314.
39. Moradpour, D., F. Penin, and C. M. Rice. 2007. Replication of hepatitis C virus. *Nat. Rev. Microbiol.* 5:453–463.
 40. Naito, T., F. Momose, A. Kawaguchi, and K. Nagata. 2007. Involvement of Hsp90 in assembly and nuclear import of influenza virus RNA polymerase subunits. *J. Virol.* 81:1339–1349.
 41. Neckers, L. 2002. Hsp90 inhibitors as novel cancer chemotherapeutic agents. *Trends Mol. Med.* 8:S55–S61.
 42. Neddermann, P., M. Quintavalle, C. Di Pietro, A. Clementi, M. Cerretani, S. Altamura, L. Bartholomew, and R. De Francesco. 2004. Reduction of hepatitis C virus NS5A hyperphosphorylation by selective inhibition of cellular kinases activates viral RNA replication in cell culture. *J. Virol.* 78:13306–13314.
 43. Okamoto, K., Y. Mori, Y. Komoda, T. Okamoto, M. Okochi, M. Takeda, T. Suzuki, K. Moriishi, and Y. Matsuura. 2008. Intramembrane processing by signal peptide peptidase regulates the membrane localization of hepatitis C virus core protein and viral propagation. *J. Virol.* 82:8349–8361.
 44. Okamoto, K., K. Moriishi, T. Miyamura, and Y. Matsuura. 2004. Intramembrane proteolysis and endoplasmic reticulum retention of hepatitis C virus core protein. *J. Virol.* 78:6370–6380.
 45. Okamoto, T., Y. Nishimura, T. Ichimura, K. Suzuki, T. Miyamura, T. Suzuki, K. Moriishi, and Y. Matsuura. 2006. Hepatitis C virus RNA replication is regulated by FKBP8 and Hsp90. *EMBO J.* 25:5015–5025.
 46. Okamoto, T., H. Omori, Y. Kaname, T. Abe, Y. Nishimura, T. Suzuki, T. Miyamura, T. Yoshimori, K. Moriishi, and Y. Matsuura. 2008. A single-amino-acid mutation in hepatitis C virus NS5A disrupting FKBP8 interaction impairs viral replication. *J. Virol.* 82:3480–3489.
 47. Pietschmann, T., V. Lohmann, A. Kaul, N. Krieger, G. Rinck, G. Rutter, D. Strand, and R. Bartenschlager. 2002. Persistent and transient replication of full-length hepatitis C virus genomes in cell culture. *J. Virol.* 76:4008–4021.
 48. Prapapanich, V., S. Chen, E. J. Toran, R. A. Rimerman, and D. F. Smith. 1996. Mutational analysis of the hsp70-interacting protein Hip. *Mol. Cell Biol.* 16:6200–6207.
 49. Pratt, W. B., and D. O. Toff. 1997. Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocr. Rev.* 18:306–360.
 50. Ridley, A. J., H. F. Paterson, C. L. Johnston, D. Diekmann, and A. Hall. 1992. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* 70:401–410.
 51. Rieder, C. L., and S. S. Bowser. 1985. Correlative immunofluorescence and electron microscopy on the same section of epon-embedded material. *J. Histochem. Cytochem.* 33:165–171.
 52. Sanchez, E. R., D. O. Toff, M. J. Schlesinger, and W. B. Pratt. 1985. Evidence that the 90-kDa phosphoprotein associated with the untransformed L-cell glucocorticoid receptor is a murine heat shock protein. *J. Biol. Chem.* 260:12398–12401.
 53. Sato, S., N. Fujita, and T. Tsuruo. 2000. Modulation of Akt kinase activity by binding to Hsp90. *Proc. Natl. Acad. Sci. USA* 97:10832–10837.
 54. Stancato, L. F., A. M. Silverstein, J. K. Owens-Grillo, Y. H. Chow, R. Jove, and W. B. Pratt. 1997. The hsp90-binding antibiotic geldanamycin decreases Raf levels and epidermal growth factor signaling without disrupting formation of signaling complexes or reducing the specific enzymatic activity of Raf kinase. *J. Biol. Chem.* 272:4013–4020.
 55. Stravopodis, D. J., L. H. Margaritis, and G. E. Voutsinas. 2007. Drug-mediated targeted disruption of multiple protein activities through functional inhibition of the Hsp90 chaperone complex. *Curr. Med. Chem.* 14:3122–3138.
 56. Taguwa, S., T. Okamoto, T. Abe, Y. Mori, T. Suzuki, K. Moriishi, and Y. Matsuura. 2008. Human butyrate-induced transcript 1 interacts with hepatitis C virus NS5A and regulates viral replication. *J. Virol.* 82:2631–2641.
 57. Tellinghuisen, T. L., K. L. Foss, and J. Treadaway. 2008. Regulation of hepatitis C virion production via phosphorylation of the NS5A protein. *PLoS Pathog.* 4:e1000032.
 58. Tellinghuisen, T. L., J. Marcotrigiano, and C. M. Rice. 2005. Structure of the zinc-binding domain of an essential component of the hepatitis C virus replicase. *Nature* 435:374–379.
 59. Terasawa, K., M. Minami, and Y. Minami. 2005. Constantly updated knowledge of Hsp90. *J. Biochem.* 137:443–447.
 60. Tomei, L., C. Failla, E. Santolini, R. De Francesco, and N. La Monica. 1993. NS3 is a serine protease required for processing of hepatitis C virus polyprotein. *J. Virol.* 67:4017–4026.
 61. Tu, H., L. Gao, S. T. Shi, D. R. Taylor, T. Yang, A. K. Mircheff, Y. Wen, A. E. Gorbalenya, S. B. Hwang, and M. M. Lai. 1999. Hepatitis C virus RNA polymerase and NS5A complex with a SNARE-like protein. *Virology* 263:30–41.
 62. Wakita, T., T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H. G. Krausslich, M. Mizokami, R. Bartenschlager, and T. J. Liang. 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat. Med.* 11:791–796.
 63. Wang, C., M. Gale, Jr., B. C. Keller, H. Huang, M. S. Brown, J. L. Goldstein, and J. Ye. 2005. Identification of FBL2 as a geranylgeranylated cellular protein required for hepatitis C virus RNA replication. *Mol. Cell* 18:425–434.
 64. Wasley, A., and M. J. Alter. 2000. Epidemiology of hepatitis C: geographic differences and temporal trends. *Semin. Liver Dis.* 20:1–16.
 65. Watashi, K., N. Ishii, M. Hijikata, D. Inoue, T. Murata, Y. Miyanari, and K. Shimotohno. 2005. Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. *Mol. Cell* 19:111–122.
 66. Whitesell, L., and S. L. Lindquist. 2005. HSP90 and the chaperoning of cancer. *Nat. Rev. Cancer.* 5:761–772.
 67. Wochnik, G. M., J. C. Young, U. Schmidt, F. Holsboer, F. U. Hartl, and T. Rein. 2004. Inhibition of GR-mediated transcription by p23 requires interaction with Hsp90. *FEBS Lett.* 560:35–38.
 68. Wolk, B., B. Buchele, D. Moradpour, and C. M. Rice. 2008. A dynamic view of hepatitis C virus replication complexes. *J. Virol.* 82:10519–10531.
 69. Yang, G., D. C. Pevear, M. S. Collett, S. Chunduru, D. C. Young, C. Benetatos, and R. Jordan. 2004. Newly synthesized hepatitis C virus replicon RNA is protected from nuclease activity by a protease-sensitive factor(s). *J. Virol.* 78:10202–10205.
 70. Zhong, J., P. Gastaminza, G. Cheng, S. Kapadia, T. Kato, D. R. Burton, S. F. Wieland, S. L. Uprichard, T. Wakita, and F. V. Chisari. 2005. Robust hepatitis C virus infection in vitro. *Proc. Natl. Acad. Sci. USA* 102:9294–9299.

Human VAP-C Negatively Regulates Hepatitis C Virus Propagation[∇]

Hiroshi Kukihara,¹ Kohji Moriishi,¹ Shuhei Taguwa,¹ Hideki Tani,¹ Takayuki Abe,¹
Yoshio Mori,¹ Tetsuro Suzuki,² Takasuke Fukuhara,^{1,3} Akinobu Taketomi,³
Yoshihiko Maehara,³ and Yoshiharu Matsuura^{1*}

Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka,¹ Department of Virology II, National Institute of Infectious Diseases, Tokyo,² and Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka,³ Japan

Received 3 May 2009/Accepted 2 June 2009

Human vesicle-associated membrane protein-associated protein (VAP) subtype A (VAP-A) and subtype B (VAP-B) are involved in the regulation of membrane trafficking, lipid transport and metabolism, and the unfolded protein response. VAP-A and VAP-B consist of the major sperm protein (MSP) domain, the coiled-coil motif, and the C-terminal transmembrane anchor and form homo- and heterodimers through the transmembrane domain. VAP-A and VAP-B interact with NS5B and NS5A of hepatitis C virus (HCV) through the MSP domain and the coiled-coil motif, respectively, and participate in the replication of HCV. VAP-C is a splicing variant of VAP-B consisting of the N-terminal half of the MSP domain of VAP-B followed by the subtype-specific frameshift sequences, and its biological function has not been well characterized. In this study, we have examined the biological functions of VAP-C in the propagation of HCV. VAP-C interacted with NS5B but not with VAP-A, VAP-B, or NS5A in immunoprecipitation analyses, and the expression of VAP-C inhibited the interaction of NS5B with VAP-A or VAP-B. Overexpression of VAP-C impaired the RNA replication of the HCV replicon and the propagation of the HCV JFH1 strain, whereas overexpression of VAP-A and VAP-B enhanced the replication. Furthermore, the expression of VAP-C was observed in various tissues, whereas it was barely detected in the liver. These results suggest that VAP-C acts as a negative regulator of HCV propagation and that the expression of VAP-C may participate in the determination of tissue tropism of HCV propagation.

Hepatitis C virus (HCV) is a major causative agent of chronic liver disease and thus a major public health problem, infecting at least 3% of the world population (47). HCV infection proceeds to the persistent stage in approximately 80% of patients, leading to the development of cirrhosis in 20% to 50% of patients, of whom approximately 5% eventually develop hepatocellular carcinoma (12). HCV encompasses a single-stranded positive-sense RNA genome of approximately 9.6 kb, which encodes a large precursor polyprotein comprising approximately 3,000 amino acids (26). The structural proteins are cleaved from the N-terminal one-fourth of the polyprotein by the host signal peptidase and signal peptide peptidase (23, 32, 33), resulting in the maturation of the capsid protein, two envelope proteins and viroporin p7. The NS2 protease cleaves after the carboxyl terminus, and then NS3 cleaves the appropriate downstream positions to produce NS4A, NS4B, NS5A, and NS5B (8, 42), all of which form the replication complex along with several host proteins (5, 21). NS5B is the RNA-dependent RNA polymerase, which is a main enzymatic component of the replication complex of HCV (3), while NS5A is a membrane-anchored zinc-binding phosphoprotein that appears to possess diverse functions, including the suppression of host defense and the regulation of the virus's replication (1, 4, 6, 41), although its biological function remains unclear.

The NS5A protein has been shown to interact with several host proteins, including vesicle-associated membrane protein (VAMP)-associated protein (VAP) subtype A (VAP-A) (44) and subtype B (VAP-B) (9), FKBP8 (34), MyD88 (1), FBL2 (46), human butyrate-induced transcript 1 (hB-ind1) (40), and so on (25). VAP-A and VAP-B also bind to NS5B, although it remains unclear whether these interactions modulate HCV replication positively or negatively (9, 44). VAP-A and VAP-B have been shown to associate with the cytoplasmic face of the endoplasmic reticulum (ER) and the Golgi apparatus (38) and to consist of the major sperm protein (MSP) domain, the coiled-coil domain, and the transmembrane (TM) region, in that order (30, 39), as shown in Fig. 1A. VAP was originally reported as a protein binding to VAMP, which is a synaptic vesicle SNARE protein required for synaptic-vesicle fusion in the nematode *Aplysia californica*, and was designated the 33-kDa VAMP-associated protein, VAP-33 (39). Two mammalian homologues, VAP-A and VAP-B, were subsequently identified (30, 38). The transcription of VAP-A and VAP-B is ubiquitously detected in mammalian organs, including the heart, placenta, lung, liver, skeletal muscle, and pancreas (30), suggesting that VAP family proteins are involved in diverse cellular functions other than neurotransmitter release (30, 38, 49). Several VAP-interacting proteins share the FFAT motif (two phenylalanines in an acidic tract), which has the consensus amino acid sequence EFFDAXE, as determined by a comparison among oxysterol binding proteins (OSBPs), OSBP-related proteins (ORPs) (20), and the ceramide transport protein CERT (10, 19), contributing to the regulation of fatty acid metabolism. The interaction of VAP family proteins with

* Corresponding author. Mailing address: Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, 3-1, Yamadaoka, Suita, Osaka 565-0871, Japan. Phone: 81-6-6879-8340. Fax: 81-6-6879-8269. E-mail: matsuura@biken.osaka-u.ac.jp.

[∇] Published ahead of print on 10 June 2009.

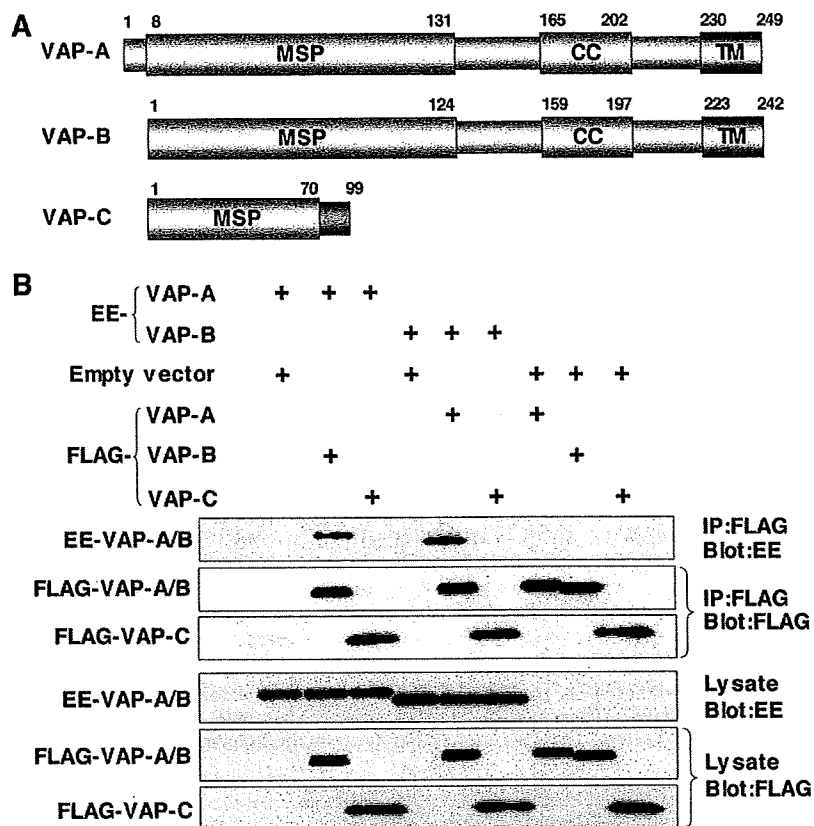


FIG. 1. VAP-C interacts with neither VAP-A nor VAP-B. (A) Structures of VAP family proteins. The MSP domain, the coiled-coil domain, and the TM region are indicated as MSP, CC, and TM, respectively. (B) Interaction among VAP family proteins. The expression plasmids encoding VAP proteins or empty vector (1 μ g each) were transfected into 293T cells, FLAG-tagged VAP proteins coexpressed with EE-tagged VAP-A or VAP-B were immunoprecipitated (IP) with anti-FLAG antibody, and the resulting precipitates were examined by immunoblotting using anti-FLAG or anti-EE antibody. One percent of the volume of the lysate was used as an input control. The data in each panel are representative of the results of three independent experiments. +, present.

other host proteins, including VAMP and tubulin, is independent of the FFAT motif (16, 36, 38, 50). The third subtype of VAP is VAP-C, which is an alternative spliced isoform of VAP-B, consisting of the N-terminal half of the MSP domain and the subtype-specific 29 amino acids (Fig. 1A). However, its tissue distribution and physiological function remain largely unknown.

Glutathione *S*-transferase pulldown and immunoprecipitation analyses revealed that both VAP-A and VAP-B interact with NSSB and NS5A through the MSP domain and the coiled-coil domain, respectively (9, 44), and the MSP domains of VAP-A and VAP-B exhibit 82.3% homology. Although VAP-C possesses the N-terminal-half region of the MSP domain of VAP-B, the biological significance of VAP-C in the propagation of HCV has not yet been clarified. In this study, we examined the expression of VAP-C in human tissues and the effects of VAP-C expression on the RNA replication, translation, and particle formation of HCV.

MATERIALS AND METHODS

Cell lines. Cells of the human hepatoma cell line Huh-7, cell line Huh7OK1, and embryonic kidney cell line 293T were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) containing 10% fetal calf

serum (FCS) and nonessential amino acids (NEAA), while Huh 9-13 cells, which possess a subgenomic HCV RNA replicon of genotype 1b (21), were cultured in DMEM supplemented with 10% FCS, NEAA, and 1 mg/ml G418. The Huh7OK1 cell line exhibits the highest efficiency of propagation of strain JFH1 virus, as described previously (35). All cell lines were cultured at 37°C in a humidified atmosphere with 5% CO₂.

Antibodies. Chicken anti-human VAP-B antibody was described previously (9). Rabbit anti-human VAP-C antibody was prepared by immunization using synthetic peptides of the amino acid residues from 86 to 98, QPHFSISP_NW EGR, which region does not share the homology to VAP-A and VAP-B. The mouse monoclonal antibody to human VAP-A was purchased from BD Pharmingen (San Diego, CA). Mouse monoclonal antibodies to influenza virus hemagglutinin (HA) and the GluGlu (EE) tag were from Covance (Richmond, CA). Mouse and rabbit anti-FLAG antibodies and mouse anti- β -actin monoclonal antibody were from Sigma. Rabbit polyclonal antibody to NS5A was prepared as described previously (34). Mouse anti-NS5A monoclonal antibody was from Austral Biologicals (San Ramon, CA).

Plasmids. A cDNA clone encoding NS5A was amplified from HCV genotype 1b strain J1 (9) (GenBank database accession number D89815) by PCR, using *Pfu* turbo DNA polymerase (Stratagene, La Jolla, CA). The fragments were then cloned into the appropriate sites in pEF-FLAG pGBK puro (13). The DNA fragment encoding NSSB of the J1 strain was generated by PCR and cloned into pCAGGS-PUR (31). The DNA fragment encoding human VAP-A was amplified by PCR from a human fetal-brain library (Clontech, Palo Alto, CA) and was introduced into pEF-FLAG pGBK puro and pEF-EE hygro (13), as described previously (9). A DNA fragment encoding VAP-C was amplified from cDNA of hepatoma cell line Huh-7 and was introduced into pEF-FLAG pGBK puro. Pro⁵⁶-to-Ser (P56S) mutants of VAPs were generated by site-directed mutagen-

esis (11). All PCR products were confirmed by sequencing with an ABI Prism 3130 genetic analyzer (Applied Biosystems, Tokyo, Japan).

Transfection, immunoblotting, and immunoprecipitation. Cells were seeded onto a six-well tissue culture plate 24 h before transfection. The plasmids were transfected into cells by liposome-mediated transfection using TransIT LT1 (Mirus Bio, Madison, WI). These transfected cells were harvested at 36 h posttransfection, washed three times with 1 ml of ice-cold phosphate-buffered saline (PBS), and suspended in 0.2 ml lysis buffer (20 mM Tris-HCl, pH 7.4, containing 135 mM NaCl and 1% Triton X-100) supplemented with protease inhibitor cocktail (Roche, Indianapolis, IN). The cell lysates were sonicated at 4°C for 5 min, incubated for 30 min at 4°C, and centrifuged at 15,000 rpm for 30 min at 4°C. The supernatant was subjected to immunoprecipitation analyses as described previously (27). The immunoprecipitated proteins were boiled in 30 µl of loading buffer and then subjected to sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and then reacted with primary antibody and secondary horseradish peroxidase-conjugated antibody. The immunocomplexes were visualized with Super Signal West Femto substrate (Pierce, Rockford, IL) and detected by using an LAS-3000 image analyzer (Fujifilm, Tokyo, Japan). The distribution of VAPs in human organs was determined by using premade human tissue lysates (Protein medleys; Clontech), which are aliquots of various organ lysates prepared from samples from several people, and liver tissues obtained during surgery after approval of the ethical committee of Kyushu University Graduate School of Medicine.

Real-time PCR. The HCV genomic RNA was determined by the method described previously (40). Total RNA was prepared from cells by using an RNeasy mini kit (Qiagen, Tokyo, Japan). First-strand cDNA was synthesized using an RNA LA PCR kit (Takara Bio, Inc., Shiga, Japan) and random primers. Expression of the appropriate gene was estimated by using platinum SYBR green quantitative PCR SuperMix UDG (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Fluorescent signals were estimated by using an ABI Prism 7000 system (Applied Biosystems). The 5' untranslated region of HCV and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were amplified using primer pairs described previously (40). The amount of HCV genomic RNA was normalized with that of GAPDH mRNA.

Focus-forming assay. The viral RNA of the JFH1 strain was introduced into the Huh7OK1 cell line according to the method of Zhong et al. (51). The culture supernatant was collected at 7 days posttransfection and used as the infectious HCV particles. Huh7OK1 cells in DMEM containing 10% FCS were seeded at 5×10^4 cells per well into a 24-well plate 12 h before infection. The cells were infected with the JFH1 strain at a multiplicity of infection (MOI) of 0.05 and incubated at 37°C for 2 h. The medium was replaced with fresh DMEM containing 10% FCS and NEAA at 2 h postinfection. The cells were fixed with 4% paraformaldehyde at 96 h postinfection and permeabilized with PBS containing 0.2% Triton X-100. These fixed and permeabilized cells were stained with the anti-NS5A mouse monoclonal antibody and Alexa Fluor (AF) 488-conjugated antibody to mouse immunoglobulin G (Molecular Probes, Eugene, OR). Clusters of infected cells stained with the NS5A antibody were derived from a single infectious focus, and virus titers were represented as focus-forming units/ml.

Quantification of the HCV core protein by ELISA. The HCV core protein was quantified by using an Ortho HCV antigen enzyme-linked immunosorbent assay (ELISA) test (Ortho Clinical Diagnostics, Tokyo, Japan) according to the manufacturer's instructions. To determine the intracellular expression of core protein, Huh7OK1 cells were infected with the infectious HCV particles described above, lysed with the lysis buffer on ice, and applied to the ELISA after 100- to 10,000-fold dilution with PBS. Total protein was quantified by using a Micro BCA protein assay reagent kit (Pierce). The intracellular and extracellular levels of expression of the core protein were normalized by the total amount of protein.

Effect of the VAP expression on the cap-independent translational activity of the viral IRES. The cDNA fragment encoding a firefly luciferase was excised from a pGL3 basic plasmid (Promega, Madison, WI) and introduced into the downstream region of the *Renilla* luciferase gene of pRL-CMV (cytomegalovirus) (Promega). Then, the cDNA fragments encoding the internal ribosome entry site (IRES) of the HCV strains Con1 and JFH1 were introduced between the *Renilla* and firefly luciferase genes, and the resulting plasmids were designated pRL-CMV-HCVCon1 and pRL-CMV-HCVJFH1, respectively (see Fig. 4A). The IRES region of HCV was replaced with that of poliovirus (PV) or encephalomyocarditis virus (EMCV), and the plasmids designated pRL-CMV-PV and pRL-CMV-EMCV, respectively (see Fig. 4B). Each reporter plasmid was introduced into Huh7OK1 cells that had been transfected with the expression plasmid encoding FLAG-green fluorescent protein (GFP), FLAG-VAP-A, FLAG-VAP-B, or FLAG-VAP-C 24 h previously, and cells were harvested at 48 h posttransfection. Luciferase activities in cells were measured by

using a dual-luciferase reporter assay system (Promega). The activity of firefly luciferase was normalized with that of *Renilla* luciferase and represented as relative luciferase activity (RLU).

Indirect immunofluorescence assay. The Huh 9-13 cells were cultured on glass slides and transfected with the expression plasmids encoding FLAG-tagged VAPs, P56S VAP mutants, or empty vector. The resulting cells were fixed at 72 h posttransfection with 4% paraformaldehyde in PBS at room temperature for 30 min. After being washed twice with PBS, cells were permeabilized for 20 min at room temperature with PBS containing 0.25% saponin and blocked with PBS containing 1% bovine serum albumin (BSA-PBS) for 60 min at room temperature. The cells were then incubated with BSA-PBS containing rabbit anti-FLAG and mouse anti-NS5A antibodies at 37°C for 60 min, washed three times with PBS containing 1% Tween 20 (PBS-T), and incubated with BSA-PBS containing AF 488-conjugated goat anti-rabbit immunoglobulin G and AF 594-conjugated goat anti-mouse antibodies at 37°C for 60 min. Finally, the cells were washed three times with PBS-T and observed with a FluoView FV1000 laser-scanning confocal microscope (Olympus, Tokyo, Japan).

RESULTS

VAP-C interacts with neither VAP-A nor VAP-B. The length of VAP-A was originally reported to be 242 amino acids but was recently corrected to 249 amino acids in the GenBank database due to the detection of 7 extra amino acids in the N terminus (Fig. 1A). VAP-C is a splicing variant of VAP-B that shares the N-terminal half of the MSP domain with VAP-B but lacks the coiled-coil motif and TM region (Fig. 1A). The region spanning residues 71 to 99 of VAP-C exhibits no homology to VAP-A and VAP-B, due to the frameshift. VAP-A and VAP-B form homo- or heterodimers via their TM domains, which is required for HCV replication (9, 44). To examine whether VAP-C is capable of interacting with VAP-A and VAP-B, FLAG-tagged VAP-A, -B, or -C was coexpressed with EE-tagged VAP-A or -B in 293T cells and was immunoprecipitated with the anti-FLAG antibody. Although EE-tagged VAP-A and VAP-B were coprecipitated with FLAG-tagged VAP-B and VAP-A, as reported previously, FLAG-VAP-C was precipitated with neither EE-VAP-A nor EE-VAP-B (Fig. 1B). These results indicate that VAP-C does not interact with VAP-A and VAP-B.

VAP-C binds to NS5B and interrupts the interaction of VAP-A and VAP-B with NS5B. VAP-A and VAP-B were identified as NS5A-binding proteins by yeast two-hybrid screening (9, 44). The coiled-coil domains of VAP-A and VAP-B were involved in the binding to NS5A, contributing to the efficiency of HCV replication (9, 44). However, VAP-C does not have the coiled-coil domain (Fig. 1A) and, therefore, VAP-C was expected not to interact with NS5A. To examine whether or not interaction between VAP-C and NS5A actually occurred, HA-tagged NS5A was coexpressed with FLAG-tagged VAP-A, -B, or -C in 293T cells and was immunoprecipitated with anti-HA antibody (Fig. 2). The results showed that the expression level of FLAG-VAP-C in the transfected cells was comparable to that of FLAG-VAP-A or FLAG-VAP-B (Fig. 2A, left). Although FLAG-tagged VAP-A and VAP-B were coprecipitated with HA-NS5A, no precipitation of FLAG-VAP-C with NS5A was detected (Fig. 2A, right), indicating that VAP-C does not interact with NS5A.

The RNA-dependent RNA polymerase NS5B was shown to interact with VAP-A through the MSP domain (44). The region spanning residues 1 to 70 of VAP-C is the same as the N-terminal-half region of the MSP domain of VAP-B and exhibits 77% homology to that of VAP-A (Fig. 1A). To exam-

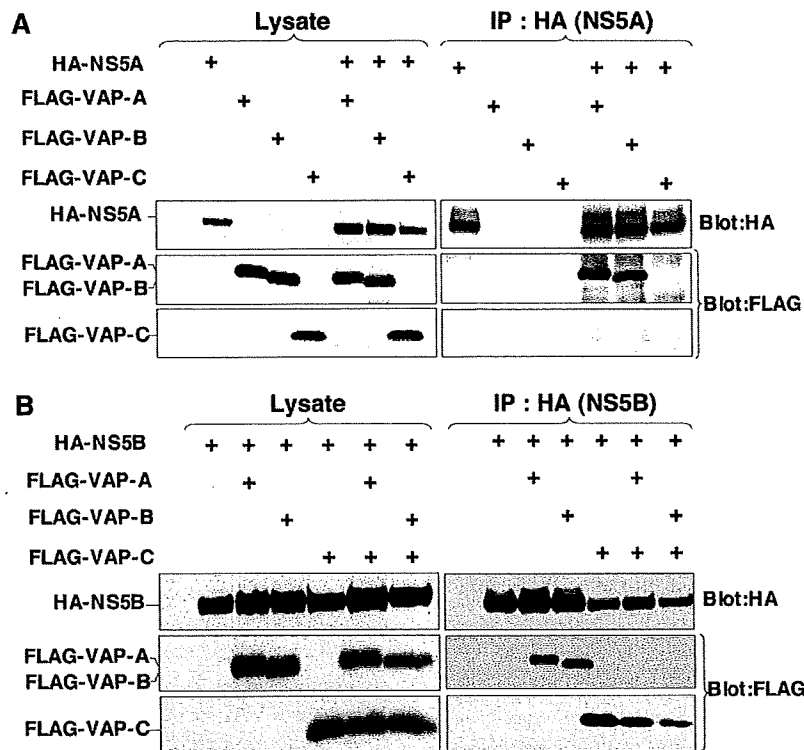


FIG. 2. VAP-C binds to NS5B but not NS5A and interrupts the interaction of VAP-A and VAP-B with NS5B. (A) The expression plasmids encoding NS5A or VAP proteins (1 μ g each) were transfected into 293T cells after adjusting the total amounts of DNA to 2.0 μ g with empty plasmid. HA-tagged NS5A was coexpressed with either FLAG-tagged VAP-A, VAP-B, or VAP-C in 293T cells and immunoprecipitated (IP) with anti-HA antibody, and the resulting precipitates were immunoblotted using anti-FLAG or anti-HA antibody. (B) The expression plasmids encoding NS5B or VAP proteins (1 μ g each) were transfected into 293T cells after adjusting the total amounts of DNA to 3.0 μ g with empty plasmid. HA-tagged NS5B was coexpressed with either FLAG-tagged VAP-A or VAP-B in the presence or absence of FLAG-tagged VAP-C in 293T cells and immunoprecipitated (IP) with anti-HA antibody, and the resulting precipitates were immunoblotted using anti-FLAG or anti-HA antibody. One percent of the lysate was used as an input control. The data in each panel are representative of the results of three independent experiments. +, present.

ine whether VAP-C is capable of interacting with NS5B, as are VAP-A and VAP-B, HA-NS5B was coexpressed with FLAG-VAP-A, FLAG-VAP-B, or FLAG-VAP-C in 293T cells and was immunoprecipitated with anti-HA antibody (Fig. 2B). Although substantial amounts of FLAG-tagged VAP-A, VAP-B, and VAP-C were coexpressed, and although all three were coprecipitated with HA-NS5B at comparable levels, the interaction of HA-NS5B with FLAG-tagged VAP-A or VAP-B was impaired by the coexpression of VAP-C, while FLAG-VAP-C was coprecipitated with HA-NS5B instead of FLAG-tagged VAP-A or VAP-B. These results suggest that VAP-C is capable of binding to NS5B and that the expression of VAP-C interrupts the interactions of NS5B with VAP-A and VAP-B.

Expression of VAP-C impairs the replication of HCV. VAP-A and VAP-B are known to support the replication of HCV RNA (2, 7). To examine the effect of VAP-C on the replication of HCV, FLAG-VAP-C was expressed in HCV replicon cells, Huh 9-13, in which a subgenomic HCV RNA of the genotype 1b strain Con1 was autonomously replicating. Huh 9-13 cells transfected with a plasmid encoding FLAG-VAP-C were harvested periodically up to 72 h posttransfection. The levels of replication of viral RNA and expression of NS5A were determined by real-time PCR and immunoblot-

ting, respectively (Fig. 3). The expression of VAP-C reduced the intracellular RNA of the subgenomic HCV replicon in accordance with the incubation period after transfection with the expression plasmid of FLAG-VAP-C; the empty plasmid did not reduce the intracellular RNA (Fig. 3A). The expression of NS5A was gradually decreased and was undetectable at 72 h posttransfection, in contrast to the increase of VAP-C expression (Fig. 3B).

Next, to determine the effects of VAP-C expression on the replication of HCV, Huh 9-13 cells were transfected with 0 to 4 μ g of the expression plasmid encoding VAP-A, VAP-B, or VAP-C and the replication of the subgenomic HCV RNA was determined at 48 h posttransfection. Although the HCV replicon cells transfected with 4 μ g of a plasmid encoding FLAG-VAP-B exhibited enhancement of the RNA replication, those transfected with an equivalent amount of plasmid encoding FLAG-VAP-A or empty vector showed a slight reduction of HCV RNA replication. In contrast, the replicon cells transfected with a plasmid encoding FLAG-VAP-C exhibited a clear reduction of the HCV RNA replication in a dose-dependent manner (Fig. 3C). The expression of FLAG-tagged VAP-A, VAP-B, or VAP-C in the replicon cells was increased in correspondence with the amount of the transfected plasmid

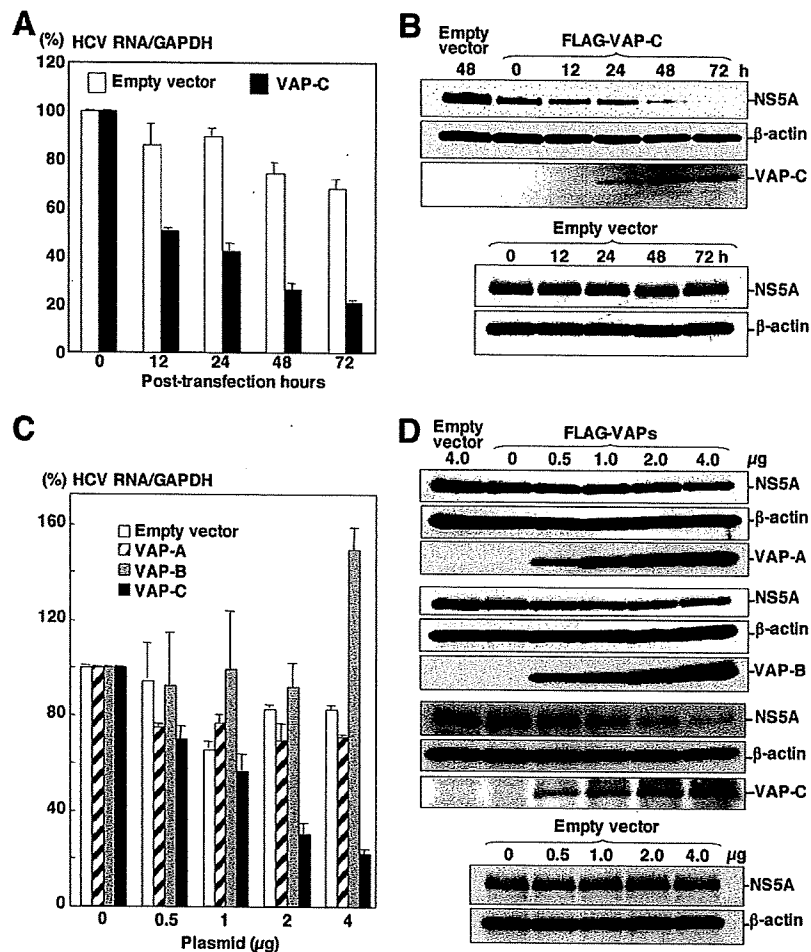


FIG. 3. Expression of VAP-C impairs the replication of HCV. (A) HCV replicon cells (Huh 9-13) were transfected with 4 µg of the expression plasmids encoding FLAG-tagged VAP-C or empty vector, and the level of intracellular HCV RNA was determined at 0, 12, 24, 48, or 72 h posttransfection by real-time PCR after normalization with GAPDH mRNA. The value of HCV RNA at 0 h posttransfection in the cell line transfected with the empty plasmid is represented as 100%. Data in this panel are shown as means ± standard deviations. (B) Huh 9-13 cells were transfected with 4 µg of the plasmid encoding FLAG-tagged VAP-C or empty plasmid, and the levels of expression of NS5A, β-actin, and VAP-C were determined at 0, 12, 24, 48, or 72 h posttransfection by immunoblotting using anti-NS5A, anti-β-actin, or anti-FLAG tag antibody. (C) Huh 9-13 cells were transfected with 0 to 4 µg of the plasmids encoding FLAG-tagged VAP-A, VAP-B, or VAP-C or empty vector, and the level of intracellular HCV RNA was determined at 72 h posttransfection as described for panel A. Data in this panel are shown as means ± standard deviations. (D) Huh 9-13 cells treated as described for panel C were harvested at 72 h posttransfection, and the levels of expression of NS5A, β-actin, VAP-A, VAP-B, and VAP-C were determined by immunoblotting. The data in each panel are representative of the results of three independent experiments.

(Fig. 3D), and the expression of NS5A was suppressed in accordance with the expression of FLAG-VAP-C, whereas the expression of FLAG-VAP-A and FLAG-VAP-B exhibited no effect on the expression of NS5A. These results suggest that the expression of VAP-C impairs the replication of HCV RNA.

VAP-C exhibits no effect on the IRES-dependent translation. The expression of VAP-C was shown to suppress the replication of the HCV RNA replication of the replicon cells. Next, to determine the effect of VAPs on the translation of HCV RNA, the reporter plasmid encoding the *Renilla* luciferase gene under the control of the CMV promoter and the firefly luciferase gene under the IRES of HCV, PV, or EMCV,

in that order, was prepared as shown in Fig. 4. These reporter plasmids were introduced into Huh7OK1 cells 24 h after transfection of the expression plasmids encoding VAP-A, VAP-B, or VAP-C and harvested at 48 h posttransfection, and then the RLU were determined. Although VAP-C exhibited a slight increase in the IRES-dependent translations of the HCV strains Con1 and JFH1, no significant effect of the expression of the VAPs on the HCV IRES-dependent translation was observed (Fig. 4A). Similarly, the expression of each of the VAPs in Huh7OK1 cells exhibited no significant effect on the IRES-dependent translation of PV or EMCV (Fig. 4B). These results indicate that the suppression of HCV RNA replication by the expression of

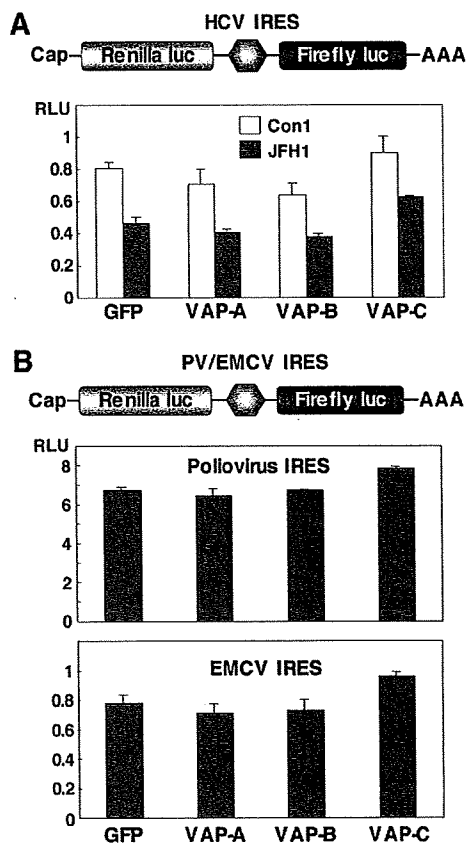


FIG. 4. VAP-C exhibits no effect on the viral IRES-dependent translation. (A) Top: structure of a reporter plasmid encoding the *Renilla* luciferase gene under the control of the CMV promoter and the firefly luciferase gene under the HCV IRES, in order. Bottom: the reporter plasmid was introduced into Huh7OK1 cells 24 h after transfection of the expression plasmids encoding VAP-A, VAP-B, or VAP-C, the cells harvested at 48 h posttransfection, and the RLU values determined after standardization with the expression of *Renilla* luciferase. (B) Top: structure of a reporter plasmid encoding the *Renilla* luciferase gene under the control of the CMV promoter and the firefly luciferase gene under the PV or EMCV IRES, in order. Bottom: each of the reporter plasmids was introduced into Huh7OK1 cells, and the RLU values were determined as described for panel A. Data in this figure are shown as the means \pm standard deviations.

VAP-C was not due to the suppression of the IRES-dependent translation of the viral RNA genome.

VAP-C impairs HCV propagation. To examine the effect of VAP expression on HCV propagation, Huh7OK1 cells transfected with the expression plasmids encoding VAP-A, VAP-B, or VAP-C were infected with JFH1 virus, and the levels of production of the viral RNA, core protein, and infectious particles were determined at 96 h postinfection. The production of intracellular and extracellular viral RNA was increased up to 10 to 30 times and 2 to 3 times, respectively, by the expression of VAP-A or VAP-B whereas it was clearly decreased in a dose-dependent manner by the expression of VAP-C (Fig. 5A). Although the extracellular core protein was increased from 0.6 to 2.6 nmol/liter by the expression of VAP-A or VAP-B, as seen in the production of viral RNA, the intracellular core protein showed only a marginal increase (40 to 65

nmol/liter) (Fig. 5A). Although the reason for the discrepancy between the intracellular production of viral RNA and core protein is not known at the moment, some mechanisms other than RNA translation might be involved, because VAP expression exhibited no effect on the HCV IRES-dependent translation, as shown in Fig. 4A. In contrast to the enhancement of core protein production by the expression of VAP-A or VAP-B, the expression of VAP-C significantly reduced both the intracellular and extracellular expression of the core protein (Fig. 5A). Furthermore, the production of infectious particles in the culture supernatants of Huh7OK1 cells infected with JFH1 virus was slightly enhanced by the expression of VAP-A or VAP-B, whereas it was suppressed by the expression of VAP-C (Fig. 5A). To further confirm the effects of VAPs on the expression of HCV proteins, Huh7OK1 cells transfected with various amounts of the expression plasmids of VAP-A, VAP-B, or VAP-C and infected with the JFH1 virus were examined by immunoblotting (Fig. 5B). Although the expression of VAP-A or VAP-B exhibited no effect on NS5A expression, VAP-C expression clearly decreased the expression of NS5A in a dose-dependent manner. These results clearly indicate that the expression of VAP-C negatively regulates HCV propagation. Overexpression of VAP-C did not affect the endogenous expression of VAP-A or VAP-B (Fig. 5C), suggesting that suppression of HCV propagation by VAP-C is not due to the reduction of VAP-A or VAP-B expression.

Lack of VAP-C expression in human livers. VAP-C consists of the first 70 amino acid residues of VAP-B and the subtype-specific 29 amino acid residues derived from frameshift (Fig. 1A). The VAP-C-specific antibody generated by immunization with the peptide corresponding to the residues from 86 to 98 clearly detected VAP-C but neither VAP-A nor VAP-B in cells transfected with expression plasmids encoding FLAG-tagged VAP-A, VAP-B, or VAP-C (Fig. 6A). To determine the distribution of VAPs in human organs, the pool lysates of various organs prepared from several people were examined by immunoblotting (Fig. 6B). Expression of VAP-A was detected clearly in the kidney, lung, prostate, and liver; slightly in the duodenum, uterus, vagina, and bladder; and barely in the small intestine and stomach. VAP-B was detected clearly in the bladder, kidney, and prostate and slightly in the duodenum, small intestine, uterus, vagina, and liver. Expression of VAP-C was detected clearly in the stomach, uterus, kidney, and bladder; slightly in the duodenum, small intestine, and prostate; and barely detected in the vagina, lung, and liver. Several bands smaller than the expected size of VAP-C were observed in the stomach, duodenum, small intestine, uterus, vagina, prostate, and bladder. Because the main target of HCV replication is thought to be the liver, we next examined the expression of VAPs in individual human liver samples. VAP-A and VAP-B were clearly detected in the liver tissues obtained from chronic hepatitis C patients and a healthy donor, but no expression of VAP-C was detected (Fig. 6C). These results suggest that the expression of VAP-C may participate in the determination of tissue tropism of HCV propagation.

Substitution of Ser for Pro⁵⁶ in VAPs leads to suppression of HCV replication. A single mutation of Pro⁵⁶ to Ser (P56S) of VAP-B has been reported to be highly associated with amyotrophic lateral sclerosis (ALS), and the P56S mutation of VAP-B but not of VAP-A has been shown to induce large

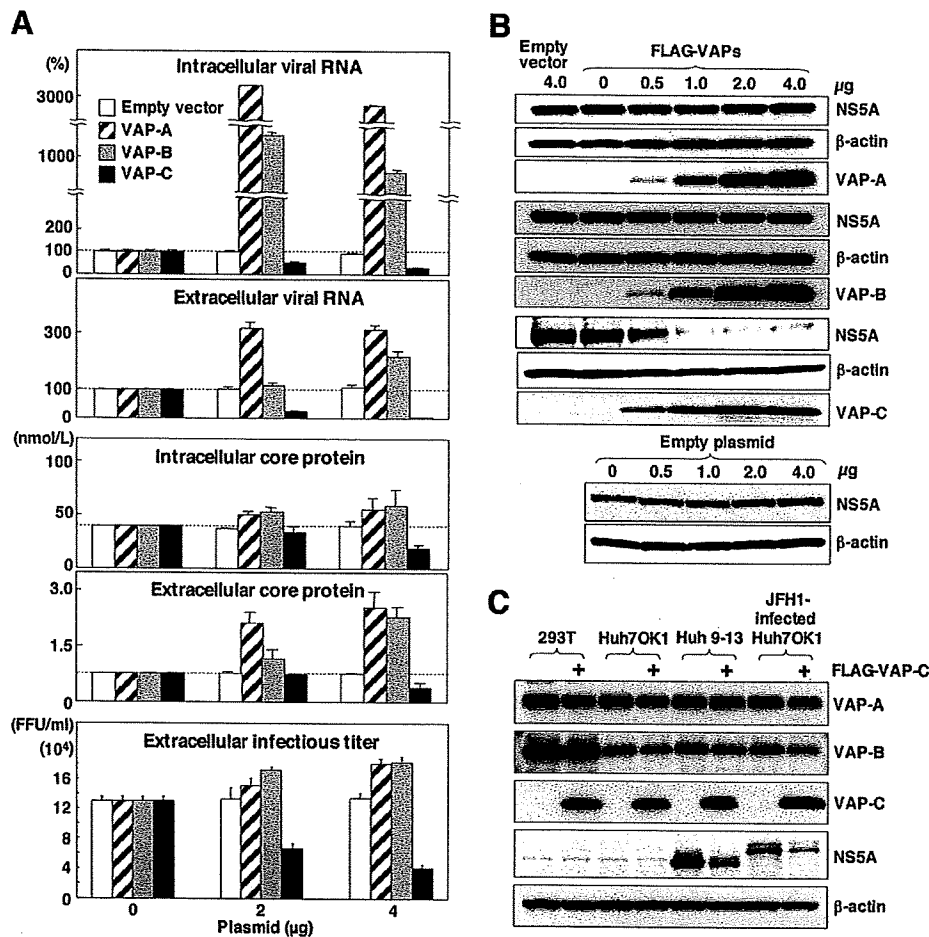


FIG. 5. VAP-C impairs HCV propagation but does not affect endogenous expression of VAP-A or VAP-B. Huh7OK1 cells transfected with 0 to 4 μ g of plasmid encoding the FLAG-tagged VAP-A, VAP-B, or VAP-C or empty vector were infected with strain JFH1 at an MOI of 0.05 at 14 h posttransfection and then harvested at 96 h postinfection. (A) The intracellular and extracellular expression levels of viral RNA (top) and core protein (middle) were determined by real-time PCR and ELISA, respectively. Infectious viral titers in the culture supernatants were determined by focus-forming assay (bottom). Data in this panel are shown as the means \pm standard deviations. (B) The expression levels of NS5A, β -actin, VAP-A, VAP-B, and VAP-C were determined by immunoblotting using anti-NS5A, anti- β -actin, or anti-FLAG tag antibody. (C) The embryonic kidney cell line (293T), the cured hepatoma cell line (Huh7OK1), and the replicon cell line (Huh 9-13) were transfected with 2 μ g of the plasmid encoding FLAG-tagged VAP-C (+) or empty plasmid. In the case of the infected cells, Huh7OK1 cells were infected with strain JFH1 at an MOI of 0.05, reseeded onto the tissue culture plate at 96 h postinfection, and then transfected with 2 μ g of the plasmids. These cells were harvested at 36 h posttransfection and examined by immunoblotting using antibodies to VAP-A, VAP-B, FLAG, NS5A, and β -actin. The data in each panel are representative of the results of three independent experiments.

aggregations of ER in culture cells and to sequester the wild-type protein into ubiquitinated inclusions (29, 37). To examine the effects on the replication of HCV of the P56S mutation in VAPs, FLAG-tagged VAP mutants were expressed in the HCV replicon cells. RNA replication of the subgenomic replicon in Huh 9-13 cells was impaired by the expression of each of the mutant VAPs (Fig. 7A, left). The expression of NS5A in the replicon cells was decreased by the expression of the mutant VAPs in a dose-dependent manner (Fig. 7A, right). Next, to examine the effect of the expression of the P56S VAP mutants on HCV propagation, Huh7OK1 cells expressing the FLAG-tagged VAP mutants were infected with JFH1 virus. The production of intracellular and extracellular viral RNA at 96 h postinfection was decreased by the expression of the P56S mutation in VAPs (Fig. 7B). Although the results of a previous

study indicated that the expression of the P56S mutant of VAP-B but not that of VAP-A induced a large aggregation of ER in hamster ovary cell line CHO (37), the P56S mutants of VAP-A and VAP-B but not that of VAP-C exhibited accumulation of membranous aggregates in Huh 9-13 cells (Fig. 7C). These results indicate that the P56S mutation in both VAP-B and VAP-A induces aggregation of ER in human hepatoma cells, which in turn leads to the suppression of HCV propagation.

DISCUSSION

The replication of HCV has been shown to require several host proteins, including VAP-A/VAP-B (6, 9, 44), FBL2 (46), FKBP8 (34), hB-ind1 (40), Hsp90 (28, 34, 45), and cyclophilins

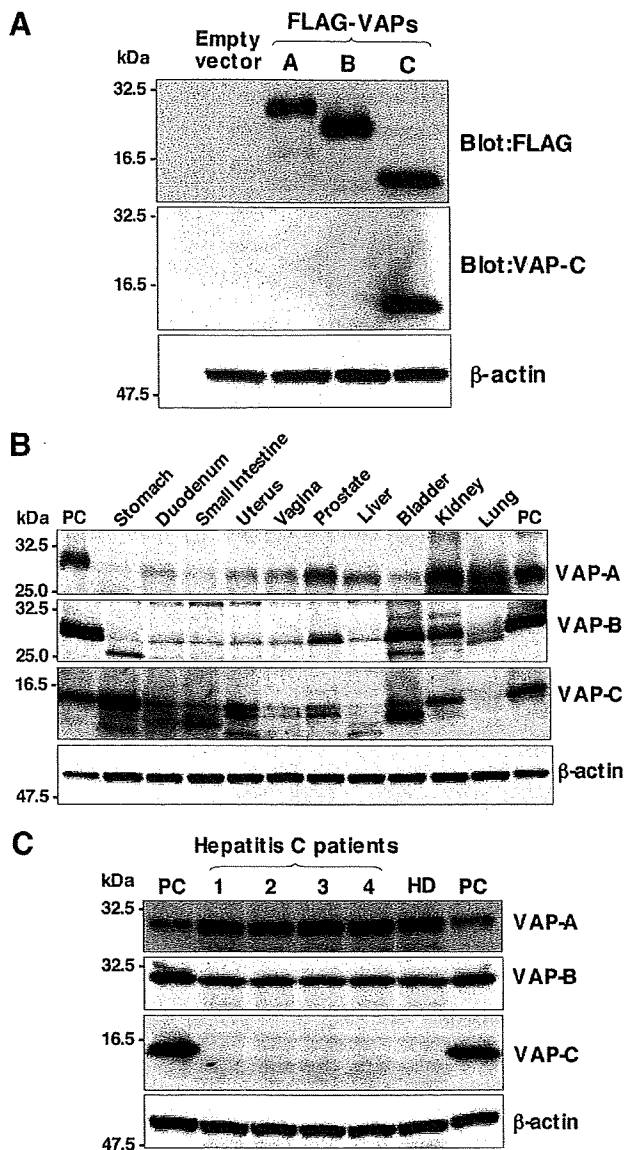


FIG. 6. Distribution of VAPs in human tissues. (A) Anti-VAP-C antibody specifically recognizes VAP-C. Human embryonic kidney 293T cells transfected with expression plasmid encoding FLAG-tagged VAP-A, VAP-B, or VAP-C or empty vector were harvested at 48 h posttransfection and examined by immunoblotting using anti-FLAG tag, anti-VAP-C, and anti- β -actin antibodies. (B) The premade human tissue lysates "Protein medleys" (20 μ g each; Clontech) were examined by immunoblotting using antibodies against VAP-A, VAP-B, VAP-C, or β -actin. (C) Expression of VAP family proteins in human liver tissues. Liver samples obtained from four hepatitis C patients (1 to 4) and one healthy donor (HD) were examined by immunoblotting as described above. The data in each panel are representative of the results of three independent experiments. PC indicates 293T cells transfected with expression plasmid encoding VAP-A, VAP-B, and VAP-C.

(15, 48). VAP-A has been detected in a detergent-resistant membrane fraction that was shown to be capable of replicating HCV RNA *in vitro*, and the interaction of VAP-A with NS5A is required for the efficient replication of HCV genomic RNA

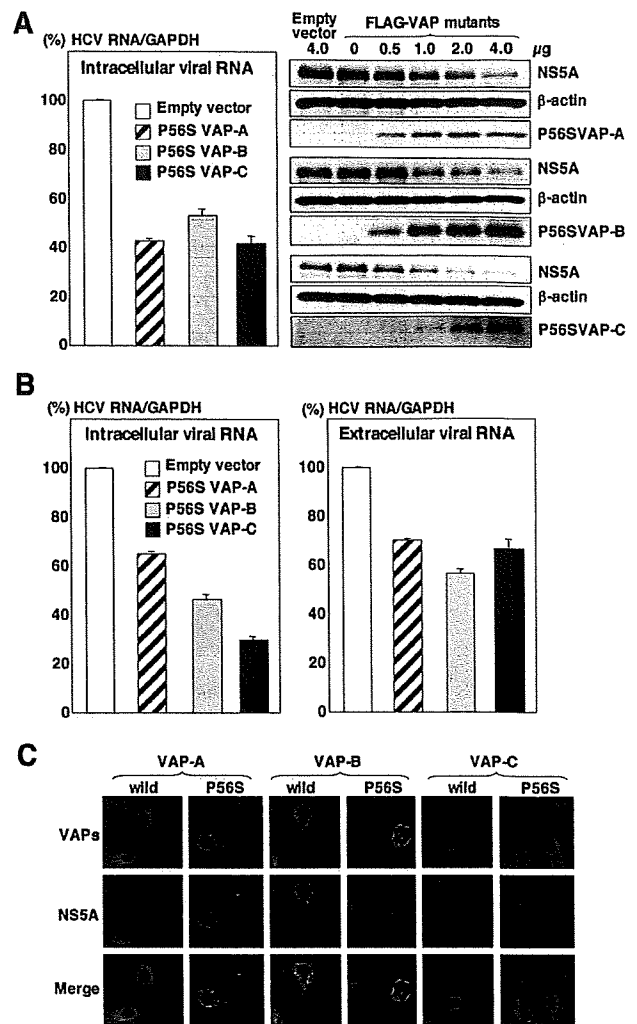


FIG. 7. Substitution of Ser for Pro⁵⁶ in VAPs leads to suppression of HCV replication. (A) Left: Huh 9-13 cells were transfected with 4 μ g of the expression plasmids encoding FLAG-tagged P56S VAP mutants or empty vector, and the level of intracellular HCV RNA was determined at 72 h posttransfection by real-time PCR after normalization with GAPDH mRNA. The value for HCV RNA at 0 h posttransfection in the cell line transfected with the empty plasmid is represented as 100%. Data in this panel are shown as the means \pm standard deviations. Right: Huh 9-13 cells were transfected with 0 to 4 μ g of the FLAG-tagged P56S VAP mutant plasmids or empty vector, and the levels of expression of NS5A, β -actin, and the mutant VAPs were determined by immunoblotting at 72 h posttransfection. The data in each panel are representative of the results of three independent experiments. (B) Huh7OK1 cells transfected with 4 μ g of the expression plasmids encoding FLAG-tagged P56S VAP mutants or empty vector were infected with strain JFH1 at an MOI of 0.05 at 14 h posttransfection, and the intracellular (left) and extracellular (right) expression levels of viral RNA were determined by real-time PCR after normalization with GAPDH mRNA at 96 h postinfection. Data in this panel are shown as the means \pm standard deviations. (C) Levels of expression of wild-type VAPs, P56S mutant VAPs, and NS5A in Huh 9-13 cells at 72 h after transfection with the expression plasmids encoding FLAG-tagged VAPs or P56S VAP mutants were determined by immunofluorescent assay. The data in each panel are representative of the results of three independent experiments.

(2, 7) and is modulated by the phosphorylation of NS5A (4, 6). VAP-B also participates in HCV replication through the formation of homo- and/or heterodimers with VAP-A (9). VAP-A and VAP-B form hetero- and homodimers through their TM regions and interact with NS5A and NS5B through the coiled-coil domain and MSP domain, respectively (9, 44). VAP-C is a splicing variant of VAP-B, consisting of the N-terminal half of VAP-B and the subtype-specific amino acid residues generated by the frameshift. However, the biological significance of VAP-C in the life cycle of HCV has not been determined. In this study, we have demonstrated that VAP-C is capable of binding to HCV NS5B but not to NS5A, VAP-A, and VAP-B due to the lack of the coiled-coil and TM regions. The expression of VAP-C inhibited the interaction of VAP-A and VAP-B with NS5B, impaired the RNA replication and particle formation of HCV, and was barely detected in human liver cells. These results suggest that VAP-C acts as a negative regulator for HCV propagation and is partly involved in the determination of the tissue specificity of HCV replication.

Overexpression of VAP-A but not of VAP-B inhibited the incorporation of the vesicular stomatitis virus (VSV) envelope glycoprotein G (VSV-G) into ER vesicles in CHO cells, resulting in impairment of membrane protein transport from the ER to the Golgi apparatus (37). VAP-B was shown to be involved in the unfolded protein response, which is an ER reaction to suppress the accumulation of misfolded proteins, and the expression of the P56S VAP-B mutant was suggested to nullify the unfolded protein response induced by VAP-B, to produce a large aggregation of ER, and to be involved in the development of ALS (17, 37). These data suggest that VAP-A and VAP-B possess different physiological functions; however, the contributions of the proteins to the life cycle of HCV have not been characterized. The expression of VAP-B but not of VAP-A resulted in an enhancement of the replication of the subgenomic HCV RNA of the genotype 1b strain Con1, whereas the expression of either VAP-A or VAP-B clearly enhanced viral RNA replication in cells infected with the genotype 2a strain JFH1 virus, suggesting that the contributions of VAP-A and VAP-B to viral RNA replication might differ among the genotypes of HCV. The expression of VAP-B or VAP-A enhanced RNA replication in the HCV replicon cells and the secretion of viral RNA, core protein, and infectious particles into the culture supernatants of Huh7OK1 cells infected with JFH1 virus, whereas the expression of these proteins had no effect on the expression of NS5A or on IRES-dependent translation. Thus, further studies will be needed to clarify the molecular mechanisms underlying the posttranslational enhancement of HCV production by the expression of VAP-A and VAP-B. In contrast to the expression of VAP-A and VAP-B, the expression of VAP-C clearly suppressed the RNA replication of both the genotype 1b RNA replicon cells and the genotype 2a strain JFH1 virus, by which both the expression of the viral proteins and the viral particle production were drastically impaired. Furthermore, the expression of the P56S mutants of VAP-A and VAP-B reduced RNA replication in HCV replicon cells and propagation of the JFH1 virus, probably due to the induction of aggregation of the ER. The reason why ER aggregation was induced by the expression of the P56S VAP-A mutant in Huh7 cells but not in CHO cells (17, 37) is not known at the moment.

The phosphorylation state of NS5A was suggested to control the interaction between VAP-A and NS5A and the replication efficiency of HCV RNA (6). Introduction of the adaptive mutations originally identified in the genotype 1b strain Con1 into NS5A of genotype 1a suppressed the hyperphosphorylation of NS5A, potentiated interaction with VAP-A, and enhanced the RNA replication (6). However, we have previously shown that NS5A of genotype 1a could bind to VAP-A and VAP-B at a level similar to that of genotype 1b despite the adaptive mutations (9). In this study, overexpression of each of the VAP proteins exhibited no effect on the mobility of NS5A in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 3 and 5), suggesting that there is no correlation between the VAP-dependent regulation of HCV propagation and the phosphorylation state of NS5A.

FKBP8 exhibits peptidyl prolyl *cis-trans* isomerase activity and interacts with NS5A and Hsp90 through the tetratricopeptide repeat (TPR) domain, and these interactions are suggested to be involved in the correct folding of the HCV replication complex (34). Treatment of cells with inhibitors of the ATPase activity of Hsp90, such as geldanamycin and its derivatives, impairs the RNA replication and particle production of HCV (28, 34, 45). The MSP domain of VAP-A was shown to interact with the TPR1 protein, which has a TPR domain and forms the chaperone complex with Hsp90 (22). Knockdown of the TPR1 protein or treatment with Hsp90 inhibitors in mammalian cells has been shown to inhibit the transport of VSV-G, leading to accumulation of the glycoprotein in the Golgi apparatus (22). The VAP-A- or VAP-B-induced enhancement of virus production might be attributable to the recruitment of Hsp90 into the replication complex through the interaction with the MSP domain.

VAP-A is well known to interact through the MSP domain with a number of mammalian and yeast proteins sharing the FFAT motif, including OSBPs, ORPs (20), and CERT (10, 19), and to be involved in the regulation of biosynthesis or trafficking of sterols and lipids. HCV replication and infection have been shown to be regulated by lipid components and to be capable of being inhibited by treatment with several inhibitors targeting lipid biosynthesis (14, 18). The intracellular membranous web structure observed in HCV replicon cells was shown to be resistant to detergent treatment, suggesting that the lipid raft-like structure abundant in cholesterol and sphingolipid is generated by the replication of HCV RNA (2, 24). Therefore, it might be feasible to speculate that VAP-A and VAP-B are involved in the construction of the HCV replication complex consisting of viral proteins and host cellular lipid components and that VAP-C interrupts the VAP-A and VAP-B functions and negatively regulates HCV propagation. Although the molecular mechanisms and the biological significance remain to be clarified, the MSP domain of VAP proteins was processed in human leukocytes and secreted into human serum (43). Further studies are needed to clarify the biogenesis and biological functions of the truncated VAP proteins in the replication of HCV.

In summary, we have shown that VAP-C is capable of suppressing the RNA replication and particle production of HCV by inhibiting the binding of VAP-A and VAP-B to NS5B through the N-terminal half of its MSP domain. The clear suppression of HCV propagation by the expression of VAP-C

further suggests the possibility of developing a novel therapeutic measure to eliminate HCV by the exogenous expression of VAP-C in the hepatocytes of chronic hepatitis C patients.

ACKNOWLEDGMENTS

We thank H. Murase for her secretarial work. We also thank R. Bartenschlager and T. Wakita for providing cell lines and plasmids.

This work was supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare; the Ministry of Education, Culture, Sports, Science, and Technology; the Global Center of Excellence Program; and the Foundation for Biomedical Research and Innovation.

REFERENCES

- Abe, T., Y. Kaname, I. Hamamoto, Y. Tsuda, X. Wen, S. Tagawa, K. Moriishi, O. Takeuchi, T. Kawai, T. Kanto, N. Hayashi, S. Akira, and Y. Matsuura. 2007. Hepatitis C Virus nonstructural protein 5A modulates Toll-like receptor-MyD88-dependent signaling pathway in the macrophage cell lines. *J. Virol.* 81:8953–8966.
- Aizaki, H., K. J. Lee, V. M. Sung, H. Ishiko, and M. M. Lai. 2004. Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts. *Virology* 324:450–461.
- Behrens, S. E., L. Tomei, and R. De Francesco. 1996. Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus. *EMBO J.* 15:12–22.
- Blight, K. J., A. A. Kolykhalov, and C. M. Rice. 2000. Efficient initiation of HCV RNA replication in cell culture. *Science* 290:1972–1974.
- Egger, D., B. Wolk, R. Gosert, L. Bianchi, H. E. Blum, D. Moradpour, and K. Bienz. 2002. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J. Virol.* 76:5974–5984.
- Evans, M. J., C. M. Rice, and S. P. Goff. 2004. Phosphorylation of hepatitis C virus nonstructural protein 5A modulates its protein interactions and viral RNA replication. *Proc. Natl. Acad. Sci. USA* 101:13038–13043.
- Gao, L., H. Aizaki, J.-W. He, and M. M. C. Lai. 2004. 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.* 78:3480–3488.
- Grakoui, A., D. W. McCourt, C. Wychowski, S. M. Feinstone, and C. M. Rice. 1993. Characterization of the hepatitis C virus-encoded serine proteinase: determination of proteinase-dependent polyprotein cleavage sites. *J. Virol.* 67:2832–2843.
- Hamamoto, I., Y. Nishimura, T. Okamoto, H. Aizaki, M. Liu, Y. Mori, T. Abe, T. Suzuki, M. M. Lai, T. Miyamura, K. Moriishi, and Y. Matsuura. 2005. Human VAP-B is involved in hepatitis C virus replication through interaction with NS5A and NS5B. *J. Virol.* 79:13473–13482.
- Hanada, K., K. Kumagai, S. Yasuda, Y. Miura, M. Kawano, M. Fukasawa, and M. Nishijima. 2003. Molecular machinery for non-vesicular trafficking of ceramide. *Nature* 426:803–809.
- Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease. 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 77:51–59.
- Hoofnagle, J. H. 2002. Course and outcome of hepatitis C. *Hepatology* 36:S21–S29.
- Huang, D. C., S. Cory, and A. Strasser. 1997. Bcl-2, Bcl-XL and adenovirus protein E1B19kD are functionally equivalent in their ability to inhibit cell death. *Oncogene* 14:405–414.
- Ikeda, M., K. Abe, M. Yamada, H. Dansako, K. Naka, and N. Kato. 2006. Different anti-HCV profiles of statins and their potential for combination therapy with interferon. *Hepatology* 44:117–125.
- Inoue, K., T. Umehara, U. T. Ruegg, F. Yasui, T. Watanabe, H. Yasuda, J. M. Dumont, P. Scalfaro, M. Yoshida, and M. Kohara. 2007. Evaluation of a cyclophilin inhibitor in hepatitis C virus-infected chimeric mice in vivo. *Hepatology* 45:921–928.
- Kaiser, S. E., J. H. Brickner, A. R. Reilein, T. D. Fenn, P. Walter, and A. T. Brunger. 2005. Structural basis of FFAT motif-mediated ER targeting. *Structure* 13:1035–1045.
- Kanekura, K., I. Nishimoto, S. Aiso, and M. Matsuoka. 2006. Characterization of amyotrophic lateral sclerosis-linked P56S mutation of vesicle-associated membrane protein-associated protein B (VAPB/ALS8). *J. Biol. Chem.* 281:30223–30233.
- Kapadia, S. B., and F. V. Chisari. 2005. Hepatitis C virus RNA replication is regulated by host geranylgeranylation and fatty acids. *Proc. Natl. Acad. Sci. USA* 102:2561–2566.
- Kawano, M., K. Kumagai, M. Nishijima, and K. Hanada. 2006. 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.* 281:30279–30288.
- Loewen, C. J., A. Roy, and T. P. Levine. 2003. A conserved ER targeting motif in three families of lipid binding proteins and in Opi1p binds VAP. *EMBO J.* 22:2025–2035.
- Lohmann, V., F. Korner, J. Koch, U. Herian, L. Theilmann, and R. Bartenschlager. 1999. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 285:110–113.
- Lotz, G. P., A. Brychzy, S. Heinz, and W. M. Obermann. 2008. A novel HSP90 chaperone complex regulates intracellular vesicle transport. *J. Cell Sci.* 121:717–723.
- McLauchlan, J., M. K. Lemberg, G. Hope, and B. Martoglio. 2002. Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. *EMBO J.* 21:3980–3988.
- Miyazari, Y., M. Hijikata, M. Yamaji, M. Hosaka, H. Takahashi, and K. Shimotohno. 2003. Hepatitis C virus non-structural proteins in the probable membranous compartment function in viral genome replication. *J. Biol. Chem.* 278:50301–50308.
- Moriishi, K., and Y. Matsuura. 2007. Host factors involved in the replication of hepatitis C virus. *Rev. Med. Virol.* 17:343–354.
- Moriishi, K., and Y. Matsuura. 2003. Mechanisms of hepatitis C virus infection. *Antivir. Chem. Chemother.* 14:285–297.
- Moriishi, K., T. Okabayashi, K. Nakai, K. Moriya, K. Koike, S. Murata, T. Chiba, K. Tanaka, R. Suzuki, T. Suzuki, T. Miyamura, and Y. Matsuura. 2003. Proteasome activator PA28gamma-dependent nuclear retention and degradation of hepatitis C virus core protein. *J. Virol.* 77:10237–10249.
- Nakagawa, S., T. Umehara, C. Matsuda, S. Kuge, M. Sudoh, and M. Kohara. 2007. Hsp90 inhibitors suppress HCV replication in replicon cells and humanized liver mice. *Biochem. Biophys. Res. Commun.* 353:882–888.
- Nishimura, A. L., M. Mitne-Neto, H. C. Silva, A. Richieri-Costa, S. Middleton, D. Cascio, F. Kok, J. R. Oliveira, T. Gillingwater, J. Webb, P. Skehel, and M. Zatz. 2004. A mutation in the vesicle-trafficking protein VAPB causes late-onset spinal muscular atrophy and amyotrophic lateral sclerosis. *Am. J. Hum. Genet.* 75:822–831.
- Nishimura, Y., M. Hayashi, H. Inada, and T. Tanaka. 1999. Molecular cloning and characterization of mammalian homologues of vesicle-associated membrane protein-associated (VAMP-associated) proteins. *Biochem. Biophys. Res. Commun.* 254:21–26.
- Niwa, H., K. Yamamura, and J. Miyazaki. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* 108:193–199.
- Okamoto, K., Y. Mori, Y. Komoda, T. Okamoto, M. Okochi, M. Takeda, T. Suzuki, K. Moriishi, and Y. Matsuura. 2008. Intramembrane processing by signal peptide peptidase regulates the membrane localization of hepatitis C virus core protein and viral propagation. *J. Virol.* 82:8349–8361.
- Okamoto, K., K. Moriishi, T. Miyamura, and Y. Matsuura. 2004. Intramembrane proteolysis and endoplasmic reticulum retention of hepatitis C virus core protein. *J. Virol.* 78:6370–6380.
- Okamoto, T., Y. Nishimura, T. Ichimura, K. Suzuki, T. Miyamura, T. Suzuki, K. Moriishi, and Y. Matsuura. 2006. Hepatitis C virus RNA replication is regulated by FKBP8 and Hsp90. *EMBO J.* 25:5015–5025.
- Okamoto, T., H. Omori, Y. Kaname, T. Abe, Y. Nishimura, T. Suzuki, T. Miyamura, T. Yoshimori, K. Moriishi, and Y. Matsuura. 2008. A single-amino-acid mutation in hepatitis C virus NS5A disrupting FKBP8 interaction impairs viral replication. *J. Virol.* 82:3480–3489.
- Pennetta, G., P. R. Hiesinger, R. Fabian-Fine, I. A. Meinertzhagen, and H. J. Bellen. 2002. *Drosophila* VAP-33A directs bouton formation at neuromuscular junctions in a dosage-dependent manner. *Neuron* 35:291–306.
- Prosser, D. C., D. Tran, P. Y. Gougeon, C. Verly, and J. K. Ngsee. 2008. FFAT rescues VAPA-mediated inhibition of ER-to-Golgi transport and VAPB-mediated ER aggregation. *J. Cell Sci.* 121:3052–3061.
- Skehel, P. A., R. Fabian-Fine, and E. R. Kandel. 2000. Mouse VAP33 is associated with the endoplasmic reticulum and microtubules. *Proc. Natl. Acad. Sci. USA* 97:1101–1106.
- Skehel, P. A., K. C. Martin, E. R. Kandel, and D. Bartsch. 1995. A VAMP-binding protein from *Aplysia* required for neurotransmitter release. *Science* 269:1580–1583.
- Tagawa, S., T. Okamoto, T. Abe, Y. Mori, T. Suzuki, K. Moriishi, and Y. Matsuura. 2008. Human butyrate-induced transcript 1 interacts with hepatitis C virus NS5A and regulates viral replication. *J. Virol.* 82:2631–2641.
- Tellinghuisen, T. L., J. Marcotrigiano, and C. M. Rice. 2005. Structure of the zinc-binding domain of an essential component of the hepatitis C virus replicase. *Nature* 435:374–379.
- Tomei, L., C. Failla, E. Santolini, R. De Francesco, and N. La Monica. 1993. NS3 is a serine protease required for processing of hepatitis C virus polyprotein. *J. Virol.* 67:4017–4026.
- Tsuda, H., S. M. Han, Y. Yang, C. Tong, Y. Q. Lin, K. Mohan, C. Haueter, A. Zoghbi, Y. Harati, J. Kwan, M. A. Miller, and H. J. Bellen. 2008. The amyotrophic lateral sclerosis 8 protein VAPB is cleaved, secreted, and acts as a ligand for Eph receptors. *Cell* 133:963–977.
- Tu, H., L. Gao, S. T. Shi, D. R. Taylor, T. Yang, A. K. Mircheff, Y. Wen, A. E. Gorbalenaya, S. B. Hwang, and M. M. Lai. 1999. Hepatitis C virus RNA polymerase and NS5A complex with a SNARE-like protein. *Virology* 263:30–41.

45. Ujino, S., S. Yamaguchi, K. Shimotohno, and H. Takaku. 2009. Heat-shock protein 90 is essential for stabilization of the hepatitis C virus non-structural protein NS3. *J. Biol. Chem.* 284:6841–6846.
46. Wang, C., M. Gale, Jr., B. C. Keller, H. Huang, M. S. Brown, J. L. Goldstein, and J. Ye. 2005. Identification of FBL2 as a geranylgeranylated cellular protein required for hepatitis C virus RNA replication. *Mol. Cell* 18:425–434.
47. Wasley, A., and M. J. Alter. 2000. Epidemiology of hepatitis C: geographic differences and temporal trends. *Semin. Liver Dis.* 20:1–16.
48. Watashi, K., N. Ishii, M. Hijikata, D. Inoue, T. Murata, Y. Miyanari, and K. Shimotohno. 2005. Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. *Mol. Cell* 19:111–122.
49. Weir, M. L., A. Klip, and W. S. Trimble. 1998. 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.* 333:247–251.
50. Weir, M. L., H. Xie, A. Klip, and W. S. Trimble. 2001. VAP-A binds promiscuously to both v- and tSNAREs. *Biochem. Biophys. Res. Commun.* 286:616–621.
51. Zhong, J., P. Gastaminza, G. Cheng, S. Kapadia, T. Kato, D. R. Burton, S. F. Wieland, S. L. Uprichard, T. Wakita, and F. V. Chisari. 2005. Robust hepatitis C virus infection in vitro. *Proc. Natl. Acad. Sci. USA* 102:9294–9299.

Enhanced ability of regulatory T cells in chronic hepatitis C patients with persistently normal alanine aminotransferase levels than those with active hepatitis

I. Itose,¹ T. Kanto,¹ N. Kakita,¹ S. Takebe,¹ M. Inoue,¹ K. Higashitani,¹ M. Miyazaki,¹ H. Miyatake,¹ M. Sakakibara,¹ N. Hiramatsu,¹ T. Takehara,¹ A. Kasahara² and N. Hayashi¹

¹Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, Suita, Japan; and ²Department of General Medicine, Osaka University Hospital, Suita, Japan

Received December 2008; accepted for publication February 2009

SUMMARY. In hepatitis C virus (HCV) infection, the Th1-type immune response is involved in liver injury. A predominance of immunosuppressive regulatory T cells (Treg) is hypothesized in patients with persistently normal alanine aminotransferase (PNALT). Our aim was to clarify the role of Treg in the pathogenesis of PNALT. Fifteen chronically HCV-infected patients with PNALT, 21 with elevated ALT (CH) and 19 healthy subjects (HS) were enrolled. We determined naturally-occurring Treg (N-Treg) as CD4+CD25^{high}+FOXP3+ T cells. The expression of FOXP3 and CTLA4 in CD4+CD25^{high}+ cells was quantified by real-time reverse transcriptase-polymerase chain reaction. Bulk or CD25-depleted CD4+ T cells cultured with HCV-NS5 loaded dendritic cells were assayed for their proliferation and

cytokine release. We examined CD127–CD25–FOXP3+ cells as distinct subsets other than CD25+ N-Treg. The frequencies of N-Treg in patients were significantly higher than those in HS. The FOXP3 and CTLA4 transcripts were higher in PNALT than those in CH. The depletion of CD25+ cells enhanced HCV-specific T cell responses, showing that co-existing CD25+ cells are suppressive. Such inhibitory capacity was more potent in PNALT. The frequency of CD4+CD127–CD25–FOXP3+ cells was higher in CH than those in PNALT. Treg are more abundant in HCV-infected patients, and their suppressor ability is more potent in patients with PNALT than in those with active hepatitis.

Keywords: HCV, PNALT, regulatory T cell.

INTRODUCTION

Hepatitis C virus (HCV) causes a wide range of chronic liver diseases in infected hosts, including chronic hepatitis (CH), liver cirrhosis and hepatocellular carcinoma (HCC).

Abbreviations: ALT, alanine aminotransferase; CH, chronic hepatitis; CTL, cytotoxic T lymphocyte; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HS, healthy subjects; IFN, interferon; IL, interleukin; IU, international units; MoDC, monocyte-derived dendritic cell; N-Treg, naturally occurring regulatory T cell; PNALT, persistently normal ALT; RT-PCR, reverse transcriptase-polymerase chain reaction; SLE, systemic lupus erythematosus; TGF, transforming growth factor; Treg, regulatory T cell.

Correspondence: Norio Hayashi, MD, PhD, Department of Gastroenterology and Hepatology, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita, Japan. E-mail: hayashin@gh.med.osaka-u.ac.jp

One of the critical determinants promoting the development of HCV-induced liver disease is sustained liver inflammation, explaining the therapeutic rationale of alleviating this condition to help prevent liver cancer [1]. Among chronically infected individuals, approximately 20–30% display persistently normal serum alanine aminotransferase levels [2,3]. Although it is reported that 40–50% of them progress to the active stage of liver inflammation within 5 years of observation [4], the incidence of HCC in the remaining patients continues to be lower than in those with elevated serum ALT levels [5]. Cumulative studies have revealed that HCV is not directly cytopathic to hepatocytes. It has been demonstrated that a Th1-type or cytotoxic T lymphocyte (CTL) response is critically involved in HCV-mediated liver injury [6,7]. Therefore, it is conceivable that some suppressor mechanisms exist against Th1-type immune responses in patients with persistently normal ALT levels (PNALT), which may be distinct from those in patients with active liver inflammation.

© 2009 The Authors.

Regulatory T cells (Treg) are a unique subset of T cells with inhibitory capacity against auto-reactive T cells [8]. Substantial data have been reported about the involvement of Treg in the pathogenesis of various diseases, including autoimmune, cancer or infectious diseases [9–13]. Currently, the existence of several types of Treg has been reported [14]. Naturally occurring Treg (N-Treg) are derived from the thymic stromal environment from progenitor cells and suppress auto-reactive T cells in antigen-specific and antigen-nonspecific manner. Forkhead/winged helix transcription factor (FOXP3) is one of the specific markers of N-Treg, the expression of which is well correlated with the gain of a suppressor function [15,16]. As cells with high expression of CD25 also display FOXP3, it is generally accepted that CD25+FOXP3+ is the most reliable marker for Treg. In HCV infection, several reports have described a higher frequency of N-Treg in the periphery and the liver [17–20], suggesting their active role in HCV persistence. It has also been demonstrated that CD25+FOXP3+ regulatory cells are inducible in the periphery [21]. Owing to the lack of a specific phenotypic marker of these induced regulatory cells, referred to as adaptive Treg, their role in the pathogenesis of HCV infection has not been clearly understood. A recent study has demonstrated that the expression of interleukin (IL)-7 receptor (CD127) is downregulated in Treg to a degree that is inversely correlated with FOXP3 expression [22]. These findings offer the possibility that adaptive Treg are traceable, not all but in part, by the combination of CD127 and FOXP3 independent of CD25 expression.

In this study, our aim was to elucidate whether or not Treg are involved in the pathogenesis of PNALT patients, by comparing the frequency and function of these cell subsets with those in active hepatitis patients or healthy subjects. A

distinct equilibrium was found between N-Treg and CD127–CD25–FOXP3+ T cells according to differences in liver inflammation.

MATERIALS AND METHODS

Subjects

Among chronically HCV-infected patients who had been followed at Osaka University Hospital, 15 patients with PNALT levels and 21 patients with elevated or fluctuating ALT levels (the CH group) were enrolled in this study. As controls, 19 healthy subjects (HS) who were negative for HCV and hepatitis B virus (HBV) markers were examined. The study protocol was approved by the ethical committee of Osaka University Graduate School of Medicine. At enrolment, written informed consent was obtained from each subject. In this study, PNALT patients were defined as those whose ALT levels remained within the normal range (<30 IU/mL) without any medications for more than 1 year. At enrolment, the patients were confirmed to be positive for both serum anti-HCV and HCV RNA, but were negative for other viral infections, including HBV and human immunodeficiency virus. The presence of other causes of liver disease, such as autoimmune, alcoholic and metabolic disorders was excluded by the use of laboratory and imaging analyses. Liver biopsy was carried out in some of the patients. Histological examination was performed according to the METAVIR scoring system. In all patients, a combination of repetitive biochemical tests, ultrasonography or computed tomography scans ruled out the presence of cirrhosis and liver tumours. The clinical background of the subjects are shown in Table 1.

Table 1 Baseline clinical characteristics of the patients

	Chronic hepatitis patients	Patients with PNALT	Healthy subjects*	
<i>n</i>	21	15	19	
Sex (M/F)	8/13	5/10	ND	NS
Age	50.6 ± 11.6	47.8 ± 12.7	ND	NS
ALT (IU/L)	88.3 ± 41.4	20.9 ± 6.9	ND	<i>P</i> < 0.0001 [†]
Plt (10 ⁴ /μL)	13.5 ± 5.4	20.0 ± 3.9	ND	<i>P</i> < 0.01 [†]
HCV RNA (Meq/mL)	8.6 ± 11.3	9.7 ± 7.8	ND	NS

*The background data of healthy subjects (blood donors) were not accessible owing to the confidentiality regulations of the blood centre, but their serum ALT levels were confirmed to be within the normal range. [†]Statistical significance was analysed by Mann–Whitney *U* test between chronic hepatitis patients and patients with PNALT. The values are expressed as mean ± SD. PNALT, persistently normal alanine aminotransferase level; ND, not determined; NS, not significant; plt, platelet count.

Frequency analyses of Treg cells

For the numerical analyses of Treg cells, heparinized venous blood was obtained from all subjects. Peripheral blood mononuclear cells were collected by density-gradient centrifugation on a Ficoll-Hypaque cushion. The cells were subsequently stained with a combination of various fluorescence-labelled anti-human mouse monoclonal antibodies for phenotypic markers. The antibodies for CD25 (clone B1.49.9) and CD4 (clone 13B8.2) were purchased from Beckman Coulter (Fullerton, CA, USA), that for CD127 (clone 40131) from R&D Systems (Minneapolis, MN, USA) and that for FOXP3-PE (clone PCH101) from eBioscience (San Diego, CA, USA), respectively. The cells were stained in phosphate-buffered saline containing 1% fetal bovine serum (FBS) with various antibodies or isotype controls for 15 min at room temperature. Intracellular staining of FOXP3 was performed using a human FOXP3 staining kit (eBioscience) according to the manufacturer's instructions. The cells were analysed by FACSCalibur (BD Biosciences, San Jose, CA, USA) and CellQuest software.

Functional analysis of CD4+CD25+ T cells in HCV-specific CD4+ T cell response

We first examined the HCV-specific CD4+ T cell response in the presence or absence of CD4+CD25+ T cells. Monocyte-derived dendritic cells (MoDC) were generated from CD14+ cells as reported previously. In brief, CD14+ cells were cultured in Iscove's modified Dulbecco's medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% FBS, 50 IU/mL of penicillin, 50 mg/mL of streptomycin, 2 mM of L-glutamine, 10 mM of HEPES buffer, 10 mM of nonessential amino acids in the presence of 50 ng/mL of granulocyte/macrophage colony-stimulating factor (PeproTech, Rocky Hill, NJ, USA) and 10 ng/mL of IL-4 (PeproTech) for 7 days at 37 °C and 5% CO₂. On day 6 of the culture, MoDC were pulsed with 10 µg/mL of recombinant HCV NS5 (amino acid position: NS5B 1-544; kindly provided by Japan Tobacco, Inc., Tokyo, Japan) and cultured for 24 h. The antigen-pulsed MoDC were then cultured with autologous bulk CD4+ T cells or CD4+CD25- T cells in 96-well flat-bottom plates (Corning, NY, USA) for 5 days. Enrichment of CD4+ T cells or CD4+CD25- T cells was performed using a CD4+CD25+ Regulatory T cell Isolation kit (Miltenyi Biotec, Auburn, CA, USA) according to the manufacturer's instructions. On day 6 of the co-culture, the cells were pulsed with 1 µCi of [3H]-thymidine during the last 16 h of incubation. The supernatants were collected before pulsing with [3H]-thymidine and subjected to cytokine enzyme-linked immunosorbent assay (ELISA). The incorporation of [3H]-thymidine in CD4+ T cells was measured using a β-counter (Wallac-Perkin-Elmer, Wallac, Finland).

Enzyme-linked immunosorbent assay

The concentrations of IL-10, TGF-β1 and interferon (IFN)-γ in the culture supernatants were determined by ELISA. We used matched pairs of relevant monoclonal antibodies (Endogen, Woburn, MA, USA) for IL-10 and IFN-γ, and the DuoSet ELISA development system (R&D Systems) for TGF-β1, according to the manufacturer's instructions. The detection thresholds of IL-10, TGF-β1 and IFN-γ were 10, 10 and 16 pg/mL, respectively.

Real time reverse transcriptase-polymerase chain reaction (RT-PCR)

In order to analyse the expression of FOXP3 and CTLA-4 in N-Treg, we collected CD4+CD25high T cells by using FACSARIA. The purity of the isolated cells was more than 95% as determined by FACS. Total RNA was extracted from sorted CD4+CD25high T cells using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Complementary DNA was synthesized using the SuperScript III First-Strand synthesis system (Invitrogen, Carlsbad, CA, USA). Assays-on-demand primers and probes (PE Applied Biosystems, Foster City, CA, USA) were used to quantify FOXP3 and CTLA4 expression. The mRNA levels were evaluated using ABI PRISM 7900 Sequence Detection System (Applied Biosystems). The thermal cycling conditions for all genes were as follows: the reaction was started with a 10-min denaturing cycle at 95 °C, followed by 40 cycles of PCR performed with 15 s of denaturing at 95 °C, then 1 minute at 60 °C for annealing and extension. We identified a calibrator sample from the healthy volunteers. The expressions of molecules were given as the relative values to the calibrator samples. To standardize the amount of total RNA added to each reaction mixture, we quantified β-actin mRNA from each sample as a control of internal RNA and corrected all values with this.

Statistical analysis

Statistical analyses were performed using StatView 5.0 software (SAS Institute Inc., Cary, NC, USA). Mann-Whitney *U*-test was used to compare differences in unpaired samples. For all analyses, a *P*-value of less than 0.05 was considered to be statistically significant.

RESULTS

Peripheral N-Treg are increased in HCV-infected patients

We compared the frequency of Treg between HCV-infected patients and healthy donors. In HCV-positive individuals, they were further categorized into PNALT and CH groups according to the difference in their serum ALT levels. The clinical backgrounds of these groups were not different except for

serum ALT levels and platelet counts (Table 1). N-Treg were defined as the cells with CD4+CD25^{high}+FOXP3+ cells. As the cut-off value between CD25^{high}+ and CD25^{intermediate}+ cells is a critical determinant for Treg analyses, we defined CD4+CD25^{high}+ as the cells with CD25 levels higher than those of CD4-CD25+ cells (Fig. 1a). We first compared the frequency of CD4+FOXP3+ T cells. The frequency of FOXP3+ cells in the CD4+ T cell population in HCV-infected patients was significantly higher than those in the HS (Fig. 1b). However, no difference was observed in FOXP3+ cells between the PNALT and CH patients (Fig. 1b). The frequency of CD4+CD25^{high}+FOXP3+ T cells in CH or PNALT patients were significantly higher than those in HS, whereas those in HCV-positive patients did not differ regardless of their ALT levels (Fig. 1c). Similar results were obtained for the frequency of CD4+CD25-FOXP3+ T cells (Fig. 1d).

Next, we examined whether or not the frequency of N-Treg is correlated with clinical parameters. Among all HCV-infected patients, no correlation was observed between the frequency of N-Treg (CD4+CD25^{high}+FOXP3+ T cells) and serum ALT, HCV RNA levels, age or platelet counts (data not shown). In the analyses of patients who had undergone liver biopsy, the frequency of N-Treg was not correlated with METAVIR grade/stage scores (data not shown).

The expressions of FOXP3 and CTLA4 are higher in N-Treg from PNALT patients compared with those from the CH group

FOXP3 is the master gene of Treg in the development and gaining of suppressor functions. Alternatively, CTLA4 is one of the key molecules of Treg in exerting inhibitory function. We thus evaluated FOXP3 and CTLA4 mRNA expression in sorted N-Treg (CD4+CD25^{high}+ T cells) by means of real-time RT-PCR. The expression of FOXP3 in PNALT or CH patients was significantly higher than those in HS (Fig. 2a). Of note is the higher expression of FOXP3 in N-Treg from the PNALT group than in those from the CH group (Fig. 2a). In contrast, the expression of CTLA4 in N-Treg from the PNALT was higher than those in the CH, while it did not differ between the CH and HS groups (Fig. 2b).

CD4+CD25+ T cells from PNALT patients have more suppressive capacity in the HCV-specific CD4+ T cell response than those from CH patients

In order to compare the ability of N-Treg to inhibit the antigen-specific CD4+ T cell response, we used autologous MoDC pulsed with HCV proteins as antigen-presenting cells. We examined CD4+ T cell proliferation or cytokine

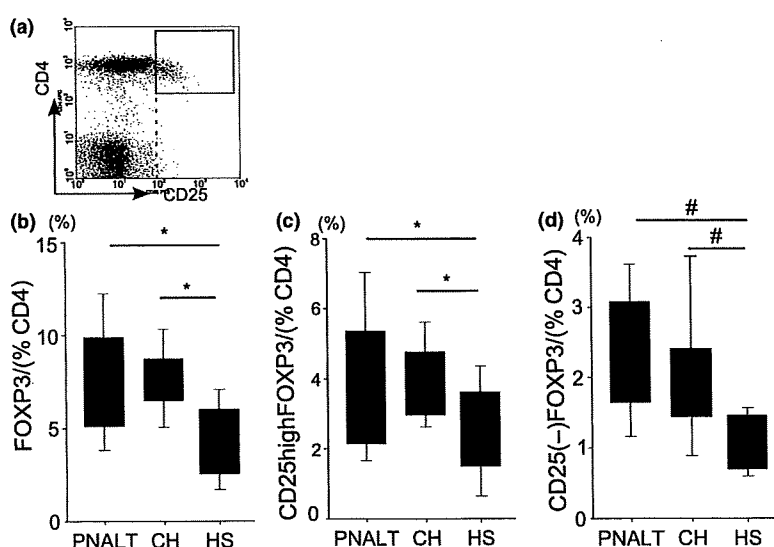


Fig. 1 Comparison of frequencies of naturally-occurring regulatory T cells (N-Treg) and FOXP3-positive cells among the groups. (a) Gating of CD4+CD25^{high}+ T cells under FACS analysis. The cut-off value of CD25^{high} expression is set at a level that is more than that of CD4-CD25+ cells (dotted line); CD4+CD25^{high}+ T cells are shown in the rectangle drawn in the representative dot plot. (b) Frequencies of FOXP3+ cells, (c) N-Treg (CD25^{high}+FOXP3+ cells) and (d) CD25-FOXP3+ cells in CD4+ T cells were compared among the groups. Boxes represent lower and upper quartiles with the median value (solid line) between boxes, while the whiskers represent the minimum and maximum values. *, $P < 0.05$; #, $P < 0.0001$ by Mann-Whitney *U*-test. Abbreviations: PNALT, hepatitis C virus (HCV)-infected patients with persistently normal alanine aminotransferase (ALT) levels; CH, HCV-infected patients with elevated ALT levels; HS, healthy subjects.

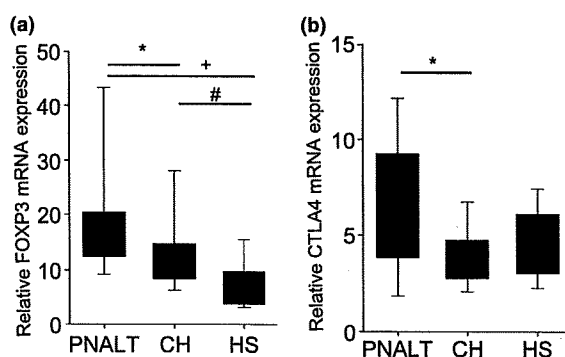


Fig. 2 Comparison of mRNA expression of FOXP3 and CTLA4 in CD4+CD25^{high}+ T cells among the groups. The expression of FOXP3 (a) and CTLA4 (b) in separated CD4+CD25^{high}+ T cells were analysed by real-time reverse transcriptase-polymerase chain reaction as described in Materials and methods. Boxes represent lower and upper quartiles with the median value (solid line) between boxes, while the whiskers represent the minimum and maximum values. *, $P < 0.05$; , $P < 0.01$; +, $P < 0.001$. For definitions of PNALT, CH and HS, see Fig. 1.

production stimulated with antigen-pulsed DC. We compared such responses between samples with or without CD4+CD25+ T cells. In PNALT patients, HCV NS5-specific T cell proliferation or IFN- γ production of CD25-depleted CD4+ T cells was significantly higher than those of the bulk CD4+ T cells (Fig. 3a,b). In contrast, in CH patients, such restoration did not occur significantly even when CD4+CD25+ T cells had been depleted (Fig. 3a,b). There was no difference in the production of IL-10 and TGF- β between bulk CD4+ T cells and CD25-depleted CD4+ T cells in both CH and PNALT patients (Fig. 3c,d). These results suggest that co-existing CD4+CD25+ T cells play an inhibitory role in the HCV-specific CD4+ T cell response, in which suppression was more potent in the PNALT than in the CH group.

CD127-FOXP3+ cells, regardless of their CD25 expression, are increased in patients with HCV infection

In the analyses of N-Treg, the frequency of CD4+CD25-FOXP3+ T cells in HCV-infected patients was higher than those in the healthy donors (Fig. 1d). These results suggest that CD4+FOXP3+ T cells, regardless of the degree of CD25

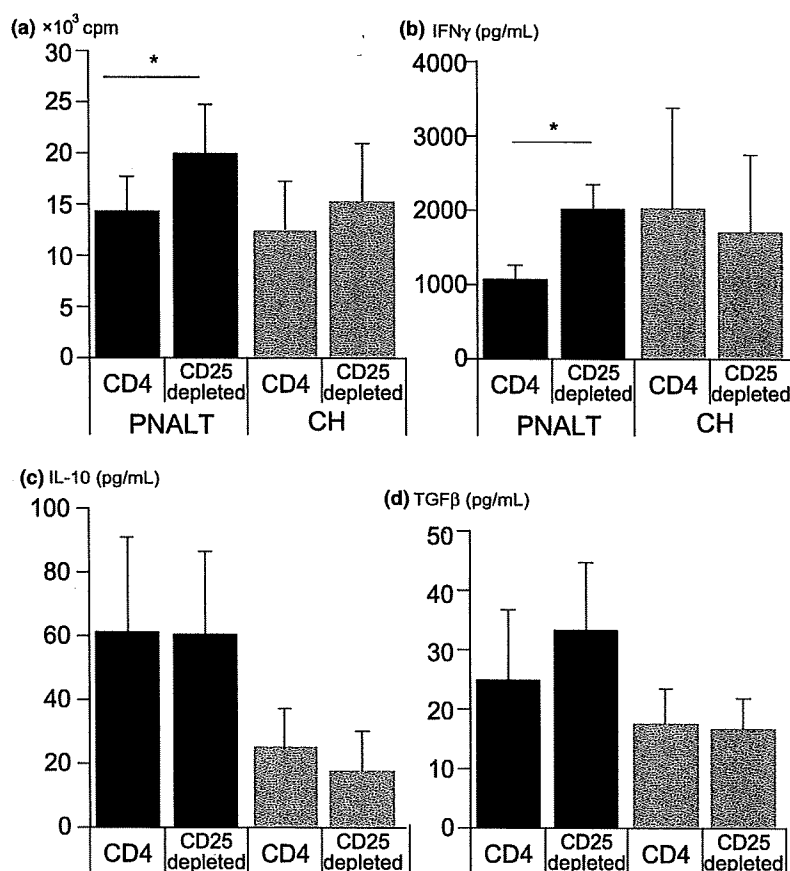


Fig. 3 Changes of hepatitis C virus (HCV)-specific CD4+ T cell responses with or without depletion of CD25+ T cells. Bulk CD4+ T cells or those depleted of CD25+ cells were cultured with autologous monocyte-derived dendritic cells in the presence of HCV-NS5 protein for 5 days as described in Materials and methods. (a) On day 4, [³H]-thymidine was pulsed and the thymidine incorporation was counted with a β -counter. Before the pulsing, the culture supernatants were harvested and subjected to enzyme-linked immunosorbent assay for interferon- γ (b), interleukin-10 (c) and TGF- β (d), respectively. *, $P < 0.05$ by Mann-Whitney U -test. For definitions of PNALT and CH, see Fig. 1.

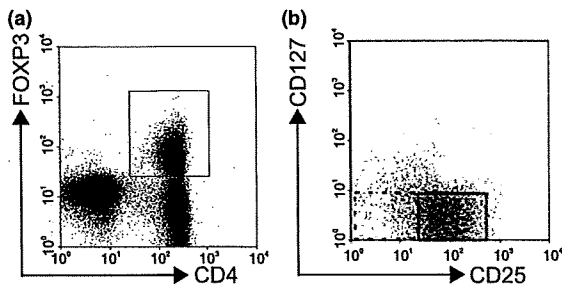


Fig. 4 Gating of CD4+CD127-FOXP3+ cells with variable CD25 expression under FACS analysis. After setting the gate on CD4+FOXP3+ cells [rectangle in the dot plot (a)], were displayed on the CD25 and CD127 axis (b). The presence of CD25+ (bold rectangle) and of CD25- cells (dotted rectangle) in CD4+FOXP3+ cells are shown in plot (b). The frequencies of these cells were analysed.

expression, increase in chronic HCV infection. Alternatively, it implies that higher expression of CD25 is not a universal marker for identifying FOXP3+ cells with regulatory activity. It has been reported that CD127 expression on CD4+ T cells is inversely correlated with FOXP3 expression, suggesting that CD127low/negative cells consist of those with regulatory activity. In order to analyse regulatory T cell subsets more precisely, we first examined FOXP3 expression on CD127- or CD127+ cells paired with CD25 expression in patients with HCV infection (Fig. 4). As a result, the majority of CD4+FOXP3+ T cells belonged to the CD127- population irrespective of CD25 expression (Fig. 4). Next, we compared the frequency of CD4+CD127-FOXP3+ cells, which consist

of CD25+ and CD25- cells, among the subject groups (Fig. 5a). The frequency of CD4+CD127-FOXP3+ cells was similar in the CH and the PNALT groups, both of which were significantly higher than those in the HS (Fig. 5a). Finally, in order to estimate the profile of CD4+CD127-FOXP3+ cells according to CD25 expression, we compared the percentage of CD25+CD127-FOXP3+ or CD25-CD127-FOXP3+ cells in CD4+ T cells among the groups. The percentage of CD25+CD127-FOXP3+ T cells in CD4+ T cells was comparable for PNALT and CH (Fig. 5b). In clear contrast, the percentage of CD25-CD127-FOXP3+ T cells in the PNALT was lower than those in the CH (Fig. 5c). The frequencies of these cells were higher in the HCV-infected patients than in HS (Fig. 5b,c). When we set the focus on the proportion of CD25+CD127- or CD25-CD127- cells in the FOXP3+ cells in the periphery as a whole, we found that the proportion of CD25+CD127- cells in the PNALT was higher than that in the CH group (Fig. 5d). On the other hand, the proportion of CD25-CD127- cells in FOXP3+ cells was lower in the PNALT than in the CH group (Fig. 5e). Therefore, the phenotypic profiles of FOXP3+ T cells are distinct between PNALT and CH patients, with regard to the expression of CD127 and CD25.

DISCUSSION

Approximately 30–40% of chronically HCV-infected patients continue to display PNALT for decades. We previously reported the possible contribution of certain human leukocyte antigen haplotypes [23] or DC dysfunction in the maintenance of the PNALT state [24]. However, the precise mechanisms behind this important issue are yet to be

Fig. 5 Comparison in the frequencies of CD127- regulatory T cell subsets among the groups. Frequencies of CD127-FOXP3+ (a), CD127-CD25+FOXP3 (b) and CD127-CD25-FOXP3+ (c) cells among CD4+ T cells were determined by FACS analysis. The proportion of CD127-CD25+ (d) or CD127-CD25- (e) cells in CD4+FOXP3+ cells were also determined. Boxes represent lower and upper quartiles with the median value (solid line) between boxes, while the whiskers represent the minimum and maximum values. *, $P < 0.05$; , $P < 0.01$; **, $P < 0.005$; ##, $P < 0.001$; +, $P < 0.0001$ by Mann-Whitney *U*-test. For definitions of PNALT, CH and HS, see Fig. 1.

