

incorporation into baculovirus particles is enhanced by C-terminal truncation of the cytoplasmic domain is not known, one possibility might be a change in SLAM localization. The gp64 protein localizes to the cell surface but is excluded from lipid raft microdomains (72). As VSVG and CD46 also do not associate with lipid rafts (35, 57), proteins localized to lipid raft microdomains may be excluded during virus assembly and budding. Although the cellular localization of SLAM is not known, further studies will be necessary to clarify the relationship between cell surface localization and incorporation into baculovirus particles and to test the involvement of lipid raft microdomains in this process.

Mangor et al. demonstrated that gp64-null baculoviruses pseudotyped with VSVG were not morphologically distinguishable from budded wild-type AcMNPV particles (34). We confirmed that Ac Δ 64/VSVG/CAIuc, as well as Ac Δ 64/CD46/CAIuc and Ac Δ 64/GFP/CAIuc, exhibited a morphology similar to that of AcMNPV. These results indicate that expression of gp64 is not required for the morphogenesis of a rod-shaped structure for budded AcMNPV particles. Immunogold labeling of Ac Δ 64/VSVG/CAIuc and Ac Δ 64/CD46/CAIuc revealed that the VSVG and CD46 proteins were incorporated into and distributed throughout the whole viral surface. These results are consistent with previous observations that VSVG fusion proteins were distributed throughout the stalk and head domains of baculovirus particles, in contrast to gp64, which was primarily localized in the head domain (11, 44).

In this study, we have demonstrated the capability for ligand-directed gene delivery by pseudotype baculoviruses in vitro. For future in vivo applications of baculovirus vectors for gene targeting to specific organs or virus-infected cells as a method of treatment of inherited or infectious diseases, it is imperative to exhaustively study the transcription of baculoviral genes in mammalian cells for certification of safety. In addition, further studies are needed to establish replication-competent cell lines capable of supporting the propagation of pseudotype viruses without the possibility of replication-competent virus breakthrough by incorporation of gp64 and to optimize the conditions necessary for the efficient incorporation of ligands into recombinant baculovirus particles.

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Nuclear Localization of Japanese Encephalitis Virus Core Protein Enhances Viral Replication†

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Japanese encephalitis virus (JEV) core protein was detected in both the nucleoli and cytoplasm of mammalian and insect cell lines infected with JEV or transfected with the expression plasmid of the core protein. Mutation analysis revealed that Gly⁴² and Pro⁴³ in the core protein are essential for the nuclear and nucleolar localization. A mutant M4243 virus in which both Gly⁴² and Pro⁴³ were replaced by Ala was recovered by plasmid-based reverse genetics. In C6/36 mosquito cells, the M4243 virus exhibited RNA replication and protein synthesis comparable to wild-type JEV, whereas propagation in Vero cells was impaired. The mutant core protein was detected in the cytoplasm but not in the nucleus of either C6/36 or Vero cell lines infected with the M4243 virus. The impaired propagation of M4243 in mammalian cells was recovered by the expression of wild-type core protein *in trans* but not by that of the mutant core protein. Although M4243 mutant virus exhibited a high level of neurovirulence comparable to wild-type JEV in spite of the approximately 100-fold-lower viral propagation after intracerebral inoculation to 3-week-old mice of strain Jcl:ICR, no virus was recovered from the brain after intraperitoneal inoculation of the mutant. These results indicate that nuclear localization of JEV core protein plays crucial roles not only in the replication in mammalian cells *in vitro* but also in the pathogenesis of encephalitis induced by JEV *in vivo*.

Japanese encephalitis virus (JEV) belongs to the genus *Flavivirus* within the family *Flaviviridae*. Members of the genus *Flavivirus* are predominantly arthropodborne viruses and frequently cause significant morbidity and mortality in mammals and birds (6). JEV is distributed in the south and southeast regions of Asia and kept in a zoonotic transmission cycle between pigs or birds and mosquitoes (6, 50, 57). JEV spreads to dead-end hosts, including humans, through the bite of JEV-infected mosquitoes and causes infection of the central nervous system, with a high mortality rate (6, 57). JEV has a single-stranded positive-strand RNA genome approximately 11 kb in length, which is capped at the 5' end but lacks modification of the 3' terminus by polyadenylation (34). The genomic RNA encodes a single large open reading frame, and a polyprotein translated from the genome is cleaved co- and posttranslationally by host and viral proteases to yield three structural proteins, the core, precursor membrane (prM), and envelope (E) proteins, and seven nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (53). Although the core protein has very little amino acid homology to other flaviviruses—for example, the core protein of JEV has only 25% homology to that of tick-borne encephalitis virus (TBEV)—the structural properties, such as the hydrophobicity profile, abundances of basic amino acid residues, and second-

ary structures, are very similar (11, 20, 36). The flavivirus core proteins commonly contain two hydrophobic sequences in the center and a carboxyl-terminal end, and the carboxyl-terminal hydrophobic region serves as a signal sequence of prM. The signal-anchor sequence is cleaved off by the viral protease NS2B-3, and this cleavage is required for the subsequent liberation of the amino terminus of prM by the host signal peptidase (35, 52, 63). The mature core protein, released from the endoplasmic reticulum (ER) membrane, is believed to bind to the genomic RNA via the basic amino acid clusters at the amino and carboxyl termini and forms nucleocapsids (23). The central hydrophobic region of the core protein may be associated with the ER membrane, and this interaction is believed to facilitate the assembly of nucleocapsid and two membrane proteins, prM and E, and to bud into the ER lumen as virions (39). The removal of the central hydrophobic region of the TBEV core protein increased the production of the subviral particles that consist of (pr)M and E proteins but that lack a core protein and genomic RNA (26, 27).

In addition to their role as structural proteins, core proteins of dengue virus (DEN) and Kunjin virus (KUN) are localized not only in the cytoplasm but also in the nucleus, especially in the nucleoli of several infected cell lines (4, 38, 55, 59, 61). Transport from the cytoplasm to the nucleus occurs through nuclear pore complexes that penetrate the double lipid layers of the nuclear envelope. Small molecules up to 9 nm in diameter (<50 kDa) can freely diffuse through the nuclear pore complexes, while most macromolecules require an active transport process via nuclear import receptor proteins such as importin- α (37). In general, cargo proteins contain mono- or bi-

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partite cluster sequences of basic amino acids termed nuclear localization signals (NLSs) to bind to nuclear import receptor proteins (5, 21). As flavivirus core proteins are relatively small (approximately 14 kDa), they may diffuse into the nucleus. However, the successful translocation of DEN core protein fused with three copies of green fluorescent protein (GFP) (96 kDa in total) into the nucleus indicates that the DEN core protein is actively translocated into the nucleus by an energy-dependent pathway, and an NLS was assigned to the region of carboxyl-terminal residues from amino acids 85 to 100 (59). Despite the many studies investigating this matter, the biological significance of the nuclear localization of core proteins in the virus replication cycle remains unclear.

In this study, we showed that the JEV core protein is also localized in both the cytoplasm and the nucleus, particularly in the nucleolus, of mammalian and mosquito cell lines and determined that an NLS is present in the core protein. We generated a mutant JEV, replaced the NLS in the core protein with Ala, and confirmed the elimination of the nuclear localization of the mutant core protein in both mammalian and mosquito cells. The characterization of the mutant JEV indicates that the nuclear localization of the core protein plays important roles in the viral replication in mammalian cells and in the pathogenesis of encephalitis in vivo. Finally, we discuss the biological significance of the nuclear localization of the JEV core protein.

MATERIALS AND METHODS

Cells. The mammalian cell lines Vero (African green monkey kidney), 293T (human kidney), BHK (hamster kidney), HeLa (human cervix cancer), HepG2 (human hepatoma), SK-N-SH (human neuroblastoma), and N18 (mouse neuroblastoma) were maintained in Dulbecco's modified Eagle's minimal essential medium (D-MEM) supplemented with 10% fetal bovine serum (FBS). A mosquito cell line, C6/36 (*Aedes albopictus*), was grown in Eagle's minimal essential medium supplemented with 10% FBS.

Plasmids. The mammalian expression vector pEGFP-C3 was purchased from Clontech (Palo Alto, Calif.). The plasmid pEGFP-JEVC105 was constructed by insertion of cDNA encoding the mature form of the JEV core protein without the C-terminal signal sequence (amino acids 2 to 105 of the AT31 strain) amplified by PCR into pEGFP-C3 as described previously (42). All of the expression vectors coding the enhanced GFP (EGFP)-fused mutant JEV core proteins were constructed based on pEGFP-JEVC105. Briefly, the gene encoding the JEV core protein with amino acids 38 to 44 deleted was amplified by splicing the overlapping extension (16, 17). For alanine scanning in putative NLS regions (amino acids 38 to 44 and 85 to 105), a series of point mutants of the JEV core protein were synthesized by PCR-based mutagenesis (14). All of the mutant genes were cloned into EcoRI and BamHI sites of pEGFP-C3. The plasmid that has a full-length cDNA of the JEV AT31 strain under the control of a T7 promoter was constructed and designated pMWJEATG1 (Z. Zhao, T. Date, Y. Li, T. Kato, M. Miyamoto, K. Yasui, and T. Wakita, submitted for publication). Guanine-to-cytosine and cytosine-to-guanine point mutations were introduced into pMWJEATG1 at nucleotides 220 and 222 of the JEV gene, respectively, by PCR-based mutagenesis to change Gly⁴² and Pro⁴³ of the core protein to Ala. The constructed plasmid was designated pMWJEAT/GP4243AA. For the mutant viral replication complementation experiments, the genes coding the C-terminal hemagglutinin (HA)-tagged core proteins derived from pMWJEATG1 and pMWJEAT/GP4243AA were cloned into pCAG-GS vector (43), and the resulting plasmids were designated pCAG-WC-HA and pCAG-MC-HA, respectively.

Antibodies. cDNA encoding the JEV core protein (amino acids 2 to 105) was inserted into pGEX-2TK (Amersham Biosciences, Piscataway, N.J.) and transformed into *Escherichia coli* strain DH5 α . The glutathione-S-transferase-fused JEV core protein expressed in the bacteria was purified with a column with glutathione Sepharose 4B (Amersham Biosciences) and intradermally injected five times into a Japanese white rabbit purchased from KITAYAMA LABES (Nagano, Japan). The collected antiserum was absorbed with glutathione-S-transferase-binding glutathione Sepharose 4B. Anti-JEV monoclonal antibodies

(MAb), anti-E 10B4 (E. Konishi, unpublished data) and anti-NS3 34B1 (K. Yasui, unpublished data), were used in immunostaining. Anti-nucleolin MAb, MS-3, and antiactin goat serum were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). Rabbit antiserum to PA28 α was purchased from AFFINITY (Exeter, United Kingdom).

Transfection of plasmids. Plasmid vectors were transfected by Superfect (QIAGEN, Tokyo, Japan) for Vero cells or Lipofectamine 2000 (Invitrogen, Carlsbad, Calif.) for 293T, BHK, N18, HeLa, HepG2, and SK-N-SH cells. To examine the intracellular localization of the EGFP or EGFP-fused proteins, the cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and permeabilized with 0.5% Triton X-100 in PBS at 24 h after transfection. After treatment with 1 μ g of RNase A (QIAGEN)/ml, the nuclei were stained with 500 μ M propidium iodide (Molecular Probes, Eugene, Oreg.). Endogenous nucleolin, a major nucleolar protein (51), was immunostained by an anti-nucleolin monoclonal antibody and Alexa Flour 564-conjugated anti-mouse immunoglobulin G (IgG) antiserum (Molecular Probes). All samples were visualized with a laser scanning confocal microscope (Bio-Rad, Hercules, Calif.).

Generation of JEV from plasmid. The wild-type and mutant (designated M4243) JEVs were generated from plasmids, pMWJEATG1, and pMWJEAT/GP4243AA, respectively, by previous methods (Zhao et al., submitted) with some modifications. Briefly, the plasmid DNAs digested by restriction enzyme KpnI were used as templates for RNA synthesis. Capped full-length JEV RNAs were synthesized in vitro by an mMESSAGE mMACHINE T7 kit (Ambion, Austin, Tex.), purified by precipitation with lithium chloride, and used for electroporation. Trypsinized Vero cells were washed with PBS and resuspended at 10⁷ cells/ml in PBS. RNA (10 μ g) was mixed with 500 μ l of cell suspension and transferred to an electroporation cuvette (Thermo Hybrid, Middlesex, United Kingdom). Cells were then pulsed at 190 V and 950 μ F by the use of a Gene Pulser II apparatus (Bio-Rad). Transfected cells were suspended in a culture medium and transferred to 10-cm-diameter culture dishes. After 3 or 4 days of incubation, the culture supernatants were collected as viral solutions. Due to a low viral yield, these viruses were amplified by a single passage in C6/36 cells. Viral infectivities were determined as focus-forming units (FFUs) by an immunostaining focus assay of Vero, C6/36, and 293T cells. Briefly, viruses were serially diluted and inoculated onto cell monolayers. After 1 h of adsorption, the cells were washed with serum free D-MEM three times and cultured in D-MEM containing 5% FBS and 1.25% methylcellulose 4000. At 2 or 3 days later, the cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. Infectious foci were stained with an anti-JEV E monoclonal antibody and visualized with a VECTASTAIN Elite ABC anti-mouse IgG kit with a VIP substrate (Vector Laboratories, Burlingame, Calif.). Vero and C6/36 cells infected with wild-type or M4243 JEV were fixed with cold acetone at 48 h postinoculation and stained with the rabbit anti-JEV core protein antiserum and Alexa Flour 488-conjugated goat anti-rabbit IgG (Molecular Probes) antibody. After treatment with 1 μ g of RNase A/ml, nuclei were stained with 500 μ M propidium iodide. Samples were examined with a laser scanning confocal microscope.

Subcellular fractionation. At 48 h postinoculation, 2 \times 10⁶ Vero cells were fractionated into cytoplasm and nucleus by using a Nuclear/Cytosol Fractionation kit (BioVision, Mountain View, Calif.) according to the manufacturer's instructions. Finally, 210 μ l of the cytoplasmic extracts and 100 μ l of the nuclear extracts were recovered and 10 μ l of each of the extracts was subjected to electrophoresis on an acrylamide gel. The JEV core protein was detected by Western blotting using the anti-JEV core protein rabbit polyclonal antibody. Endogenous PA28 α (3, 42) and nucleolin were detected as controls for the cytoplasmic and nuclear fractions, respectively.

Growth kinetics of mutant JEV in culture cells. Vero or C6/36 cells (2 \times 10⁵) in 24-well plates were infected with wild-type or M4243 virus at a multiplicity of infection (MOI) of 5 for 1 h at 4°C, washed three times with a medium to remove unbound viruses, and incubated with a medium supplemented with 5% FBS for a total duration of 30 h. The culture supernatants were used for titration of infectious virus, and cells were used for detection of viral proteins by Western blotting and for detection of negative-strand viral RNA by real-time reverse transcription-PCR (RT-PCR). Total RNAs were extracted from the cells by using an RNeasy Mini kit (QIAGEN) and quantified with a Gene Quant RNA/DNA calculator (Amersham Biosciences). RNA samples (5 μ l) were reverse transcribed at 52°C for 30 min with TaqMan reverse transcription reagents (Applied Biosystems, Foster, Calif.) by the use of a negative-strand-specific "tagged" primer corresponding to nucleotides (nt) 9307 to 9332 (5'-GCG TCA TGG TGG CGT ATT TAC CAG AAC TGA TTT AGA AAA TGA A-3'). The tagged sequence, which is underlined, had no correlation to JEV or other flaviviruses. The reverse transcripts were applied to a real-time PCR assay using a TaqMan PCR core reagents kit with sense (5'-GCG TCA TGG TGG CGT

ATT TA-3') and antisense (5'-TGG ACA GCG ATG TTC GTG AA-3') primers corresponding to the tagged sequence and nt 9519 to 9538 of the JEV AT31 strain, respectively. The kinetics of cDNA amplification were monitored with an ABI PRISM 7000 sequence detection system (Applied Biosystems) using a reporter probe corresponding to nt 9363 to 9380 of the JEV AT31 strain (5'-CAC CGC ATG CTC GCC CGA-3') conjugated with 6-carboxyfluorescein at the 5' terminal and 6-carboxy-tetramethylrhodamine at the 3' terminal. As references for the real-time RT-PCR, positive- and negative-strand RNAs were synthesized by *in vitro* transcription from plasmids containing nt 8907 to 9955 of JEV cDNA inserted in the forward and backward directions under the control of a T7 promoter.

Characterization of viral particles. Vero and C6/36 cells were inoculated with wild-type or M4243 viruses at an MOI of 0.1, and culture fluids harvested after 2 (Vero cells) or 3 (C6/36 cells) days postinoculation were clarified by centrifugation at $6,000 \times g$ for 30 min and precipitated with 10% polyethylene glycol (molecular mass, approximately 6,000 kDa). The precipitate was collected by centrifugation at $10,000 \times g$ for 45 min and resuspended in TN buffer (10 mM Tris-HCl [pH 8.0], 100 mM NaCl). The infectious titers of the concentrated viral particles were determined on Vero cells. The hemagglutination (HA) titers were determined at pH 6.6 by the method of Clarke and Casals (9). The viral particles (400 HA units) were applied on 10 to 40% of sucrose gradients and were centrifuged at $147,000 \times g$ for 90 min. Fractions collected from the bottom were examined by the HA test.

Complementation of mutant virus replication in mammalian cells. pCAG-WC-HA, pCAG-MC-HA, or pCAG-GS (1 μ g) was transfected into 293T cells in a 24-well plate (5×10^4 cells). At 4 h after transfection, the cells were washed three times with a serum-free medium and infected with the wild-type or M4243 JEV at an MOI of 5. At 12, 18, and 24 h after inoculation, the culture supernatants were harvested and infectivity was determined on Vero cells. The infected cells were harvested, and expression levels of the core proteins and replication of viral RNA were determined by Western blotting and real-time RT-PCR, respectively.

Mouse experiments. Female ICR mice of strain Jcl:ICR (3 weeks old) were purchased from CLEA Japan (Osaka, Japan). All mice were kept in pathogen-free environments. Groups of mice ($n = 10$) were inoculated intracerebrally (ic) with 30 μ l of 10-fold-diluted solutions of wild-type or M4243 virus. The virus dilution solution (D-MEM) was administered to 10 mice as a control. The mice were observed for 2 weeks after inoculation to determine survival rates. The value of the 50% lethal dose (LD_{50}) for each virus was determined by the method by Reed and Muench (47). Groups of mice ($n = 10$ or 11) were inoculated intraperitoneally (ip) with 10^5 FFU (100 μ l) of the viruses. The mice were observed for 3 weeks after inoculation to determine survival rates. To examine viral growth in the brain, 100 FFU (ic) or 10^5 FFU (ip) of the viruses were administered to the mice. At 1 to 7 days after inoculation, the mice were euthanized, and the brains were collected. The infectious viral titers in the homogenates of the brains were determined in Vero cells as described above.

RESULTS

Determination of amino acids essential for nuclear or nucleolar localization of the JEV core protein. To examine the subcellular localization of the mature JEV core protein without the C-terminal signal sequence in mammalian cells, pEGFP-JEVC105 encoding the EGFP-fused core protein or parental vector, pEGFP-C3, was transfected into Vero cells. EGFP was diffusely distributed in both the cytoplasm and nucleus, while the EGFP-fused core protein exhibited a diffuse distribution in the cytoplasm but granular localization in the nucleus (Fig. 1A). The fusion JEV core protein in the nucleus was colocalized with nucleolin, a major nucleolar component, indicating that the core protein is accumulated at the nucleoli (Fig. 1B). A similar subcellular localization of the fusion core protein was observed in all of the cell lines examined, including neuronal (N18 and SK-N-SH) and nonneuronal (293T, BHK, HeLa, and HepG2) cells (data not shown). Wang et al. (59) reported that the DEN core protein possessed a bipartite NLS in residues 85 to 100 (RKEIGRMLNLRKR). A computer program, PSORTII (Institute of Medical Science, Tokyo Uni-

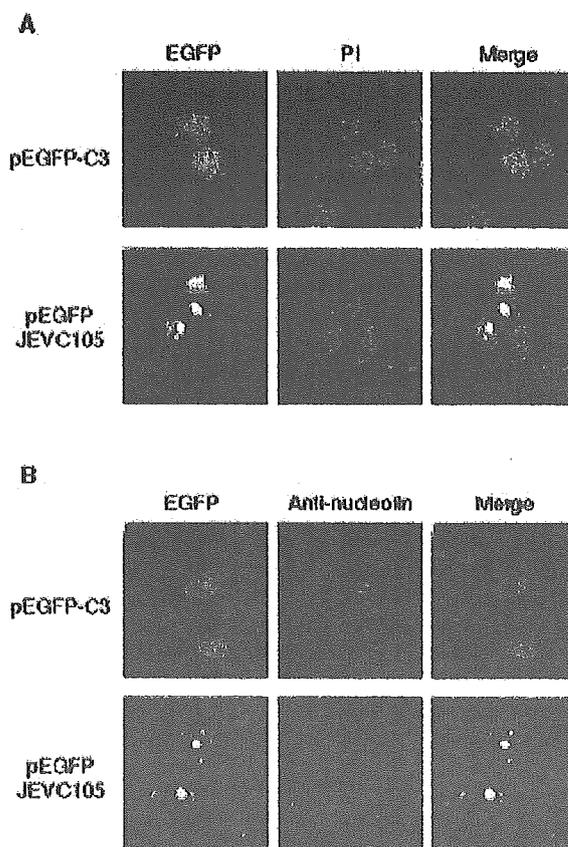


FIG. 1. Intracellular localization of EGFP-fused JEV core protein. Vero cells were transfected with expression plasmids encoding EGFP or EGFP-fused JEV core protein. At 24 h after transfection, cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. (A) Nuclei were stained with propidium iodide. (B) A representative nucleolar protein, nucleolin, was stained with anti-nucleolin monoclonal antibody. All samples were observed with a confocal microscope.

versity [<http://psort.ims.u-tokyo.ac.jp/helpwww2.html>], predicted that the JEV core protein also had an NLS at the corresponding region (residues 85 to 101 [KRELGLTIDAVNKRGRK]). To confirm whether the region functions as an NLS, an expression vector for the EGFP-fused mutant core protein in which all of the six basic amino acids (Arg and Lys) that were key amino acids in the NLS motifs were replaced by Ala (AAELGLTIDAVNAAAGAA) was transfected into Vero cells. However, these mutations did not affect the nuclear or nucleolar localization of the JEV core protein (data not shown), suggesting that this region of the JEV core protein does not participate as an NLS.

Alternatively, we found another candidate for an NLS in the JEV core protein. The NLS of the core protein of hepatitis C virus (HCV), a member of the same family *Flaviviridae*, has been mapped to the amino acid residues 38 to 43 (54). This domain of the HCV core protein is found to be homologous with flaviviruses, including JEV, St. Louis encephalitis virus, KUN, West Nile virus (WNV), Murray Valley encephalitis virus, and DEN (type 1 to 4) (Fig. 2A). In particular, the two amino acids Gly and Pro are completely conserved among

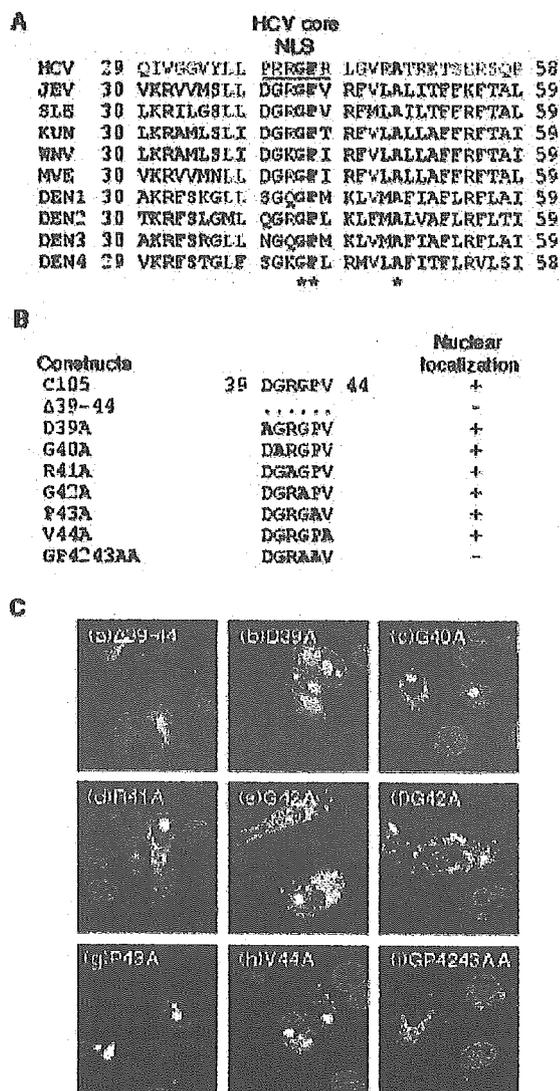


FIG. 2. Role of an HCV core protein NLS in nuclear localization of JEV core protein. (A) A partial alignment of amino acid sequences of core proteins of HCV (HCVJ1 strain, genotype 1b) and flaviviruses, including JEV, SLE (St. Louis encephalitis virus), KUN, WNV, MVE (Murray Valley encephalitis virus), and DENs. Amino acid sequences of the HCV core protein and identical amino acids in flaviviruses are indicated with red letters. Amino acids that were completely conserved among the viruses are indicated by asterisks and red boldface letters. The NLS of HCV core protein previously reported by Suzuki et al. (54) is underlined. (B) Expression plasmids encoding EGFP-fused JEV core proteins mutated in the NLS of the HCV core protein. The presence (+) or absence (-) of nuclear localizations of the EGFP-fused JEV core proteins is indicated. Dots indicate the deleted amino acids. Boldface letters indicate the substituted amino acids. (C) Intracellular localization of EGFP-fused JEV core proteins with deletion or substitution in the NLS region of HCV core protein in Vero cells. Panels (except for panel f) indicate merged images of EGFP and nuclear staining by propidium iodide. Panel f shows merged images of EGFP-fused JEV core protein with a G⁴²-to-Ala substitution and a major nucleolar protein nucleolin. All samples were observed with a confocal microscope.

these flaviviruses and HCV. Therefore, we next analyzed the effect of mutation in this region on the nuclear localization of the JEV core protein. The EGFP-fused JEV core protein with residues 39 to 44 deleted was localized only in the cytoplasm but not in the nucleus (Fig. 2B and panel a in Fig. 2C). To further identify the essential amino acids for the nuclear localization, a series of point mutants were constructed (Fig. 2B). No single-amino-acid substitution of the core proteins abolished nuclear localization except for a mutant of Gly⁴² in which the mutant core protein did not colocalize with nucleolin and was distributed as filamentous structures in the nuclei (Fig. 2C, panels e and f). However, double substitutions of the most conserved Gly⁴² and Pro⁴³ to Ala completely eliminated the nuclear localization of the JEV core protein (Fig. 2C, panel i). These results indicate that Gly⁴² and Pro⁴³, which are well conserved among flaviviruses and HCV (Fig. 2A), are important for nuclear and nucleolar localization of the JEV core protein.

Mutant JEV lacking the nuclear localization of core protein. To generate a mutant JEV incapable of localizing the core protein in the nucleus, synthetic RNA transcribed from pMWJEAT/GP4243AA encoding a full-length cDNA of mutant JEV M4243 under the T7 promoter was electroporated into Vero cells. The wild type, which was similarly generated from pMWJEATG1, and M4243 viruses were amplified in C6/36 cells after recovery from Vero cells because of a low viral yield of M4243 virus in Vero cells after electroporation (2×10^3 FFU/ml at 3 days after transfection) and used in subsequent experiments. The entire genomic cDNAs of the recovered viruses were confirmed to be identical to those of the infectious clones by direct sequencing. Intracellular localization of core proteins of the wild-type and mutant JEVs was examined in Vero and C6/36 cells by an immunofluorescence assay. In both cell lines, the core protein of the wild-type virus was localized in both the cytoplasm and nuclei whereas the core protein of M4243 was detected only in the cytoplasm and not in nuclei in both cell lines, as we expected (Fig. 3A). To confirm the intracellular localization of the core proteins, cytoplasmic and nuclear fractions of Vero cells infected with the viruses were analyzed by Western blotting (Fig. 3B). The wild-type core protein was fractionated in both cytoplasmic and nuclear fractions, while the mutant core protein was detected in the cytoplasmic fraction but not in the nuclear fraction.

Growth properties of the mutant JEV in vitro. To examine the roles of the nuclear localization of the core protein in viral propagation, one-step growth kinetics of the viruses in Vero and C6/36 cells were determined after inoculation at an MOI of 5 (Fig. 4A). The M4243 virus exhibited impaired propagations, with the infectious titers being 773- and 31-fold lower than those of wild-type JEV at 30 h postinoculation in Vero and C6/36 cells, respectively. These results indicate that Gly⁴² and Pro⁴³ in the JEV core protein were important for viral propagation, especially in Vero cells. The size of infectious foci in Vero cells produced by the M4243 virus recovered from the culture supernatants at 1 day postinfection of Vero cells was markedly smaller than that of the wild-type virus (Fig. 4B, left and middle panels). However, supernatants of Vero cells recovered 3 days after infection with M4243 produced larger foci than those obtained after incubation for 1 day (Fig. 4B, right panel). This phenomenon was not observed in C6/36 cells. To

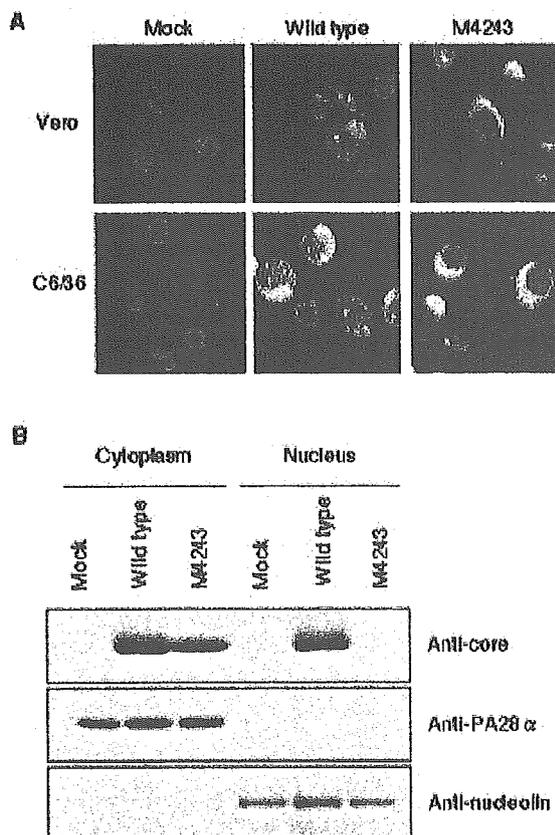


FIG. 3. Intracellular localization of core proteins in cells infected with wild-type or M4243 virus. (A) Intercellular localization of wild-type and mutant JEV. Vero or C6/36 cells were infected with wild-type or M4243 virus, fixed at 2 days postinoculation and immunostained with anti-JEV core rabbit serum. Nuclei were stained with propidium iodide. All samples were observed with a confocal microscope. (B) Intracellular fractionation of Vero cells infected with the viruses. The core proteins in the cytoplasmic and nuclear fractions were detected by Western blotting with the anti-JEV core rabbit serum. Endogenous proteins PA28 α and nucleolin were detected as controls for the cytoplasmic and nuclear fractions, respectively.

assess the possibility of the emergence of revertant viruses, the nucleotide sequences of two independent clones obtained at 3 days postinfection with M4243 in Vero cells were determined by direct sequencing. The majority of viruses carried a single-amino-acid reversion from Ala to Gly (GCG to GGG) at residue 42 in both clones. The single mutation of Pro⁴³ to Ala of the EGFP-fused JEV core protein did not abolish the nuclear or nucleolar localization, as shown in Fig. 2C (panel g). These results also support the idea that nuclear—especially nucleolar—localization of the JEV core protein is important for viral propagation in Vero cells.

Characterization of the released particles. It has been established that the flavivirus core protein is involved in the assembly and budding of infectious particles as a structural protein (34). Mutations in the core protein might possess the possibility to inhibit the release of infectious particles and, inversely, increase production of defective particles as described in previous reports (25, 26). Therefore, we determined the ratios between the infectivities and quantities of the par-

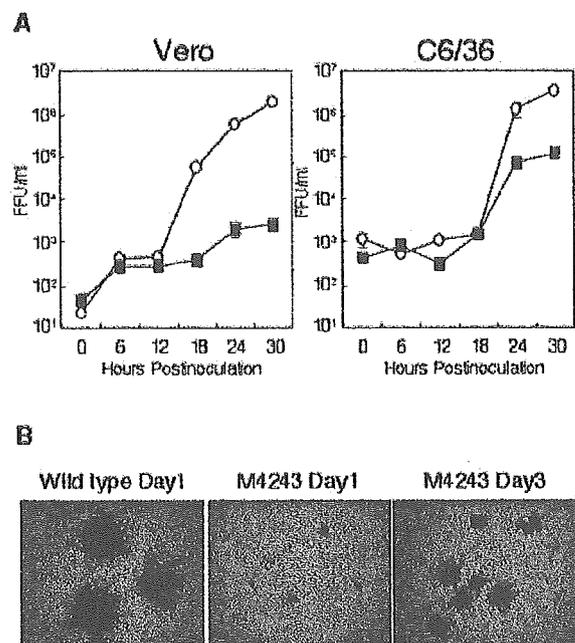


FIG. 4. Growth properties of wild-type and M4243 viruses. (A) Growth kinetics of the viruses in Vero and C6/36 cells. Both cell lines were infected with wild-type or M4243 virus at an MOI of 5. Culture supernatants were harvested at the indicated times postinoculation, and infectious titers were determined by focus-forming assays using Vero cells. Open circles and closed squares indicate the wild-type and M4243 viruses, respectively. Means of three experiments are indicated. (B) Infectious focus formation of the wild-type and M4243 viruses on Vero cells. Culture supernatants recovered at 1 or 3 days postinoculation in Vero cells were inoculated onto Vero cells and incubated for 3 days with methylcellulose overlay medium. The infectious foci were immunostained as described in Materials and Methods.

ticles released from Vero and C6/36 cells infected with the wild-type or M4243 JEV. The HA assay is able to detect viral particles irrespective of infectivity, because HA activity of the flavivirus is associated with E protein (28, 29). As shown in Table 1, the FFU/HA ratios of the wild-type JEV were significantly higher than those of the M4243 virus in both Vero and C6/36 cells, indicating that the M4243 virus produced a larger amount of defective particles than the wild-type virus. Although the ratios of defective particle production were equivalent between Vero and C6/36 cells, the mutant virus exhibited an HA titer comparable to that of the wild-type virus in C6/36 cells but significantly lower than that of the wild-type virus in Vero cells. In addition, the marked difference of infectious

TABLE 1. Infectious and HA titers of wild type and M4243 viruses derived from Vero and C6/36 cells

Cell	Virus ^a	FFU/ml	HA/ml	FFU/HA
Vero	Wild type	1.4×10^{10}	25600	5.5×10^5
	M4243	1.1×10^6	400	2.8×10^3
C6/36	Wild type	1.9×10^9	200	9.5×10^6
	M4243	4.0×10^7	800	5.0×10^4

^a Viruses were 100-fold concentrated by a polyethylene glycol precipitation and determined the infectivities and HA activities.

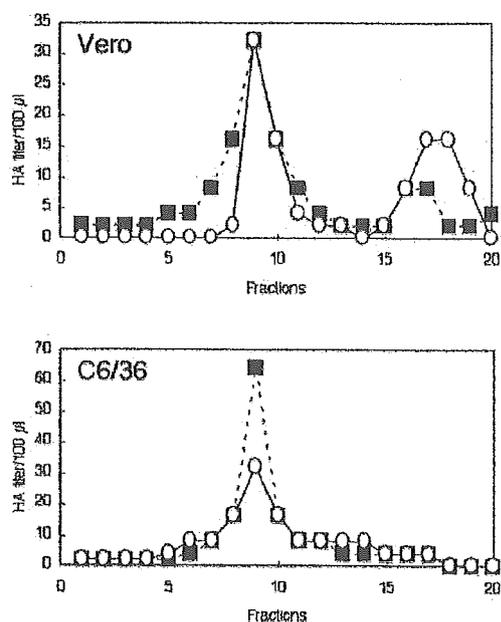


FIG. 5. Gradient fractionation of viral particles of the wild-type and M4243 viruses. The viral particles (400 HA units) derived from Vero or C6/36 cells were applied to 10 to 40% (wt/wt) sucrose gradient and centrifuged at $147,000 \times g$ for 90 min. Twenty fractions were collected from bottom to top and quantified by the HA test. Open circles and closed squares indicate the wild-type and M4243 viruses, respectively. The representative data from three experiments are indicated.

titers between the wild-type and M4243 viruses in Vero cells (Fig. 4A) indicates that there may be another mechanism(s) underlying the low-growth properties of the M4243 virus in Vero cells besides the increased production of defective particles. To examine the production of subviral particles in culture supernatants of cells infected with M4243 virus, we carried out gradient fractionations and quantifications of viral particles by HA assay. As indicated in Fig. 5, the patterns of the fractionations of the particles of the M4243 virus were similar to those of the wild-type virus in both Vero and C6/36 cells, and subviral particles were detected in the fractions (fractions 16 to 19) of the supernatants of Vero cells infected with the wild-type or M4243 JEV.

Effect of nuclear localization of core protein on RNA replication and protein synthesis. To clarify the reasons for the impaired growth of the M4243 virus in Vero cells, we measured viral RNA replication and protein synthesis in Vero and C6/36 cells infected with wild-type and M4243 viruses. It has been reported that the ratio of the positive strands to the negative strands of viral RNA in JEV-infected cells was 3:1 to 11.7:1 (58). Real-time RT-PCR specific for the negative-strand viral RNA used in this study is capable of detecting more than 10^2 copies/2 μ l of the negative-sense viral RNA in the absence of the positive-strand RNA (Fig. 6A). The amounts of negative-strand RNA in the presence of a 100- or 1,000-fold excess amount of the positive-strand RNA were less than 10-fold different compared with those determined in the absence of the positive-strand RNA (Fig. 6A), indicating that the PCR system is specific enough to measure the negative-strand viral

RNA levels in cells infected with JEV. We then measured the synthesis of the negative-strand viral RNAs in Vero and C6/36 cells infected with wild-type or mutant JEV at an MOI of 5 by RT-PCR. Although similar levels of negative-strand RNA synthesis were observed in C6/36 cells infected with either virus, M4243 exhibited 3- and 18-fold-lower RNA replication than the wild type in Vero cells at 18 and 30 h postinoculation, respectively (Fig. 6B). Metabolic labeling of the host proteins indicated that there were no significant differences between the viabilities of Vero cells infected with wild-type and M4243 virus (data not shown). To determine the level of impairment of RNA translation of M4243 in Vero cells, viral protein syn-

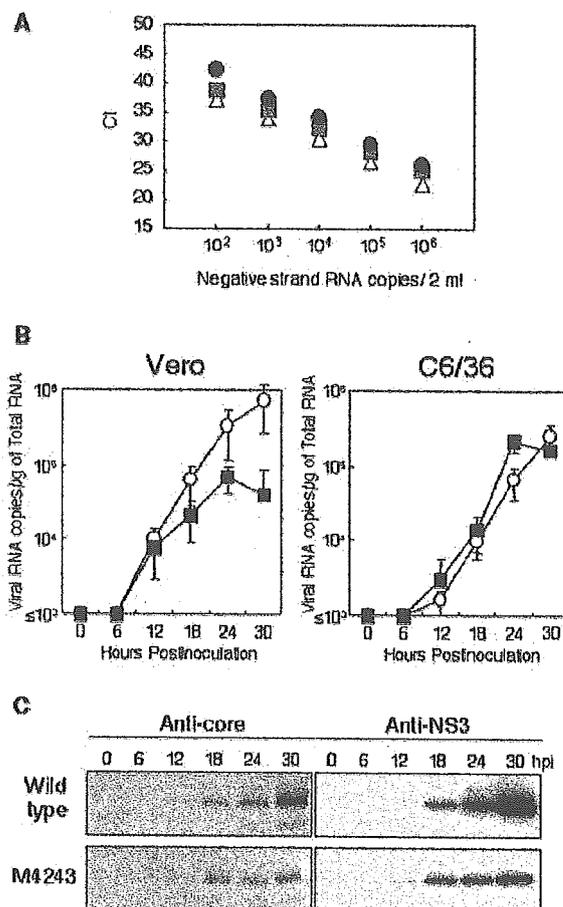


FIG. 6. Viral RNA and protein syntheses in Vero and C6/36 cells infected with wild-type or M4243 virus. (A) Establishment of negative-strand JEV RNA-specific real-time RT-PCR. A series of 10-fold dilutions of synthetic negative-strand RNA in the absence (closed circles) or presence of 100-fold (gray squares) or 1,000-fold (open triangles) synthetic positive-strand RNAs were applied to the real-time RT-PCR. The Ct value represents the first PCR cycle to detect the increase in signal associated with an exponential growth of PCR product. (B) Viral negative-strand RNAs were quantified in the infected cells by the real-time RT-PCR. Open circles and closed squares indicate wild-type and M4243 viruses, respectively. The detection limit was 10^3 copies of viral RNA/ μ g of total RNA. Means of three experiments are indicated. (C) Core and NS3 proteins were detected by Western blotting with anti-JEV core rabbit serum and anti-JEV NS3 MA b 34A1, respectively. A total of 4 μ g of each sample was loaded.

theses in Vero cells infected with the wild-type or M4243 virus were analyzed by Western blotting (Fig. 6C). Although comparable amounts of core and NS3 proteins were detected at an early phase of infection (12 and 18 h postinoculation) in Vero cells infected with either virus, saturation of protein syntheses by a mutant virus was observed at 24 h postinfection, in contrast to cells infected with the wild-type virus, in which protein synthesis increased until 30 h postinfection. The early saturation of viral protein synthesis of M4243 in Vero cells is quite consistent with that of RNA replication. These results suggest that nuclear localization of the core protein plays a crucial role in the maintenance of replication-translation of viral RNA in mammalian cells but not in mosquito cells, in which the mutant virus replicates at a rate similar to that of the wild-type JEV.

Complementation of mutant virus replication by expression of the wild-type core protein. We next examined the growth of a mutant virus in cells transiently expressing the wild-type or mutant core protein. The efficiency of gene transduction into Vero cells is very low, and we therefore selected 293T cells for their high efficiency of foreign-gene transduction and used them to transiently express the JEV core protein. HA-tagged wild-type and mutant core proteins (approximately 16 kDa) and the viral core protein derived from M4243 (approximately 14 kDa) were detected in 293T cells transfected with the expression plasmids and infected with M4243 virus (Fig. 7A). Expression of the wild-type core protein, but not that of the mutant core protein, drastically enhanced viral growth of the M4243 virus up to the level of wild-type virus growth (Fig. 7B). However, the expression of the core proteins did not affect the replication of the wild-type virus. Furthermore, the negative-strand RNA synthesis of the mutant virus was increased three-fold by the expression of the wild-type core protein, but not by that of the mutant protein, compared with mock-transfection results (Fig. 7C). These results indicate that the expression of the wild-type JEV core protein is able to compensate for the propagation of the M4243 virus.

Neurovirulence and neuroinvasiveness of M4243. To examine the neurovirulence characteristics of the wild-type and mutant viruses, we determined the LD₅₀ values by intracerebral inoculation of the viruses into 3-week-old ICR mice. The LD₅₀ values for the wild-type and the mutant viruses were 2.1 and 0.5 FFU, respectively. No significant differences in symptoms, mean duration period of diseases (wild versus mutant, 1.1 versus 0.9 days), and mean day of death (7.5 versus 7.6 days postinoculation) were observed between mice inoculated with 100 FFU of the wild-type virus and those inoculated with an equivalent dose of M4243 virus. To examine the growth kinetics of the viruses in the brain, 100 FFU of each virus was intracerebrally injected, and the viruses recovered from the brain homogenates were titrated. The growth of the M4243 virus was approximately 100 times lower than that of the wild-type virus (Fig. 8A, left panel), and revertant viruses exhibiting medium-sized plaques (Fig. 4B) were not recovered from the brains inoculated with the M4243 virus. The neuroinvasiveness of encephalitis flaviviruses is thought to be a reflection of their ability to grow in the peripheral organs, to breach the blood-brain barrier, and to infect central nervous systems following peripheral inoculation. To examine the neuroinvasiveness of wild-type and M4243 viruses, ICR mice were intraperitoneally

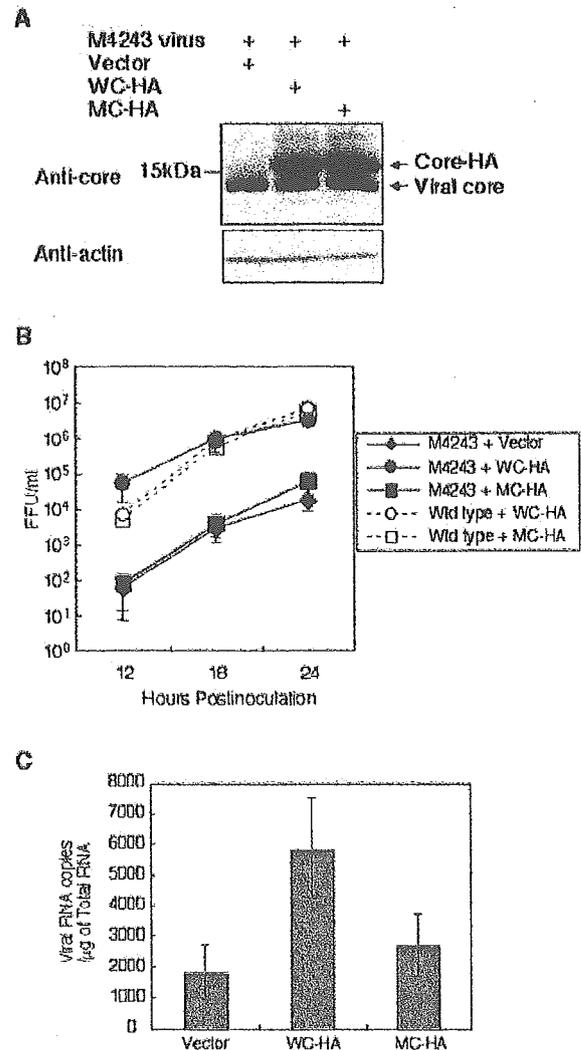


FIG. 7. Complementation of M4243 replication by expression of core proteins in 293T cells. At 4 h after transfection with pCAG-GS (Vector), pCAG-WC-HA (WC-HA), or pCAG-MC-HA (Gly⁴² and Pro⁴³ to Ala) (MC-HA), 293T cells were infected with wild-type or M4243 virus at an MOI of 5. At 12, 18, or 24 h postinoculation, culture supernatants and cells were harvested to apply to focus-forming assays and Western blotting or real-time RT-PCR, respectively. (A) Western blotting of 293T cells transfected with plasmids expressing HA-tagged wild-type or mutant core protein and infected with M4243 virus. Molecular mass marker was indicated in the left of the panel. (B) Growth of wild-type and M4243 viruses in 293T cells transfected with the plasmids. Viral titers were determined by focus-forming assays in Vero cells. (C) Complementation of M4243 in 293T cells transfected with the plasmids at 24 h postinoculation. Viral RNA levels were determined by the negative-strand-specific real-time RT-PCR. Means from three experiments are indicated.

inoculated with 10⁵ FFU of each virus. Only 1 of 11 mice inoculated with the M4243 virus had died by 9 days postinoculation, while on average 10 of the 11 mice inoculated with the wild-type virus had died by 9.6 days postinoculation (Fig. 8B). Over 5 days after inoculation, the viruses were recovered from the brain of mice inoculated with wild-type JEV but not from those inoculated with M4243 (Fig. 8A, right panel). These re-

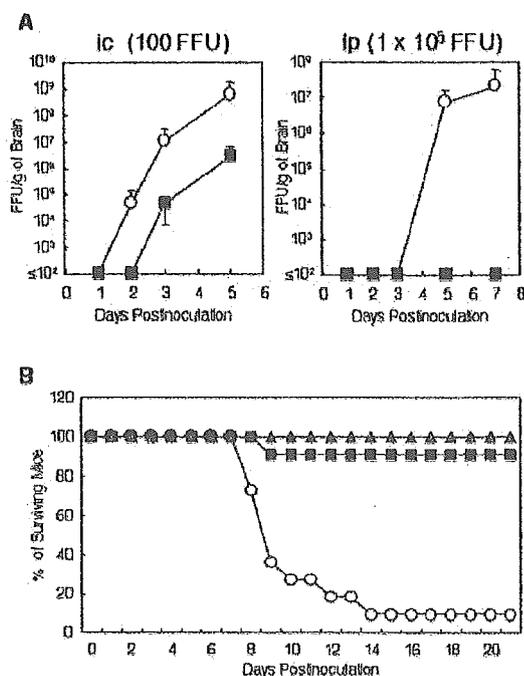


FIG. 8. Virulence of wild-type and M4243 viruses for ICR mice. (A) The infectious titers of wild-type and M4243 viruses in the brains of mice after inoculation with 100 FFU of the viruses intracerebrally (ic) or 10⁵ FFU intraperitoneally (ip). Open circles and closed squares indicate wild-type and M4243 viruses, respectively. The detection limit is 10² FFU/gram of brain. Means of titers from four mice are indicated. (B) Percentages of surviving mice (10 to 11 mice per group) inoculated with 10⁵ FFU of wild-type or M4243 viruses by an intraperitoneal route. Open circles, closed squares, and gray triangles indicate mice infected with the wild-type virus, infected with the M4243 virus, or mock infected, respectively.

sults indicated that the mutant virus exhibited a level of neurovirulence equivalent to that of the wild type but that its neuroinvasiveness was severely impaired in 3-week-old-ICR mice.

DISCUSSION

Like most animal RNA viruses, except for those of the families *Orthomyxoviridae*, *Bornaviridae*, and *Retroviridae*, members of the *Flaviviridae* replicate in the cytoplasm of host cells (34). However, it has been reported that the core proteins of DEN, KUN, and HCV are observed not only in the cytoplasm but also in the nucleus (4, 38, 55, 59, 61). In this study, we demonstrated that the JEV core protein was translocated into the nucleus and accumulated in the nucleolus of cells infected with JEV or transfected with an expression plasmid for the core protein. We revealed that Gly⁴² and Pro⁴³ were important for the nuclear localization and that Gly⁴² was essential for the nucleolar accumulation of the JEV core protein. The two amino acids Gly and Pro are well conserved not only among mosquito-borne flaviviruses such as JEV, KUN, WNV, and DEN but also among HCVs. According to the three-dimensional structures of KUN and DEN, the two amino acids are mapped to the unsheltered loop domain between α -helices 1 and 2 (11, 36). Substitutions of Gly⁴² and Pro⁴³ with Ala completely abolished the nuclear localization of the JEV core pro-

tein as well as that of the DEN core protein (data not shown). However, a previous study showed that deletion of the N-terminal 45 amino acids of the DEN core protein did not eliminate nuclear localization of the protein (59). Although the reason for this discrepancy is not presently clear, our data suggest that the well-conserved Gly and Pro are important for nuclear or nucleolar localization of the core protein of flaviviruses. The two amino acids and the flanking sequences exhibited no similarity to the well-known classical NLSs, such as a large T-antigen of simian virus 40 (PKKKRKV) (21), nucleoplasmin (KRPAATKKAGQAKKKK) (5), and nucleolar localization signals, such as the Rex protein of human T-cell leukemia virus type I (MPKTRRRPRRSQRKRPTTP) (44). This domain may be required for recognition of novel nuclear import receptor protein(s) and nucleolar component(s). Recently, Dokland et al. demonstrated that tetramers of the KUN core protein extended as filamentous ribbon structures (11). The change of Gly⁴² to Ala in the JEV core protein, therefore, may abolish the binding activity to nucleolar compartment(s) and exhibit a filamentous structure in the nuclei (Fig. 2C).

The recovery of the M4243 virus in which Ala was substituted for Gly⁴² and Pro⁴³ of the core protein suggests that nuclear localization of the core protein is not a necessary condition for viral propagation. However, replication of the mutant virus was impaired in mammalian cells but not in mosquito cells. The impairment of propagation of the M4243 virus in mammalian cells may be due to either of two phenomena: the decrease in production of infectious particles with a simultaneous increase of defective particles or the low efficiency of viral RNA replication. Since budding of flavivirus takes place through the interaction of prM and E proteins independently of the association with the core protein, disfunctions of core protein may reduce the production of infectious particles and enhance the production of subviral particles. In fact, mutations at the cleavage site of host signal peptidase in the core-prM junction of Murray Valley encephalitis virus caused an increase of subviral particle production (35). Furthermore, TBEV core proteins with deletions of the central hydrophobic region, containing Gly³⁶ corresponding to the Gly⁴² essential for nucleolar localization of the JEV core protein, increased the production of subviral particles due to the lack of association with the ER membrane, where budding of the flavivirus takes place (26, 27). In contrast, the mutations on the Gly⁴² and Pro⁴³ region of the JEV core protein caused the production of the defective particles different from subviral particles. Although the mechanisms underlying the production of the defective particles remain unclear, the mutations might affect the functions except for assembly and budding, such as maturation or uncoating. As far as we know, biological functions except for nuclear localization in the Gly⁴² and Pro⁴³ region of the flavivirus core proteins have not been studied. The mutations might collaterally disrupt the conformation of the core protein essential for their functions of JEV. Meanwhile, it might be feasible that the nuclear localization of the core protein directly participates in the viral infectivity. Tijms et al. (56) demonstrated that the capsid protein of equine arteritis virus mostly shuffled between the cytoplasm and nucleus prior to cytoplasmic viral assembly, suggesting that the nuclear localization is crucial for viral assembly. In any case, production of the defective particles by the mutant JEV was enhanced in both Vero and C6/36 cells, and

thus, a decrease of infectious particles cannot explain the impairment of M4243 in Vero cells.

It is believed that the core protein is not required for RNA replication, since the RNA replicon of flavivirus, which does not contain the whole core gene, has been shown to be capable of replicating (10, 13, 24). Therefore, it is noteworthy that RNA replication of M4243 was impaired in Vero cells but not in mosquito cells. Although a *cis*-acting nucleotide sequence element essential for RNA replication has been mapped to the flavivirus core genes (10, 22), the nucleotide changes were not involved in the impairment of replication of M4243 due to the compensation of RNA replication by the expression of wild-type core protein in *trans*. The kinetics study of viral RNA and protein syntheses suggested that the JEV core protein translated at the early step of infection was translocated into the nucleus and enhanced RNA replication at the late phase of infection, although further studies are needed to clarify the precise mechanism. Earlier studies (30, 31) resulted in reports that flaviviruses, including JEV, but not alphaviruses failed to propagate and produce viral antigens in cells enucleated by cytochalasin B, suggesting the involvement of host nucleus factors in flavivirus replication. The DEN core protein was reported to interact with a nuclear transcription factor, heterogeneous nuclear ribonucleoprotein K, and regulate the C/EBP- β -mediated transcription (7). Furthermore, the HCV core protein was also shown to associate with host nuclear proteins such as heterogeneous nuclear ribonucleoprotein K (18) and PA28 γ (42) and was suggested to regulate the transcription of host cells (18, 45). The nuclear localization of core proteins of *Flaviviridae* might change the suitability of the host-cell environment for viral propagation by producing factors that enhance RNA replication or by suppressing those that reduce it.

Other cytoplasmic RNA viruses, such as members of the families *Picornaviridae* (1, 2, 12), *Coronaviridae* (8, 15, 62), *Arteriviridae* (48, 56), *Togaviridae* (41), and *Rhabdoviridae* (46), may also feed their proteins into the nucleus to facilitate viral propagation. For example, it is suggested that the protein 2A of encephalomyocarditis virus is localized to the nucleoli and inhibits cellular mRNA transcription (1) and that point mutations within the NLS of another nucleolar protein, 3D^{pol}, were lethal due to the inhibition of viral RNA replication (2). The coronavirus nucleoproteins were also found to be localized in the nucleoli of the host cells (8, 15, 62), and the expression of the coronavirus nucleoproteins by transfection inhibits host cell division (8, 62).

Surprisingly, the M4243 virus exhibited a high level of neurovirulence in mice comparable to that of the wild-type JEV despite the fact that the M4243 virus had a 100-fold-lower replication efficiency than the wild-type JEV *in vivo*. Encephalitis induced by flavivirus infection is thought to arise from direct injury of brain neurons by viral replication (49, 64) or indirect injury of brain neurons by immune responses (60). However, the idea that the direct injury of neurons is responsible for encephalitis induced by flavivirus is difficult to reconcile with the results showing that the virulence of the M4243 virus is equivalent to that of the wild-type virus. A previous study indicated that a low dose of WNV-induced encephalitis was associated with inflammatory cell infiltration in mice (60). Alternatively, the defective particles contained in the inoculum or produced by M4243 infections might stimulate a signal path-

way via reactive oxygen species in neuronal cells and induce cell death, as described by Lin et al. (33).

In contrast to the neurovirulence results, a striking difference in levels of neuroinvasiveness was observed between the wild-type and M4243 viruses. Although the magnitude and duration of viremia were suggested to be major determinants for neuroinvasion (19, 32), the precise mechanism by which flaviviruses breach the blood-brain barrier and enter the brain tissue remains uncertain. Encephalitis flaviviruses injected by peripheral routes are thought to replicate in lymphatic tissues, such as peripheral lymph nodes or spleen, and to induce further viremia (32, 40). Although we had no evidence that JEV replicated in peripheral tissues prior to neuroinvasion as described by others (32), the wild-type virus, but not M4243, was present in small amounts (100 to 200 FFU/ml) in blood samples at 1 and 3 days postinoculation (data not shown). In this context, it might be possible that the M4243 virus was unable to replicate in peripheral tissues at a level sufficient to develop viremia and breach the blood-brain barrier, resulting in the low level of neuroinvasiveness. The novel attenuation of neuroinvasiveness observed in M4243 may be applicable to the development of new live vaccines against flavivirus infection.

The life cycles of most flaviviruses are sustained between arthropods and vertebrates. The present finding that nuclear localization of the core protein enhances viral replication in mammalian cells may lead to an improved understanding of the evolutionary adaptation strategy of flaviviruses in expanding their host range from arthropods to vertebrates. It might also be possible to speculate that JEV transgressed its host barrier by translocating the core protein into the nucleus of porcine cells and incorporated the pig as an amplifier in the life cycle of JEV. Furthermore, it is of interest for evolutionary studies on the family *Flaviviridae* that the NLSs of core proteins were well conserved between mosquito-borne flaviviruses and bloodborne and human-adapted HCV.

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Reduction of hepatitis C virus NS5A phosphorylation through its interaction with amphiphysin II [☆]

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Abstract

Hepatitis C virus non-structural protein 5A (NS5A) is a pleiotropic protein with key roles in viral RNA replication, modulation of cellular-signaling pathways and interferon (IFN) responses. To search for possible host factors involved in mediating these functions of NS5A, we adopted an affinity purification approach coupled with mass spectrometry to examine protein–protein interactions, and found that human amphiphysin II (also referred to as Bin1) specifically interacts with NS5A in mammalian cells. Pull-down assays showed that the Src homology 3 (SH3) domain of amphiphysin II is required for NS5A interaction and that c-Src also interacts with NS5A in cells. IFN- α treatment reduced the interaction of NS5A with c-Src, but not amphiphysin II, suggesting that the latter is independent of the IFN-signaling pathway. NS5A is a phosphoprotein and its phosphorylation status is considered to have an effect on viral RNA replication. *In vitro* kinase assays demonstrated that its interaction with amphiphysin II inhibits phosphorylation of NS5A. These results suggest that amphiphysin II participates in the HCV life cycle by modulating the phosphorylation of NS5A.
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Keywords: Proteome; NS5A; HCV; Amphiphysin II; Phosphorylation

Chronic infection with hepatitis C virus (HCV) carries with it a substantial risk of severe liver conditions such as chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. The interactions between cellular proteins and HCV gene products may provide clues for novel approaches to interfere with viral propagation and the accompanying pathogenesis. NS5A is a non-structural HCV protein thought to confer resistance to IFN therapy in HCV infection [1,2]. The NS5A protein in certain HCV isolates asso-

ciates with IFN-induced double-stranded RNA-activated protein kinase (PKR) and inhibits PKR activity [3,4]. In addition, PKR-independent repression of IFN activity by NS5A, such as via the induction of IL-8 transcription, has been reported [5]. Several investigators have examined the role of NS5A in IFN resistance *in vivo* and *in vitro*, and have suggested the possibility that the number of mutations in the ISDR of NS5A is related to IFN responsiveness [1–4]. Thus, NS5A could be an important protein for the development of therapies against HCV infection.

The NS5A protein has been reported to interact with Grb2, p53, Cdk1, Pitx1, TRAF-2, karyopherin β 3, and the SNARE-like protein, hVAP-33 (the human homologue of the 33-kDa vesicle-associated membrane protein-associated protein) [6–13]. To assess the effect of NS5A on cell function, it is important to identify proteins that interact

[☆] **Abbreviations:** NS5A, hepatitis C virus non-structural protein 5A; ISDR, interferon sensitivity determining region; SH3, Src homology region 3; PAGE, polyacrylamide gel electrophoresis; LC-MS/MS, liquid chromatography–mass spectrometry/mass spectrometry.

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with NS5A. In the present study, we investigated NS5A-protein binding by a protein–protein interaction assay using cultured cells. Using LC–MS/MS analysis, we identified amphiphysin II as an NS5A-interacting protein in HeLa cells transfected with NS5A. Amphiphysin II has been reported to interact with c-myc and thereby possess anti-oncogenic function [14]. Zech et al. [15] previously identified amphiphysin II as an NS5A-interacting protein using an *in vitro* GST pull-down assay and demonstrated that IFN- α treatment abrogated this interaction by reducing NS5A levels in a HuH7 replicon system. However, the effect of this interaction on cellular function was not explored. Here, we determined the consequences of NS5A–amphiphysin II interactions for NS5A phosphorylation *in vitro*. NS5A is known to be the only phosphoprotein among the HCV non-structural proteins [16,17] and its hyperphosphorylation inhibits HCVRNA replication [18,19]. We demonstrated that its interaction with amphiphysin II reduces NS5A phosphorylation, which may help explain the mechanisms by which host defenses interact with the HCV during its life cycle.

Materials and methods

Cell lines and reagents. HeLa S3, HuH7, and 293T cells were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Human IFN- α was purchased from Toray Co. Ltd.

Plasmid construction and stable transfectant production. Wild type NS5A cDNA was constructed from NIHJ1 (mutant NS5AcDNA) by modifying the ISDR [20,21]. Wild type and mutant NS5AcDNA were inserted into the *HindIII*–*KpnI* sites of a p3xFLAG-CMV-14 expression vector (Sigma). The wild type NS5A expression vector (designated p5AFLAG) was transfected into HeLa S3 cells using Fugene6 (Roche Biochemicals) and after 2-weeks' culture with G418 (500 μ g/ml), drug-resistant HeLa cells were pooled as stably transfected cells. Wild type HA-amphiphysin II cDNA and HA-amphiphysin II Δ 10 cDNA (amphiphysin II lacking exon 10) were constructed from the amphiphysin II cDNA and subcloned into the *BamHI*–*XbaI* sites of the pcDNA3.1(+) vector (Invitrogen) [22]. HA-amphiphysin II Δ SH3 cDNA was subcloned into the *BamHI*–*XhoI* site of the pcDNA3.1(+) vector. The amphiphysin II mutants amphiphysin II Δ 10, amphiphysin II Δ SH3, and amphiphysin II Δ N lacked the amino acids 250–266, 377–451, and 1–47, respectively [22].

Analysis by LC–MS/MS. HeLa cells stably transfected with NS5A-flag expression vector (p5AFLAG) were cultured in a 1 L spinner flask for 5 days. Cell extracts from HeLa cells were incubated with anti-flag M2-agarose and washed, and bound products were then eluted with flag peptide (0.2 mg/ml). The eluted fraction was electrophoresed on SDS–10% PAGE and stained with Silver Quest (Invitrogen). Individual protein bands were excised from the silver-stained gel and destained with a solution of 30 mM potassium ferricyanide/100 mM sodium thiosulfate (1:1) (v/v). Trypsin-digested sample solutions were analyzed by LC–MS/MS.

Preparation of cell extracts, M2-agarose, and HA-agarose pull-down assay, and Western blot analysis. 293T cells were transfected with full length amphiphysin II pcDNA3.1, amphiphysin II Δ 10 pcDNA3.1, amphiphysin II Δ SH3 pcDNA3.1 or amphiphysin II Δ N pcDNA3.1 together with p5AFLAG. The transient transfection of 293T cells was carried out with Fugene 6 (Roche Biochemicals). The cells were harvested, washed with PBS, and lysed in lysis buffer M containing 50 mM Tris–HCl (pH 8.0), 100 mM NaCl, 0.1% NP-40, 1 mM EDTA, 1 mM dithiothreitol, and protein inhibitor cocktail (Sigma). Total cell lysates were incubated with M2-agarose (Sigma) or HA-agarose (Sigma) for 2 h on a rotator at 4 °C. After washing the agarose with lysis buffer M three times, the bound

proteins were eluted with 0.2 mg/ml flag peptide (Sigma) or 1 mg/ml HA peptide (Sigma), and subjected to electrophoresis on SDS–10% PAGE, and analyzed by Western blotting with anti-flag (Sigma), anti-IRF-3 (Santa Cruz), anti-nucleolin (Santa Cruz), anti-stat1(UBI), anti-amphiphysin II(UBI), anti-c-Src (Santa Cruz), and anti-NS5A (Austral Biologicals) antibodies. The proteins were visualized by Chemiluminescence (Perkin Elmer).

***In vitro* kinase assays.** Lysate from p5AFLAG and HA-amphiphysin II pcDNA3.1 cotransfected cells was incubated with anti-flag M2-agarose, and then the washed precipitate was reacted in kinase buffer (20 mM Tris–HCl, pH 8.0, 10 mM MgCl₂, and 1 mM Na₂VO₄) and 2 μ Ci, 10 μ M [γ -³²P]ATP (Amersham) for 30 min at 37 °C. The reaction was terminated by the addition of an SDS sample buffer and boiling for 5 min at 95 °C, followed by SDS–10% PAGE and autoradiography.

Results

NS5A associates with amphiphysin II in HeLa cells

HeLa cells were stably transfected with p5AFLAG encoding NS5A conjugated with flag in the C-terminal domain. Expression of NS5A protein was confirmed by an anti-flag M2-agarose pull-down assay (Fig. 1A). Western blotting using anti-flag and anti-NS5A antibodies confirmed the production of flag-tagged NS5A protein in HeLa cells (Fig. 1A). To isolate proteins that associate with NS5A protein, cell lysate from a large-scale HeLa cell culture was incubated with anti-flag M2-agarose, and then the M2-agarose precipitate was eluted with flag peptide. The eluted fraction was electrophoresed and stained with silver. We observed that three bands a, b, and c increased compared to the level in the control (empty vector transfected) (Fig. 1B, lanes 1 and 2). These bands were cut out of the gel and digested with trypsin, and then the peptides were identified by LC–MS/MS. LC–MS/MS analysis identified band a as NS5A and band b as muscle type amphiphysin II [22]. Band c included β -tubulin, which was later identified as a non-specific protein. We focused on band b as an NS5A interaction protein. The indicated peptide coverage identified by LC–MS/MS for band b was approximately 42% (Fig. 1C). Western blot analysis using the amphiphysin II antibody confirmed that anti-flag M2-agarose precipitated amphiphysin II from HeLa cells transfected with p5AFLAG, but not control cells (Fig. 1D), further suggesting that amphiphysin II was specifically recruited to NS5A. We observed two bands by Western blotting which seemed to be phosphorylated and non-phosphorylated forms of amphiphysin II (Fig. 1D, top panel).

Several investigators have suggested that ISDR mutations in NS5A may be related to IFN responsiveness [1–4]. To examine whether the ISDR is important for interaction with amphiphysin II, an NS5A mutant NIHJ1-flag plasmid (with the ISDR modified as described previously) [20,21] was transfected together with HA-amphiphysin II pcDNA3.1 into 293T cells. Cell lysates were incubated with M2-agarose or HA-agarose, and then resin-bound products were eluted with flag peptide or HA peptide. As shown in Fig. 2, both wild type and mutant NS5A-bound

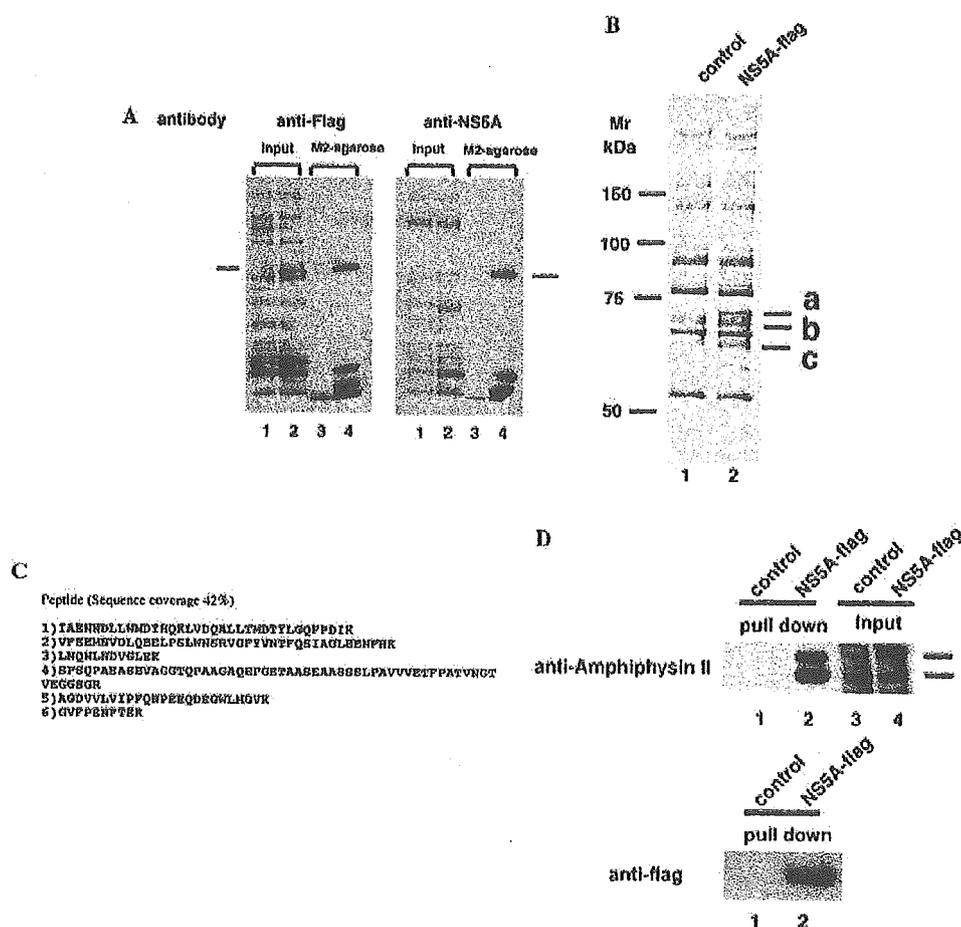


Fig. 1. Amphiphysin II associates with NS5A. (A) Expression of NS5A-flag protein in HeLa cells. NS5A-flag expression vector (p5AFLAG) (lanes 2 and 4) and a control vector (lanes 1 and 3) were transfected into HeLa cells. After a 2-week culture with geneticin 500 $\mu\text{g}/\text{ml}$, surviving cells were collected and lysed in lysis buffer. Cell lysate (lanes 1 and 2) was incubated with anti-flag M2-agarose and eluted with flag peptide (lanes 3 and 4) and then the eluate was subjected to Western blotting with anti-flag (left panel) or anti-NS5A (right panel) antibodies. (B) Cell lysates from large-scale (1 L) HeLa culture transfectants were incubated with anti-flag M2-agarose and then the flag peptide-eluted fractions from control and NS5A-transfectants were electrophoresed. Individual bands were analyzed by LC-MS/MS to identify proteins. The bands which are present in lane 2 but not lane 1 are indicated as a, b, and c. (C) Peptide coverage from trypsinized protein band b analyzed by LC-MS/MS. LC-MS/MS analysis identified band b as amphiphysin II. (D) Western blot analysis of M2-agarose immunoprecipitates. Anti-flag M2-agarose immunoprecipitates from control (lane 1) and NS5A-flag HeLa transfectants (lane 2) were electrophoresed and immunoblotted with anti-amphiphysin II and anti-flag antibodies. Aliquots of whole cell lysates from control cells (lane 3) and NS5A-flag transfectants (lane 4) were immunoblotted with the anti-amphiphysin II antibody.

amphiphysin II comparably, suggesting that the NS5A–amphiphysin II interaction is independent of the ISDR.

Next, we investigated the association of various proteins by Western blotting. As shown in Fig. 3A, c-Src protein was detected in anti-flag M2-agarose precipitates from HeLa cells transfected with p5AFLAG. IRF-3 and nucleolin were not detected as NS5A-interacting proteins, although these proteins are abundant in HeLa cells. Furthermore, in our system, we could not detect PKR or Pitx1 by Western blot analysis, although other investigators have reported that these proteins interact with NS5A [11] (data not shown).

We studied whether IFN- α treatment affects the interaction of NS5A with amphiphysin II or c-Src. Interestingly, IFN- α treatment reduced the c-Src–NS5A interaction,

but not the amphiphysin II–NS5A interaction, although the expression of c-Src and amphiphysin II was unchanged after IFN- α treatment (Fig. 4). In a control experiment, Stat1 did not interact with NS5A before or after IFN- α treatment (Fig. 4). These results show that c-Src may be related to IFN- α -signaling factors, and that the interaction of amphiphysin II and NS5A is independent of the IFN- α response.

The interaction with amphiphysin II SH3 inhibits NS5A phosphorylation

To examine which region of amphiphysin II associates with NS5A, several amphiphysin II mutants were cotransfected into 293T cells with p5AFLAG. An anti-flag M2-

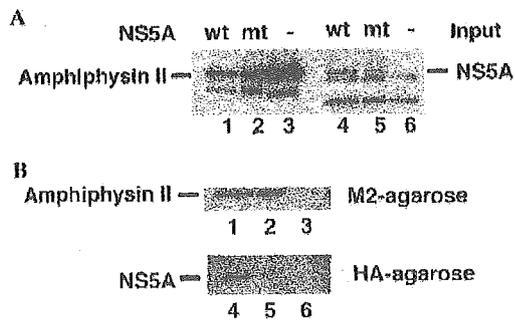


Fig. 2. NS5A associates with amphiphysin II in 293T cells. (A) p5AFLAG (lanes 1 and 4) or p5AFLAG mutant (NIHJ1 expression vector) (lanes 2 and 5) with HA-amphiphysin II pcDNA3.1 or only HA-amphiphysin II pcDNA3.1 (lanes 3 and 6) was transfected into 293T cells. Cell lysate from 293T cells was electrophoresed and immunoblotted with anti-amphiphysin II antibody (lanes 1–3) or anti-NS5A antibody (lanes 4–6). (B) Half of the cell lysate from 293T cells transfected with plasmids encoding wild type (lanes 1 and 4) or mutant NS5A (lanes 2 and 5) with HA-amphiphysin II pcDNA3.1 or only HA-amphiphysin II pcDNA3.1 (lanes 3 and 6) was incubated with anti-flag M2-agarose (lanes 1–3), and washed, and then the flag peptide-eluted fraction was immunoblotted with the anti-amphiphysin II antibody. The other half of the same 293T cell lysate was incubated with HA-agarose, and washed, and then the HA peptide-eluted fraction was immunoblotted with the anti-NS5A antibody (lanes 4–6).

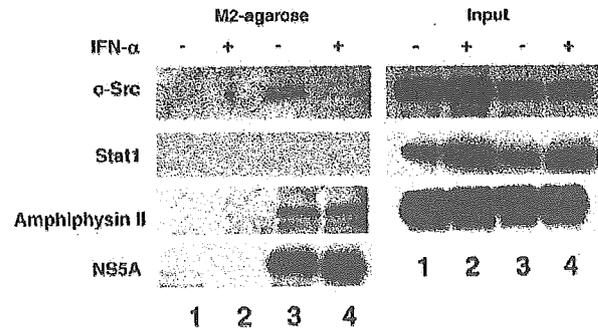


Fig. 4. IFN- α treatment inhibits the c-Src-NS5A interaction, but not the amphiphysin II-NS5A interaction. The M2-agarose pull down (left) and input (right) fractions were prepared from control HeLa cells (lanes 1 and 2) and HeLa-NS5A transfectants (lanes 3 and 4), untreated (lanes 1 and 3) or treated (lanes 2 and 4) with IFN- α (1000U) for 24 h. Immunoblot analysis was performed using anti-c-Src, anti-Stat1, anti-amphiphysin II, and anti-NS5A antibodies.

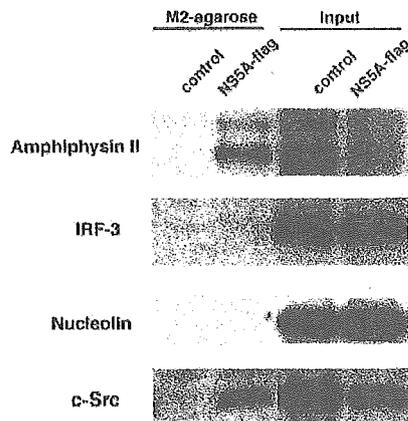


Fig. 3. Western blot analysis of NS5A-associated proteins in HeLa transfectants. Cell lysate from p5AFLAG-transfected HeLa cells was prepared, and then the flag peptide-eluted fraction from anti-flag M2-agarose immunoprecipitates was immunoblotted with anti-IRF-3, anti-nucleolin, and anti-c-Src, as well as anti-amphiphysin II antibodies.

agarose pull-down assay indicated that the amphiphysin II isoform $\Delta 10$ associated with NS5A, although the binding affinity was lower than with wild type (Fig. 5A). We also tested amphiphysin II ΔN , which is defective in the N-terminal region, and amphiphysin II $\Delta SH3$, lacking the C-terminal region, for interaction with NS5A protein. As shown in Fig. 5B, NS5A associated with amphiphysin II ΔN to the same extent as with wild type amphiphysin II, but the association of amphiphysin II $\Delta SH3$ with NS5A was markedly reduced. Together, these data suggest that the SH3 region of amphiphysin II is important for interacting with NS5A.

NS5A is known to be a phosphoprotein [17,23]. We investigated the effect of interaction of amphiphysin II with NS5A on phosphorylation of the latter. Anti-flag M2-agarose precipitates from 293T cells double-transfected with p5AFLAG and HA-amphiphysin II pcDNA3.1 were reacted in a kinase buffer containing [γ - ^{32}P]ATP followed by immunoblotting with an anti-flag antibody. When HA-agarose precipitates from 293T cells double-transfected with p5AFLAG and HA-amphiphysin II pcDNA3.1 were immunoblotted with an anti-flag antibody, we detected a strong association of NS5A with wild type amphiphysin II, but not amphiphysin II $\Delta SH3$ (Fig. 5C, top). As shown in Fig. 5C, NS5A from the M2-agarose precipitates were phosphorylated in vitro, suggesting that intracellular kinase associates with NS5A. NS5A from p5AFLAG and amphiphysin II pcDNA3.1 double-transfected 293T cells was much less phosphorylated than that from cells transfected with p5AFLAG alone (Fig. 5C, bottom). Amphiphysin II protein from HA-agarose precipitates of these double-transfected cells was also incubated in a kinase buffer, but amphiphysin II phosphorylation was not detectable (data not shown). We also tested NS5A phosphorylation using a human hepatic cell line, HuH7, because HCV is known to mainly infect hepatocytes [3]. HuH7 cells were double-transfected with p5AFLAG and amphiphysin II pcDNA3.1, and anti-flag M2-agarose precipitates were phosphorylated in vitro. We also detected NS5A phosphorylation in p5AFLAG and control vector-transfected HuH7 cells; however, NS5A from p5AFLAG and amphiphysin II pcDNA3.1-transfected HuH7 was much less phosphorylated (Fig. 5D). In addition, amphiphysin II $\Delta SH3$ pcDNA3.1 transfection did not affect NS5A phosphorylation in HuH7 cells as in 293T cells (Figs. 5C and D). Here, we observed that HCV NS5A phosphorylation was regulated by host protein amphiphysin II. We conclude that the interaction between amphiphysin II and NS5A inhibits NS5A phosphorylation.

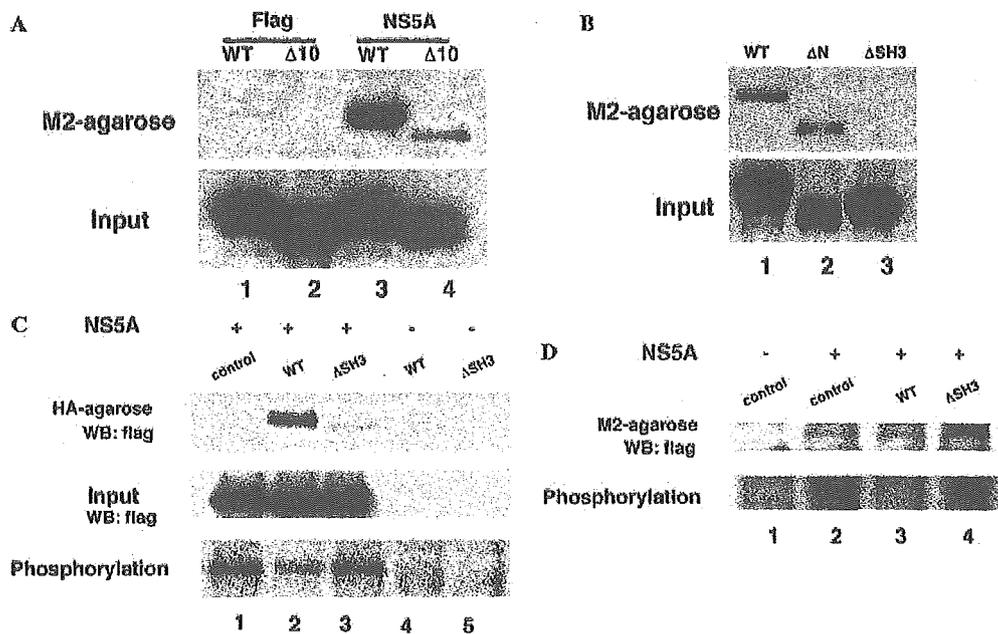


Fig. 5. NS5A associates with different amphiphysin II isoforms. (A) Full length amphiphysin II (lanes 1 and 3) or amphiphysin II $\Delta 10$ pcDNA3.1 (lanes 2 and 4) together with p5AFLAG were transfected into 293T cells and an anti-flag M2-agarose pull-down assay was performed. The flag peptide fraction was then immunoblotted with anti-amphiphysin II antibody. (B) Full length amphiphysin II (lane 1), amphiphysin II ΔN (lane 2), and amphiphysin II $\Delta SH3$ (lane 3) deletion mutant plasmids were transfected into 293T cells with p5AFLAG and an anti-flag M2-agarose pull-down assay was performed. Then the flag peptide fraction was immunoblotted with anti-amphiphysin II antibody. (C) Amphiphysin II interaction inhibits NS5A phosphorylation. Empty vector pcDNA3.1 (lane 1), amphiphysin II pcDNA3.1 (lanes 2 and 4), amphiphysin II $\Delta SH3$ pcDNA3.1 (lanes 3 and 5), and p5AFLAG (lanes 1–3) were transfected into 293T cells and half of the lysate was incubated with anti-flag M2-agarose while the other half was incubated with HA-agarose. The washed agarose was incubated with kinase reaction mixture containing $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, boiled in an SDS-sample buffer, electrophoresed, and subjected to immunoblotting with anti-flag antibody (top and middle panels) or autoradiography (bottom panel). (D) Empty vector pcDNA3.1 (lanes 1 and 2), amphiphysin II pcDNA3.1 (lane 3), amphiphysin II $\Delta SH3$ pcDNA3.1 (lane 4), and p5AFLAG (lanes 2–4) were transfected into HuH7 cells and the whole cell lysate was incubated with anti-flag M2-agarose. The washed agarose was incubated with kinase reaction mixture containing $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, boiled in SDS-sample buffer, electrophoresed, and subjected to immunoblotting with the anti-flag antibody (top panel) or autoradiography (bottom panel).

Discussion

In this study, we demonstrated that HCV protein NS5A binds amphiphysin II in mammalian cells and that this interaction down-regulates NS5A phosphorylation, which affects the HCV life cycle. Amphiphysin II function was initially identified based on its ability to interact with c-myc and inhibit malignant transformation by this oncogene [14,24]. Amphiphysin II has several isoforms [22,25] and muscle type amphiphysin II is ubiquitously expressed amongst isoforms which localize to the cytoplasm and nucleus, and which is known to activate a caspase-independent apoptotic process [14,22]. Although widely expressed in normal cells, amphiphysin II is reported to exist in a functional form in ~50% of carcinoma cell lines and primary breast carcinomas examined. It should be noted that the amphiphysin II isoform in HeLa cells has been reported to be an exon 10-defective type [26]. Wild type amphiphysin II, which has exon 10, is a differentiation-associated isoform. In contrast, the amphiphysin II $\Delta 10$ isoform, which lacks exon 10, is involved in cell growth [26]. Although we found amphiphysin II as an NS5A-associated protein in HeLa cells, amphiphysin II–

NS5A interactions in normal or non-transformed cells remain to be confirmed.

Zech et al. [15] reported that amphiphysin II and NS5A interact in HuH7 cells and showed that the binding of amphiphysin II to GST-NS5A polyP-mutant fusion protein was reduced, while a NS5A C-terminal deletion mutant was completely unable to bind to amphiphysin II. Macdonald et al. [27] demonstrated that two closely spaced Class II polyproline motifs near the C-terminus of NS5A were able to bind isolated SH3 domains of Src kinase family members *in vitro*. Using Huh7-derived cells harboring sub-genomic HCV replicons, they showed that the expression of NS5A could differentially regulate the activity of endogenous Src family kinases and perturb Src-family-regulated-signaling pathways. However, their study found that NS5A in HuH7 cells did not bind GST-SrcSH3 [27]. In our report, c-Src was found to be an NS5A-associated protein in HeLa cells, although Src peptide fragments were not detected by LC-MS/MS analysis. c-Src can perhaps bind NS5A somewhere other than the SH3 region.

The ISDR of NS5A has been reported to be important for the IFN- α response [1,2,28]. We detected comparable affinity for amphiphysin II binding between the wild type

and ISDR mutant NS5A (Fig. 1), suggesting that NS5A ISDR is not critical for NS5A–amphiphysin II interactions. In our study, IFN- α treatment inhibited c-Src association with NS5A, while in contrast the binding affinity of amphiphysin II to NS5A did not change (Fig. 3). Dumenil et al. [29] reported that IFN- α inhibits the Src-mediated pathway necessary for *Shigella*-induced cytoskeletal rearrangement in epithelial cells. Our results suggest the possibility of an interaction between IFN signaling and the Src-mediated pathway, and appear to indicate that amphiphysin II association with NS5A does not affect the IFN-signaling pathway.

Both NS5A and amphiphysin II are known to be phosphoproteins [15,23,30]. NS5A has been reported to be phosphorylated by cAMP-dependent protein kinase and casein kinase II [31,32]. We observed that the phosphorylation of NS5A was reduced on interaction with amphiphysin II in both 293T and HuH7 cells. Phosphorylation of NS5A was not inhibited in the presence of amphiphysin II Δ SH3, which did not bind to NS5A. The interaction between amphiphysin II and NS5A is important for the inhibition of NS5A phosphorylation, although it is not yet clear how the SH3 region of amphiphysin II mechanistically affects the NS5A phosphorylation sites.

Recently, Neddermann et al. [18] demonstrated that hyperphosphorylation of NS5A inhibits HCV RNA replication. Evans et al. [19] reported that NS5A hyperphosphorylation disrupts interaction with hVAP-A and negatively regulates viral RNA replication. In our study, amphiphysin II expression reduced NS5A phosphorylation in a hepatic cell line, HuH7, which suggests that it may induce HCV RNA replication in HCV-infected cells. However, it is not yet known whether NS5A phosphorylation affects the interaction with amphiphysin II. HCV is known to mainly infect hepatocytes [3]. NS5A may bind amphiphysin II in normal hepatocytes and thus induce the transformation of these cells into hepatocellular carcinoma by inhibiting the anti-oncogenic function of amphiphysin II. It is as yet unclear how NS5A phosphorylation affects cellular transformation. The NS5A–host protein interaction affects both host defense and critical steps in the HCV life cycle such as viral replication. Such information as presented here on the interaction of NS5A and amphiphysin II should contribute to a better understanding of the mechanisms of host defense against HCV, and thus facilitate the development of effective anti-HCV therapies.

Acknowledgments

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