We have reported previously that HCV core protein specifically interacts with a proteasome activator PA28γ/REGγ in the nucleus and is digested by a PA28γ-dependent proteasome activity.[7] *In vivo* experiments in a mouse model suggest that PA28γ plays a critical role in the pathogenesis induced by HCV core protein.[8,9] PA28γ forms a homoheptamer in the nucleus and enhances the proteasome-mediated cleavage after basic amino acid residues, whereas PA28α and PA28β exhibit 41% and 34% homology to PA28γ, respectively, and form a heteroheptamer in the cytoplasm to activate cleavage after hydrophobic, acidic, or basic amino acid residues.[10] Recently, several groups reported that PA28γ interacts with steroid receptor coactivator-3 and cell cycle suppressors such as $p21^{WAF1/CIP1}$, $p16^{INK4A}$, and $p19^{ARF}$, and enhances the degradation of these proteins in a ubiquitin- and adenosine triphosphate–independent manner.[11-13] Furthermore, other mechanisms of ubiquitin-independent degradation have been considered for cell cycle regulation, summarized in the review of Jariel -Encontre et al.[14] However, the precise physiological functions of PA28γ are largely unknown *in vivo*, because PA28γ-knockout mice exhibit only mild growth retardation and live approximately as long as their control littermates.[15,16]

HCV core protein is degraded in a PA28γ-dependent and ubiquitin-independent manner in the nucleus,[7,17] while E6AP is also involved in the degradation of the core protein in a ubiquitin-dependent manner.[17,18] E6AP is a member of E3 ligases, which catalyze ubiquitin ligation of host and foreign proteins. Knockdown of E6AP suppressed degradation of HCV core protein and enhanced the release of infectious particles, suggesting that E6AP negatively regulates HCV propagation.[18] However, the role of PA28γ in the propagation of HCV has not yet been characterized. In this study, we examined the biological significance of PA28γ in the propagation of HCV.

Materials and Methods

Transfection, Immunoblotting, and RNA Interference. Plasmid DNA was transfected into Huh7OK1 cells by way of liposome-mediated transfection using Lipofectamine LTX with Plus reagent (Invitrogen, Carlsbad, CA). Expression of HCV core protein was determined by way of enzyme-linked immunosorbent assay as described.[19] Immunoblotting was performed as described.[8] The small interfering RNAs (siRNAs) targeted to the PA28γ gene were purchased from Ambion (Austin, TX) and were introduced into the cell lines using Lipofectamine RNAiMax (Invitrogen). siRNAs with the Ambion siRNA ID numbers 138669 and 138670 were designated as siPA28γ1 and siPA28γ2, respectively. Antibodies and plasmids are described in the Supporting Information.

Cell Lines and Virus Infection. All cell lines were cultured at 37°C under the conditions of humidified atmosphere and 5% CO_2. The human hepatoma cell line Huh7OK1 and derivative cell lines were maintained in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) supplemented with nonessential amino acids, sodium pyruvate, and 10% fetal bovine serum. The Huh7-derived cell line harboring a subgenomic or a full-length HCV replicon RNA[20] was maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, nonessential amino acids, sodium pyruvate, and 1 mg/mL G418 (Nakarai Tesque, Kyoto, Japan). Huh7OK1 cells were transfected with pSilencer-shPA28γ4 or a control plasmid, pSilencer 2.1 U6 hygro negative control (Ambion), and drug-resistant clones were selected by treatment with hygromycin (Wako, Tokyo, Japan) at a final concentration of 100 μg/mL. Huh7OK1 cells transfected with the control plasmid were selected with puromycin and designated as shCntrl, whereas those transfected with pSilencer-shPA28γ4 were established by limited dilution,[8] and two of the resulting cell lines were designated as KD5 and KD7. Plasmids encoding wild-type or mutant PA28γ complementary DNAs resistant to siRNA against PA28γ were prepared by using the silent mutations as reported.[8] These plasmids were transfected into Huh7OK1 cells and cultivated in medium containing 0.1 μg/mL of puromycin for 2 days. The surviving cells were used for virus infection. The shCtrl and KD5 cells were transformed with pSilencer shE6AP or pSilencer 3.1 H1 puro negative control (Ambion) and treated with 0.1 μg/mL of puromycin for 2 days. The surviving cells were infected with JFH-1 virus at a multiplicity of infection (moi) of 0.05. The viral RNA derived from the plasmid pJFH1 was transcribed and introduced into Huh7OK1 cells according to the method of Wakita et al.[21] The infectivity of JFH1 strain was determined using a focus-forming assay[21] and is expressed in focus-forming units. The Huh7 cell line harboring subgenomic replicon RNA of the Con1 or JFH1 strain was prepared according to the method of Pietschmann et al.[22] The infectivity of the Japanese encephalitis virus (JEV) was determined by an immunostaining focus assay as described[23] and is expressed in focus-forming units. Colony formation and replication assays, quantitative

reverse-transcription polymerase chain reaction, and estimation of cell growth was performed as described in the Supporting Information.

Immunofluorescent Staining. Huh7OK1-derived cells were seeded at 0.5×10^4 cells/well in an eight-well chamber slide, infected with JFH-1 virus at an moi of 0.3 after incubation at 37°C for 24 hours, stained with Bodipy 558/568 C_{12} according to the method of Targett-Adams et al.[24] at 4 days postinfection, and then fixed at 4°C for 30 mintues with 4% paraformaldehyde in phosphate-buffered saline. After treatment of cells with 1 μg/mL of RNase A, nuclei were stained with 50 μM Heachest 33258. The fixed cells were permeabilized with 20 mM Tris-HCl containing 1% Nonidet P-40 and 135 mM NaCl at room temperature for 5 minutes, reacted with rabbit anti-core or anti-NS5A antibody followed by Alexa Fluor 488-goat antibody to rabbit immunoglobulin G, washed three times with phosphate-buffered saline, and observed with a FluoView FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan). The percentage of the area occupied by the core protein in nucleus and cytoplasm was calculated using Image-Pro software (Media Cybernetics). The percentage of the nuclear core protein to the total core protein was examined randomly in 10 fields of every three wells. The percentage of the nuclear NS5A to total NS5A was estimated by the same method as the ratio of the core protein.

Results

Transient Knockdown of PA28γ Prior to or After Infection With HCV Reduces Particle Production. We reported previously that Huh7OK1 cells are as permissive to JFH-1 virus infection as Huh7.5.1 cells.[25] The Huh-7OK1 cell line retained the ability to produce type I IFNs through the RIG-I–dependent signaling pathway upon infection with RNA viruses and exhibited a cell surface expression level of human CD81 comparable to that of the parental cell line. However, the mechanism through which the Huh7 OK1 cell line exhibits highly permissive to JFH-1 virus infection has not been clarified yet. Two siRNAs were used to knock down PA28γ, but only one, siPA28γ1, was used because the other had off-target effects (Supporting Fig. 1). To examine the effect of PA28γ on the propagation of HCV, siPA28γ1 was introduced into Huh7OK1 cells 24 hours before infection. The levels of viral RNA, core protein, and infectious viral titer were determined at 48 and 96 hours postinfection. Viral RNA in the culture supernatant and cells was clearly reduced by the knockdown of PA28γ at 48 and 96 hours postinfection, respectively (Fig. 1A), whereas a significant reduction of core protein expression was detected at 96 hours but not at 48

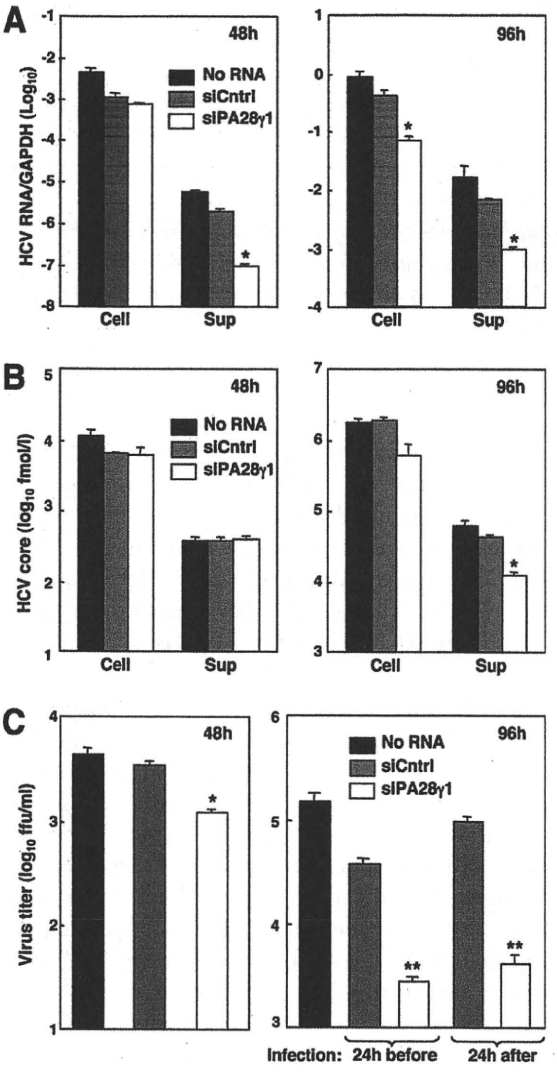

Fig. 1. Transient knockdown of PA28γ before or after infection with HCV reduces particle production. (A) Huh7OK1 cells transfected with a control siRNA (siCntrl) or PA28γ siRNA1 were infected with JFH-1 virus at 24 hours posttransfection and then harvested at 48 hours (left panel) and 96 hours postinfection (right panel). The quantity of HCV RNA in cells and supernatants was determined by way of quantitative reverse-transcription polymerase chain reaction. (B) The expression of HCV core protein in cells and supernatants at 48 hours (left panel) and 96 hours (right panel) postinfection was determined by ELISA. (C) Huh7OK1 cells that were transfected with siCntrl or PA28γ siRNA1 were infected with JFH-1 virus at 24 hours posttransfection. The infectivity of the virus in the culture supernatant was determined by a focus-forming assay at 48 hours postinfection (left panel). Those transfected with the siRNAs at 24 hours before and after infection with JFH-1 virus were determined similarly at 96 hours postinfection (right panel). $*P < 0.05$, $**P < 0.01$ versus control siRNA-transfected cells. Data are representative of three independent experiments.

hours postinfection (Fig. 1B). Infectious viral titer in the culture supernatant was significantly reduced at 48 and 96 hours postinfection by the PA28γ knockdown (Fig. 1C), consistent with the suppression of the viral RNA in the supernatant. Furthermore, a comparable suppression of the production of infectious particles in the supernatant was also achieved by introducing siPA28γ1 into cells even at 24 hours postinfection (Fig. 1C, right panel). These results suggest that PA28γ participates in the regulation of HCV propagation in postentry steps.

Stable Knockdown of PA28γ Impairs Viral Propagation. To establish the PA28γ knockdown cell lines, Huh7OK1 cells were transfected with a plasmid encoding a short hairpin RNA (shRNA) targeted to PA28γ and selected with hygromycin, resulting in two clones—KD5 and KD7—that exhibited a clear reduction of PA28γ expression (Fig. 2A). Although the suppression of PA28γ expression in KD7 cells was slightly more efficient than that in KD5 cells, the growth of KD7 cells was impaired (Fig. 2B). Viral production in the culture supernatants in cells infected with the JFH-1 virus was significantly impaired in PA28γ knockdown KD5 cells compared with control cells (Fig. 2C). The viral RNA and core protein in the supernatant were also reduced in KD5 cells (Fig. 2D). Expression of siRNA-resistant PA28γ in PA28γ knockdown KD5 and KD7 cells recovered virus production in the supernatant to a level similar to that in the control cells transfected with an empty vector, and overexpression of siRNA-resistant PA28γ in control cells slightly enhanced virus production (Fig. 2E). Our previous data suggest that capsid protein of JEV does not bind to PA28γ.[7] To examine whether PA28γ regulates JEV propagation, KD5 and shCntrl cells were infected with JEV at an moi of 0.5. The infectivity of JEV in KD5 cells was similar to that in shCntrl cells (Fig. 2F), suggesting that PA28γ does not participate in the virus production pathway of JEV. These results further support the notion that PA28γ participates in HCV propagation.

Knockdown of PA28γ Exhibits No Effect on Viral RNA Replication. Although knockdown of PA28γ resulted in the suppression of viral particle and RNA production in the culture supernatant at 48 hours postinfection with JHF-1 virus, viral RNA in the cells was not reduced (Fig. 1), suggesting that PA28γ does not participate in viral replication. To gain more insight on this point, we examined the effect of PA28γ knockdown on RNA replication in replicon cells. Transient knockdown of PA28γ through introduction of siPA28γ into the subgenomic HCV replicon cells

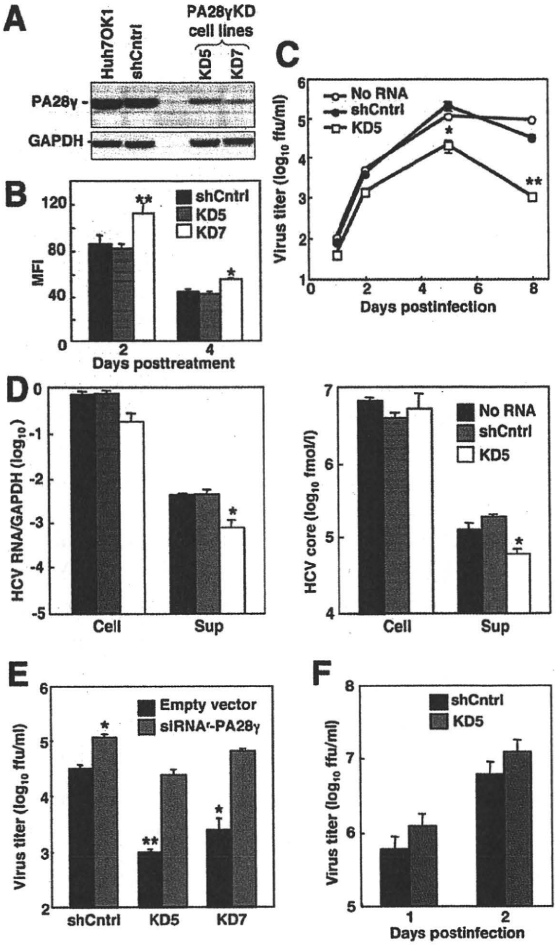

Fig. 2. Establishment of PA28γ knockdown cell lines and propagation of HCV. (A) Huh7OK1 cells were transfected with pSilencer shPA28γ or control plasmid and selected by hygromycin at 48 hours posttransfection. Two PA28γ knockdown cell lines (KD5 and KD7) and one control cell line (shCntrl) were established, and PA28γ knockdown was confirmed by way of immunoblotting. (B) Growth of the cell lines was determined by staining with carboxyfluorescein succinimidyl ester. (C,D) KD5 and shCntrl cell lines were infected with the JFH-1virus at an moi of 0.05. The infectious virus titers in the culture supernatants (C) was determined by way of focus-forming assay. The virus RNA (D, left panel) and the core protein (D, right panel) in both cell and the supernatant were determined at 5 days postinfection by way of ELISA and quantitative reverse-transcription polymerase chain reaction, respectively. (E) The plasmid encoding an siRNA-resistant PA28γ or empty vector was transfected into the cell lines, seeded at 5×10^4 cells into a six-well plate after cultivation in the presence of puromycin for 2 days, and infected with JFH-1 virus at an moi of 0.05. The viral titers were determined at 5 days postinfection. $*P < 0.05$, $**P < 0.01$ versus shCntrl cells transfected with an empty vector. (F). KD5 and shCntrl cell lines were infected with the JEV virus at an moi of 0.5. The infectivity of JEV in the supernatant was determined at 1 and 2 days postinfection. Data are representative of three independent experiments.

derived from the Con1 or JFH-1 strain induced no significant reduction of HCV RNA (Fig. 3A). Furthermore, luciferase activities in the stable PA28γ

knockdown cell line KD5 and the control cell line transfected with the subgenomic replicon RNA (WT) were gradually increased until 4 days posttransfection, whereas luciferase activities in the same two cell lines transfected with the polymerase-dead replicon RNA (GND) were decreased in a time-dependent manner (Fig. 3B). Next, to explore the effect of PA28γ knockdown on the viral replication over a longer period, replicon RNA encoding the neomycin-resistance gene was transfected into the cell lines for a colony formation assay. The numbers of colonies in the KD5 cell line after 4 weeks of selection with G418 were similar to those in the control cell line (Fig. 3C). To further clarify the roles of PA28γ on the postreplication steps, *in vitro* transcribed full-length viral RNA was transfected into Huh7OK1 cells, and siPA28γ1 was then introduced into the cells at 24 hours posttransfection of viral RNA. Intracellular core protein was increased in a time-dependent manner, but no significant difference was observed between cells transfected with control siRNA and those transfected with siPA28γ1 (Fig. 3D, left panel). However, infectious virus titers in the supernatant were significantly decreased by the transient and stable knockdown of PA28γ compared with control cells (Fig. 3D, middle and right panels). Furthermore, PA28γ did not contribute to the virus production of JEV (Fig. 2F), suggesting that the general sorting pathway of the flavivirus is functional under

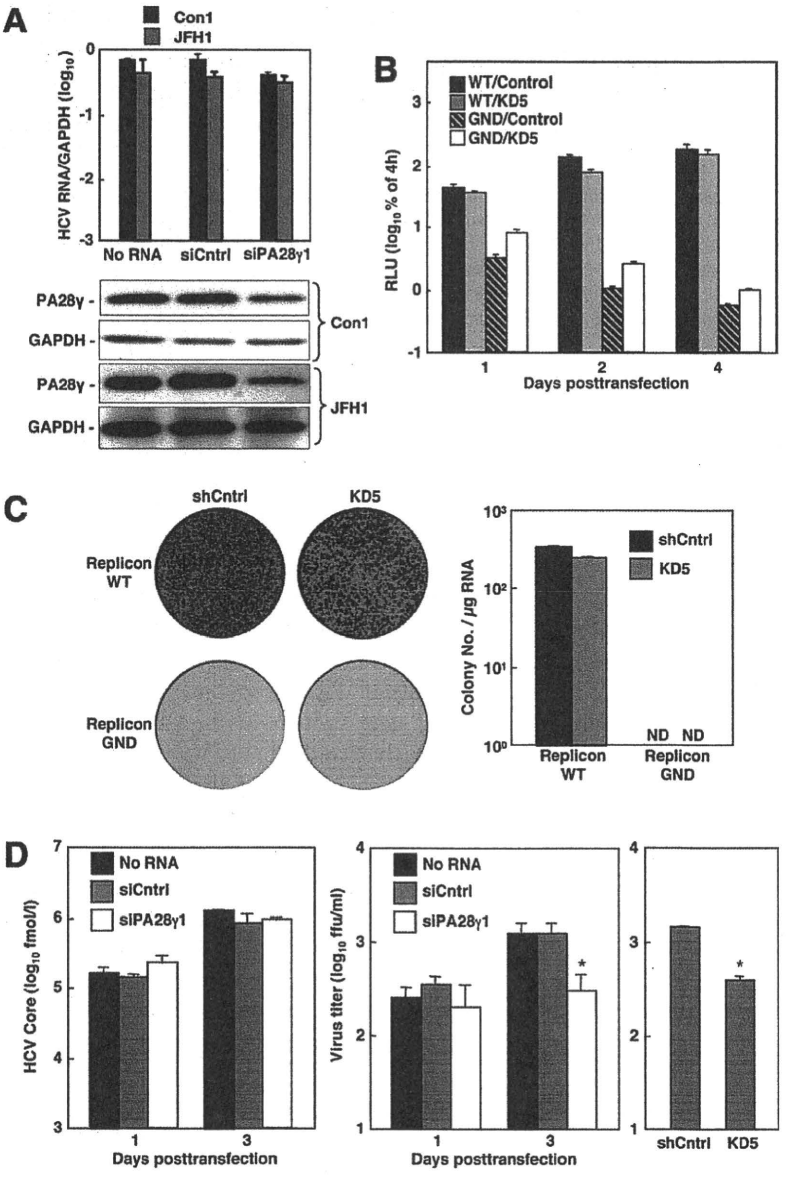

Fig. 3. Effect of PA28γ knockdown on HCV RNA replication. (A) The siCntrl or siPA28γ1 (10 nM) was transfected into the subgenomic HCV replicon cells derived from Con1 and JFH-1 strains. The transfected cells were harvested at 72 hours posttransfection. The replicon RNA was determined by quantitative reverse-transcription polymerase chain reaction at 72 hours posttransfection (upper). PA28γ or glyceraldehyde 3-phosphate dehydrogenase was detected by way of immunoblotting. Cell lysates were subjected to western blotting using antibodies to PA28γ and glyceraldehyde 3-phosphate dehydrogenase (lower). (B) The HCV replicon RNA encoding luciferase gene (WT) or the HCV replicon RNA that has a replication-deficient mutation (GND) was transfected into the shCntrl (Control) and KD5 cell lines. Relative luciferase activity was determined using the activity at 4 hours post-electroporation as a transfection efficiency. (C) Colony formation assay. Replicon RNA encoding the neomycin-resistance gene was transfected into the shCntrl and KD5 cell lines, and the remaining colonies were fixed with 4% paraformaldehyde at 4 weeks posttransfection and then stained with crystal violet. The number of colonies was counted (right). (D) Huh7OK1 cells transfected with 10 μg of *in vitro*-transcribed full-length JFH-1 viral RNA were further transfected with siCntrl or siPA28γ1 at 24 hours posttransfection of viral RNA. The level of HCV core protein in the cells was determined by way of ELISA at 1 and 3 days posttransfection (left). Infectious virus titers in the culture supernatants at 1 and 3 days posttransfection were determined by way of focus-forming assay (middle). Infectious viral titers in the shCntrl or KD5 cells transfected with 10 μg of the infectious viral RNA were determined at 5 days posttransfection (right). $^*P < 0.05$, $^{**}P < 0.01$ versus the control cells or cells transfected with siCntrl. Data are representative of three independent experiments.

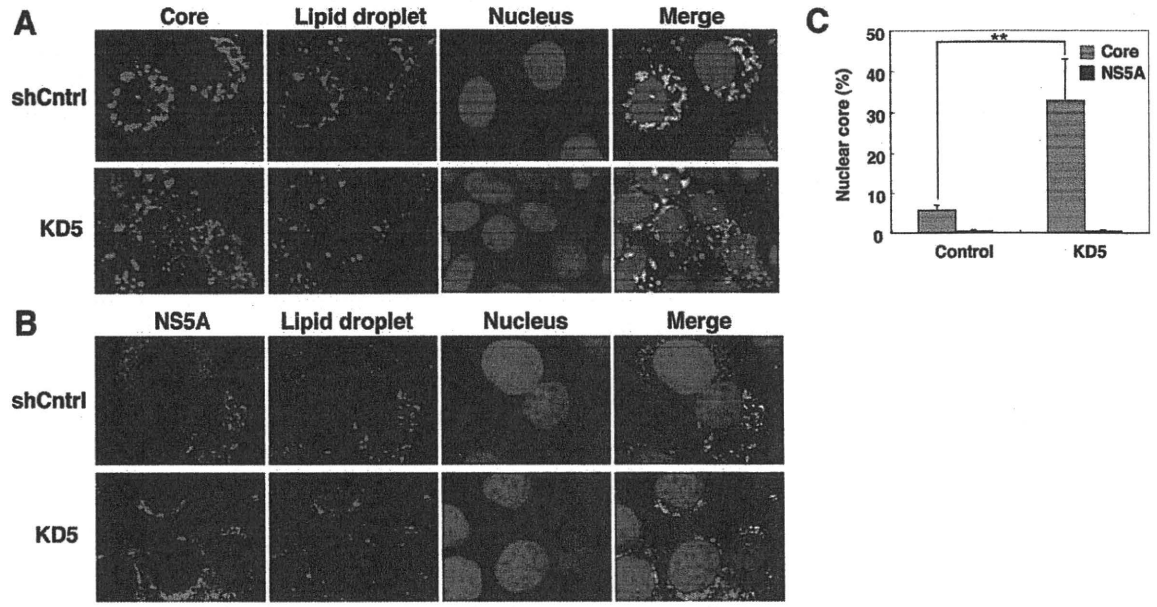

Fig. 4. Effect of PA28γ knockdown on the localization of HCV core protein and lipid droplets. The shCntrl and KD5 cell lines infected with JFH-1 virus were fixed with methanol or paraformaldehyde for 5 minutes at 4 days postinfection. HCV core (A) and NS5A (B) proteins were stained with rabbit antibodies raised against the proteins and Alexa Flour 488-conjugated goat anti-rabbit immunoglobulin G antibody. Lipid droplets were stained with Bodipy 558/568 C12. Nuclei were stained with 50 μM Heachest 33258 after treatment with 1 μg/mL of RNase A. Data are representative of three independent experiments. (C) The percentage of the area occupied by the core protein in nucleus and cytoplasm was calculated using the method described in Materials and Methods. The percentage of the nuclear NS5A to total NS5A was estimated by the same way as the ratio of the core protein. $^{**}P < 0.01$ versus control siRNA-transfected cells.

the PA28γ knockdown condition. These results suggest that PA28γ specifically regulates the postreplication steps in the life cycle of HCV.

Core Protein Is Partially Accumulated in the Nucleus of PA28γ Knockdown Cells. We reported previously that some fraction of HCV core protein migrates into the nucleus and is then degraded by a PA28γ-dependent proteasome pathway.[7] Furthermore, we have demonstrated that HCV core protein is clearly accumulated in the nucleus of the liver cells of PA28γ-knockout mice.[8] However, the role of PA28γ on the intracellular localization of HCV core protein in the infected HCV cells has not been characterized. HCV core protein was chiefly detected in cytoplasm of the control cell line infected with the JFH-1 virus, where it appeared around lipid droplets after staining with Bodipy 558/568 C12 (Fig. 4A, upper panels). In contrast, the core protein was detected not only in the cytoplasm around the surface of lipid droplets, but also in the nucleus in the KD5 cell line (Fig. 4A, lower panels). The NS5A protein was detected in the cytoplasm but not in the nucleus in both the shCntrl and KD5 cell lines (Fig. 4B). The percentage occupied by nuclear core protein to total core protein was increased by about six time levels in the KD5, while the ratio of nuclear NS5A to total NS5A exhibited no difference (Fig. 4C). These results suggest that PA28γ participates in the degradation of HCV core protein in the nucleus.

PA28γ Positively Regulates HCV Propagation by Inhibiting Ubiquitin-Dependent Degradation of Core Protein in Cytoplasm. We reported previously that HCV core protein is degraded by at least two distinct pathways: a ubiquitin-dependent proteasome pathway and a ubiquitin-independent proteasome pathway.[17] The ubiquitin E3 ligase, E6AP, can catalyze ubiquitin ligation of the core protein for ubiquitin-dependent degradation in the cytoplasm,[18] whereas PA28γ participates in the degradation of the core protein through a ubiquitin-independent pathway in the nucleus.[17] We have also demonstrated that PA28γ knockdown leads to enhanced ubiquitination of HCV core protein.[8] However, the interplay between these two pathways in cells infected with HCV has not been determined. To address this point, we examined the effects of knockdown of E6AP or PA28γ on the virus propagation and the ubiquitination of the core protein. JFH-1 virus was inoculated into E6AP- and/or PA28γ knockdown cell lines (Fig. 5A). Transfection of the plasmid encoding shRNA to E6AP into the control cells (shCntrl) increased virus production (Fig. 5A [C-E]) in comparison with that of the

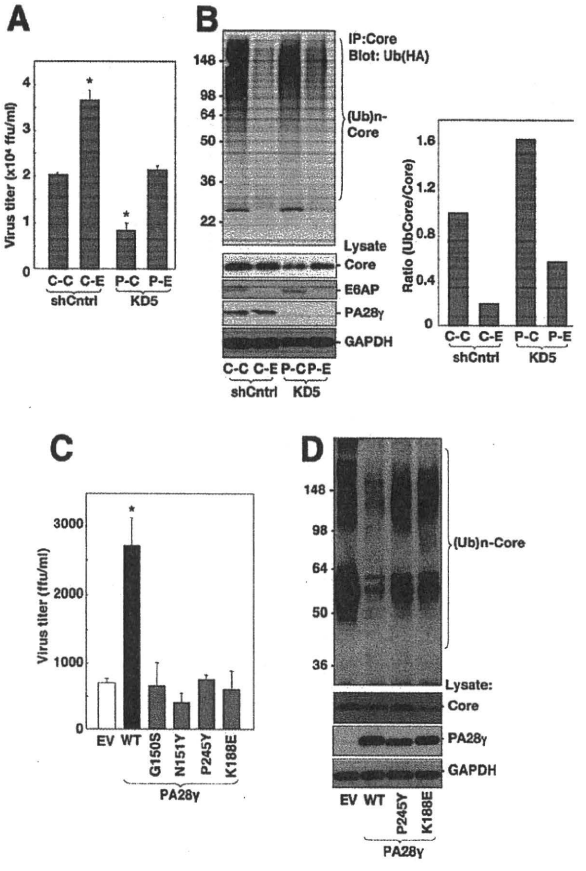

Fig. 5. PA28γ knockdown enhances E6AP-dependent ubiquitination of core protein and reduces virus titer. (A) shCntrl and KD5 cells transfected with plasmids encoding the negative control (C-C and P-C) or E6AP (C-E and P-E) shRNA were treated with puromycin for 2 days. The remaining cells seeded at 2.5×10^4 cells in a 24-well plate were infected with the JFH-1 virus at an moi of 0.05, and infectious virus titers in the supernatants were determined at 72 hours postinfection by way of focus-forming assay. (B) The cells transfected and infected as in (A) were further transfected with a plasmid encoding HA-tagged ubiquitin at 48 hours postinfection. The cells were treated with 10 μM MG132 for 5 hours at 72 hours postinfection and subjected to immunoprecipitation with anti-core monoclonal antibody and immunoblotting with anti-HA antibody. The ratio of ubiquitination of HCV core protein was assessed by the densitometries of the ubiquitinated and unubiquitinated core proteins. (C) KD5 cells transfected with plasmids encoding wild-type or mutant PA28γ were infected with the JFH-1 virus at an moi of 0.05 at 24 hours posttransfection, and the infectious titers in the supernatant were determined at 72 hours postinfection by way of focus-forming assay. (D) KD5 cells transfected with plasmids encoding HCV core protein and HA-tagged ubiquitin, together with wild-type or mutant PA28γ, were treated with 10 μM MG132 for 5 hours at 24 hours posttransfection and subjected to immunoprecipitation with anti-core monoclonal antibody and immunoblotting with anti-HA antibody. EV, empty vector; WT, plasmid encoding wild-type PA28γ. *$P < 0.05$ versus shCntrl or KD5 cells transfected with the negative control or empty vector. Data are representative of three independent experiments.

control cells transfected with the plasmid encoding control shRNA (Fig. 5A [C-C]). Furthermore, the impaired virus production in the PA28γ knockdown cells (KD5) was restored by the transfection of the plasmid encoding shRNA to E6AP (Fig. 5A [P-E]). Cells expressing hemagglutinin (HA)-tagged ubiquitin infected with the JFH-1 virus were immunoprecipitated by the anti-core antibody, and the immunoprecipitates were analyzed by immunoblotting with anti-HA antibody (Fig. 5B). E6AP knockdown decreased the ratio of ubiquitination of HCV core protein, in contrast to the increase of that by PA28γ knockdown (Fig. 5B, lanes C-E and P-C). Furthermore, E6AP knockdown in the PA28γ knockdown cells restored the ubiquitination of the core protein to a certain extent (Fig. 5B, lane P-E). It was shown that Pro^{245} of PA28γ is critical for binding to the 20S proteasome, and that Gly^{150} and Asn^{151} of PA28γ are important for activation of the proteasome.[26] To further examine the functional significance of PA28γ on HCV propagation, expression plasmids encoding siRNA-resistant PA28γ mutants in which Gly^{150}, Asn^{151}, and Pro^{245} were replaced with Ser (G150S), Tyr (N151Y), and Tyr (P245Y), respectively, were transfected into KD5 cells and inoculated with JFH-1 virus at 24 hours posttransfection. The infectious virus titers in the culture supernatant were determined at 3 days postinfection (Fig. 5C). KD5 cells transfected with the plasmid encoding wild-type PA28γ exhibited a partial recovery of virus production, although those transfected with the plasmid encoding PA28γ G150S, N151Y, or P245Y or with an empty vector exhibited no effect on virus production. Replacing Lys^{188} with Glu in PA28γ (PA28γ K188E) confers the capability of proteasome-mediated cleavage after hydrophobic, acidic, and basic residues such as those exhibited by PA28α.[27] Expression of siRNA-resistant PA28γ K188E in KD5 cells could not restore virus production (Fig. 5D). The ubiquitination of HCV core protein was inhibited by expression of the wild-type PA28γ but not expression of the PA28γ mutants (P245Y or K188E) in KD5 cells (Fig. 5D). Collectively, these results suggest that PA28γ positively regulates HCV propagation by inhibiting degradation of HCV core protein by an E6AP/ubiquitin-dependent proteasome.

Discussion

To explore the role of PA28γ on the life cycle of HCV, we examined the effects of knockdown of PA28γ in Huh7OK1 cells infected with the JFH-1 virus. Knockdown of PA28γ in Huh7OK1 cells before or after infection with the JFH-1 virus impaired

production of infectious particles but did not impair viral RNA replication. However, PA28γ knockdown did not affect the production of JEV, of which the capsid protein does not interact with PA28γ, suggesting that PA28γ knockdown does not affect the general sorting pathway of flavivirus. These results suggest that PA28γ is specifically involved in the postreplication steps of HCV life cycle. Our previous report indicated that HCV core protein was accumulated in the nucleus of the hepatocytes of HCV core transgenic/PA28γ knockout mice.[8] PA28γ is located mainly in the nucleus, although a small portion is also located in the cytoplasm[7,28] and can up-regulate trypsin-like proteasome activity, which cleaves after basic amino acid residues.[27] Previous studies have shown that some fraction of HCV core protein is translocated into the nucleus and quickly degraded in the PA28γ-dependent proteasome pathway.[7,8,29] Miyanari et al.[30] demonstrated that the core protein is localized on the surface of lipid droplets and is surrounded by nonstructural proteins, suggesting that HCV particles are assembled near the surface of the lipid droplets. In the present experiments, although HCV core protein was detected on the surface of the lipid droplets in both control and PA28γ knockdown cell lines, it was partially localized in the nucleus in PA28γ knockdown cells but not control cells. Furthermore, localization of HCV core protein on the surface of lipid droplets was impaired in PA28γ knockdown cells (Fig. 4). These results suggest that HCV core protein is partially translocated into the nucleus and degraded in the PA28γ-dependent proteasome pathway in HCV-infected cells and that PA28γ does not directly participate in the particle formation of HCV.

HCV core protein is degraded by at least two proteasome pathways: a ubiquitin-dependent pathway and a ubiquitin-independent and PA28γ-dependent pathway.[17] The E3 ligase E6AP catalyzes ubiquitin ligation to HCV core protein, resulting in enhanced degradation of the core protein in the cytoplasm.[18] Knockdown of E6AP up-regulated virus production in cells infected with the JFH-1 virus,[18] suggesting that E6AP/ubiquitin-dependent degradation of the core protein contributes to an antiviral response. In contrast, knockdown of PA28γ induced up-regulation of the ubiquitination of HCV core protein and down-regulation of the viral production, suggesting that PA28γ-dependent proteasome activity contributes to the proviral response by suppressing E6AP-dependent degradation of the core protein, thereby enhancing viral particle formation. The wild-type PA28γ enhances the trypsin-like activity of proteasome that cleaves peptide bonds after basic residues of the substrates, whereas the PA28γ K188E mutant enhances the proteasome activity that cleaves peptide bonds after hydrophobic, acidic, and basic residues in the manner of PA28α.[27] Therefore, the sizes of fragments produced by the PA28γ-dependent proteasome should be different from those produced by the PA28α/β- or ubiquitination-mediated proteasome. It might be feasible to speculate that the peptide fragments of HCV core protein generated by the PA28γ-dependent proteasome or PA28γ *per se* may be directly or indirectly involved in the suppression of the E6AP-dependent ubiquitination of the core protein. Further studies will be needed to clarify the relationship between E6AP and PA28γ in the degradation and ubiquitination of HCV core protein. Figure 6 shows a schematic diagram of our hypothesis of the regulation of HCV propagation by PA28γ.

HCV core protein was found in not only nuclei but also cytoplasm of the infected KD5 cells (Fig. 4). The down-regulation of virus production should potently reduce a total amount of the core protein in KD5 cells before a clear accumulation of the core protein in nuclei. Furthermore, a small amount of PA28γ was found in the PA28γ knockdown cells, suggesting that E6AP-dependent degradation of HCV core protein is not potently suppressed in the PA28γ knockdown cells. If HCV core protein is constitutively expressed under the PA28γ knockout cells regardless of an amount of infected virus, a clear accumulation of the core protein in nuclei should be found without cytoplasmic expression of the core protein under the PA28γ knockout condition. We reported previously that HCC and liver steatosis in mouse are induced by the HCV core protein in the presence, but not the absence, of PA28γ.[8] Although HCV core protein is predominantly detected in the cytoplasm of the liver cells of $PA28\gamma^{+/+}$ mice,[8,31] HCV core protein was clearly accumulated in the nuclei, but clearly reduced in cytoplasm, of liver cells of $PA28\gamma^{-/-}$ mouse.[8] In addition, ubiquitination of HCV core protein was increased by PA28γ knockdown in the 293T cell line.[8] These results and the data in Fig. 5 suggest that the suppression of PA28γ function enhances the E6AP-dependent degradation of HCV core protein. Hence, the reason there is no difference between $PA28\gamma^{+/+}$ and $PA28\gamma^{-/-}$ mice with respect to the amount of core protein may be due to the competitive regulation of the core protein by E6AP- and PA28γ-dependent degradation mechanisms. E6AP-dependent degradation of HCV core protein in cytoplasm may be enhanced *in vivo* under the PA28γ knockout condition.

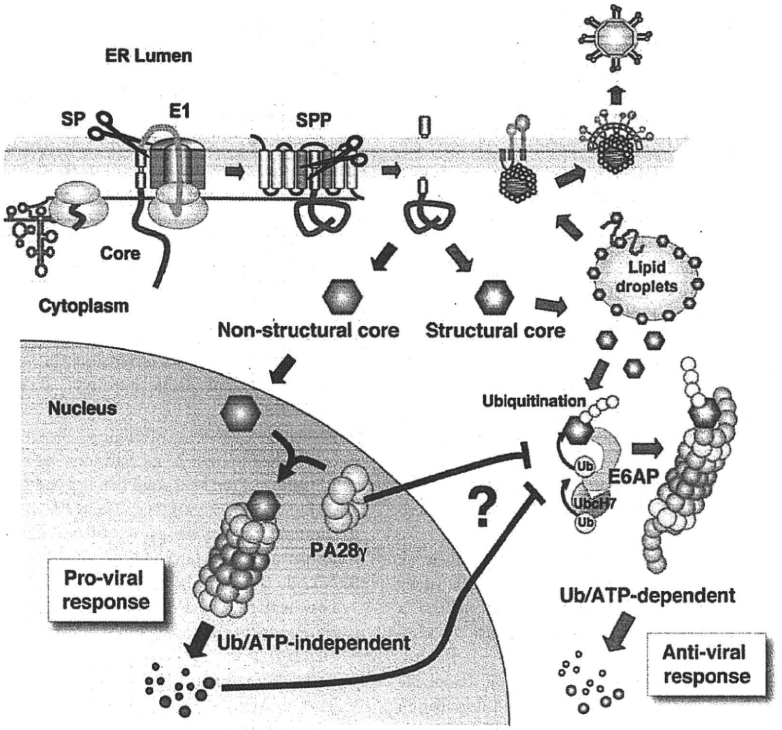

Fig. 6. Schematic diagram of the potential roles of PA28γ in HCV propagation. HCV core protein is cleaved off from the precursor polyprotein by signal peptidase (SP) and the signal sequence is further processed by signal peptide peptidase (SPP). The mature core protein mainly localizes on the lipid droplets close to the endoplasmic reticulum to form a nucleocapsid with the viral RNA genome and is incorporated into virus particles as a structural protein. In addition to the structural protein of HCV, the core protein has characteristics of a nonstructural protein. HCV core protein is degraded through ubiquitin-dependent and ubiquitin-independent proteasome pathways. E6AP catalyzes ubiquitin ligation to HCV core protein and promotes degradation in the cytoplasm, which contributes to the antiviral response. In contrast, the core protein partially migrates into the nucleus and is degraded through a ubiquitin-independent and PA28γ-dependent proteasome pathway, and the core protein fragments generated by the PA28γ pathway or PA28γ *per se* were suggested to participate in the suppression of E6AP-dependent ubiquitination of HCV core protein, which contributes to the proviral response.

In conclusion, in this study we demonstrated that the proteasome activator PA28γ positively regulates particle production of HCV by inhibiting E6AP-dependent ubiquitination of the core protein, in addition to our previous observation that PA28γ plays a crucial role in the development of liver pathology induced by HCV core protein.[8] PA28γ knockout mice exhibit only mild growth retardation.[15,16] Therefore, PA28γ may be a novel and promising antiviral target not only for elimination of HCV from hepatitis C patients but also for intervention in the progression of liver diseases induced by chronic HCV infection.

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Involvement of Ceramide in the Propagation of Japanese Encephalitis Virus▽

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Japanese encephalitis virus (JEV) is a mosquito-borne RNA virus and one of the most important flaviviruses in the medical and veterinary fields. Although cholesterol has been shown to participate in both the entry and replication steps of JEV, the mechanisms of infection, including the cellular receptors of JEV, remain largely unknown. To clarify the infection mechanisms of JEV, we generated pseudotype (JEVpv) and recombinant (JEVrv) vesicular stomatitis viruses bearing the JEV envelope protein. Both JEVpv and JEVrv exhibited high infectivity for the target cells, and JEVrv was able to propagate and form foci as did authentic JEV. Anti-JEV envelope antibodies neutralized infection of the viruses. Treatment of cells with inhibitors for vacuolar ATPase and clathrin-mediated endocytosis reduced the infectivity of JEVpv, suggesting that JEVpv enters cells via pH- and clathrin-dependent endocytic pathways. Although treatment of the particles of JEVpv, JEVrv, and JEV with cholesterol drastically reduced the infectivity as previously reported, depletion of cholesterol from the particles by treatment with methyl β-cyclodextrin enhanced infectivity. Furthermore, treatment of cells with sphingomyelinase (SMase), which hydrolyzes membrane-bound sphingomyelin to ceramide, drastically enhanced infection with JEVpv and propagation of JEVrv, and these enhancements were inhibited by treatment with an SMase inhibitor or C_6-ceramide. These results suggest that ceramide plays crucial roles in not only entry but also egress processes of JEV, and they should assist in the clarification of JEV propagation and the development of novel therapeutics against diseases caused by infection with flaviviruses.

Japanese encephalitis virus (JEV) is a small, enveloped virus belonging to the family *Flaviviridae* and the genus *Flavivirus*, which also includes *Dengue virus* (DENV), *West Nile virus* (WNV), *Yellow fever virus*, and *Tick-borne encephalitis virus* (11). JEV is the most common agent of viral encephalitis, causing approximately 50,000 cases annually, of which 15,000 will die, and up to 50% of survivors are left with severe residual neurological complications. JEV has a single-stranded positive-sense RNA genome of approximately 11 kb, encoding a single large polyprotein, which is cleaved by the host- and virus-encoded proteases into three structural proteins, capsid (C), premembrane (PrM), and envelope (E), and seven nonstructural proteins. The structural proteins are components of viral particles, and the E protein is suggested to interact with a cell surface receptor molecule(s). Although a number of cellular components, including heat shock cognate protein 70 (33), glycosaminoglycans, such as heparin or heparan sulfate (21, 41), and laminin (3), have been shown to participate in JEV infection, the precise mechanisms by which these receptor candidates participate in JEV infection remain largely unclear.

In addition to the many studies identifying and characterizing receptor molecules in numerous viruses, data suggesting the involvement of membrane lipids, such as sphingolipids and cholesterol, in viral infection have also been accumulating. Lipid rafts consisting of sphingolipids and cholesterol and distributing to the outer leaflet of the cell membrane have been shown to be involved in the infection of not only many viruses but also several bacteria and parasites (24), in addition to playing roles in various functions such as lipid sorting, protein trafficking (26, 47), cell polarity, and signal transduction (38). With respect to cholesterol itself, various aspects of the life cycle of flaviviruses have been shown to involve this lipid, including the entry of DENV (34), hepatitis C virus (HCV) (16), and WNV (27), the membrane fusion of tick-borne encephalitis virus (40), and the replication of HCV (14, 17), WNV (23), and DENV (35). Recently Lee et al. (20) showed that treatment with cholesterol efficiently impairs both the entry and replication steps of JEV and DENV-2 but enhances infection with the Sindbis virus (22).

On the other hand, sphingolipids, including sphingomyelins and glycosphingolipids, are ubiquitous components of eukaryotic cell membrane structures, providing integrity to cellular membranes. Ceramide is one of the intermediates of sphingolipids and plays roles in cell differentiation, regulation of apoptosis and protein secretion, induction of cellular senescence, and other processes (2). Ceramide is generated from the hydrolysis of sphingomyelin by sphingomyelinase (SMase) or from catalysis by serine-palmitoyl-coenzyme A (CoA) transferase and ceramide synthase. Ceramide spontaneously self-associates to form ceramide-enriched microdomains and then to form larger ceramide-enriched membrane platforms which serve as the spatial and temporal organization for cellular signalosomes and for regulation of protein functions (2). The ceramide-enriched platforms have also been used by many pathogens to facilitate entry and infection (2). The acid SMase is activated not only by multiple stimuli, including receptor molecules, gamma irradiation, and some chemicals, but also by infection with some bacteria or viruses (36). Rhinovirus activates the SMase for generation of ceramide and forms ceramide-enriched membrane platforms that serve in the infection of

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target cells (10). Sindbis virus also activates the SMase and induces apoptosis through a continuous release of ceramide (15). In contrast to these viruses, ceramide inhibits infection with HIV (7) and HCV (48). Ceramide enrichment of the plasma membrane reduces expression of HCV receptor molecules through an ATP-independent internalization and impairs entry of HCV.

Pseudotype and recombinant viruses based on the vesicular stomatitis virus (VSV) bearing foreign viral envelope proteins have been shown to be powerful tools for the investigation of viral entry and the development of vaccines. These systems have been used to study infection with viruses that do not propagate readily (31, 43) or that are difficult to handle due to their high-level pathogenicity for humans (42). In addition, the systems allow us to focus on the investigation of entry mechanisms of particular viral envelope proteins by using control viruses harboring an appropriate protein on identical particles.

In the present study, we generated pseudotype (JEVpv) and recombinant (JEVrv) VSVs bearing the JEV envelope protein in human cell lines and determined the involvement of sphingolipids, especially ceramide, and cholesterol in infection of human cell lines with JEV. Both JEVpv and JEVrv exhibited infection of target cells via pH- and clathrin-dependent endocytosis. Treatment of cells with cholesterol impaired infection with JEVpv and JEVrv, as previously found in JEV infection (20). In contrast, treatment of cells with SMase drastically enhanced infection with both JEVpv and JEVrv and the production of infectious JEVrv particles. These results indicate that ceramide plays crucial roles in the entry and egress of JEV.

MATERIALS AND METHODS

Plasmids and cells. A cDNA clone encoding the PrM and E proteins of the AT31 strain was generated by PCR amplification, cloned into pCAGGS/MCS-PM (43), and designated pCAGC105E (JEV). The plasmid used for construction of JEVrv was pVSVΔG-GFP2.6 (provided by M. A. Whitt, University of Tennessee), which has additional transcription units with multicloning sites (MCS) and green fluorescent protein (GFP) located between the M and L genes. The PrM-E gene, obtained from pCAGC105E (JEV) by digestion with BglII and EcoRI, was cloned into the SmaI site of pVSVΔG-GFP2.6 after blunting, and the construct was designated pΔG-JEV (PrM-E). Huh7, BHK, Vero, and 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) containing 10% fetal bovine serum (FBS).

Viruses and chemicals. Wild-type JEV was used as described previously (29). The virus was amplified on Huh7 cells and stored at −80°C. The infectious titer was determined by using a focus-forming assay as described below. Bafilomycin A_1 from *Streptomyces griseus* was purchased from Fluka (Sigma). Chlorpromazine hydrochloride, sphingomyelinase (SMase), phospholipase C from *Bacillus cereus*, methyl-β-cyclodextrin (MβCD), a water-soluble cholesterol, and amitriptyline hydrochloride were obtained from Sigma. C_6-ceramide and sphingomyelin were purchased from Biomol International (Plymouth Meeting, PA). Biotin-ceramide was purchased from Echelon Biosciences Inc. (Salt Lake City, UT).

Reverse genetics of VSV. Recombinant VSVs were generated by a previously described method (43) with minor modifications. Briefly, BHK cells were grown to 90% confluence on 35-mm tissue culture plates and infected with a recombinant vaccinia virus encoding T7 RNA polymerase at a multiplicity of infection (MOI) of 5. After incubation at room temperature for 1 h, the cells were transfected with 4 μg of mixed plasmids encoding each component of VSV proteins (pBS-N/pBS-P/pBS-L/pBS-G, 3:5:1:8) and 2 μg of pΔG-Luci or pΔG-JEV (PrM-E) plasmid using the *Trans*IT-LT1 transfection reagent (Mirus, Madison, WI). After 48 h of incubation, the supernatants were passed through a filter with a pore size of 0.22 μm (Milex-GS; Millipore, Tokyo, Japan) to remove vaccinia virus and inoculated into 293T cells that had been transfected with pCAGVSVG (25) 24 h previously. Recovery of progeny virus was assessed by the appearance of cytopathic effects at 24 to 36 h postinfection. VSV G-complemented (*G) recombinant viruses were stored at −80°C. The infectious titers of the recovered viruses were determined by a plaque assay.

Production and characterization of JEVpv, JEVrv, and JEV. To generate JEVpv, Huh7 cells transiently expressing the PrM and E proteins by the transfection with pCAGC105E using *Trans*IT-LT1 (Mirus) were infected with VSVΔG/Luc-*G, in which the G gene was replaced with the luciferase gene and was pseudotyped with the G protein, at an MOI of 0.1. The virus was adsorbed for 2 h at 37°C and then extensively washed four times with serum-free DMEM. After 24 h of incubation at 37°C with 10% FBS-DMEM, the culture supernatants were centrifuged to remove cell debris and stored at −80°C. To generate JEVrv, Huh7 cells were infected with VSVΔG/JEV-*G at an MOI of 5 for 2 h at 37°C and then extensively washed four times with serum-free DMEM. After 24 h of incubation at 37°C with 10% FBS-DMEM, the culture supernatants were collected and stored at −80°C. Schematic representations of the genome structures and the production of recombinant and pseudotype VSVs are shown in Fig. 1. The purification and concentration of the pseudotype or recombinant viruses were conducted as described previously (43). Purified viruses and infected cell lysates were analyzed by immunoblotting to detect the incorporation of the envelope protein with anti-JEV E mouse polyclonal antibody (E#2-1; unpublished). The infectivities of JEVpv, JEVrv, and JEV were assessed by both luciferase activity and a focus-forming assay, as described below. The relative light unit (RLU) value of luciferase was determined by using the Bright-Glo luciferase assay system (Promega Corporation, Madison, WI), following a protocol provided by the manufacturer. To examine the effects of oligosaccharide modification of the JEV E protein in cells or on the particles, the cell lysates and the purified particles were digested with endoglycosidase H (Endo H) or peptide-N-glycosidase F (PNGase F) (Boehringer Mannheim, Mannheim, Germany), following a protocol provided by the manufacturer, and analyzed by immunoblotting.

Pseudotype VSVs bearing HCVE1E2 (HCVpv), VSVG (VSVpv), and murine leukemia virus envelope (MLVpv) proteins were produced in 293T cells transfected with pCAGc60-p7 (H77), pCAGVSVG, and pFBASALF (provided by T. Miyazawa, Kyoto University), respectively, and used as controls. Recombinant HCV (HCVrv) was also used as a control as described previously (43). To neutralize infection with JEVpv, JEVrv, and JEV, viruses were preincubated with the indicated dilution of anti-JEV E monoclonal antibody (22A1; provided by E. Konishi, Kobe University) for 1 h at 37°C and then inoculated into Huh7 cells. After 1 h of adsorption, the cells were washed three times with DMEM containing 10% FBS, and infectivity was determined after 24 h of incubation at 37°C.

Focus-forming assays. Cells infected with JEV, VSV, JEVrv, or HCVrv after treatment with the indicated reagents were cultured at 37°C with 0.8% methylcellulose in 10% FBS-DMEM for 24 or 48 h and fixed with 4% paraformaldehyde solution for 1 h. Cells were washed once with phosphate-buffered saline (PBS), treated with 0.5% Triton X-100 for 20 min for permeabilization, incubated with mouse monoclonal antibody to JEV (MsX Japanese encephalitis; Chemicon International Inc., Temecula, CA) for JEV or that to VSV N (10G4; provided by M. A. Whitt) for VSV, JEVrv, and HCVrv for 1 h, and stained by using a Vectastain Elite ABC anti-mouse IgG kit with a VIP substrate (Vector Laboratories, Burlingame, CA), following a protocol provided by the manufacturer.

Effects of chemicals on the infectivities of JEVpv, JEVrv, and JEV. To examine the entry pathways of the viruses, cells treated with various concentrations of bafilomycin A_1, chlorpromazine, MβCD, SMase, phospholipase C, or amitriptyline for 1 h at 37°C were inoculated with JEVpv, HCVpv, VSVpv, or MLVpv, and infectivity was determined by luciferase activity as described above. To examine the effects of cholesterol or SMase on the viral particles, purified virions incubated with various concentrations of water-soluble cholesterol or SMase for 1 h at 37°C were inoculated into the target cells. Viruses treated with SMase were ultracentrifuged (43) and resuspended in culture media to deplete any residual amount of SMase, and infectivity was determined by luciferase or focus-forming assay. To examine the effects of ceramide on the infection, 10 mM C_6-ceramide or sphingomyelin dissolved in ethanol was diluted with medium at various concentrations and preincubated with JEVpv, HCVpv, VSVpv, or MLVpv for 1 h at 37°C. After treatment, the viruses were inoculated into Huh7 cells, washed with medium after 1 h of incubation at 37°C, and cultured for 24 h at 37°C, and the residual infectivity was determined by measuring luciferase activity. The effects of C_6-ceramide on the infection of JEV were assessed by following the same protocol, and the residual infectivity was determined by focus-forming assay.

Ceramide binding assay. To examine the interaction of JEV E protein and ceramide, purified viruses were incubated with 500 μl of lysis buffer (20 mM Tris-HCl, pH 7.4, containing 135 mM NaCl and 1% Triton X-100) supplemented

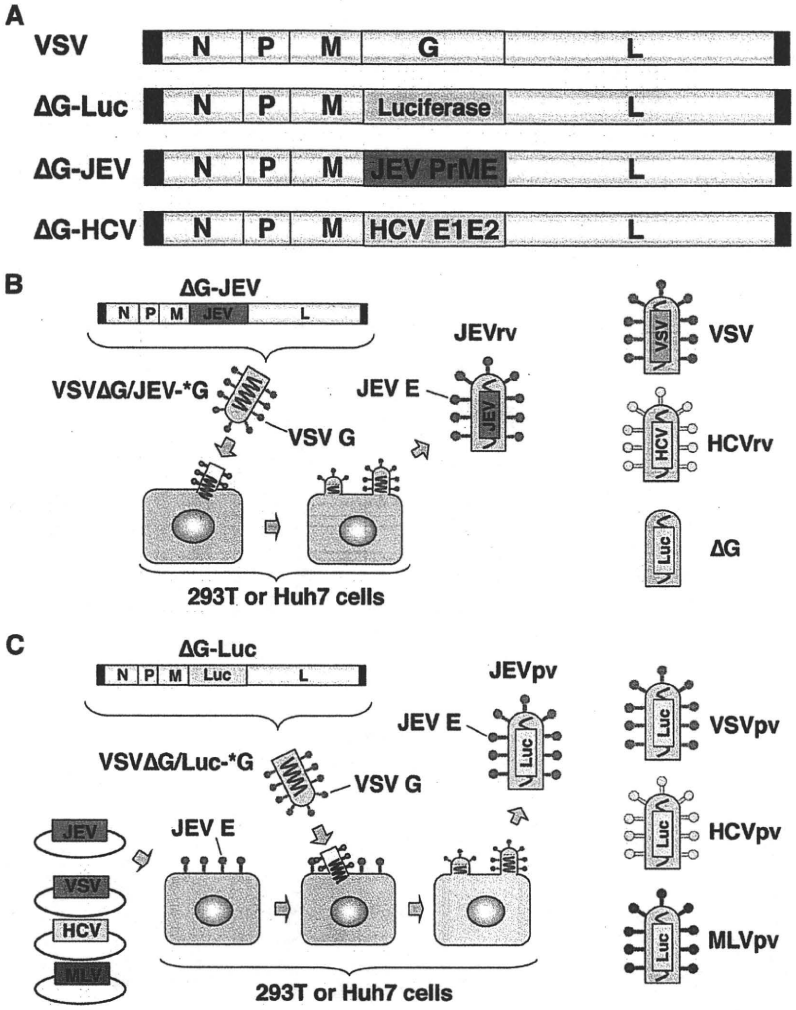

FIG. 1. Schematic representation of the genome structures and production of recombinant and pseudotype VSVs. (A) The luciferase, PrME, and E1E2 genes were inserted into the full-length cDNA clone of VSV in place of the G gene and designated ΔG-Luc, ΔG-JEV, and ΔG-HCV, respectively. (B) Recombinant VSVs, JEVrv, HCVrv, and ΔG, bearing the JEV E protein, HCV E1/E2 proteins, and no envelope, respectively, were generated in 293T or Huh7 cells by infection with the respective recombinant VSV after complementation with VSV G protein (*G). (C) Pseudotype VSVs, JEVpv, VSVpv, HCVpv, and MLVpv, were generated by infection with VSVΔG/Luc-*G in 293T or Huh7 cells transiently expressing the respective foreign protein.

with protease inhibitor cocktail (Roche, Indianapolis, IN) and 10 μl of 1-mg/ml biotin-ceramide in dimethyl sulfoxide (DMSO) for 1 h at 37°C, and then 20 μl of streptavidin-Sepharose 4B (Zymed, Invitrogen, Carlsbad, CA) was added and the solution was kept at 4°C for 4 h. After washing with the lysis buffer three times, the pellets were analyzed by immunoblotting with anti-JEV E polyclonal antibody (E#2-1).

RESULTS

Construction and characterization of recombinant and pseudotype VSVs. Recombinant VSVs were propagated in Huh7 cells by infection with VSVG-complemented (*G) recombinant VSVs possessing foreign genes of either JEV PrM/E, HCV E1/E2, or luciferase in place of the VSV G gene, as shown in Fig. 1A and B. The pseudotype VSVs, JEVpv, VSVpv, HCVpv, and MLVpv, were generated by infection with VSVΔG/Luc-*G in 293T or Huh7 cells transiently expressing the respective foreign protein (Fig. 1C).

To examine the properties of the JEV E proteins incorporated into JEV, JEVrv, and JEVpv particles, the E proteins expressed in Huh7 cells and incorporated into the viral particles were digested with Endo H or PNGase F and examined by immunoblotting (Fig. 2A). Although E proteins in the lysates of cells infected with JEV, JEVrv, or JEVpv were sensitive to both Endo H and PNGase F treatments, those incorporated into the viral particles were resistant to Endo H, suggesting that both JEVrv and JEVpv particles selectively incorporate the matured E proteins modified to the complex- or hybrid-type glycans as seen in the authentic JEV particles. Next, to examine the infectivity of JEVrv and JEVpv for the target cells, HCVpv, MLVpv, VSVpv, VSV, HCVrv, and ΔG were prepared as controls (Fig. 2B). Both JEVpv and JEVrv were infectious for Huh7, BHK, and Vero cells, whereas HCVpv and HCVrv were infectious for Huh7 cells but not for BHK

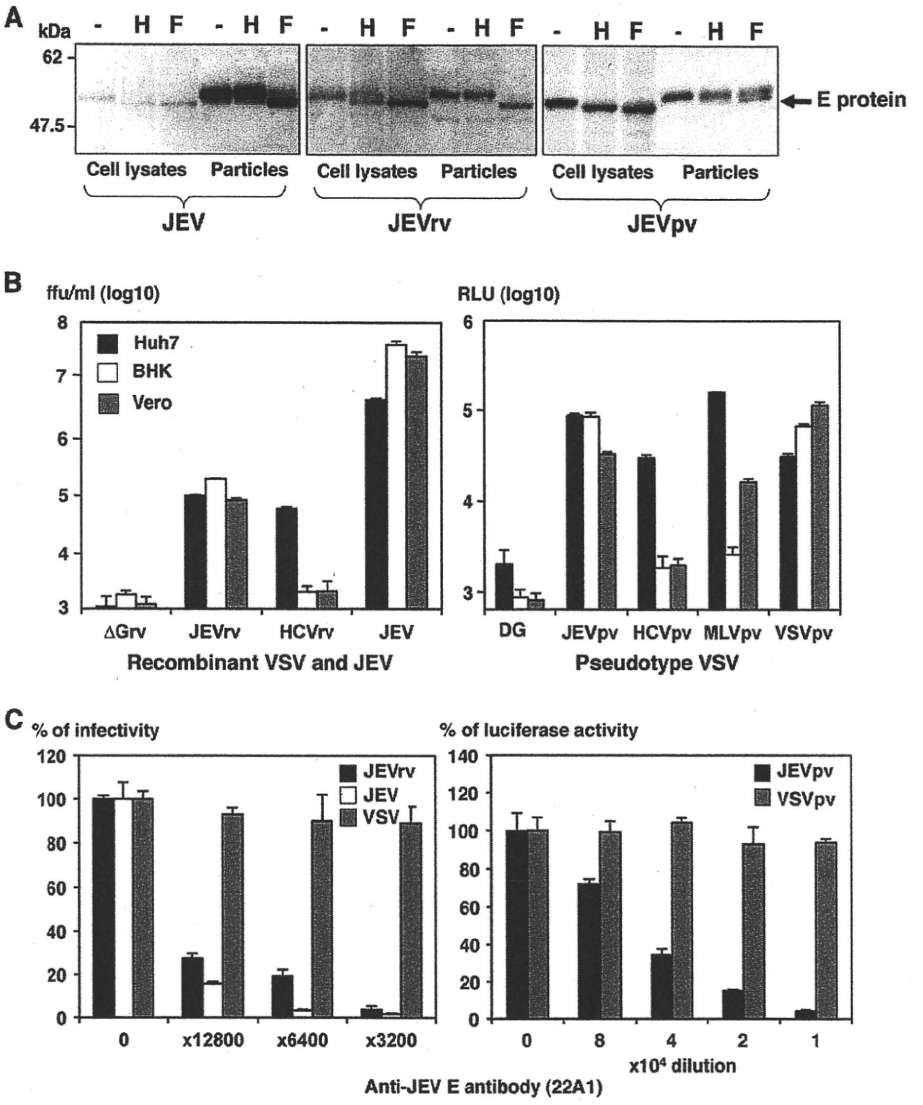

FIG. 2. Characterization of JEVrv and JEVpv. (A) JEV E proteins expressed in cells incorporated into the viral particles were treated with endoglycosidase H (H) or peptide-N-glycosidase F (F) and examined by immunoblotting using anti-E polyclonal antibody. "-" indicates an untreated sample. (B) Infectivities of recombinant viruses (left panel) and pseudotype viruses (right panel) were determined in Huh7, BHK, and Vero cells by a focus-forming assay and measurement of luciferase activity (RLU), respectively. VSV without envelope (ΔG) was used as a negative control. ffu, focus-forming units. (C) Neutralization of JEVrv (left panel) or JEVpv (right panel) infection by anti-E polyclonal antibody. Viruses were incubated with the indicated dilution of antibody for 1 h at room temperature and inoculated into Huh7 cells. Residual infectivities are expressed as percentages. VSV and VSVpv were used as controls. The results shown are from three independent assays, with error bars representing standard deviations.

and Vero cells, as previously reported (1). Although JEVpv and JEVrv generated in 293T cells were also infectious, these viruses were slightly more infective when generated in Huh7 cells, even though the efficiency of transfection of the expression plasmids into 293T cells was higher than that of transfection into Huh7 cells (data not shown). To determine the specificity of infection of JEVpv, JEVrv, and JEV, a neutralization assay was performed by using anti-E antibody (22A1). The infectivities of JEVpv and JEVrv but not of VSVpv and VSV for Huh7 cells were clearly inhibited by anti-E antibody in a dose-dependent manner (Fig. 2C). These results suggest that the JEVrv and JEVpv generated in this study had characteristics comparable to those of authentic JEV.

Entry pathways of JEVpv. Previous studies showed that JEV infection was inhibited by treatment with inhibitors of vacuolar acidification, such as ammonium chloride, concanamycin A, and bafilomycin A_1, suggesting that JEV enters target cells via pH-dependent endocytosis (30). Other flaviviruses, including WNV, DENV, and HCV, exhibit similar entry mechanisms (18, 45). To compare the entry pathway of JEV with those of other viruses, Huh7 cells were pretreated with various concentrations of bafilomycin A_1 and then the cells were inoculated

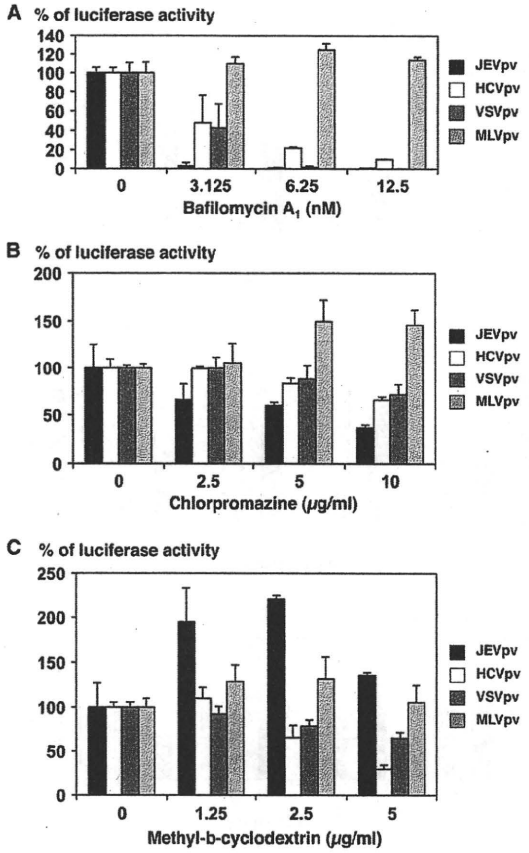

FIG. 3. Entry pathways of the pseudotype VSVs. Huh7 cells were pretreated with various concentrations of bafilomycin A_1 (A), chlorpromazine (B), or methyl-β-cyclodextrin (C) for 1 h and inoculated with the pseudotype viruses, JEVpv, HCVpv, VSVpv, and MLVpv. Luciferase activities were determined at 24 h postinfection. The results shown are from three independent assays, with error bars representing standard deviations.

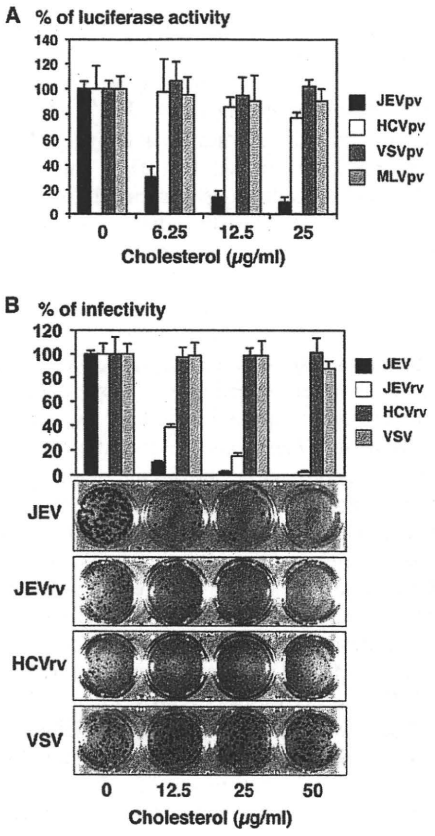

FIG. 4. Effects of cholesterol on infection with recombinant and pseudotype VSVs. (A) The pseudotype viruses were incubated with various concentrations of cholesterol for 1 h at room temperature and inoculated into Huh7 cells, and luciferase activities were determined at 24 h postinfection. (B) JEV, JEVrv, HCVrv, and VSV were incubated with various concentrations of cholesterol for 1 h at room temperature and inoculated into Huh7 cells, and residual infectivities were determined by focus-forming assay in a culture medium containing 1% methylcellulose at 48 h postinfection for JEV, JEVrv, and HCVrv and at 24 h postinfection for VSV. Foci of infected cells were detected by immunohistochemical staining (lower panel). The rate of focus formation of the viruses was analyzed by counting foci. The results shown are from three independent assays, with error bars representing standard deviations.

with JEVpv, HCVpv, VSVpv, and MLVpv (Fig. 3A). As expected, bafilomycin A_1 treatment did not affect the infectivity of MLVpv-bearing envelope proteins of MLV, which enters cells through a pH-independent direct fusion of the viral membrane and plasma membrane. In contrast, infections with HCVpv and VSVpv, which enter cells through pH-dependent endocytosis, were inhibited by treatment with bafilomycin A_1 in a dose-dependent manner. Similarly, infection with JEVpv was clearly inhibited by treatment with bafilomycin A_1 in a dose-dependent manner, suggesting that JEVpv enters cells through pH-dependent endocytosis, as seen in JEV infection.

To further examine the entry pathway of JEVpv, Huh7 cells were pretreated with various concentrations of chlorpromazine, an inhibitor of clathrin-mediated endocytosis, or MβCD, an inhibitor of caveolar/raft-mediated endocytosis, and infected with the pseudotype viruses. The infectivity of MLVpv was not affected by the treatment with either chlorpromazine or MβCD, as we expected. Treatment of cells with chlorpromazine slightly reduced the infectivity of JEVpv, HCVpv, and VSVpv in a dose-dependent manner (Fig. 3B), whereas treatment of cells with MβCD reduced the infectivity of HCVpv and VSVpv but increased the infectivity of JEVpv (Fig. 3C). These results suggest that JEVpv enters cells via clathrin-mediated endocytosis, as previously reported for infection with JEV (30), and that caveola/raft plays a different role in the entry of JEV than in the entry of HCV and VSV.

Effects of cholesterol on the entry and egress of JEV. Recently it was shown that entry of flaviviruses, including JEV and DENV, was drastically inhibited by treatment of the particles with cholesterol (20). To examine the effect of cholesterol on entry of JEV, the pseudotype viruses were inoculated into Huh7 cells after treatment with various concentrations of cholesterol. The infectivity of JEVpv but not that of HCVpv, VSVpv, or MLVpv was severely impaired by treatment with cholesterol in a dose-dependent manner (Fig. 4A). Next, to examine the effect of cholesterol on the propagation of JEV, the recombinant viruses were inoculated into Huh7 cells after

treatment with various concentrations of cholesterol. Infectivities of JEV and JEVrv but not those of VSV and HCVrv were inhibited by the treatment with cholesterol (Fig. 4B, upper panel). Suppression of the propagation of JEV and JEVrv was further confirmed by a focus-forming assay (Fig. 4B, lower panels). These results confirmed that JEV entry was suppressed by cholesterol, as previously reported (20), and raise the possibility that cholesterol participates not only in entry via the E protein but also in the assembly of the E protein. These data also support the notion that JEVpv and JEVrv are comparable to JEV in terms of the properties of the E protein involved in the entry and egress processes.

Effects of SMase on infection with JEVpv, JEVrv, and JEV. Because infection with enveloped viruses was initiated by the interaction of viral and host membrane lipids, we next examined the involvement of membrane lipids in the entry of JEV. Sphingolipid is a major component of eukaryotic lipid membranes, and sphingomyelin is one of the most abundant sphingolipids, with a wide presence across the cell membrane. SMase is known to cleave sphingomyelin, yielding phosphorylcholine and ceramide. To examine the effect of SMase on viral infection, cells were infected with viruses after treatment with various concentrations of SMase, and the infectivities of the viruses were assessed by the luciferase or focus-forming assay. Infection with JEVpv was drastically enhanced by SMase treatment of Huh7 cells, whereas such treatment exhibited no effect on infection with VSVpv and MLVpv and suppressed HCVpv infection (Fig. 5A). The enhancement of JEVpv infection by SMase treatment was also observed in other cell lines, including BHK and Vero cells (data not shown). Although the effect was not as evident as in JEVpv infection, SMase treatment exhibited a slight but substantial enhancement of the infectivity of JEV and JEVrv in Huh7 cells, in contrast to having no effect on VSV infection and a suppressive effect on HCVrv infection (Fig. 5B). The difference in the magnitude of enhancement of infectivity by treatment with SMase between infection with JEVpv and that with JEV or JEVrv might be attributable to the difference in the viral systems based on pseudotype (JEVpv) and replication-competent (JEV and JEVrv) viruses, which allow single and multiple rounds of infection, respectively. The effects of SMase may be more critical for the entry step than for other, later steps of infection. Suppression of HCVpv and HCVrv infection by treatment with SMase was consistent with previously reported data on infection of HCV-pseudotyped retroviral particles (HCVpp) and JFH1 virus (48).

Next, we examined the effect of SMase on the viral particles. Treatment of pseudotype particles of JEVpv, VSVpv, and MLVpv with various concentrations of SMase had no significant effect on their infectivity for Huh7 cells (Fig. 5C), whereas the infectivity of HCVpv particles was impaired by the treatment in a dose-dependent manner, as reported previously (1), suggesting that SMase treatment enhances the infectivity of JEVpv by modifying the molecules on target cells rather than the molecules on viral particles. To further determine the involvement of SMase in infection of JEVpv, cells were pretreated with various concentrations of amitriptyline, an inhibitor of acid SMase. The infectivity of JEVpv but not that of other viruses was decreased by the treatment with amitriptyline in a dose-dependent manner (Fig. 5D). A similar effect was observed with treatment with another SMase inhibitor, imipramine (data not shown). Collectively, these results suggest that entry of JEV into the target cells is enhanced by SMase treatment, which modifies the cell surface sphingolipids into a more competent state for interaction with the JEV envelope protein, thereby enabling its entry.

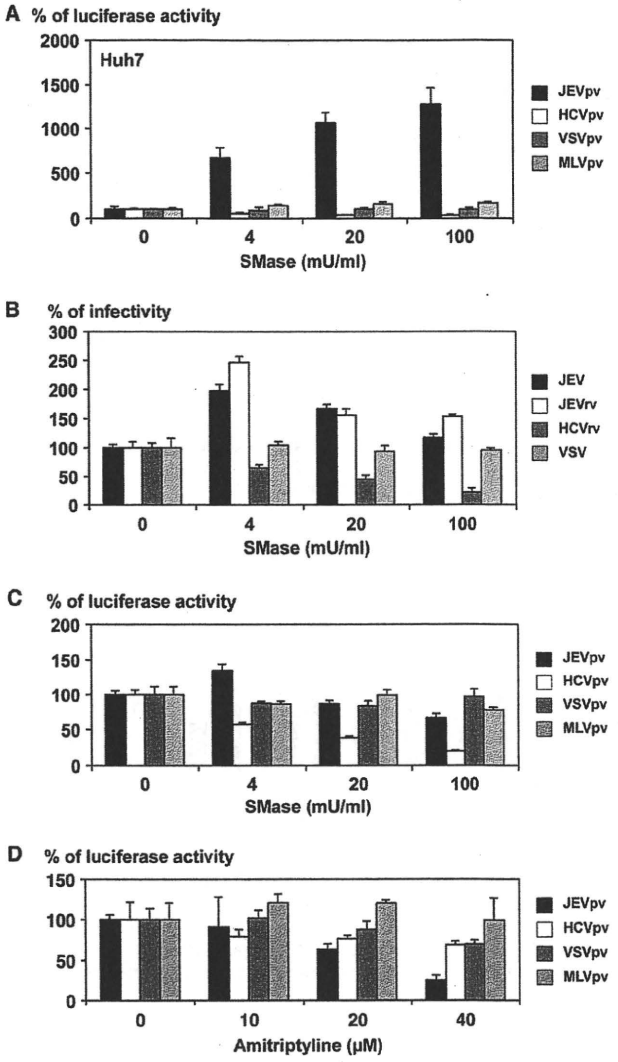

FIG. 5. Effects of SMase and amitriptyline treatment of cells on infection with pseudotype and recombinant VSVs. Huh7 cells were pretreated with various concentrations of SMase for 1 h, and then pseudotype viruses (A) or recombinant viruses (B) were inoculated. The infectivities were determined by luciferase activity measurement or focus-forming assay, and changes in infectivities are expressed as percentages. (C) The purified pseudotype particles were treated with various concentrations of SMase for 1 h and inoculated into Huh7 cells after removal of SMase by ultracentrifugation. Infectivities were determined at 24 h postinfection by measuring luciferase activity, and changes in infectivities are expressed as percentages. (D) Huh7 cells were pretreated with various concentrations of amitriptyline, an inhibitor for the acid SMase, for 1 h, and then pseudotype viruses were inoculated. Infectivities were determined at 24 h postinfection by measuring luciferase activity, and changes in infectivities are expressed as percentages. The results shown are from three independent assays, with error bars representing standard deviations.

Effects of SMase on propagation of JEVrv and JEV. We next examined the effects of SMase on the propagation of JEV. Recombinant VSV is capable of replicating by using the VSV genome and producing infectious particles bearing a foreign envelope protein encoded in place of the original G protein, and thus, it is feasible to assess the efficiency of not only entry but also egress of the recombinant viruses possessing foreign envelope genes of different origins, irrespective of their replication efficiency within the target cells. To examine the effects of SMase on viral propagation, cells were treated with various concentrations of the enzyme, inoculated with the recombinant viruses, and then cultured for up to 48 h in the presence of SMase. Production of JEVrv was dramatically enhanced by cultivation in the presence of SMase, in contrast to the suppression of HCVrv propagation (Fig. 6A). Although the effect of SMase treatment on the production of JEV was not as great as that seen in JEVrv propagation, treatment with SMase resulted in a substantial enhancement of JEV but not of VSV propagation (Fig. 6B). These results suggest that SMase treatment induces robust propagation of JEVrv mainly through enhancement of the entry step although also partly through enhancement of the egress step.

Involvement of ceramide in infection with JEV. Because treatment of cells with SMase induces production of ceramide, we next examined the effect of ceramide on the infectivity of the viruses. Treatment of the pseudotype particles with C_6-ceramide inhibited the infectivity of JEVpv for Huh7 cells in a dose-dependent manner, whereas no clear reduction of infectivity was observed with treatment of HCVpv, VSVpv, and MLVpv with ceramide (Fig. 7A). In contrast, treatment of the pseudotype particles with sphingomyelin, which is a substrate for SMase and is catalyzed into ceramide, did not affect the infectivity of the viruses, suggesting that the enhancement of infectivity of JEVpv by treatment with SMase was due to the generation of ceramide. Propagation of JEV but not of VSV was also suppressed by treatment of the viral particles with C_6-ceramide in a dose-dependent manner (Fig. 7B). Finally, to confirm the interaction of the JEV E protein with ceramide, purified JEV and JEVrv particles were incubated with biotin-ceramide and streptavidin-Sepharose 4B and examined by pull-down assay (Fig. 7C). The E proteins of both JEV and JEVrv were precipitated with the ceramide beads. These results indicate that the interaction of the JEV E protein with ceramide plays a crucial role in the entry of JEV.

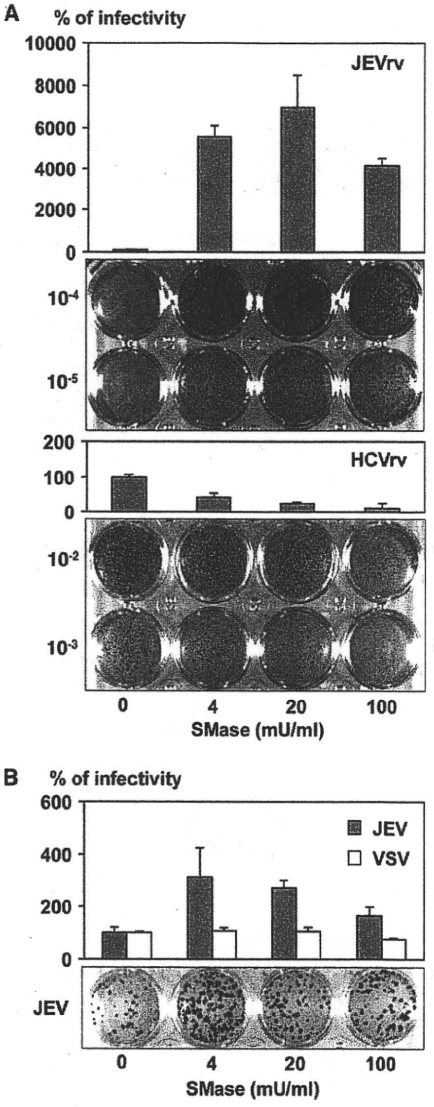

FIG. 6. Effects of SMase on the propagation of JEVrv and JEV. Huh7 cells were pretreated with various concentrations of SMase for 1 h and inoculated with JEVrv or HCVrv (A) or JEV or VSV (B), and infectivities were determined by focus-forming assay in a culture medium containing 1% methylcellulose at 48 h after infection with JEVrv, HCVrv, and JEV and at 24 h after infection with VSV. Titers were determined by counts of foci detected by immunohistochemical staining (lower panels). The results shown are from three independent assays, with error bars representing standard deviations.

DISCUSSION

Ceramide has been shown to play a crucial role in various cell signaling pathways through the clustering and activation of the receptor molecules in lipid rafts. Although the generation of ceramide inhibits the infectivity of HIV and HCV by the rearrangement of the entry receptor molecules (7, 48), rhinovirus and Sindbis virus generate ceramide by activating SMase for their entry and cell survival, respectively (10, 15). In this study, we demonstrated for the first time that ceramide plays crucial roles not only in the entry pathway of JEV but also in the egress through a direct interaction with the E envelope proteins.

To examine the roles of the E protein in the infectivity of JEV, we employed pseudotype and recombinant VSVs bearing JEV envelope proteins as surrogate systems in addition to authentic JEV. VSV assembles and buds from the plasma membrane, and therefore the surrogate viruses bearing the foreign envelope proteins being expressed on the plasma membrane exhibited more-efficient incorporation of the envelope proteins. Although the E protein of JEV, as well as that of other flaviviruses, including HCV, is mainly retained on the endoplasmic reticulum (ER) membrane, the E protein was incorporated into JEVpv and JEVrv particles and exhibited infectivity comparable to that of authentic JEV. Further studies are needed to clarify the mechanisms of incorporation of

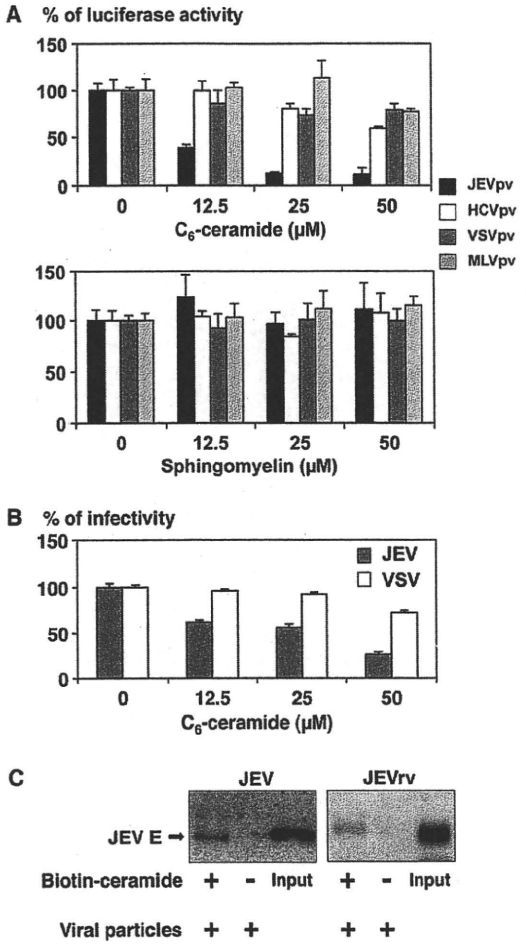

FIG. 7. Involvement of ceramide in infection with JEV. (A) Effects of C_6-ceramide or sphingomyelin in infection with JEVpv. Purified pseudotype viruses were pretreated with various concentrations of C_6-ceramide (upper) or sphingomyelin (lower) for 1 h and then inoculated into Huh7 cells. The infectivities were determined at 24 h postinfection by luciferase activity, and changes in infectivities are expressed as percentages. (B) Effects of C_6-ceramide in infection of JEVrv and JEV. JEV and VSV were pretreated with various concentrations of C_6-ceramide for 1 h, and then the viruses were inoculated into Huh7 cells. At 24 h postinfection, the infectivities were determined by focus-forming assay. (C) Binding of JEV and JEVrv to ceramide beads. Purified viruses were preincubated with (+) or without (−) biotin-ceramide resolved in DMSO and streptavidin-Sepharose 4B. After washing, residual pellets were analyzed by immunoblotting. Inputs are purified viruses. The results shown are from three independent assays, with error bars representing standard deviations.

the foreign envelope proteins on the ER membrane into VSV particles. In general, glycoproteins are modified into the complex type during the translocation from the ER to the Golgi apparatus. Although the JEV E glycoproteins were modified mainly into the high-mannose type in cells infected with JEVpv, JEVrv, or JEV, viruses possessing the E proteins were modified into the complex type within the particles. These results suggest that the E proteins of JEV and the surrogate viral particles are modified into the complex type after budding into the ER lumen during translocation into the Golgi apparatus. Recently assembly of DENV in the ER was revealed by three-dimensional architecture using electron tomography (49).

A number of viruses utilize cholesterol-rich membrane microdomains or lipid rafts for their entry, assembly, or egress processes (5). Cholesterol-rich membrane microdomains have been shown to be required for the entry but not for the replication of WNV through cholesterol depletion by treatment with MβCD (27). Entry of HCV was also shown to be partially required for cellular cholesterol (1, 16), which is consistent with the present data that infection with HCVpv was partially inhibited by treatment of cells with MβCD. Lee et al. recently reported that the infectivity of JEV, especially the replication step, was inhibited by treatment of cells with MβCD or the cholesterol chelation antibiotic filipin III (20). Furthermore, treatment of the viral particles with cholesterol inhibited the infectivity of JEV, in contrast to the enhancement of the infectivity of Sindbis virus by the same treatment (20, 22). Our data also indicated that the infectivity of JEVpv and JEVrv, as well as that of JEV, was completely inhibited by treatment of the particles with cholesterol in a dose-dependent manner, supporting the notion that the presence of an abundant amount of cholesterol increases the rigidity of the E protein of JEV particles and inhibits the membrane fusion event, as suggested by Lee et al. (20).

According to the current models, SMase alters the biophysical properties of the membrane bilayer by generating ceramide through the hydrolysis of sphingomyelin. Genetic disorders of SMase or ceramide metabolism are critically involved in human genetic diseases, such as Niemann-Pick disease (37) and Wilson's disease (19). *In vivo* studies of the function of SMase or ceramide in infections with pathogens are accumulating (9, 44, 46), and acid SMase-deficient mice have been shown to be unable to eliminate the pathogens because of failure to undergo apoptosis or phagolysosomal fusion, ultimately a massive release of cytokines and death by sepsis. It has recently been shown that acid SMase is a key regulator of cytotoxic granule secretion by primary T lymphocytes (13). The reduction of the cytolytic activity of $CD8^+$ cytotoxic T lymphocytes in acid SMase-deficient mice resulted in a significantly delayed clearance of lymphocytic choriomeningitis virus infection. Recently it was shown that entry of HCV is inhibited by SMase treatment through the downregulation of CD81, a major receptor of HCV, because enrichment of ceramide on the plasma membrane induces internalization of CD81 (48). HIV infection is also inhibited by ceramide enrichment through a restriction of the lateral diffusion of CD4 (6). Sindbis virus and rhinovirus activate the SMase and induce generation of ceramide in the endosomal membrane. Inhibition of SMase by genetic manipulation or pharmacological agents prevents infection with rhinoviruses, suggesting that SMase and ceramide-enriched membrane platforms play an important role in viral infection (10).

In this study, we have shown that entry of JEVpv, JEVrv, and JEV was specifically enhanced by treatment of cells with SMase. Treatment of cells with amitriptyline, an inhibitor interfering with the binding of SMase to the lipid bilayer, impaired the uptake of rhinovirus (10) and *Neisseria gonorrhoeae* (8). The entry of JEVpv was also inhibited by treatment with the inhibitor. Furthermore, the infections of JEVpv and JEV were inhibited by treatment with C_6-ceramide but not by treat-

ment with sphingomyelin, and JEV and JEVrv were coprecipitated with the ceramide beads, suggesting that the interaction of ceramide with the JEV E protein plays a crucial role in the early steps of infection. Ceramide is known to bind to the ceramide transport protein (CERT), which transports ceramide from the ER to the Golgi apparatus (12), and thus, it might be feasible to speculate that CERT participates in the translocation or maturation of the JEV E protein. Further studies are needed to clarify the interaction among ceramide, CERT, and the JEV E protein. Recently Aizaki et al. reported that the infectivity of HCV particles was decreased by treatment with MβCD or SMase, suggesting that cholesterols or sphingolipids incorporated into the virions are important for the infectivity of HCV (1). In this study, SMase treatment of HCVpv particles but not of JEVpv particles reduced infectivity, suggesting that incorporation of cholesterols and sphingolipids into the viral particles was different among flaviviruses.

The discrepancy between the drastic increase in the production of infectious particles of JEVrv and the marginal increase in that for JEV induced by SMase treatment in ceramide-enriched cells may indicate that ceramide enrichment enhances the entry and egress steps but negatively regulates genomic replication of JEV. Previously it was reported that digestion of sphingomyelin by SMase induces cholesterol redistribution (32), an increase in intracellular cholesterol esterification (4), and a decrease in cholesterol biosynthesis (39). Furthermore, ceramide has been shown to selectively displace cholesterol from lipid rafts and decrease the association of the cholesterol binding protein caveolin-1 (28, 50). Although we have not determined the cholesterol composition of the membranes of cells treated with SMase, cholesterol depletion induced by SMase treatment may also participate in the enhancement of JEV entry.

JEV initiates infection by interacting with receptor and/or coreceptor molecule(s), probably in cooperation with ceramide located in the ceramide-enriched platforms. The ceramide-enriched membrane domains facilitate signal transduction through reorganization and clustering of cell surface receptor molecules. Although the entry receptor(s) of JEV has not been well characterized yet, modification of the distribution, organization, and steric conformation of the receptor molecule(s) by treatment with SMase may facilitate entry of JEV. Generation of ceramide by SMase treatment has been shown to promote vesicular fusion processes and fusion of phagosomes, thereby engulfing bacteria with late endosomes and resulting in efficient intracellular bacterial killing (46).

In conclusion, we have demonstrated that the entry and egress processes of JEV were enhanced by treatment with SMase by using pseudotype and recombinant VSVs. The interaction of cellular ceramide and the E glycoproteins facilitates infection and propagation of JEV. Modification of sphingolipids on the plasma membrane of the target cells might be a novel target for the development of antivirals against JEV infection.

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Production of Infectious Hepatitis C Virus by Using RNA Polymerase I-Mediated Transcription▽

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In this study, we used an RNA polymerase I (Pol I) transcription system for development of a reverse genetics protocol to produce hepatitis C virus (HCV), which is an uncapped positive-strand RNA virus. Transfection with a plasmid harboring HCV JFH-1 full-length cDNA flanked by a Pol I promoter and Pol I terminator yielded an unspliced RNA with no additional sequences at either end, resulting in efficient RNA replication within the cytoplasm and subsequent production of infectious virions. Using this technology, we developed a simple replicon *trans*-packaging system, in which transient transfection of two plasmids enables examination of viral genome replication and virion assembly as two separate steps. In addition, we established a stable cell line that constitutively produces HCV with a low mutation frequency of the viral genome. The effects of inhibitors of N-linked glycosylation on HCV production were evaluated using this cell line, and the results suggest that certain step(s), such as virion assembly, intracellular trafficking, and secretion, are potentially up- and downregulated according to modifications of HCV envelope protein glycans. This Pol I-based HCV expression system will be beneficial for a high-throughput antiviral screening and vaccine discovery programs.

Over 170 million people worldwide have been infected with hepatitis C virus (HCV) (22, 33, 37), and persistence of HCV infection is one of the leading causes of liver diseases, such as chronic hepatitis, cirrhosis, and hepatocellular carcinoma (16, 25, 38). The HCV genome is an uncapped 9.6-kb positive-strand RNA sequence consisting of a 5′ untranslated region (UTR), an open reading frame encoding at least 10 viral proteins (Core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B), and a 3′UTR (46). The structural proteins (Core, E1, and E2) reside in the N-terminal region.

The best available treatment for HCV infection, which is pegylated alpha interferon (IFN-α) combined with ribavirin, is effective in only about half of patients and is often difficult to tolerate (25). To date, a prophylactic or therapeutic vaccine is not available. There is an urgent need to develop more effective and better tolerated therapies for HCV infection. Recently, a robust system for HCV production and infection in cultured cells has been developed. The discovery that some HCV isolates can replicate in cell cultures and release infectious particles has allowed the complete viral life cycle to be studied (23, 49, 53). The most robust system for HCV production involves transfection of Huh-7 cells with genomic HCV RNA of the JFH-1 strain by electroporation. However, using this RNA transfection system, the amount of secreted infectious viruses often fluctuate and mutations emerge in HCV genome with multiple passages for an extended period of time (54), which limits its usefulness for antiviral screening and vaccine development.

DNA-based expression systems for HCV replication and virion production have also been examined (5, 15, 21). With DNA-based expression systems, transcriptional expression of functional full-length HCV RNA is controlled by an RNA polymerase II (Pol II) promoter and a self-cleaving ribozyme(s). DNA expression systems using RNA polymerase I (Pol I) have been utilized in reverse genetics approaches to replicate negative-strand RNA viruses, including influenza virus (12, 29), Uukuniemi virus (11), Crimean-Congo hemorrhagic fever virus (10), and Ebola virus (13). Pol I is a cellular enzyme that is abundantly expressed in growing cells and transcribes rRNA lacking both a 5′ cap and a 3′ poly(A) tail. Thus, viral RNA synthesized in cells transfected with Pol I-driven plasmids containing viral genomic cDNA has no additional sequences at the 5′- or 3′ end even in the absence of a ribozyme sequence (28). The advantages of DNA-based expression systems are that DNA expression plasmids are easier to manipulate and generate stable cell lines that constitutively express the viral genome.

We developed here a new HCV expression system based on transfection of an expression plasmid containing a JFH-1 cDNA clone flanked by Pol I promoter and terminator sequences to generate infectious HCV particles from transfected cells. The technology presented here has strong potential to be the basis for *trans*-encapsidation system by transient transfection of two plasmids and for the establishment of an efficient and reliable screening system for potential antivirals.

MATERIALS AND METHODS

DNA construction. To generate HCV-expressing plasmids containing full-length JFH1 cDNA embedded between Pol I promoter and terminator se-

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