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Involvement of cyclophilin B in the replication of Japanese encephalitis virus

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ABSTRACT

Japanese encephalitis virus (JEV) is a mosquito-borne RNA virus that belongs to the *Flaviviridae* family. In this study, we have examined the effect of cyclosporin A (CsA) on the propagation of JEV. CsA exhibited potent anti-JEV activity in various mammalian cell lines through the inhibition of CypB. The propagation of JEV was impaired in the CypB-knockdown cells and this reduction was cancelled by the expression of wild-type but not of peptidylprolyl *cis-trans* isomerase (PPIase)-deficient CypB, indicating that PPIase activity of CypB is critical for JEV propagation. Infection of pseudotype viruses bearing JEV envelope proteins was not impaired by the knockdown of CypB, suggesting that CypB participates in the replication but not in the entry of JEV. CypB was colocalized and immunoprecipitated with JEV NS4A in infected cells. These results suggest that CypB plays a crucial role in the replication of JEV through an interaction with NS4A.

Introduction

The genus *Flavivirus* within the family *Flaviviridae* comprises over 70 viruses, many of which are predominantly arthropodborne viruses, such as Japanese encephalitis virus (JEV), West Nile virus (WNV), Murray Valley encephalitis virus, dengue virus (DENV), yellow fever virus (YFV), and tick-borne encephalitis virus. JEV is one of the most important flaviviruses in the medical and veterinary fields and exists in a zoonotic transmission cycle among mosquitoes, pigs, and birds mostly in Eastern and Southeast Asia. This virus spreads to dead-end hosts, including humans, through the bite of JEV-infected mosquitoes, and around 30,000–50,000 cases and up to 15,000 deaths are reported annually (Ghosh and Basu, 2009; Mackenzie et al., 2004; Solomon et al., 2003). JEV has a single-stranded positive-sense RNA genome of approximately 11 kb, which is capped at the 5′ end but lacks a 3′ polyadenine tail. The genome RNA is translated into a single large polyprotein at the endoplasmic reticulum (ER) membrane, then cleaved by the host- and virus-encoded proteases into three structural proteins, the capsid, precursor membrane (prM), and envelope (E) proteins, and seven nonstructural (NS) proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Sumiyoshi et al., 1987).

Flavivirus infection causes extensive rearrangement of cellular membranes to form two distinct membrane structures called the vesicle packet and convoluted membrane (Mackenzie et al., 1996; Miller and Krijnse-Locker, 2008). Whereas the vesicle packet is believed to contain the replication complex in which viral RNA synthesis takes place, the convoluted membrane is the putative site for viral polyprotein processing (Mackenzie et al., 1999). A recent tomography study clarified that the ER, convoluted membrane, and outer membrane of the vesicle packet were connected together to form a continuous membrane, with the vesicle packet being observed as an invagination of the ER with NS proteins and viral RNA, suggesting that viral replication occurred on the surface of the ER (Welsch et al., 2009). The structures of the convoluted membrane can be observed by infection with the WNV strain Kunjin virus or expression of the DENV NS4A protein alone (Miller et al., 2007; Roosendaal et al., 2006). Previous studies have indicated that NS4A localizes to both the vesicle packet and convoluted membrane and interacts with NS1, indicating that NS4A plays an important role as an integral scaffold of the replication complex (Lindenbach and Rice, 1999; Mackenzie et al., 1998).

In addition to NS proteins, flavivirus RNA replication is known to be regulated by several host factors, such as eEF1A, TIA/TIAR, HMGCR, and cyclophilin (Cyp) A (Davis et al., 2007; Emara and Brinton, 2007; Mackenzie et al., 2007; Qing et al., 2009). RNAi screening has identified various host factors involved in the replication of RNA viruses, including the hepatitis C virus (HCV), human immunodeficiency virus (HIV), and influenza A virus (Karlas et al., 2010; Konig et al., 2010, 2008; Tai et al., 2009). Host factors essential for viral replication might be an ideal target for antiviral development because the frequency of appearance of resistant viruses is lower by this method than when using antivirals targeted to the viral proteins.

In this study, we identified CypB as a host factor involved in the propagation of JEV. CypB is a member of the Cyp family, is ubiquitously expressed in most cells, and predominantly resides in the ER through the ER retention signal sequence in the C-terminus (Price et al., 1994,

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1991; Wang and Heitman, 2005). CypB participates in various biological functions, such as chaperone activities, immunosuppression, transcriptional regulation, apoptosis, and viral propagation (Allain et al., 1996; Kim et al., 2008; Rycyzyn and Clevenger, 2002; Watanabe et al., 2010; Watashi et al., 2005; Zhang and Herscovitz, 2003). Cyclosporin A (CsA), an inhibitor for Cyps, significantly impaired the propagation of JEV. Knockdown of CypB reduced the RNA replication in the JEV replicon cells, whereas it exhibited no effect on the infection of a pseudotype virus bearing JEV envelope proteins. Furthermore, CypB was colocalized and immunoprecipitated with the JEV NS4A protein. Collectively, these results suggest that CypB plays a crucial role in the propagation of JEV through its interaction with NS4A.

Results

CsA suppresses the production of JEV by inhibiting Cyps

CsA is an immunosuppressive agent widely used in the management of organ transplantation. In addition to this activity, it has been reported that CsA has potent antiviral effects against HCV (Chatterji et al., 2009; Kaul et al., 2009; Watashi et al., 2005; Yang et al., 2008), HIV (Franke et al., 1994; Thali et al., 1994), measles virus (MV) (Watanabe et al., 2010), influenza A virus (Liu et al., 2009), vesicular stomatitis virus (VSV) (Bose et al., 2003), and vaccinia virus (VV) (Castro et al., 2003; Damaso and Moussatche, 1998). To examine the possibility that CsA has an antiviral effect on JEV, mammalian cell lines including Huh7, BHK, and N18 cells were treated with various concentrations of CsA followed by infection with JEV. At 48 h post-infection, cells were subjected to immunoblotting. The level of expression of JEV NS1 was significantly decreased by treatment with CsA in a dose-dependent manner in all the cell lines examined (Fig. 1A). Furthermore, infectious particle production in the culture supernatant was also reduced by the treatment with CsA under the conditions employed without exhibiting any serious cytotoxic effect (Fig. 1B).

CsA exhibits three distinct inhibitory activities on, respectively, the calcineurin NF-AT signaling pathway, the peptidylprolyl *cis-trans* isomerase (PPIase) activity of Cyps, and the transport activity of p-glycoprotein (Silverman et al., 1997). To determine the antiviral activity of CsA, we used CsA derivatives and FK506, an immunosuppressant structurally different from CsA. cyclosporin D (CsD) has almost no effect on the calcineurin pathway (Sadeg et al., 1993) and cyclosporin H (CsH) has a specific inhibitory activity on the p-glycoprotein (Silverman et al., 1997). FK506 also inhibits the calcineurin NF-AT signaling pathway (Almawi and Melemedjian, 2000). Huh7 cells were infected with JEV and treated with various concentrations of the compounds at 1 h post-infection. The cells and culture supernatants were harvested at 48 h after treatment and the expression of JEV NS1 and infectivity were determined, respectively (Fig. 2). Treatment with CsA and CsD reduced the expression of the NS1 and the production of JEV in a dose-dependent manner, whereas CsH and FK506 exhibited almost no effect on the propagation of JEV (Fig. 2). These results suggest that CsA inhibits JEV propagation through the inhibition of Cyps, but not through the inhibition of calcineurin and p-glycoprotein.

CypB participates in the propagation of JEV

Cyps possessing the PPIase activity are highly conserved and ubiquitously expressed in both prokaryotic and eukaryotic cells (Wang and Heitman, 2005). Next, to determine whether the particular Cyp isoform participates in the propagation of JEV, short interference RNAs (siRNAs) targeted to CypA, CypB, or CypC were transfected into Huh7 cells and the expression of each Cyp was determined by immunoblotting or real-time PCR at 24 h post-transfection. CypA and CypB were specifically decreased by the transfection of the siRNAs (Fig. 3A). Although CypC could not be detected by immunoblotting due to the lack of a specific antibody in our laboratory, CypC mRNA was decreased by approximately 90% upon transfection with siRNA targeted

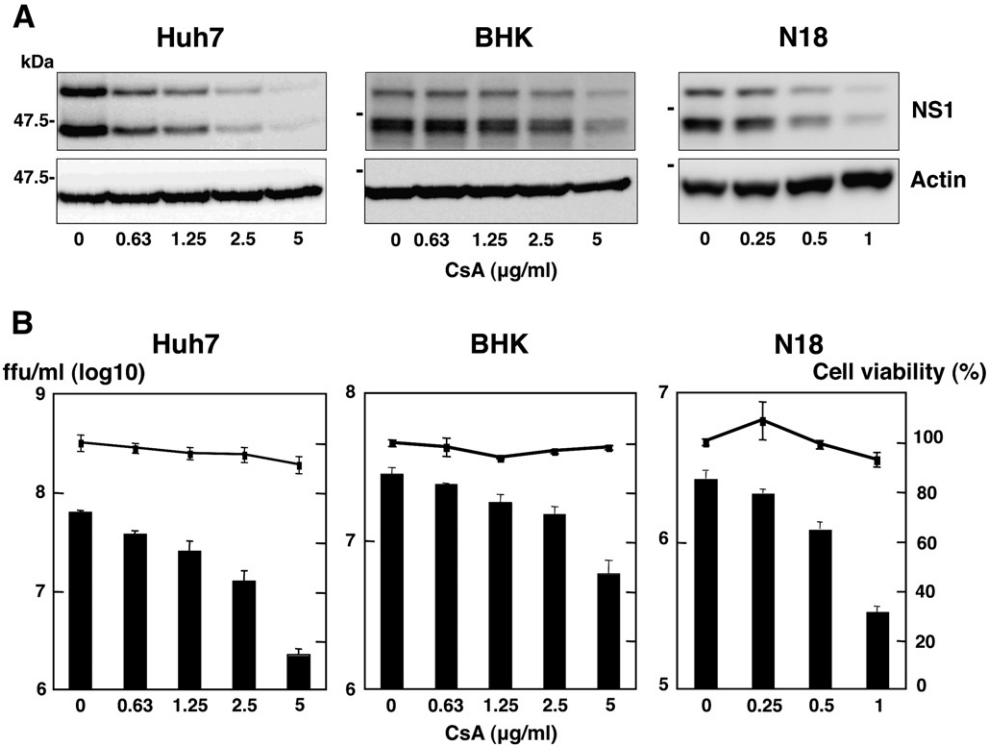


Fig. 1. Effect of CsA on the propagation of JEV in mammalian cells. (A) JEV was inoculated at an MOI of 0.1 (Huh7 and BHK cells) or 10 (N18 cells) and incubated for 1 h. Cells were washed with 10% FBS DMEM and treated with the indicated concentrations of CsA in 10% FBS DMEM for 48 h. The propagation of JEV was assessed by the expression of NS1. NS1 and actin were detected by immunoblotting. (B) The production of infectious JEV in the culture supernatant at 48 h post-infection was determined in Vero cells by a focus-forming assay. Cell viability was determined at 48 h post-incubation of CsA. The results are representative of three independent assays, with the error bars indicating the standard deviations.

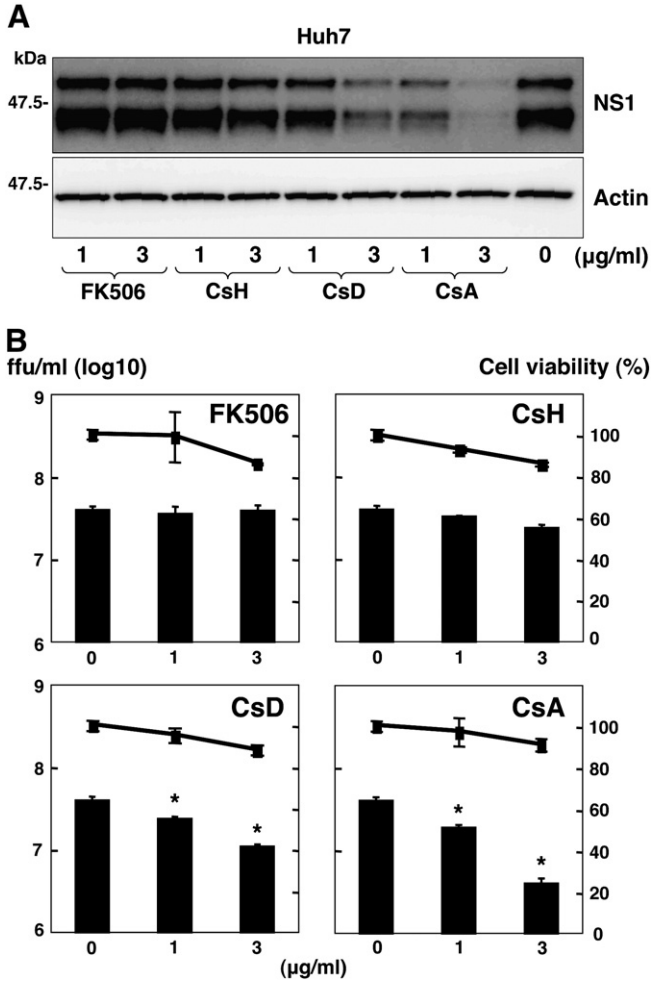


Fig. 2. CsA inhibits JEV propagation through the inhibition of Cyps. Huh7 cells were infected with JEV at an MOI of 0.1 for 1 h and then treated with 10% FBS DMEM containing the indicated concentrations of CsA, CsD, CsH, or FK506 for 48 h. The propagation of JEV was evaluated by immunoblotting (A) and focus-forming assay (B). The results are representative of three independent assays, with the error bars indicating the standard deviations. Asterisks indicate significant differences (*$P<0.01$).

to CypC compared to the level in the cells transfected with the control siRNA (Fig. 3B). JEV was inoculated into cells transfected with the siRNA at 48 h post-transfection and the cells and culture supernatants were harvested at 48 h post-infection. Expression of JEV NS1 was most effectively decreased by the knockdown of CypB, followed by CypC, and knockdown of CypA resulted in a marginal reduction of NS1 expression compared to the control siRNA (Fig. 3C). Furthermore, the production of JEV was also effectively suppressed in cells with knockdown of CypB, followed by those with knockdown of CypC and CypA (Fig. 3D). These results suggest that CypB plays an important role in the propagation of JEV. To further confirm the effect CypB on the propagation of JEV, we established stable knockdown cell lines expressing a short hairpin RNA (shRNA) targeted to CypB. Consistent with the data from transient knockdown experiments, both expression of NS1 and virus production were significantly reduced in the CypB-knockdown cell lines (Bose et al., 2003; Castro et al., 2003) in accordance with the reduction of CypB (Fig. 4A and B). There was no significant difference in cell growth among the cell lines (Fig. 4C).

PPIase activity of CypB is crucial for the propagation of JEV

The PPIase activity of Cyps is suggested to catalyze the proper folding of certain proteins (Andreotti, 2003; Wang and Heitman, 2005). It has been demonstrated that PPIase activity of Cyps is required for HCV replication (Chatterji et al., 2009; Kaul et al., 2009; Watashi et al., 2005). To examine the effect of the PPIase activity of CypB on the propagation of JEV, we constructed an expression plasmid encoding a PPIase-defective CypB in which the Arg^{62} was replaced with Ala, because the Arg^{62} in CypB has been shown to be critical for PPIase catalytic activity (Carpentier et al., 1999). Each of the expression plasmids encoding the FLAG-tagged wild- or Ala^{62}-CypB carrying the silent mutations resistant to the siRNA was introduced into the stable CypB-knockdown cell line (Bose et al., 2003) and cultured for a week in the presence of neomycin. Although expression of both endogenous and exogenous CypB was detected at a similar level (Fig. 4D), JEV production was partially rescued by introducing the wild-CypB but not the Ala^{62}-CypB (Fig. 4E). These results indicate that the PPIase activity of CypB is crucial for the propagation of JEV.

CypB participates in the replication but not in the entry of JEV

To further examine the effect of CsA on the JEV life cycle, we generated a subgenomic replicon of JEV to assess the effect of CsA on the JEV RNA replication (Fig. 5A). The replicon cells treated with CsA for 6 days exhibited a significant reduction of NS1 expression compared to the non-treated cells (Fig. 5B). The replicon RNA transcribed from the pJErepIRESpuro was transfected into the stable CypB-knockdown (#4) or control cell lines and incubated for 3 weeks in the presence of puromycin. A few colony formation was detected in the CypB-knockdown cell line, in contrast to the abundant colony formation in the control cell line (Fig. 5C). These results suggest that CypB is required for the efficient replication of JEV.

Next, to examine the impact of CypB on the entry of JEV, we generated pseudotype VSVs bearing envelope proteins of JEV (JEVpv) or VSV (VSVpv). Because these viruses possess the luciferase gene, the infectivity can be assessed by the luciferase activity (Tani et al., 2010). Huh7 cells pretreated with various concentrations of CsA were infected with JEVpv or VSVpv, and the infectivity was assessed by the expression of luciferase. There was no significant effect of CsA on the infection of either pseudotype virus (Fig. 5D). Similarly, no effect was observed on the infection of the pseudotype viruses in the CypB-knockdown cell lines (Fig. 5E). Collectively, these results clearly indicate that CypB participates in the replication but not in the entry of JEV.

CypB interacts with the JEV NS4A protein

Many viruses have been shown to utilize Cyps through the interaction with their viral proteins. For example, HCV recruits CypA and CypB to enhance viral RNA replication through the interaction with NS5A and NS5B, respectively (Chatterji et al., 2009; Kaul et al., 2009; Watashi et al., 2005; Yang et al., 2008). To determine whether the JEV proteins interact with CypB, we prepared expression plasmids encoding each of the JEV nonstructural proteins involved in the viral RNA replication. FLAG-tagged CypB was co-expressed with each of the HA-tagged JEV nonstructural proteins in 293T cells and immunoprecipitated with anti-HA antibody. The precipitates were subjected to immunoblotting by using either anti-FLAG or anti-HA antibodies. CypB was co-precipitated with the JEV NS4A protein but not with other proteins (Fig. 6A). Furthermore, interaction of CypB with NS4A was reduced in the immunoprecipitation analysis in the presence of CsA (Fig. 6B). To gain more insight into the interaction between CypB and NS4A, the intracellular localization of these proteins was examined by confocal microscopy. Huh7 cells were transfected with an expression plasmid encoding HA-tagged NS4A or an empty vector and fixed at 48 h post-transfection. Endogenous CypB was detected in the perinuclear region together with NS4A protein. In addition, NS4A colocalized with ER marker protein, calnexin (Fig. 6C). These results suggest that NS4A protein interacts with CypB at the replication complex localized in the ER.

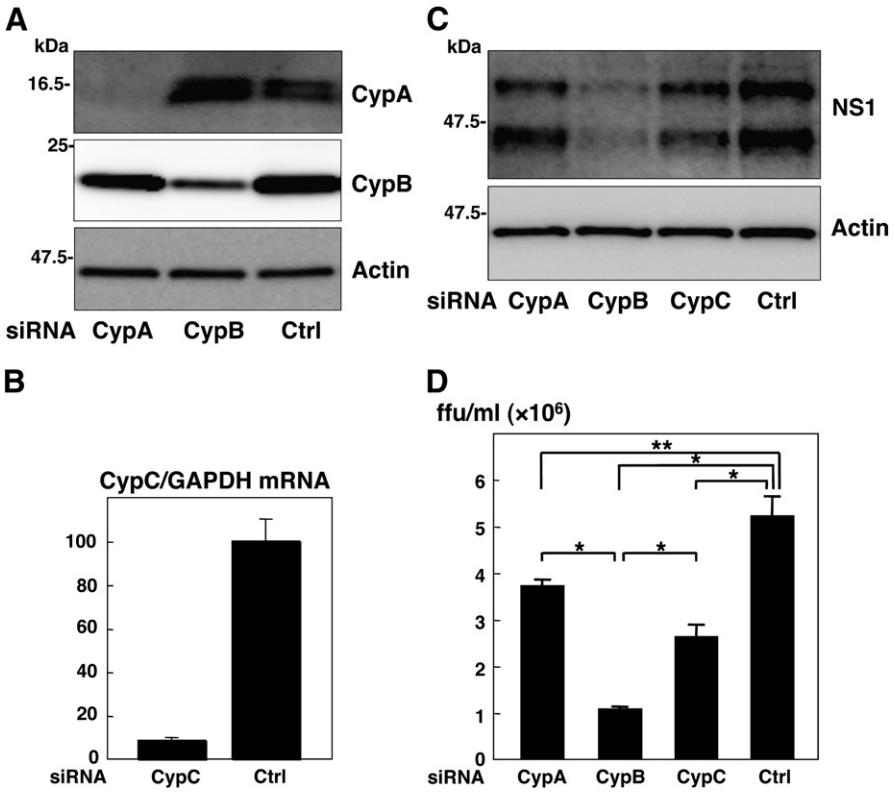


Fig. 3. CypB plays an important role in the propagation of JEV. (A) Knockdown of endogenous CypA and CypB by siRNA. Huh7 cells were transfected with 35 nM of siRNA targeted to CypA, CypB, or a non-specific control. Cell lysates after 96 h post-transfection were analyzed for expression of CypA, CypB, or actin by immunoblotting. (B) Huh7 cells transfected with 35 nM of siRNA targeted to CypC or a non-specific control were harvested at 24 h post-transfection. CypC mRNA levels were determined by quantitative real-time PCR. The level of CypC mRNA was normalized to the amount of GAPDH mRNA and expressed as a percentage of the control value. (C, D) Huh7 cells were transfected with siRNA targeted to CypA, CypB, or CypC and infected with JEV at an MOI of 0.1 at 48 h post-transfection. The propagation of JEV was determined by immunoblotting (C) and focus-forming assay (D). The results are representative of three independent assays, with the error bars indicating the standard deviations. Asterisks indicate significant differences (*$P<0.01$; **$P<0.05$).

Discussion

In this study, we have shown that CsA inhibits the replication of JEV through the inhibition of the PPIase activity of Cyps. A previous study showed that CsA does not induce interferon in Huh7 cells (Nakagawa et al., 2005), suggesting that the antiviral activity of CsA on the propagation of JEV relies on the inhibition of Cyps. Cyps are highly conserved PPIases that catalyze the *cis-trans* isomerization of peptide bonds to facilitate certain protein foldings (Andreotti, 2003; Wang and Heitman, 2005) and are involved in the correct folding of host and viral proteins. Among the Cyp isoforms, CypA and CypB are the most abundantly expressed in cells and play key roles in the propagation of various viruses. CypA is incorporated into HIV, influenza A virus, VSV, and VV to regulate their replication (Bose et al., 2003; Castro et al., 2003; Damaso and Moussatche, 1998; Franke et al., 1994; Liu et al., 2009; Thali et al., 1994). CypB is incorporated into MV particles to facilitate an efficient infection (Watanabe et al., 2010). Both CypA and CypB have been shown to serve as host factors involved in the replication of HCV through the interaction with NS5A and NS5B (Chatterji et al., 2009; Kaul et al., 2009; Watashi et al., 2005; Yang et al., 2008).

Recently, Qing et al. reported that CypA plays an important role in the replication of flaviviruses such as WNV, YFV, and DENV. The PPIase activity of CypA was shown to be crucial for the efficient replication of the viruses, indicating that CypA acts as a molecular chaperone for the viral and host proteins required for an effective RNA replication (Qing et al., 2009). Indeed, knockdown of CypA suppressed the JEV propagation in this study, but that of CypB exhibited more potent impairment of the JEV propagation, suggesting that CypB plays a crucial role in the propagation of JEV. However, we could not exclude the possibility of the involvement of other Cyps in the replication of JEV. Multiple Cyps have been shown to involved in the life cycle of HCV (Gaither et al., 2010; Nakagawa et al., 2005) and the knockdown experiment of Cyps in this study suggests that not only CypB, but also CypC and CypA are involved in the propagation of JEV. At least 16 Cyps have been shown to participate in various cellular functions in humans (Wang and Heitman, 2005), and therefore, further studies to clarify the precise function of these Cyps in the life cycle of the flaviviruses are needed.

In addition to Cyps, flavivirus recruits several host chaperones for an efficient propagation. HSP70 and HSP90 have been identified as comprising the DENV receptor complex in human cell lines. These chaperones presumably facilitate the viral envelope dimer–trimer transition after the binding of the envelope protein to the cellular receptor (Reyes-Del Valle et al., 2005). Moreover, inhibition of the interaction between the ER chaperone calnexin and JEV glycoproteins has been suggested to affect the folding of viral proteins, leading to a reduction in the mortality rate in a mouse model of lethal infection (Wu et al., 2002). It has been reported that ER chaperones including BiP, calnexin, and calreticulin interact with the DENV envelope protein, and that knockdown of these chaperones decreased viral production (Limjindaporn et al., 2009). In addition, BiP was shown to be upregulated in cells infected with DENV to facilitate viral production (Wati et al., 2009), and BiP and calreticulin have been associated with CypB (Zhang and Herscovitz, 2003). Therefore, these ER resident chaperones are considered to play important roles in the flavivirus replication through the proper folding of the viral and host proteins making up the viral RNA replication complex.

Lack of recovery of JEV propagation in the CypB-knockdown cell lines by the expression of the PPIase-deficient CypB mutant suggests that PPIase activity is crucial for the JEV production. Although the PPIase activity of CypA has been shown to be required for flavivirus replication

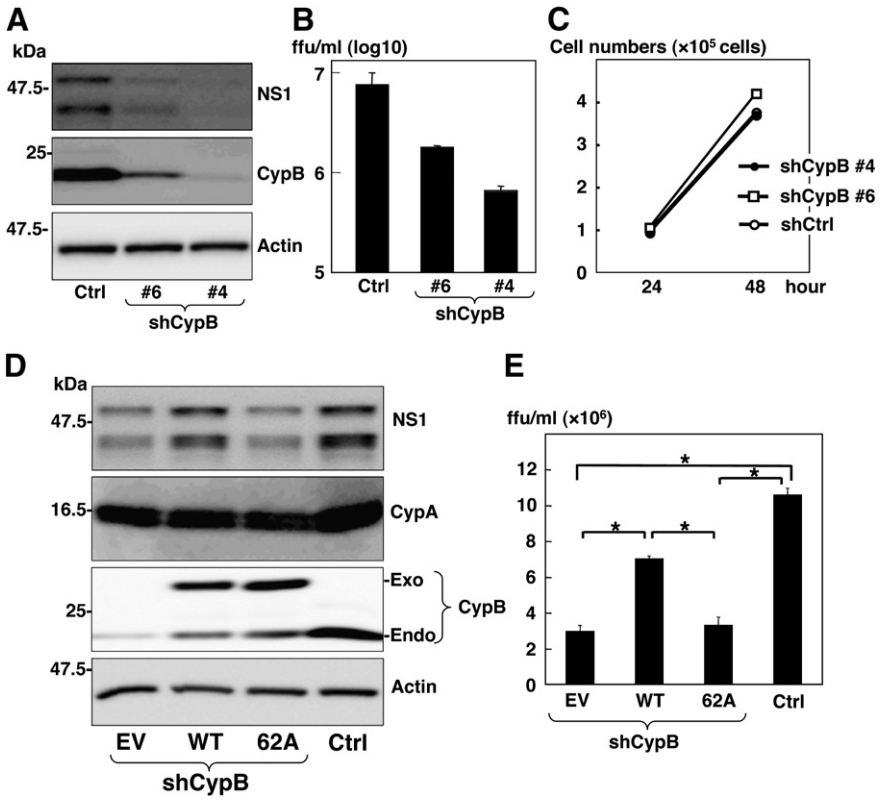


Fig. 4. PPIase activity of CypB is crucial for the propagation of JEV. Huh7 cell lines expressing shRNA targeted to CypB or the control were infected with JEV at an MOI of 0.1 for 1 h and cultured in 10% FBS DMEM for 48 h. The expressions of NS1, CypB, and actin were detected by immunoblotting (A). The propagation of JEV was determined by focus-forming assay (B). Growth kinetics of the stable CypB-knockdown cell lines were determined by the method of trypan blue dye exclusion (C). The stably knocked-down cell lines were transfected with the siRNA-resistant FLAG-tagged wild- or Ala^{62}-CypB, or empty vector and cultured for 1 week in the presence of 1 μg/ml puromycin. The remaining cells were infected with JEV at an MOI of 1. The expressions of NS1, CypA, endogenous and exogenous CypBs, and actin were detected by immunoblotting (D). Virus production in the culture supernatant at 36 h post-infection was determined by a focus-forming assay (E). The results are representative of three independent assays, with the error bars indicating the standard deviations. Asterisks indicate significant differences (*P<0.01).

through the interaction with the NS5 polymerase (Qing et al., 2009), CypB was colocalized and specifically co-immunoprecipitated with JEV NS4A. CypA is abundantly expressed in the cytoplasm of mammalian cells (Galigniana et al., 2004) and NS5 is predominantly detected on the cytoplasmic side of the ER (Zhang et al., 1992). Thus, it is conceivable that an interaction between CypA and NS5 occurs on the cytoplasmic side of the ER. On the other hand, CypB is localized in the ER lumen and targeted to the secretory pathway via its ER signal sequence (Price et al., 1994, 1991). NS4A is predicted to be a three-transmembrane protein with its C-terminal end localized in the ER lumen (Miller et al., 2007). Therefore, it is plausible that CypB interacts with NS4A within the ER lumen and confers proper folding to form the RNA replication complex of JEV. Expression of DENV NS4A alone has been shown to induce rearrangement of the cytoplasmic membrane to form the convoluted membrane required for viral replication (Roosendaal et al., 2006). It might be feasible to speculate that JEV NS4A undergoes conformational change through the interaction with CypB and induces formation of the convoluted membrane in the ER essential for genome replication of JEV. It was reported that HCV NS5A from CsA resistant mutant exhibits an enhanced interaction with CypB and NS5B facilitates a stronger binding of the mutant NS5A to endogenous CypB than wild-type in cell culture (Fernandes et al., 2010). Study of the molecular mechanism underlying the CsA resistant of JEV may shed light on the complex interaction among Cyps and viral proteins.

In conclusion, we have demonstrated that CsA suppresses the propagation of JEV by inhibiting the interaction between CypB and NS4A, which is required for viral RNA replication. Further studies are needed to elucidate the precise molecular mechanism underlying the involvement of cellular Cyps in the efficient propagation of JEV. Three inhibitors of the PPIase activity of Cyps, DEBIO-025, SCY635, and NIM811, are currently under clinical trial for the treatment of hepatitis C patients (Puyang et al., 2010). The PPIase inhibitor may be an attractive therapeutic target for the treatment of patients infected with not only HCV but also other flaviviruses.

Materials and methods

Plasmids

The human CypB gene was amplified from the total cDNA of Huh7 by PCR using *LA taq* (Takara Bio Inc., Shiga, Japan) and cloned into pcDNA3.1 and pCAGPM (Mori et al., 2007). The plasmids encoding the NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 of the JEV AT31 strain were generated by PCR and cloned into pCAGPM. The pSilencer-CypB, carrying an shRNA targeted to CypB under the control of the U6 promoter, was constructed by cloning of the oligonucleotide pair 5′-GATCCGGTGGAGAGCACCAAGACATTCAAGAGATGTCTTGGTGCTCTC-CACCTTTTTTGGAAA-3′-5′-AGCTTTTCCAAAAAAGGTGGAGAGCACCAA-GACATCTCTTGAATGTCTTGGTGCTCTCCACCG-3′ between the *Bam*HI and *Hin*dIII sites of pSilencer 2.1-U6 hygro (Ambion, Austin, TX). A plasmid coding a mutant CypB resistant to shRNA was prepared by insertion of four silent mutations (the nucleotides at positions 543, 549, 555, and 561 were changed from G to A, G to A, C to G, and A to C, respectively) into CypB cDNA by the method of splicing by overlap extension (Ho et al., 1989). The pSilencer negative-control plasmid (Ambion) has no homology to any human gene. The pJErep plasmid was kindly provided by Dr. Konishi (Kobe University, Kobe, Japan). A puromycin-resistant gene under the internal ribosomal entry site (IRES) of encephalomyocarditis virus was inserted into pJErep and designated as pJErepIRESpuro.

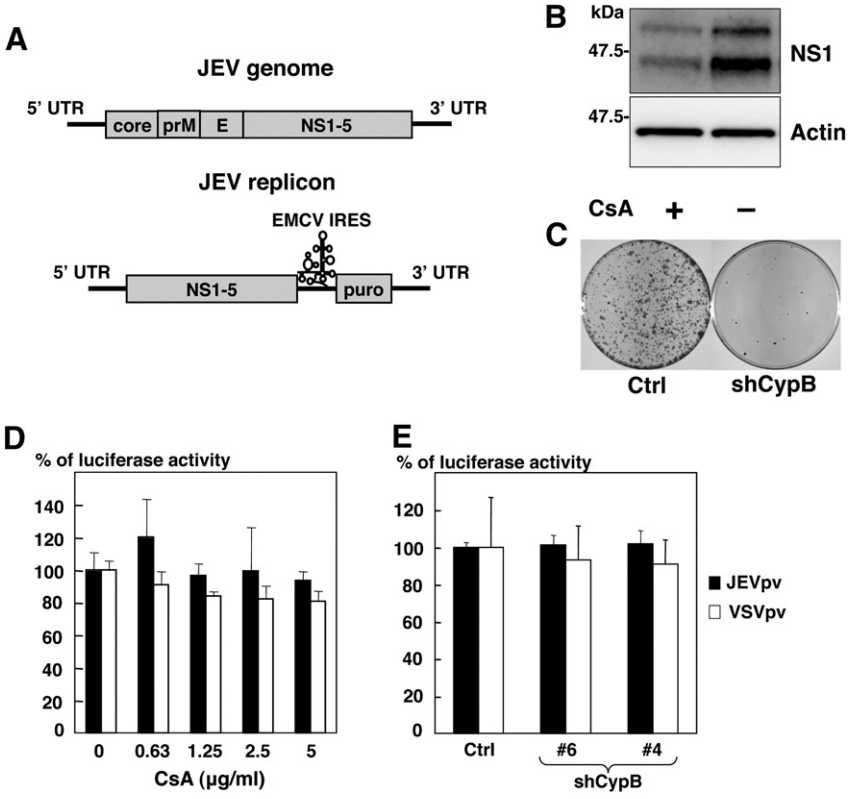


Fig. 5. CypB participates in the replication but not in the entry of JEV. (A) Schematic representations of the JEV genome and its subgenomic replicon. (B) JEV replicon cells were treated with CsA (1 μg/ml) for 6 days, and the expressions of NS1 and actin were detected by immunoblotting. (C) The stable CypB-knockdown and control cell lines were electroporated with the JEV replicon RNA and cultured for 3 weeks in the presence of 1 μg/ml of puromycin. The remaining cells were fixed with 4% paraformaldehyde and stained with crystal violet. (D) Huh7 cells treated with the indicated concentrations of CsA for 1 h were infected with the pseudotype viruses, JEVpv and VSVpv, and luciferase activities were determined at 24 h post-infection. (E) The stable CypB-knockdown and control cell lines were incubated with the pseudotype viruses, and the luciferase activities were determined. The results shown are representative of three independent assays, with error bars indicating standard deviations.

Cells and viruses

All cell lines were cultured at 37 °C under the condition of a humidified atmosphere and 5% CO_2. The human embryonic kidney cell line, 293T, African green monkey kidney cell line, Vero, hepatocellular carcinoma cell line, Huh7, mouse neural cell line, N18, and baby hamster kidney cell line, BHK, were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, non-essential amino acid (Sigma), and 10% fetal bovine serum (FBS). The mosquito C6/36 cell line (*Aedes albopictus*) was cultured at 27 °C and maintained in modified Eagle's medium (MEM) (Sigma). Huh7 cells were transfected with pSilencer-CypB or control plasmid and drug-resistant clones were selected by treatment with hygromycin B (Wako, Tokyo, Japan) at a final concentration of 50 μg/ml. Huh7 cells were electroporated with *in vitro*-transcribed RNA from pJErepIRESpuro and drug-resistant clones were selected by treatment with puromycin (InvivoGen, San Diego, CA) at a final concentration of 1 μg/ml. Wild-type JEV strain AT31 was used as described previously (Tani et al., 2010). The wild-type JEV was amplified on C6/36 cells and stored at −80 °C. Pseudotype VSVs bearing JEV PrM and E proteins (JEVpv) and VSVG (VSVpv) were produced in 293T cells transfected with pCAG105E and pCAGVSVG, respectively, as described previously (Tani et al., 2010). The infectivities of JEV and the pseudotype VSVs were assessed by both a focus-forming assay and luciferase activity as described previously (Tani et al., 2010). Cell viability was determined by using CellTiter-Glo (Promega Corporation, Madison, WI) according to the manufacturer's protocol.

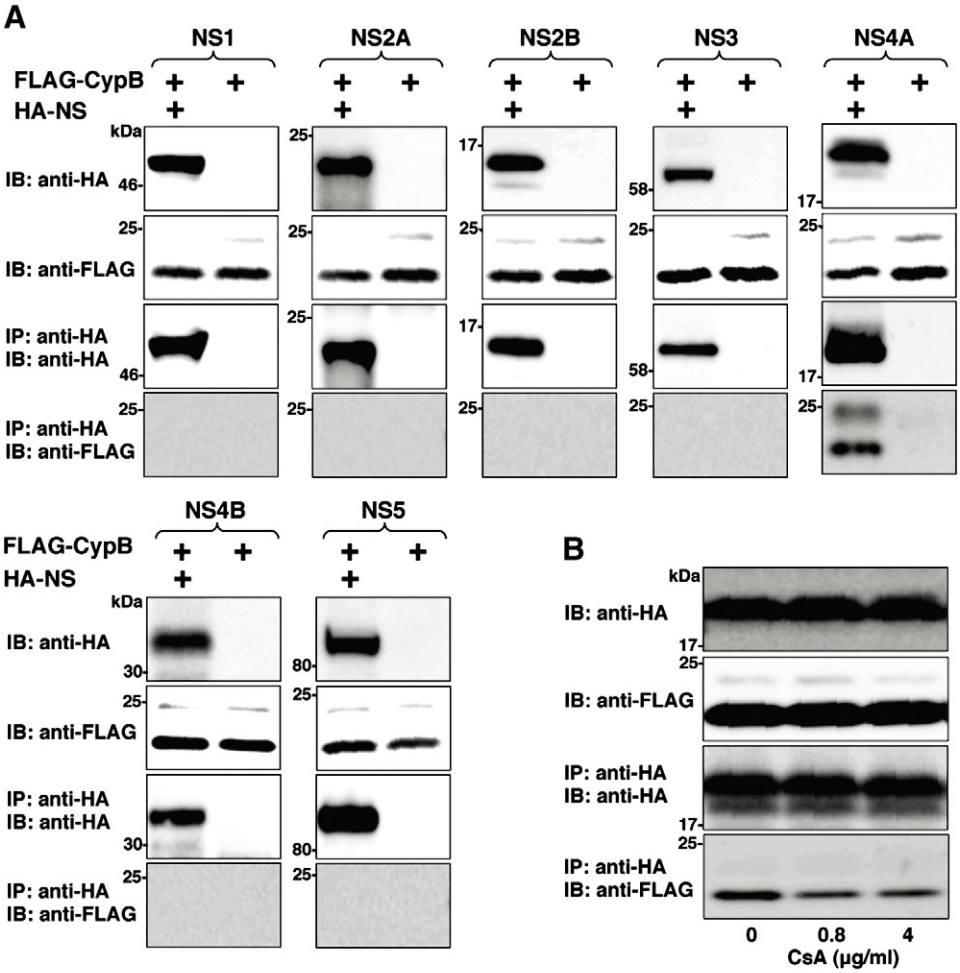
Reagents and antibodies

CsA and FK506 were purchased from Sigma, and CsD and CsH from Eton Bioscience Inc. (San Diego, CA). Mouse monoclonal antibodies to tags of HA and FLAG and β-actin were previously described (Taguwa et al., 2009). Rabbit polyclonal antibodies to CypA and CypB were purchased from Upstate Cell Signaling (Lake Placid, NY) and Affinity BioReagents (Golden, CO), respectively. Rabbit polyclonal antibody to calnexin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody to JEV NS1 protein (34A1) was kindly provided by Dr. Yasui.

Transfection, immunoblotting, and immunoprecipitation

Transfection and immunoprecipitation were carried out as described previously (Taguwa et al., 2009). Immunoprecipitates boiled in loading buffer were subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene

Fig. 6. NS4A protein recruits CypB to the replication complex in the JEV-infected cells. (A) FLAG-tagged CypB was co-expressed with HA-tagged NS1, NS2A, NS2B, NS3, NS4A, NS4B, or NS5 in 293T cells and immunoprecipitated with anti-HA antibody. The immunoprecipitates were subjected to immunoblotting by using either anti-FLAG or anti-HA antibody. (B) FLAG-tagged CypB was co-expressed with HA-tagged NS4A in 293T cells. The cell lysates obtained after lysis with the buffer containing CsA were immunoprecipitated with anti-FLAG antibody. The immunoprecipitates were subjected to immunoblotting by using either anti-FLAG or anti-HA antibody. (C) Huh7 cells transfected with an expression plasmid encoding HA-tagged NS4A or empty vector were fixed at 48 h post-transfection, permeabilized, and stained with the appropriate antibodies to HA (green), calnexin (red), and CypB (red). Cell nuclei were stained with DAPI (blue). Intracellular localization of CypB and NS4A was examined by confocal microscopy.


A
NS1
NS2A
NS2B
NS3
NS4A
FLAG-CypB
HA-NS
kDa
IB: anti-HA
IB: anti-FLAG
IP: anti-HA
IB: anti-HA
IP: anti-HA
IB: anti-FLAG
NS4B
NS5
B
CsA (μg/ml)
0
0.8
4
C
Nuc
NS4A
CypB
Merge
Empty
Calnexin

difluoride membranes (Millipore, Bedford, MA) and were reacted with the appropriate antibodies. The immune complexes were visualized with Super Signal West Femto substrate (Pierce, Rockford, IL) and detected by an LAS-3000 image analyzer system (Fujifilm, Tokyo, Japan).

Gene silencing by siRNA

The siRNAs against CypA and CypB were 5′-AAGCATACGGGTCCTGGCATC-3′ and 5′-AAGGTGGAGAGCACCAAGACA-3′, respectively (QIAGEN, Tokyo, Japan). FlexTube siRNAs against CypC and the negative control were purchased from QIAGEN. The cells were grown on 6-well plates and transfected with 35 nM siRNA by using Dharmafect (Dharmacon, Buckinghamshire, UK) according to the manufacturer's protocol. The transfected cells were incubated in DMEM supplemented with 10% FBS.

Quantitative RT-PCR

RNA was determined by the method described previously (Taguwa et al., 2009). The total RNA was prepared from cells by using an RNeasy mini kit (QIAGEN). First-strand cDNA was synthesized using an RNA LA PCR™ *in vitro* cloning kit (Takara Bio Inc.) and random primers. Each cDNA was determined by Platinum SYBR Green qPCR SuperMix UDG (Invitrogen, San Diego, CA) according to the manufacturer's protocol. Fluorescent signals were analyzed by an ABI PRISM 7000 (Applied Biosystems, Tokyo, Japan).

In vitro transcription and RNA transfection

Plasmid pJErepIRESpuro linearized at the *Swa* I site was transcribed *in vitro* using an mMESSAGE mMACHINE (Ambion) according to the manufacturer's protocol. The *in vitro*-transcribed RNA was introduced into Huh7 cells at 5 million cells/0.5 ml by electroporation at 270 V and 960 μF using Gene Pulser™ (Bio-rad, Hercules, CA).

Colony formation assay

Colony formation was determined as previously described (Taguwa et al., 2009). Briefly, *in vitro*-transcribed RNA was electroporated into Huh7 cells and plated on DMEM containing 10% FBS and non-essential amino acids. The medium was replaced with fresh DMEM containing 10% FBS, non-essential amino acids, and 1 μg/ml puromycin at 24 h post-transfection. The remaining colonies were fixed with 4% paraformaldehyde (PFA) and stained with crystal violet at 3 weeks after electroporation.

Indirect immunofluorescence assay

Cells cultured on glass slides were fixed with 4% PFA in phosphate buffered saline (PBS) at room temperature for 30 min. After washing three times with PBS, the cells were permeabilized for 20 min at room temperature with PBS containing 0.25% saponin and blocked with phosphate buffer containing 2% BSA for 1 h at room temperature. The cells were incubated with blocking buffer containing mouse anti-HA or rabbit anti-CypB at room temperature for 1 h, then washed three times with PBS and incubated with blocking buffer containing AF488-conjugated anti-mouse IgG and AF594-conjugated anti-rabbit IgG at room temperature for 1 h. Cell nuclei were stained blue with DAPI. Finally, the cells were washed three times with PBS and observed a FluoView FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan).

Statistical analysis

Results are expressed as the means ± standard deviation. The significance of differences between the means was determined by Student's *t*-test.

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