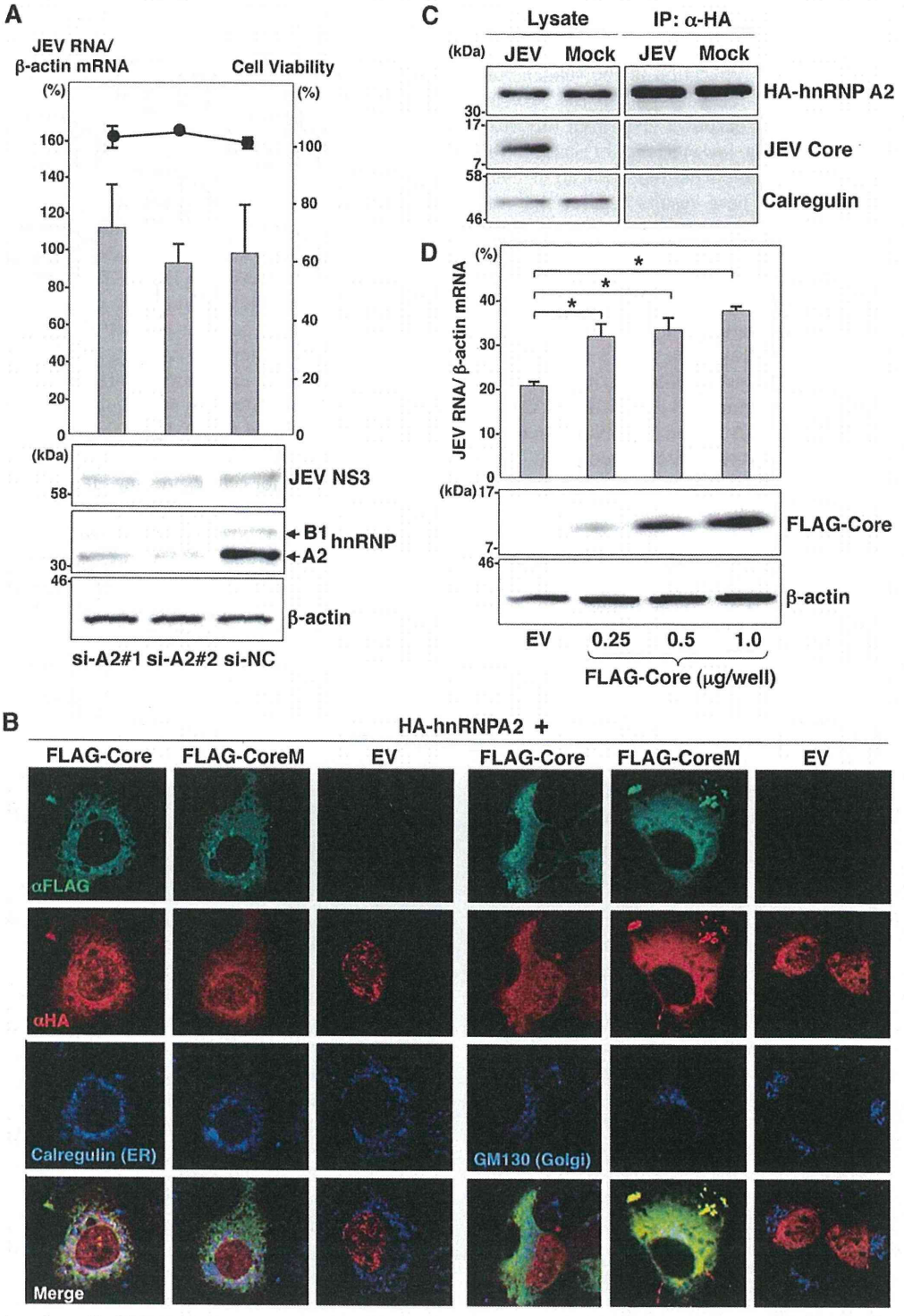


FIG. 4. Translocation of hnRNP A2 from the nucleus to the ER by expression of JEV core protein enhances viral RNA replication. (A) JEV subgenomic replicon (JEV-SGR-293T) cells were transfected with either si-A2#1, si-A2#2, or si-NC and harvested at 48 h posttransfection. (Top) The level of JEV RNA (NS5) was determined by real-time PCR and calculated as a percentage of the control β-actin mRNA level (bar graph). Cell viability was determined 48 h after transfection with each siRNA and was calculated as a percentage of the viability of cells treated with si-NC (line graph). (Bottom) Cell lysates collected at 48 h posttransfection were subjected to immunoblotting with mouse MAbs to JEV NS3, hnRNP A2 (DP3B3), and β-actin. (B) JEV subgenomic replicon (JEV-SGR-Huh7) cells were transfected with an expression plasmid for HA-hnRNP A2 together with that for FLAG-Core or FLAG-CoreM (a mutant defective in nuclear localization) or with an empty vector (EV) and then fixed with cold acetone at 24 h posttransfection. FLAG-Core was stained with either mouse anti-FLAG MAb (M2) and AF488-conjugated anti-mouse IgG or rabbit anti-FLAG PAb and AF488-conjugated anti-rabbit IgG. HA-hnRNP A2, calregulin, and GM130 were stained with rat anti-HA MAb (3F10), rabbit anti-calregulin PAb, and mouse anti-GM130 MAb, followed by AF594-conjugated anti-rat IgG, AF633-conjugated anti-rabbit IgG,

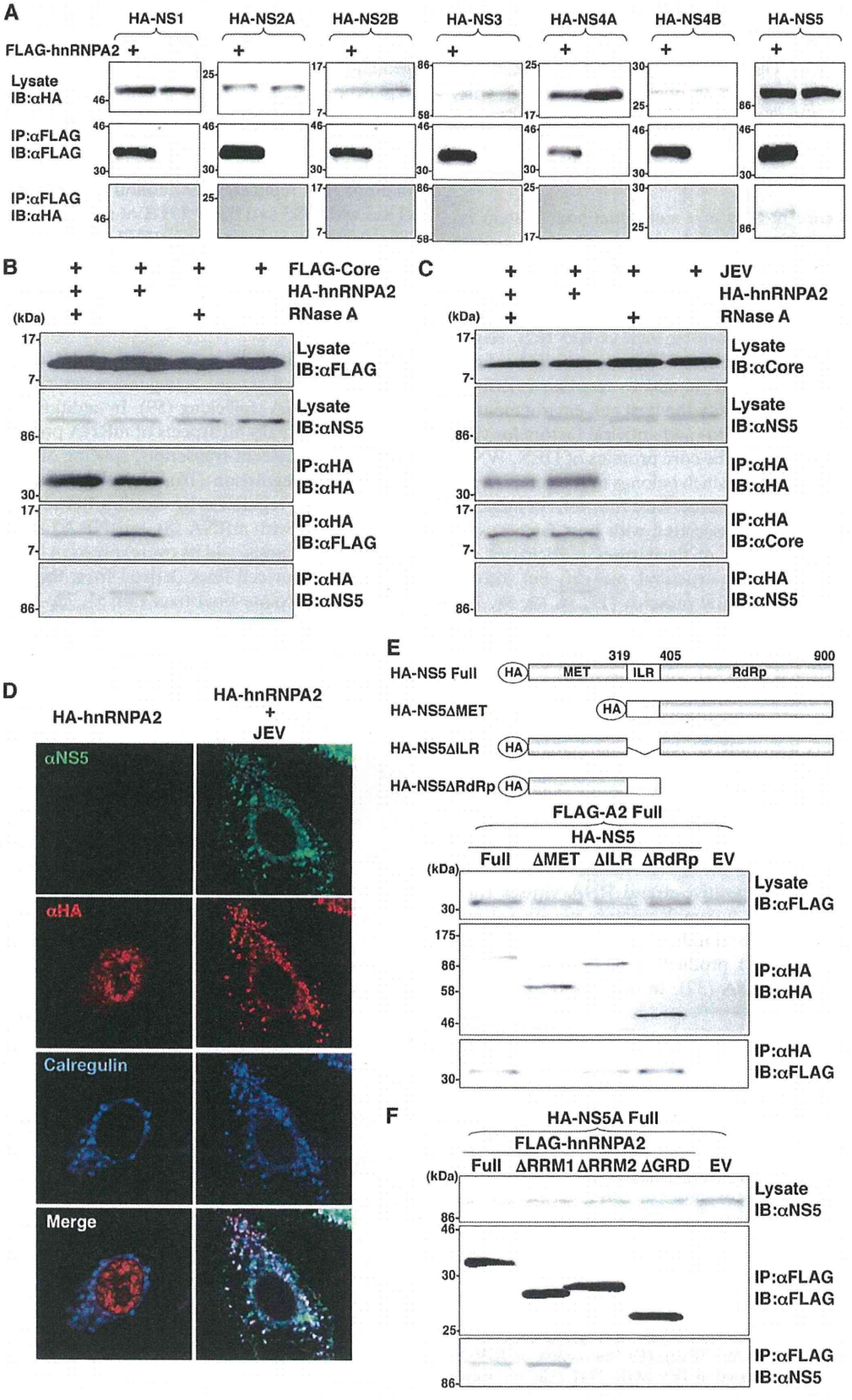
expressed in JEV-SGR-293T cells. As shown in Fig. 4D, the levels of JEV RNA were increased up to <2-fold, in accord with the expression levels of the core protein. The effect of JEV core expression on RNA replication in the subgenomic replicon cells was weak compared with that in the JEV-infected cells with hnRNP A2 knockdown (Fig. 3A); the weak effect might be attributable to the low efficiency of access of the exogenously expressed core protein to the replication complexes in the replicon cells. These results suggest that the passive ER retention of hnRNP A2 by interaction with the JEV core protein enhances viral replication.

hnRNP A2 interacts with JEV NS5 through interaction with viral RNA, in contrast to protein-protein interaction with JEV core protein. The viral RNA replication of flaviviruses takes place in a replication complex consisting of NS proteins and host proteins in the ER (45). To determine the interaction of hnRNP A2 with JEV NS proteins, FLAG-hnRNP A2 was coexpressed with each of the HA-tagged JEV NS proteins in JEV-SGR-293T cells and immunoprecipitated. with anti-FLAG antibody. Among the JEV NS proteins we examined, only NS5 was coimmunoprecipitated with hnRNP A2 (Fig. 5A). More importantly, the interaction between hnRNP A2 and NS5 was observed in JEV-SGR-293T cells but not in 293T cells expressing both of the proteins (data not shown). Like the core protein, both NS5 and hnRNP A2 are RNA-binding proteins (23, 59, 74), and thus it might be feasible to speculate that viral RNA mediated the interplay between these proteins. To determine the role of viral RNA in these interactions, HA-hnRNP A2 and FLAG-Core were coexpressed in JEV-SGR-293T cells, and the cell lysates were treated with RNase A before immunoprecipitation (Fig. 5B). Coprecipitation of NS5 with hnRNP A2 in the replicon cells was abolished by the treatment with RNase A, while that of JEV core protein with hnRNP A2 was rather resistant to the treatment. The RNA-mediated interaction between NS5 and hnRNP A2 was also observed in the JEV-infected cells, but not that between the core protein and hnRNP A2 (Fig. 5C). These results indicate that hnRNP A2 interacts with JEV NS5 through the interaction with viral RNA, in contrast to protein-protein interaction with the JEV core protein. Next, to determine the subcellular localization of NS5 and hnRNP A2, Vero cells expressing HA-hnRNP A2 were infected with JEV and examined by confocal microscopy at 24 h postinfection. Both hnRNP A2 and NS5 were colocalized in the ER of cells infected with JEV (Fig. 5D). These results suggest that hnRNP A2 interacts with JEV core protein and NS5A around the ER (Fig. 4B) and in the ER, respectively. To determine the region in NS5 responsible for the interaction with hnRNP A2, three HA-NS5 mutants, lacking the MTase region (ΔMET), the internal linker region (ΔILR), and the RdRp region (ΔRdRp), were coexpressed with FLAG-hnRNP A2 in JEV-SGR-293T cells. FLAG-hnRNP A2 was immunoprecipitated with each of the NS5 constructs except for the mutant lacking the MTase region (Fig. 5E). On the other hand, NS5 was coprecipitated with full-length hnRNP A2 (FLAG-A2 Full) and with a mutant lacking the RRM1 domain (FLAG-A2 ΔRRM1) but not with mutants lacking either RRM2 or GRD (FLAG-A2 ΔRRM2 or FLAG-A2 ΔGRD) (Fig. 5F). These results indicate that RRM2 and GRD in hnRNP A2 participate in the interaction with the MTase region in NS5 as well as with the C-terminal region of the JEV core protein, as described above (Fig. 1E).

hnRNP A2 interacts with the 5′ UTR of the negative-sense JEV RNA and facilitates viral RNA synthesis. Next, to determine the interaction between hnRNP A2 and JEV RNA, 293T cells expressing HA-hnRNP A2 were inoculated with JEV, and the cell lysates were immunoprecipitated with an anti-HA antibody at 24 h postinfection. RNAs extracted from the precipitates were subjected to RT-PCR to detect JEV RNA. JEV NS1 RNA was detected in the precipitates obtained by anti-HA antibody for cells expressing HA-hnRNP A2 (Fig. 6A), suggesting that hnRNP A2 associates with JEV RNA. Several hnRNPs have been shown to interact with the UTR of the viral RNA of positive-strand RNA viruses, such as poliovirus and enterovirus 71 (5, 35). To determine the region in the UTRs of JEV responsible for the interaction with hnRNP A2, biotin-labeled 5′ and 3′ UTRs of the positive- and negative-sense JEV RNAs were synthesized *in vitro* (Fig. 6B), and a pulldown assay was carried out using streptavidin beads to capture the biotinylated viral RNA associated with HA-hnRNP A2 in 293T cells. HA-hnRNP A2 was pulled down with the 5′ UTR of the negative-strand viral RNA but not with other viral RNAs. To further confirm the interaction between hnRNP A2 and the viral RNA, GST-hnRNP A2 prepared in *E. coli* was incubated with the biotinylated 5′ UTR of the negative-strand JEV RNA or 3′ UTR of the positive-strand JEV RNA, and the viral RNA interacting with GST-hnRNP A2 was detected by Northern blot analyses using streptavidin. The 5′ UTR of the negative-strand JEV RNA was detected in the complex (Fig. 6C). These results indicate that hnRNP A2 interacts directly with the 5′ UTR of the negative-strand JEV RNA.

As described for Fig. 3, knockdown of hnRNP A2 suppresses JEV propagation. To further examine the roles of hnRNP A2 in viral RNA replication in more detail, syntheses of the positive- and negative-strand viral RNAs were determined for cells transfected with si-A2#2 targeted to hnRNP A2 and inoculated with JEV at 24 h posttransfection. Total RNAs extracted from the infected cells at various time points were reverse transcribed by using strand-specific primers for either the 5′ UTR of the negative-strand JEV RNA or the 3′ UTR of the positive-strand JEV RNA, with oligo(dT) primers used for

and AF633-conjugated anti-mouse IgG antibodies, respectively. (C) HA-hnRNP A2 was expressed in 293T cells, which were infected with JEV at an MOI of 1.0 and subjected to immunoprecipitation with mouse anti-HA MAb (HA11) at 24 h postinfection. The immunoprecipitates were subjected to immunoblotting using rat anti-HA MAb (3F10), rabbit anti-core PAb, or rabbit anti-calregulin PAb. (D) JEV-SGR-293T cells were transfected with empty vector (EV) or a plasmid encoding FLAG-Core, and the level of JEV RNA (NS5) was determined by real-time PCR at 48 h posttransfection and calculated as a percentage of the control β-actin mRNA level. The data are representative of three independent experiments. Error bars indicate the standard deviations of the means. The significance of differences between the means was determined by Student's *t* test.



β-actin mRNA as an internal control, and JEV RNA and β-actin mRNA levels were determined by real-time PCR. Syntheses of both the positive- and negative-strand viral RNAs were delayed from 12 h postinfection in the hnRNP A2 knockdown cells (Fig. 6D). These results suggest that hnRNP A2 facilitates viral RNA synthesis through interaction with core, NS5, and the 5′ UTR of negative-strand viral RNA.

DISCUSSION

The flavivirus core protein is a multifunctional protein involved in viral replication and pathogenesis. The core protein binds to the viral RNA and forms a nucleocapsid in the cytoplasm as a structural protein (23). Furthermore, some portion of the core protein of flaviviruses localizes in the nucleus and associates with various host factors, such as B23 (62), Hsp70 (54), Daxx (50), and Jab1 (53). The DEN core protein has been shown to interact with hnRNP K and to regulate C/EBP-β-mediated transcription to modify the host cell environment by regulating the expression of pro- and antiviral factors for viral propagation (7). In addition, the core proteins of DEN, WNV, and hepatitis C virus (HCV), which belongs to the genus *Hepacivirus* within the family *Flaviviridae*, have functions of inducing or inhibiting the apoptosis associated with host factors, suggesting that the core protein of flaviviruses participates not only in viral assembly (as a structural protein) but also in pathogenesis (as a nonstructural protein) (13, 34, 43, 58, 70). The flavivirus core protein is not essential for RNA replication, since NS proteins alone are sufficient for efficient replication of the subgenomic viral RNA (24), while the core protein has been suggested to augment viral RNA replication (48).

In this study, we demonstrated that the JEV core protein specifically interacts with hnRNP A2 and participates in viral replication. Many RNA-binding proteins, including members of the hnRNP complex, have been shown to participate in the life cycles of several positive-strand RNA viruses, such as poliovirus (5), enterovirus 71 (35, 36), Sindbis virus (36), HCV (25), and DEN (7, 20, 51), through an interaction with viral RNA. The RdRp of the positive-strand RNA viruses transcribes the viral RNA into a complementary negative-strand RNA and generates double-stranded RNA, which serves as a replicative intermediate for production of a large excess of positive-strand genomic RNA (32). In the case of poliovirus, hnRNP C1/C2 has been shown to be involved in the initiation of viral RNA synthesis through the interaction with viral replication polypeptides and the 3′ UTR of the negative-strand RNA (5). In this study, we have shown that the JEV core protein interacts with hnRNP A2 and participates in RNA replication through recruitment of hnRNP A2 to the ER; however, enhancement of JEV replication could not be explained by the interaction of the core protein with hnRNP A2. Therefore, we further examined the factors involved in the enhancement of JEV replication and found that hnRNP A2 also associates with NS5 and the 5′ UTR of negative-strand viral RNA. These findings suggest that hnRNP A2 participates in positive-strand RNA synthesis through the interaction with viral proteins and RNA. Further studies are needed to clarify the molecular mechanisms by which hnRNP A2 promotes viral RNA replication.

hnRNP A2 is the most abundant of the hnRNP family proteins and is the first *trans*-acting factor described to be involved in neural mRNA trafficking (59). In addition, hnRNP A2 participates in virtually all aspects of mRNA processing, including packaging of nascent transcripts, splicing of pre-mRNAs, and translational regulation (16), and plays crucial roles in post-transcriptional regulation by shuttling between the nucleus and the cytoplasm with mRNA (8). hnRNP A2 also participates in telomere biogenesis, and its overexpression has been described for many cancer cell lines derived from the breast, pancreas, liver, and gastrointestinal tract (30, 31, 72, 75). In virus infections, hnRNP A2 has been shown to enhance mouse hepatitis virus RNA synthesis (60) and to regulate the trafficking of genomic RNA of human immunodeficiency virus (33). Although in this study we have demonstrated that hnRNP A2 interacts with the 5′ UTR of negative-strand JEV RNA, it has been shown previously that hnRNP A2 interacts with the 3′ UTR of positive-strand DEN RNA (55). This discrepancy might be attributable to differences in sequence and/or structure of the UTRs between JEV and DEN, as reported previously (21). Localization of hnRNP A2 was changed from the nucleus to the cytoplasm upon infection with JEV, as seen in many other virus infections (5, 25, 35). In addition, expression of the JEV core protein alone induced retention of hnRNP A2 in the ER and facilitated RNA replication in the replicon cells, suggesting that interaction of the core protein with hnRNP A2 is important for RNA replication of JEV.

FIG. 5. hnRNP A2 forms a complex with JEV core protein and NS5 via JEV RNA. (A) FLAG-hnRNP A2 was coexpressed with HA-JEV NS proteins in JEV subgenomic replicon (JEV-SGR-293T) cells and immunoprecipitated (IP) with mouse anti-FLAG MAb (M2). The immunoprecipitates were subjected to immunoblotting (IB) using rabbit anti-FLAG PAb. (B) FLAG-Core and HA-hnRNP A2 were coexpressed in JEV-SGR-293T cells, and cell lysates were treated with or without RNase A (10 μg/ml) for 30 min at 4°C and immunoprecipitated with mouse anti-HA MAb (HA11). The immunoprecipitates were subjected to IB with rabbit anti-FLAG PAb, mouse anti-NS5 MAb, or rat anti-HA MAb (3F10). (C) HA-hnRNP A2 was expressed in 293T cells, which were infected with JEV at an MOI of 1.0 and subjected to IP with mouse anti-HA MAb (HA11) at 24 h postinfection. The cell lysates were pretreated with or without RNase A (10 μg/ml) for 30 min at 4°C. The immunoprecipitates were subjected to IB using rabbit anti-core PAb, mouse anti-NS5 MAb, or rat anti-HA MAb (3F10). (D) Vero cells infected with JEV at an MOI of 1.0 and fixed with cold acetone at 24 h postinfection. JEV NS5, HA-hnRNP A2, and calregulin, which is an ER marker, were stained with mouse anti-NS5 MAb, rat anti-HA MAb (3F10), and anti-calregulin rabbit PAb (H-170), followed by AF488-conjugated anti-mouse IgG, AF594-conjugated anti-rat IgG, and AF633-conjugated anti-rabbit IgG, respectively. (E) Interaction of hnRNP A2 with deletion mutants of JEV NS5. FLAG-hnRNP A2 and a series of deletion mutants of HA-NS5 were cotransfected into JEV-SGR-293T cells. Deletion mutants of HA-NS5 in cell lysates were immunoprecipitated with mouse anti-HA MAb (HA11), and the immunoprecipitates were subjected to IB with mouse anti-FLAG MAb (M2) or rat anti-HA MAb (3F10). (F) Interaction of JEV NS5 with deletion mutants of hnRNP A2. A series of deletion mutants of FLAG-hnRNP A2 were expressed in JEV-SGR-293T cells and were immunoprecipitated with mouse anti-FLAG MAb (M2). The immunoprecipitates were subjected to IB with rabbit anti-FLAG PAb or mouse anti-NS5 MAb.

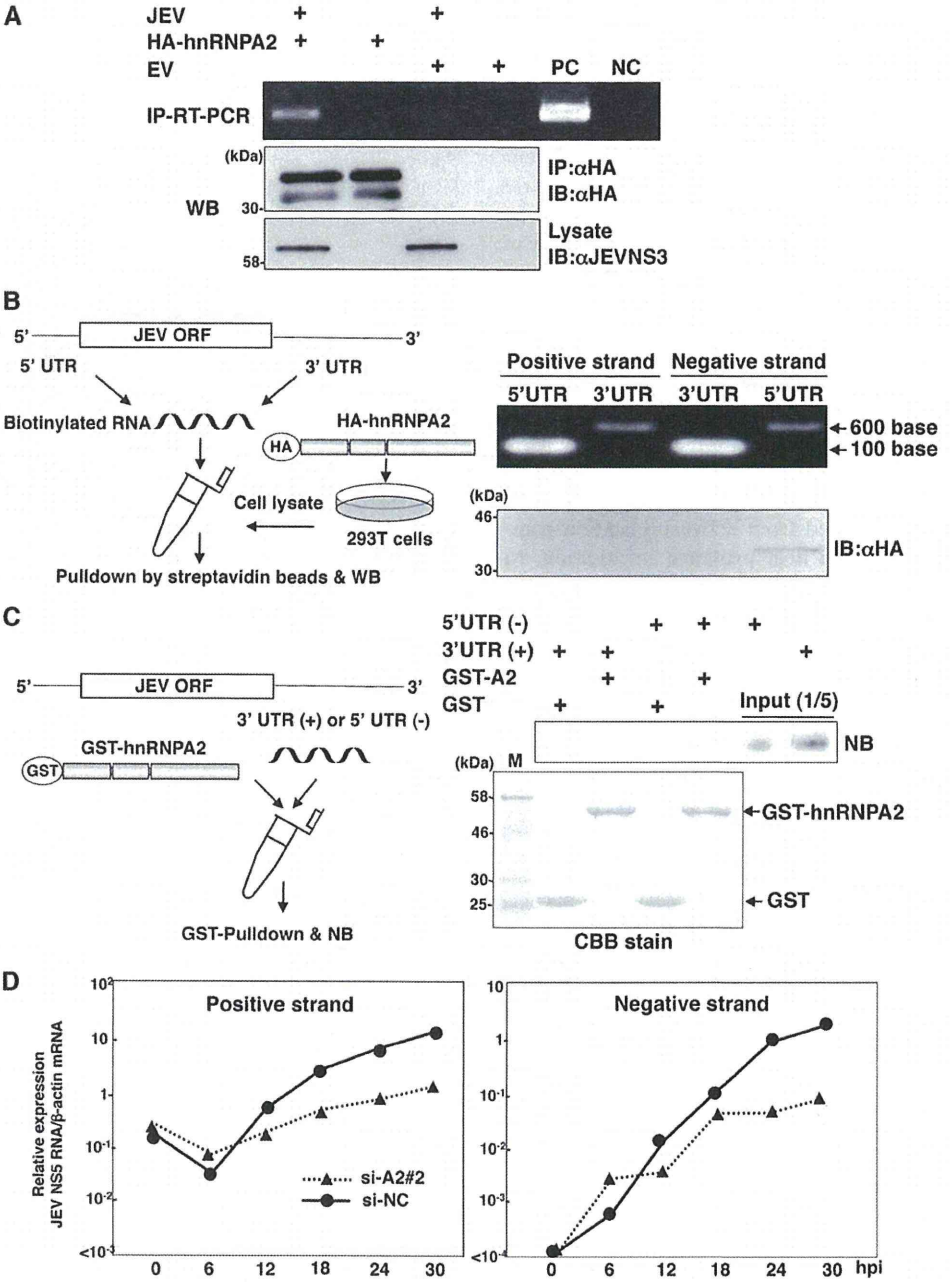


FIG. 6. hnRNP A2 interacts with the 5′ UTR of the negative-strand JEV RNA and facilitates viral RNA synthesis. (A) A plasmid encoding HA-hnRNP A2 was transfected into 293T cells, which were infected with JEV at an MOI of 1.0 at 24 h posttransfection. Cell lysates harvested at 24 h postinfection were immunoprecipitated with mouse anti-HA MAb (HA11) and subjected to IB with rat anti-HA MAb (3F10). RNAs extracted from the immunoprecipitates were subjected to RT. JEV RNA was detected by PCR using primers targeting the NS1 region. (B) Cell lysates from 293T cells transfected with a plasmid encoding HA-hnRNP A2, prepared at 24 h posttransfection, were incubated with the biotin-labeled 5′ or 3′ UTR of the positive- or negative-sense JEV RNA for 15 min at 30°C. After pulldown by streptavidin, protein complexes were subjected to IB with mouse anti-HA MAb (HA11). (C) GST-fused hnRNP A2 prepared in bacteria was incubated with the biotin-labeled 3′ UTR of positive-sense JEV RNA or 5′ UTR of negative-sense JEV RNA for 15 min at 30°C. RNAs extracted from the precipitates obtained by GST pulldown were subjected to Northern blotting (NB) with streptavidin. CBB, Coomassie brilliant blue. (D) Total RNA extracted from 293T cells transfected with si-A2#2 or si-NC and infected with JEV at an MOI of 10 at 24 h posttransfection was subjected to RT using strand-specific primers and an oligo(dT) primer. The levels of positive- and negative-strand JEV RNAs (NS5) were determined by real-time PCR and calculated as percentages of the control β-actin mRNA level. Closed triangles and circles indicate the relative RNA levels in cells transfected with si-A2#2 and si-NC, respectively. The data are representative of three independent experiments.

Although replication and packaging of the viral genome remain obscure steps in the life cycle of flaviviruses, coupling between RNA replication and particle assembly has been suggested to occur in several positive-strand RNA viruses (22, 52). Replication of flaviviruses takes place in virus-induced intracellular membrane structures on the ER known as replication complexes, which contain NS proteins, viral RNA, and host factors essential for replication, and is suggested to circumvent the activation of the host immune response triggered by viral RNA (45). The invagination of the ER membrane induced by the expression of NS proteins is connected to the cytoplasm through pores, which allows entry of nucleotides and other factors required for RNA replication and for exit of the newly synthesized RNA to the sites for translation and particle assembly (10, 44, 65). The majority of the viral RNA species in the replication complex exist as double-stranded replicative intermediates, and newly transcribed genomic RNA is exported efficiently from the replication complex to the assembly sites (10). In addition, to minimize the production of defective viral RNA transcribed from the error-prone viral RdRp (22), viral RNA must be synthesized from active replication complexes consisting of viral and host proteins; in addition, to circumvent the induction of innate immunity, the viral genome should be packaged immediately into viral particles. Therefore, structural and nonstructural viral proteins participate coordinately in viral RNA replication and particle formation, as reported for the participation of the NS3 protein of YFV in viral assembly (56). Although the biological significance of the interaction between the JEV core protein and hnRNP A2 for viral replication is unclear, it might be feasible to speculate that the core protein recruits hnRNP A2 to the replication complex to promote viral RNA replication.

The property of hnRNP family proteins shuttling between the cytoplasm and the nucleus has been suggested to participate in the maintenance of cellular homeostasis in response to stress stimuli such as heat shock (57), amino acid starvation (41), mitochondrial dysfunction (11), and nucleolar stresses (73). Infection of positive-strand RNA viruses modulates the host environment for efficient viral propagation through the remodeling of host proteins. For instance, the core protein of WNV suppresses the expression of the ER stress-protective protein OASIS and inhibits the antiviral response through induction of ER stress (2, 63), while poliovirus infection increases nuclear envelope permeability and replaces nuclear proteins required for efficient viral replication in the cytoplasm (3). In this study, hnRNP A2 was localized in both the nucleus and the cytoplasm upon infection with JEV or expression of the JEV core protein, suggesting that the JEV core protein plays an important role in the replication of JEV RNA through a modification of the host cellular environment.

Viruses are obligatory intracellular parasites, and therefore they are completely dependent on infected cells to supply energy, chemicals, and much of the machinery required for their replication. In the present study, we identified hnRNP A2 as one of the host factors participating in JEV propagation. The ER retention of hnRNP A2 through an interaction with the core protein leads to the interaction of hnRNP A2 with NS5 and the negative-strand viral RNA, resulting in the promotion of JEV RNA replication. This may help to open up a new area of inquiry into virus-cell interactions and could lead to an improved understanding of the mechanism of flavivirus RNA replication.

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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

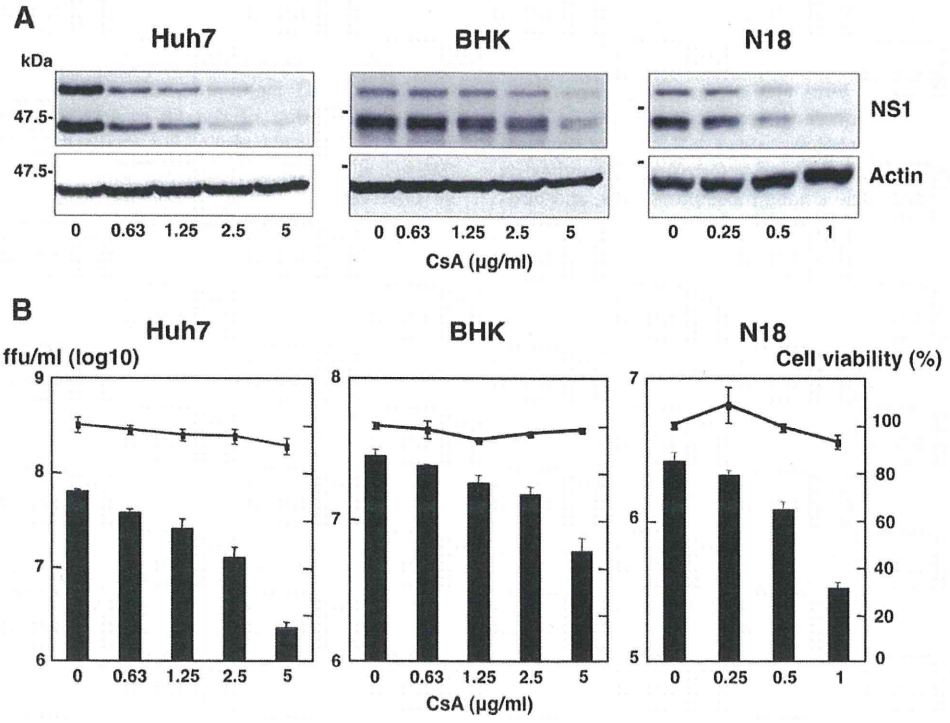


Fig. 1. Effect of CsA on the propagation of JEV in mammalian cells. (A) JEV was inoculated at an MOI of 0.1 (Huh7 and BHK cells) or 10 (N18 cells) and incubated for 1 h. Cells were washed with 10% FBS DMEM and treated with the indicated concentrations of CsA in 10% FBS DMEM for 48 h. The propagation 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.

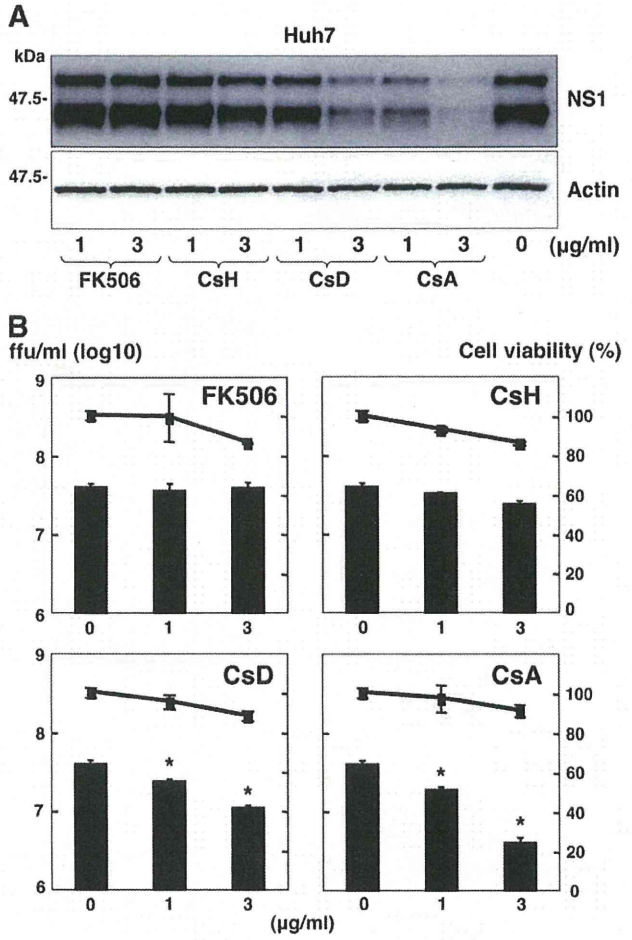


Fig. 2. CsA inhibits JEV propagation through the inhibition of Cyps. Huh7 cells were infected with JEV at an MOI of 0.1 for 1 h and then treated with 10% FBS DMEM containing the indicated concentrations of CsA, CsD, CsH, or FK506 for 48 h. The propagation of JEV was evaluated by immunoblotting (A) and 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 pJEreplRESpuro 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.

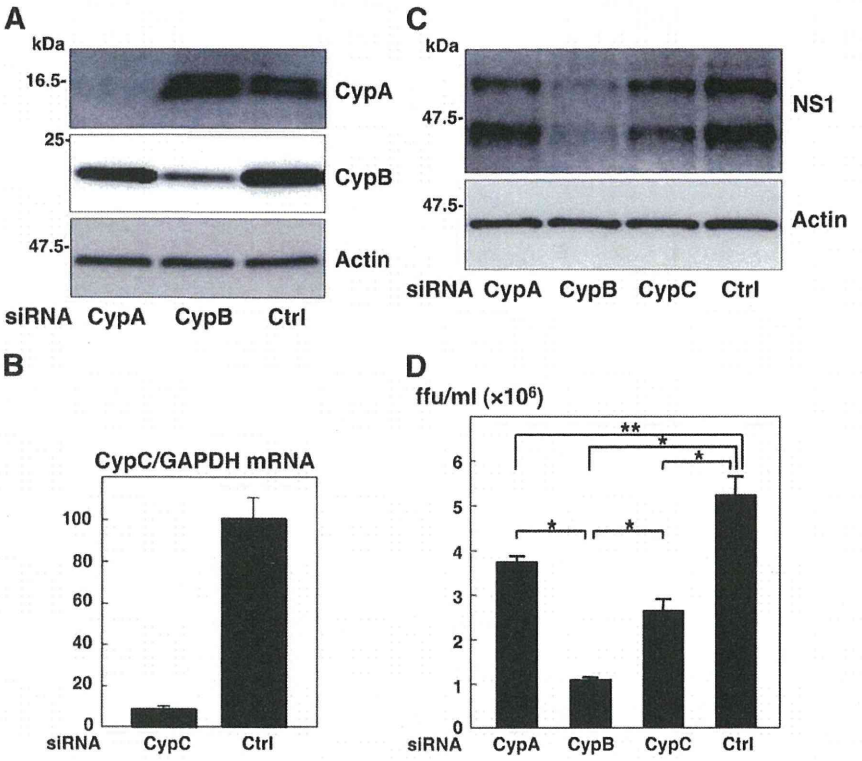


Fig. 3. CypB plays an important role in the propagation of JEV. (A) Knockdown of endogenous CypA and CypB by siRNA. Huh7 cells were transfected with 35 nM of siRNA targeted to CypA, CypB, or a non-specific control. Cell lysates after 96 h post-transfection were analyzed for expression of CypA, CypB, 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

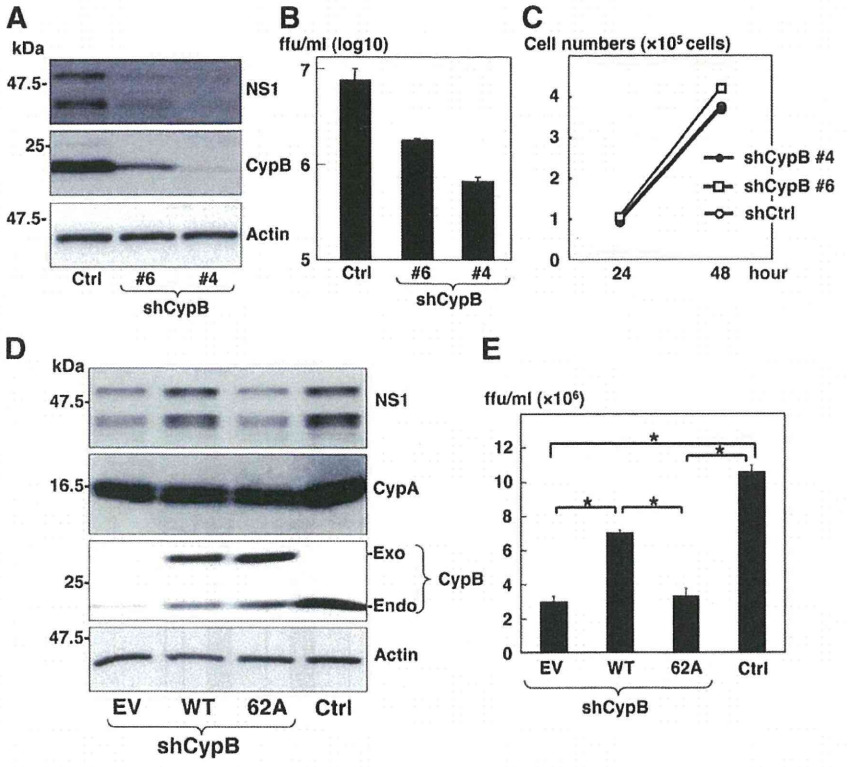


Fig. 4. PPIase activity of CypB is crucial for the propagation of JEV. Huh7 cell lines expressing shRNA targeted to CypB or the control were infected with JEV at an MOI of 0.1 for 1 h and cultured in 10% FBS DMEM for 48 h. The expressions of NS1, CypB, and actin were detected by immunoblotting (A). The 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.

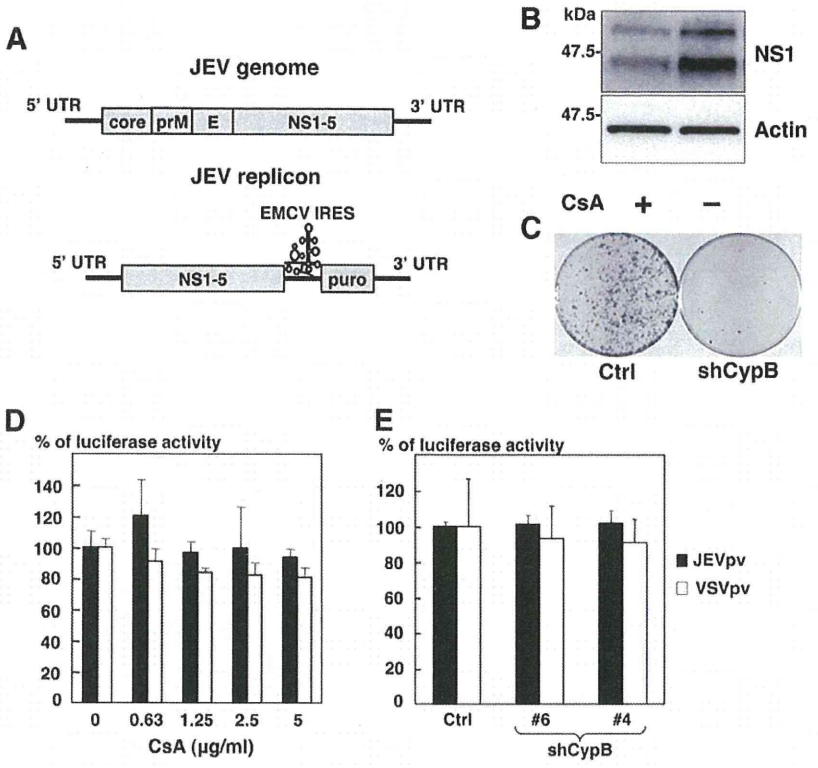


Fig. 5. CypB participates in the replication but not in the entry of JEV. (A) Schematic representations of the JEV genome and its subgenomic replicon. (B) JEV replicon cells were treated with CsA (1 μg/ml) for 6 days, and the expressions of NS1 and actin were detected by immunoblotting. (C) The stable 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.

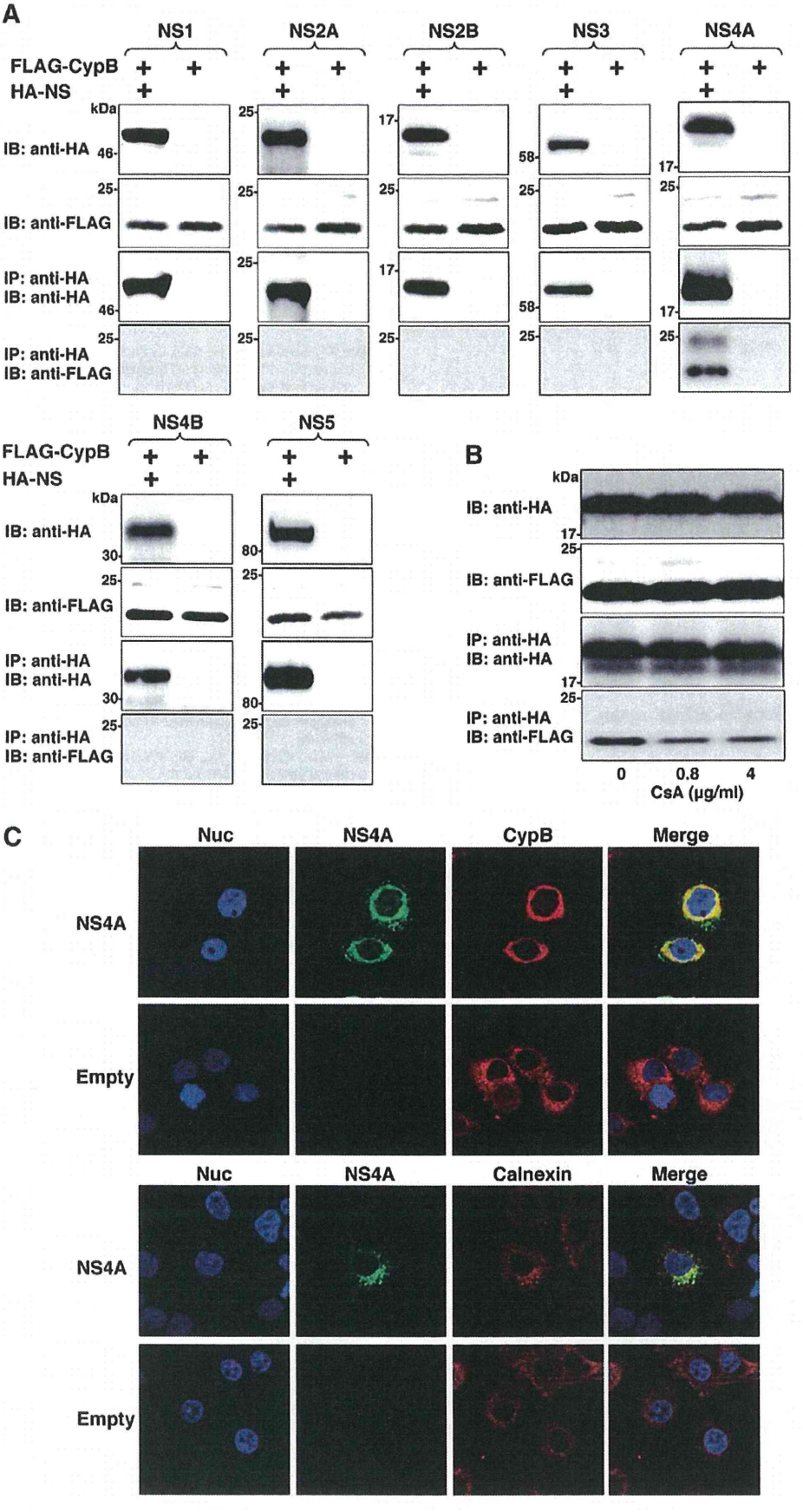
Reagents and antibodies

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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Elimination of Hepatitis C Virus from Hepatocytes by a Selective Activation of Therapeutic Molecules

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Abstract

To eliminate hepatitis C virus (HCV) from infected hepatocytes, we generated two therapeutic molecules specifically activated in cells infected with HCV. A dominant active mutant of interferon (IFN) regulatory factor 7 (IRF7) and a negative regulator of HCV replication, VAP-C (Vesicle-associated membrane protein-associated protein subtype C), were fused with the C-terminal region of IPS-1 (IFNβ promoter stimulator-1), which includes an HCV protease cleavage site that was modified to be localized on the ER membrane, and designated cIRF7 and cVAP-C, respectively. In cells expressing the HCV protease, cIRF7 was cleaved and the processed fragment was migrated into the nucleus, where it activated various IFN promoters, including promoters of IFNα6, IFNβ, and IFN stimulated response element. Activation of the IFN promoters and suppression of viral RNA replication were observed in the HCV replicon cells and in cells infected with the JFH1 strain of HCV (HCVcc) by expression of cIRF7. Suppression of viral RNA replication was observed even in the IFN-resistant replicon cells by the expression of cIRF7. Expression of the cVAP-C also resulted in suppression of HCV replication in both the replicon and HCVcc infected cells. These results suggest that delivery of the therapeutic molecules into the liver of hepatitis C patients, followed by selective activation of the molecules in HCV-infected hepatocytes, is a feasible method for eliminating HCV.

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Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver diseases. A high risk of chronicity is the major concern of HCV infection, since chronic HCV infection often leads to liver cirrhosis and hepatocellular carcinoma [1,2]. Although the proportion of patients achieving a sustained virological response (SVR) has been increased by the recent used of combination therapy with pegylated-interferon-α (PEG-IFNα) and ribavirin (RBV), half of patients still exhibit no response to this therapy, suggesting that the IFN signaling pathway is modulated by HCV infection. In addition, various side effects have been reported in more than 20% of patients treated with this combination therapy [3].

HCV belongs to the family *Flaviviridae* and possesses a single positive-stranded RNA genome that encodes a single polyprotein composed of about 3,000 amino acids. The HCV polyprotein is processed into 10 viral proteins by host and viral proteases. Viral structural proteins, including the capsid protein and two envelope proteins, are located in the N-terminal one third of the polyprotein, followed by nonstructural proteins. The NS2 protease cleaves its own carboxyl terminus and NS3 cleaves the downstream positions to produce NS4A, NS4B, NS5A and NS5B. Although laboratory strains of HCV propagating in cell culture (HCVcc) have been established based on the full-length genome of the genotype 2a JFH1 strain [4], establishment of a robust cell culture system capable of propagating serum-derived HCV from hepatitis C patients has not yet been achieved.

Type I IFN exhibits potent antiviral effects through the regulation of hundreds of IFN-stimulated genes (ISGs) which encode proteins involved in the establishment of antiviral state in cells [5]. IFNs induce transcription of ISGs through activation of the Jak-STAT pathway [6]. Binding of type I IFN to the IFN receptor induces phosphorylation of the receptor-associated tyrosine kinases, Jak1 and Tyk2, and then these kinases activate STAT1 and STAT2. The phosphorylated STATs migrate into the nucleus and activate ISG promoters through binding to the specific responsible elements. HCV infection has been suggested to impair the IFN production through multiple pathways. The IFN-induced Jak-STAT signaling is inhibited in cells and transgenic mice expressing HCV proteins and in the liver biopsy samples of chronic hepatitis C patients [7–9].

Induction of type I IFN upon infection with pathogens is crucial for innate immunity, and it is mediated by the activation of pattern-recognition receptors, including Toll-like receptors (TLRs) and cytosolic receptors, such as RIG-I and MDA5 [10–12]. The induction of type I IFN is primarily controlled at the gene transcriptional level, wherein a family of transcription factors known as IFN regulatory factors (IRFs) play a pivotal role. IRF3

and IRF7 are known to be essential for the RIG-I-, MDA5-, and TLR-mediated type I IFN production pathways. IRF3 is induced primarily by a response to initiate IFNβ production, whereas IRF7 is induced by IFNβ and participates in the late phase for IFNβ induction [13]. All TLRs, except for TLR3, activate the MyD88-dependent pathway, whereas TLR3 and TLR4 activate the TRIF-dependent pathway. HCV NS3/4A protease has been shown to impair the production of IFNβ as well as the subsequent IFN-inducible genes through the inactivation of the adaptor molecules involved in the TLR-dependent and -independent signaling pathways [14–18]. On the other hand, Vilasco *et al.* suggested that impairment of IKKi - which, along with TBK1, is one of the important factors participating in IRF3 phosphorylation and activation - in the HCV replicon cells plays at least a partial role in the restoration of type I IFN signaling pathways [19]. In addition, IRF7 was shown to participate in the positive feedback of type I IFN signaling through the IFN receptor [13]. Therefore, we tried to examine the effect of exogenous expression of IRF7 under the assumption that IRF7 is a potent type I IFN inducer and capable of modulating the viral propagation in hepatocytes infected with HCV.

In this study, we generated two therapeutic molecules consisting of a dominant active mutant of IRF7 or VAP-C, a negative regulator of HCV replication [20], followed by the C-terminal region of IFN promoter stimulator 1 (IPS-1), including the cleavage site of the HCV NS3/4A protease, which was modified so that the cleavage site localized on the ER membrane [21]. The expression of the plasmids encoding these molecules in the HCV replicon and HCVcc-infected cells resulted in a substantial suppression of HCV propagation, suggesting the possibility that these or other similar molecules could be used therapeutically to eliminate HCV from hepatocytes infected with HCV.

Results

IRF7m, a dominant active mutant of IRF7, activates the IFN promoters in cells replicating HCV

Previous studies have shown that an IRF7 mutant, IRF7m, lacking the amino acid residues from 284 to 454, a region that includes the auto-inhibitory domain (from amino acid residue 305 to 467), and an IRF3 mutant, IRF3m, carrying the substitution of Ser^{396} to Asp in the carboxyl terminal region (Fig. 1A), induced a potent activation of type I IFN promoter in non-hepatic cell lines irrespective of viral infection [22–25]. We first examined the effect of the expression of the IRF dominant active mutants on the inhibition of HCV RNA replication through the production of type I IFN. HCV replicon cells and Huh7OK1 cells infected with HCVcc were transfected with the plasmids encoding either wild-type or dominant active mutant of IRF3 or IRF7 together with the reporter plasmids encoding a luciferase gene under the control of the promoters of IFNα6, IFNβ and ISRE, respectively. Among these examined constructs, we observed significant activation of the promoters of IFNα6 and ISRE in the replicon and HCVcc-infected cells compared with naïve and mock-infected cells upon expression of IRF7m, while we observed no activation of the IFNα6 promoter in cells expressing IRF3m (Figs. 1 B and 1C). Potent stimulation of the IFNβ promoter was observed in the replicon cells expressing IRF7m but not in cells infected with HCVcc. Next we examined the antiviral activity of the IRF constructs in both replicon (Fig. 1D) and HCVcc-infected cells (Fig. 1E). The expression of the plasmid encoding IRF7m resulted in potent suppression of viral protein and viral RNA syntheses in both cell types. Although expression of IRF3m induced a slight suppression of viral propagation in cells infected with HCVcc, expression of the IRF constructs except for IRF7m did not induce the significant suppression of viral replication and propagation. These results suggest the possibility of elimination of HCV through a specific induction of type I IFN by the expression of IRF7m in HCV-infected cells.

cIRF7, a chimeric construct of IRF7m, specifically activates the IFN promoters in cells replicating HCV

To induce IFNs in cells infected with HCV but not in uninfected cells through a selective activation of IRF7m, we constructed a chimeric IRF7 (cIRF7) consisting of the IRF7m fused with FLAG-tag and the C-terminal amino acid residues from 503 to 540 of IPS-1 modified to be localized on ER (Fig. 2A upper) [21]. HCV NS3/4A protease cleaves the carboxyl site of Cys^{508} in the C-terminal domain of IPS-1. Although cIRF7 is anchored in the ER and exhibits no activation in uninfected cells, cIRF7 would be cleaved by the NS3/4A protease in cells infected with HCV and the released N-terminal fragment would migrate into the nucleus and activate various IFN promoters (Fig. 3). Immunoblot analyses revealed that cIRF7 was cleaved in 293T cells expressing HCV NS3/4A protease of a wild type but not in those expressing the mutant protease NS3/4A(S139A), and a mutant cIRF7(C508A) which has a substitution of Cys^{508} to Ala, exhibited resistance to the cleavage by the HCV protease (Fig. 2A bottom). To assess a specific activation of the IFN promoters after cleavage of the cIRF7 by HCV NS3/4A, 293T cells expressing FLAG-tagged HCV proteases were transfected with the plasmids encoding the luciferase gene under the control of the promoter of IFNα6, IFNβ or ISRE together with the plasmid encoding either cIRF7 or cIRF7(C508A). Expression of cIRF7 but not of cIRF7(C508A) induced the activation of the IFNα6, IFNβ and ISRE promoters in cells expressing HCV NS3/4A protease but not in those expressing the mutant protease NS3/4A(S139A) (Fig. 2B). Next we examined the activation of the IFN promoters associated with the expression of the plasmid encoding cIRF7 in the replicon and HCVcc-infected cells. Expression of cIRF7 but not of cIRF7(C508A) induced the activation of the IFN promoters in both cell types (Figs. 2C and 2D). On the other hand, these promoters were not activated by the expression of cIRF7 in the replicon cells harboring subgenomic RNA of Japanese encephalitis virus (JEV) and Huh7 cells infected with JEV (Fig. 2E). These results suggest that the cIRF7 expression is a feasible method for specifically activating the IFN promoters in cells infected with HCV.

Specificity of activation of the IFN promoters by the expression of cIRF7

To further examine the specificity of the activation of the IFN promoters by the expression of cIRF7 in cells replicating HCV, a plasmid encoding either cIRF7 or IRF7m was co-transfected with that encoding the luciferase gene under the ISRE promoter into the HCV replicon or HCVcc-infected cells and cultured in the presence or absence of inhibitors for HCV replication. Treatment with an HCV protease inhibitor (BILN2061) or cyclosporine A (CsA) inhibited the activation of the ISRE promoter by the expression of cIRF7 in the HCV replicon and HCVcc-infected cells in a dose-dependent manner, in contrast to the resistance to the treatments in cells expressing the IRF7m (Fig. 4A and Fig. 4B). Recently, it was shown that an NS3/4A protease of GB virus B (GBV-B), which is the virus genetically related most closely to HCV, also impairs the dsRNA-induced IFN production through a cleavage of IPS-1[26]. Therefore, to assess the possibility of activation of cIRF7 by other flaviviral proteases, cleavage of cIRF7

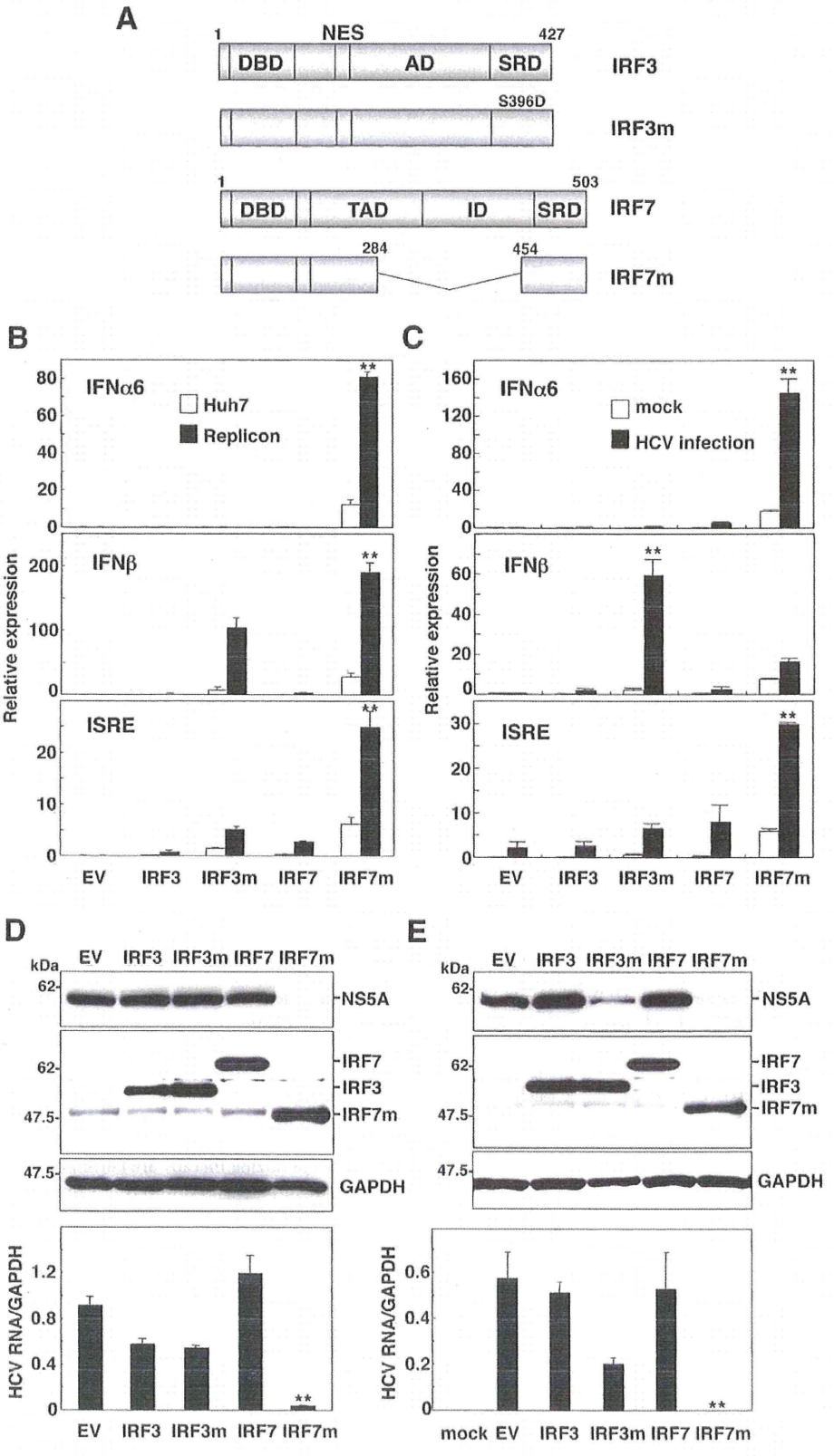

Figure 1. Dominant active mutant of IRF7 activates IFN promoters in cells replicating HCV. (A) Structures of IRF3, IRF7 and the dominant active mutants, IRF3m and IRF7m. The DNA-binding domain, nuclear export sequence, transactivation domain, association domain, inhibitory domain, and signal response domain are indicated as DBD, NES, TAD, AD, ID, and SRD, respectively. Huh7 cells and HCV replicon cells (1×10^5 cells/

well) (B), and Huh7OK1 cells (7.5×10^4 cells/well) infected with HCVcc at an moi of 1 and incubated for 72 h (C) were transfected with 100 ng of plasmid encoding the luciferase gene under the control of the IFNα6, IFNβ, or ISRE promoter together with an empty vector (EV) or a plasmid encoding each of the IRF constructs. The relative luciferase activity of cell lysates was determined at 24 h post-transfection. HCV replicon cells (3×10^5 cells/well) (D) and Huh7OK1 cells (1.5×10^5 cells/well) infected with HCVcc at an moi of 1 and incubated for 72 h (E) were transfected with EV or a plasmid encoding each of the IRF constructs and the expressions of NS5A, IRFs, and GAPDH (upper panel) and synthesis of viral RNA (lower panel) at 72 h post-transfection were determined by immunoblotting and real-time PCR after standardization with GAPDH, respectively. The data shown in this figure are representative of three independent experiments. The error bars represent the standard deviations. Asterisks indicate significant differences (**$P<0.01$) versus the control cells or mock-infected cells.
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and activation of the IFN promoters were evaluated in 293T cells expressing the viral proteases of HCV, GBV and JEV. Immunoblot analyses revealed that cIRF7 was processed by the viral proteases of HCV and GBV but not by that of JEV and the activation of the IFN promoters was well correlated with the cleavability of the cIRF7 (Fig. 4C). Although the GBV protease exhibited an efficient activation of cIRF7 comparable to HCV protease, processing of cIRF7 and activation of the IFN promoters by the GBV protease was not inhibited by the pretreatment with the HCV protease inhibitor (Figs. 4D and 4E). These results indicate that cIRF7 is capable of activating the IFN promoters through a specific cleavage by the protease in cells infected with HCV.

Nuclear localization of cIRF7 in cells expressing HCV protease

From these results, it was suggested that cIRF7 is cleaved by the HCV protease and the processed fragment migrates into the nucleus and activates IFN promoters (Fig. 3). To confirm the nuclear localization of the cleaved cIRF7, we constructed an EGFP-cIRF7 and determined its subcellular localization in cells expressing the HCV protease and in the HCV replicon cells by confocal microscopy. Nuclear accumulation of the cIRF7 was observed in cells expressing EGFP-cIRF7 together with NS3/4A, but not in those with NS3/4A(S139A) or NS5A and also not in cells co-expressing EGFP-cIRF7(C508A) and NS3/4A (Fig. 5A). Furthermore, expression of EGFP-cIRF7 but not of EGFP-cIRF7(C508A) induced a nuclear accumulation of cIRF7 in the HCV replicon cells, and nuclear localization of the cIRF7 abrogated the expression of viral antigen (NS3), in contrast to the co-localization of EGFP-cIRF7(C508A) and the ER marker PDI, which had no discernible antiviral effect (Fig. 5B). These results suggest that cIRF7 is capable of suppressing HCV replication through an HCV protease-dependent cleavage, migration into the nucleus and activation of the IFN promoters.

Suppression of HCV replication by the expression of cIRF7

To examine the inhibitory effect of the expression of cIRF7 on HCV replication, a plasmid encoding either cIRF7 or cIRF7(C508A) was transfected into the HCV replicon and HCVcc-infected cells, and HCV replication was evaluated by immunoblotting and real-time PCR. The expression of cIRF7 but not of cIRF7(C508A) resulted in cleavage by the HCV protease, and a clear reduction of viral protein and RNA syntheses in both replicon and HCVcc-infected cells (Figs. 6A and 6B). In addition, we examined the effect of cIRF7 on the replication of HCV in the 4βR replicon cells [27,28], which have been shown to exhibit more resistant to the IFNα treatment than Con1 replicon cells (Fig. 6C upper left). Expression of the cIRF7 in the 4βR replicon cells but not in those cured HCV RNA (4βRc cells) induced an activation of the ISRE promoter (Fig. 6C lower left). Expression of cIRF7 but not of cIRF7(C508A) also resulted in processing by the HCV protease and suppression of viral protein and RNA syntheses in the 4βR replicon cells (Fig. 6C right panels).

Suppression of HCV replication by the expression of cVAP-C

Human vesicle-associated membrane protein-associated protein subtype A (VAP-A) and B (VAP-B) are known to be involved in the regulation of membrane trafficking, lipid transport and metabolism, and the unfolded protein response [29]. VAP-A and VAP-B have been shown to be involved in the replication of HCV, and we have shown recently that human VAP-C, a splicing variant of VAP-B, negatively regulates HCV replication by interfering with the interaction of VAP-A and VAP-B with HCV NS5B [20]. We next examined the possibility of using a selective activation of VAP-C to suppress HCV replication in cells infected with HCVcc. We generated expression plasmids encoding a chimeric VAP-C fused with the IPS-1 sequence (cVAP-C), a cVAP-C(C508A) which is made resistant to the HCV protease by a substitution in the cleavage site similar to the substitutions made in cIRF7(C508A), or VAP-C (Fig. 7A). The cVAP-C was cleaved in cells infected with HCVcc, and expression of cVAP-C and VAP-C suppressed expression of NS5A, in contrast to the weak reduction of NS5A in the infected cells expressing cVAP-C(C508A), probably due to a slight cleavage of cVAP-C(C508A) (Fig. 7B, top). Furthermore, the production of viral RNA and infectious particles in the culture supernatants of cells infected with HCVcc was also impaired by the expression of cVAP-C and VAP-C, but not of cVAP-C(C508A) in a dose-dependent manner (Fig. 7B, middle and bottom). Collectively, these results suggest that delivery of the therapeutic molecules into liver of hepatitis C patients, followed by selective activation of the molecules in HCV-infected hepatocytes, is a feasible method for eliminating HCV.

Discussion

An effective prophylactic vaccine against HCV has not been developed yet. Although combination therapy consisting of PEG-IFNα and RBV has been introduced for the treatment of hepatitis C patients, and 50% of individuals infected with genotype 1 achieved a SVR, this treatment is sometimes associated with serious side effects, including depression and anemia [3]. Therefore, new anti-HCV drugs targeted to HCV protease and polymerase and capable of optimizing therapy are currently in the early stages of the development [30,31]. However, it is difficult to achieve a complete removal of viruses by antiviral drugs targeted to the viral enzymes from patients persistently infected with RNA viruses that exhibit a quasispecies nature, such as human immunodeficiency virus (HIV) and HCV. Viral quasispecies are not a simple collection of diverse mutants but a group of interactive variants capable of adapting to new environments [32]. Furthermore, introduction of antiviral drugs may induce an emergence of drug-resistant breakthrough viruses as seen in the case of HIV infection. Therefore, a novel therapeutic approach for hepatitis C patients in addition to the current chemotherapies is required to overcome serious adverse effects and improve the ratio of patients achieving SVR.

In this study, we have generated two therapeutic molecules, cIRF7 and cVAP-C, which are selectively activated in cells