

Stabilizing the Pro-Apoptotic BimBH3 Helix (BimSAHB) Does Not Necessarily Enhance Affinity or Biological Activity

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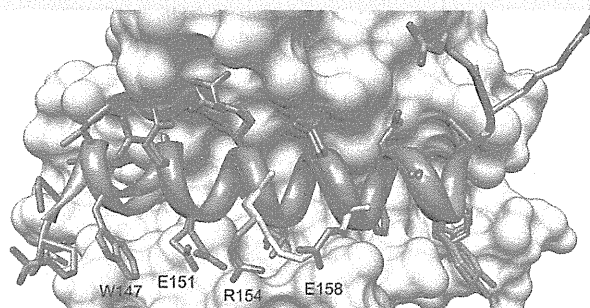
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Supporting Information

ABSTRACT: An attractive approach for developing therapeutic peptides is to enhance binding to their targets by stabilizing their α -helical conformation, for example, stabilized BimBH3 peptides (BimSAHB) designed to induce apoptosis. Unexpectedly, we found that such modified peptides have reduced affinity for their targets, the pro-survival Bcl-2 proteins. We attribute this loss in affinity to disruption of a network of stabilizing intramolecular interactions present in the bound state of the native peptide. Altering this network may compromise binding affinity, as in the case of the BimBH3 stapled peptide studied here. Moreover, cells exposed to these peptides do not readily undergo apoptosis, strongly indicating that BimSAHB is not inherently cell permeable.



Stabilized helical peptides are designed to mimic an α -helical structure through a constraint imposed by covalently linking two residues on the same helical face (e.g., residue i with $i + 4$). “Stapling” the peptide into a preformed helix might be expected to lower the energy barrier for binding by reducing entropic costs, with a concomitant increase in binding affinity. Additionally, stabilizing the peptide may reduce degradation by proteases^{1,2} and, in the case of hydrocarbon linkages, reportedly enhance transport into cells,³ thereby improving bioavailability and their potential as therapeutic agents.^{3–5} The findings we present here for the stapled BH3 peptide (BimSAHB), however, do not support these claims, particularly in regards to affinity and cell permeability. We observe a reduction in binding upon BimBH3 stapling, which we attribute to the loss of a network of stabilizing intramolecular interactions on the peptide. Thus, in addition to the primary consideration for staple placement in peptide design, that of avoiding key binding interfaces, our observations reveal a new consideration, that staples should also avoid disruption of favorable interactions within the peptide itself.

It has previously been reported that a stapled version of the BimBH3 peptide (dubbed: “stabilized α -helices of Bcl-2 domains”, BimSAHB), where two native residues were replaced with (*S*)-pentenyl alanine derivatives and covalently joined through a metathesis reaction,^{2,3} kills cells by directly activating Bax through an interface involving residue Lys21.⁶ This

stabilized peptide has also been reported to have enhanced binding for the pro-survival proteins.⁷ When mouse embryonic fibroblasts (MEFs) (Supplementary Figure 1) or Jurkat cells (data not shown) were treated with BimSAHB, we observed no cell death, in contrast to the potent killing induced by the well-characterized activator of apoptosis, etoposide. This was true for both the 20-mer BimSAHB peptide used below and the 21-mer BimSAHB peptide previously used in cellular assays.⁸

Given that no cell killing was observed when BimSAHB was added to cells in culture, we decided to test the ability of these peptides to induce mitochondrial cytochrome *c* release *in vitro* from cells permeabilized with a low concentration of the detergent digitonin. This would determine if the absence of killing activity could be attributed to a lack of cellular uptake. In such assays, we found that BimSAHB was indeed capable of liberating mitochondrial cytochrome *c* (Figure 1a,b), consistent with the conclusion that BimSAHB, under the conditions tested, does not readily enter cells in sufficient amounts to induce apoptosis (although proteolytic degradation or non-specific binding effects could also account for this observation).

We next tested the role of the Bax interface encompassing Lys21 proposed to be essential for its activation.⁶ To undertake

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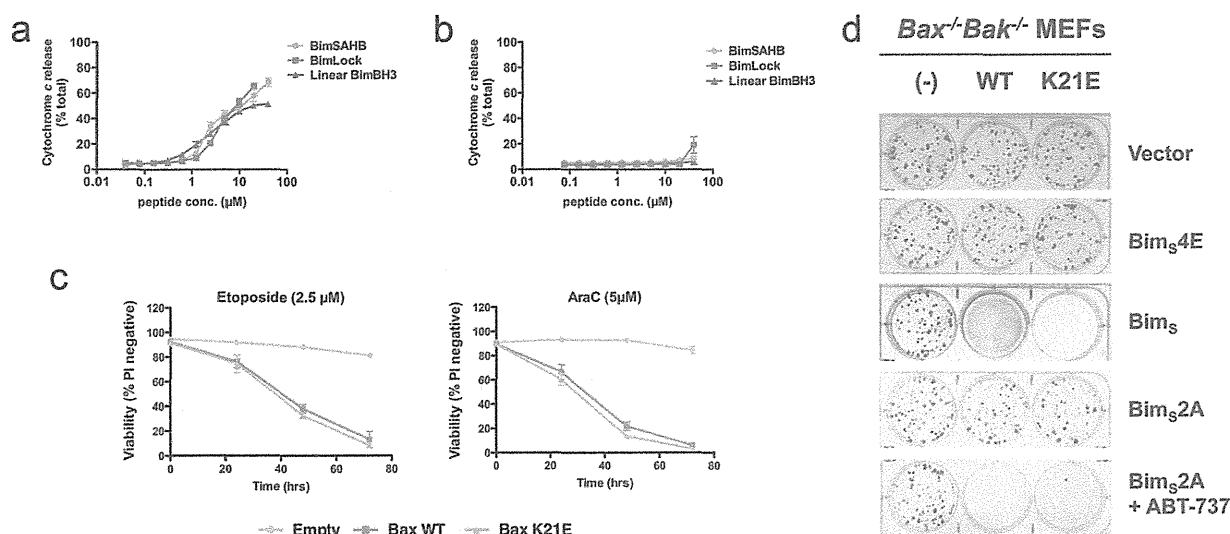


Figure 1. Bioactivity of constrained BimBH3 peptides. Cytochrome *c* release experiments were performed on permeabilized cells (MEFs) that were derived from either (a) wild-type or (b) from $Bax^{-/-}Bak^{-/-}$ mice. The cell pellets, containing intact mitochondria, were treated with increasing concentrations of the indicated peptides. The quantity of cytochrome *c* release was assessed by ELISA (R&D Systems) analyses of supernatants postpeptide treatment as compared to mitochondria treated with the permeabilizing agent 1% Triton X-100 (100% activity). (c) Killing assay of $Bax^{-/-}Bak^{-/-}$ MEF cells reconstituted with either wild-type Bax (WT) or Bax(K21E) in response to DNA damaging agents etoposide or Ara-C. Cell viability was monitored by propidium iodide (PI) exclusion determined by flow cytometry. Expression of wild-type Bax and BaxK21E were confirmed by Western blot analysis (Supplementary Figure 2). (d) Colony formation assays of $Bax^{-/-}Bak^{-/-}$ MEF cells reconstituted with either wild-type Bax (WT) or Bax (K21E) and transfected with vector encoding an inactive Bim variant (Bim_{4E}),²⁰ wild-type Bim₅ (targeting all pro-survival proteins), a Bim, variant targeting only Mcl-1 (Bim_{2A}),²¹ or Bim_{2A} in combination with ABT-737 treatment. Error bars in panels a–c represent SEM of 2 independent experiments.

this, we reconstituted MEFs lacking the essential cell death mediators Bax and Bak with wild-type (WT) Bax or the K21E mutant that was reported to be inert.⁶ Unexpectedly, cells reconstituted with either WT or K21E mutant Bax behaved identically in short- or long-term survival assays (Figure 1c,d), suggesting that this interface was not required for Bax activation. Equivalent cell death to that observed for BimBH3 overexpression was observed when ABT-737 (to target Bcl-2, Bcl-x_L, and Bcl-w⁷) was combined with Bim₅2A (to target Mcl-1⁹), suggesting that apoptosis in these cells is primarily the result of inhibition of pro-survival proteins (Figure 1d).¹⁰ By implication, the mechanism by which BimBH3 peptides initiate cytochrome *c* release in our experiments using isolated mitochondria could be accounted for by relieving the pro-survival proteins restraining Bax and Bak¹⁰ and possibly by direct activation of Bax via an alternative interface such as that shown for Bak.¹¹

In sharp contrast to the reported studies,³ our studies suggest that BimSAHB is not inherently cell permeable. Moreover, the linear control BimBH3 of precisely the same length as the stapled peptide was just as active at inducing cytochrome *c* release *in vitro* (Figure 1a). As the proposed interface for Bax activation by BimSAHB did not appear to play a major role (Figure 1c,d), we undertook a detailed biochemical and structural analysis of the constrained BimSAHB and its interactions with pro-survival proteins. To undertake these studies, we also employed a second approach, which we termed BimLOCK, to link the side-chains of a glutamate with a lysine through a lactam bridge.^{12,13} These modified residues were identical to the (*S*)-pentenyl alanines used to construct BimSAHB.

Circular dichroism (CD) was employed to confirm that covalent linkages enhanced helical content (Figure 2a). Both

BimSAHB (39% helix) and BimLOCK (49%) displayed enhanced helical content in an aqueous solution compared to an equivalent linear peptide (21%). Additionally, we determined crystal structures for both constrained peptides in complex with the pro-survival Bcl-2 family protein Bcl-x_L (BCL2L1) (Figure 2b,c). These structures revealed that both constrained peptides bind to Bcl-x_L analogously to the linear BimBH3 peptide and that neither the hydrocarbon staple nor the lactam bridge interacts with residues on Bcl-x_L. Because of the increased helicity, we anticipated an approximate 4-fold increase in binding affinity (see Supporting Information). Instead, these peptides have reduced affinities for pro-survival proteins (Table 1a). Direct binding assays confirmed this observation for two pro-survival proteins, Bcl-x_L and Mcl-1. The penalty imposed by the staple for Bcl-x_L is due to slower on- and faster off-rates (1.8- and 13-fold, respectively). Similarly, the loss in affinity for Mcl-1 resulted predominantly from a faster off-rate (2.8-fold increase, compared to a 1.3-fold reduction in on-rate) (Table 1b).

Inspection of the structure of BimBH3-peptide in complex with Bcl-x_L (PDB code, 3FDL¹⁴) reveals how the side-chain groups of residues on the exposed face of the peptide form a series of interactions with one another; Glu151 and Glu158 (numbering based on human Bim_{EL}) form a pair of salt bridges with Arg154, and the aliphatic methylene moiety of Glu151 packs tightly with the indole side-chain of Trp147. Additionally, Arg154 of Bim forms a water-mediated interaction with Arg103 of Bcl-x_L (Supplementary Figure 3a). Critically, none of these interactions are observed in either structure of the stapled peptides in complex with Bcl-x_L (Supplementary Figure 3b,c).

We have calculated the pairwise interaction energy between the side-chain groups of each of these residues employing the AUTODOCK empirical function: the AUTODOCK potential

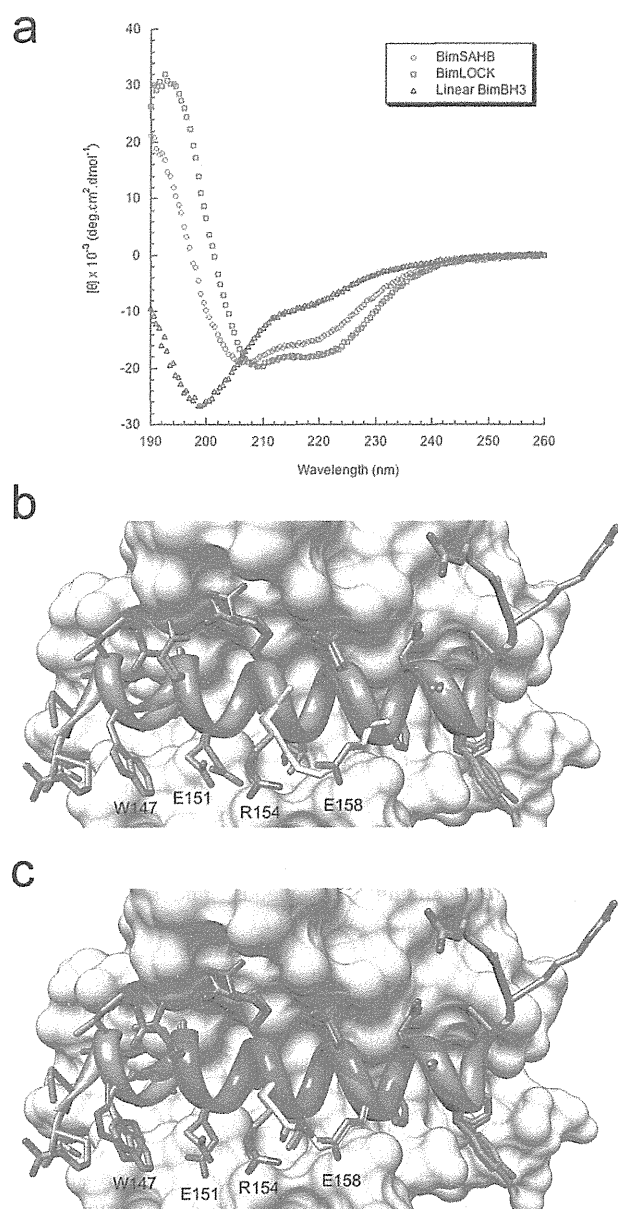


Figure 2. Characterization of constrained BimBH3 peptides. (a) Circular dichroism was used to measure the percentage of helical content for BimSAHB, BimLOCK, and an equivalent linear BimBH3 peptide. Both constrained BimBH3 peptides were found to have significant helical content (BimSAHB 39% helix; BimLOCK 49% helix), while the linear peptide was found to have a modest secondary structure content (21% helix). (b) Structure of the Bcl- x_L :BimSAHB complex overlaid with linear BimBH3 peptide in complex with Bcl- x_L (PDB code, 3FDL;¹⁴ note that the peptide used for the 3FDL structure is longer than those used in this study). Peptide regions of BimSAHB are colored green and the hydrocarbon staple yellow. (c) Structure of the Bcl- x_L :BimLOCK complex overlaid with BimBH3. Peptide regions of BimLOCK are colored magenta and the lactam bridge yellow. BimBH3 is colored blue, and Bcl- x_L is represented in surface format in both complexes. Peptide sequences are as detailed in Table 1 unless otherwise stated.

includes five major contributions to the total free energy of interaction: the van der Waals, hydrogen-bond, electrostatic, conformational entropy, and desolvation energies.¹⁵ The individual energies of interaction calculated were, between

Trp147–Glu151, -0.3 kJ mol⁻¹, Glu151–Arg154, -7.0 kJ mol⁻¹, and Arg154–Glu158, -2.7 kJ mol⁻¹ (using the Bcl- x_L /Bim coordinates), yielding a total interaction energy between all four residues of -10.0 kJ mol⁻¹. Assuming these side-chains do not interact with one another in the free peptide, the cumulative effect of increased helicity of the peptide and estimated loss in binding energy due to missing side-chain interactions, the anticipated reduction in binding affinity for BimSAHB and BimLOCK is 24-fold and 16-fold, respectively. These estimates compare favorably with the observed 26-fold reduction from the competition assay and 8-fold reduction from the direct binding assay for BimSAHB (Table 1, Supplementary Table 2) and, likewise, 3-fold reduction from the competition assay for BimLOCK.

For Mcl-1 and A1, where the reduction in binding affinity observed by the introduction of the constraint is not as large as that observed for Bcl-2 and Bcl- x_L , either the side-chain groups may maintain some interaction with one another or the side-chain groups may interact with the pro-survival receptor to improve binding affinity. To further assess the contribution of the intramolecular bonding network on the exposed face of the peptide and to ensure loss of affinity could not be attributed to steric hindrance imposed by the staple, we tested the binding affinity of an unstapled Bim peptide (linear Bim with Glu151 and Arg154 substituted with pentenylalanine residues but not subjected to ring closing metathesis) (Table 1b). This peptide bound Bcl- x_L with a further reduced affinity as compared to the stapled Bim, as would be anticipated with the combined effects of a reduction in helicity (due to loss of the staple) and the loss of the stabilizing intramolecular network.

Upon binding their pro-survival receptor, the BH3 peptides adopt an α -helical geometry, stabilized by the interactions between receptor and peptide and by the intramolecular interactions within the peptide, specifically the classical hydrogen bond between the carbonyl oxygen and the NH groups (i with $i + 4$) and also the interactions between the side-chain groups. Disrupting the latter of these interactions results in a decrease in binding affinity; in our estimate, this is ~ 10 kJ mol⁻¹ in the case of the BimBH3 peptide. The introduction of the staple into the peptides has two consequences: (i) while the staple is able to preorganize the helix and reduce the entropic penalty for the association, the introduction of a covalent bond between side-chains removes the contribution to the binding energy that comes about when the two residues interact (in the salt bridge formed between Arg154 and Glu158, we estimate this to be 2.7 kJ mol⁻¹), and (ii) the chemical nature of the staple may not be conducive to forming stable interactions with the side-chain group of the residue one turn away. Loss of a stable interaction with the staple can be perpetuated down the chain; in both stapled peptides here, the result is the loss of all stabilizing interactions between side-chains, most critically the loss of the salt-bridge between Glu151 and Arg154. The binding free energy reflects the change in free energy between unbound and bound states, and since the covalent linkage is present in both states, there is no change in free energy of interaction across the staple upon binding. In contrast, in the native peptide, we calculate a significant favorable change in intramolecular energy of the ligand upon binding.

The structures of the peptides in complex with pro-survival Bcl- x_L reveal that they bind analogously to unrestrained BimBH3, neither modification interacting directly with the target protein unlike that observed with another stabilized peptide in complex with Mcl-1¹⁶ where the staple itself makes

Table 1. Analyses of Binding of BimBH3 Peptides to Pro-Survival Proteins Using Biacore-Based Assays

a					
K_i (nM)	Bcl-2	Bcl-x _L	Bcl-w	Mcl-1	A1
BimSAHB	460 ± 80	300 ± 37	370 ± 61	3.4 ± 0.1	4.1 ± 0.3
BimLOCK	800 ± 27	35 ± 3.7	37 ± 3.0	3.7 ± 0.3	4.7 ± 0.1
Linear Bim	6.8 ± 1.6	11 ± 0.6	25 ± 2.3	<1.4	<2.5
b					
	on-rate K_a (1/MS)		off-rate K_d (1/s)		affinity K_D
Bcl-x _L					
BimSAHB	3.2 × 10 ⁶		1.5 × 10 ⁻¹		45 nM
unstapled BimSAHB	7.9 × 10 ⁵		7.5 × 10 ⁻²		95 nM
linear BimBH3	1.7 × 10 ⁶		1.1 × 10 ⁻²		5.7 nM
Mcl-1					
BimSAHB	3.7 × 10 ⁶		2.7 × 10 ⁻³		0.73 nM
linear BimBH3	4.9 × 10 ⁶		9.7 × 10 ⁻⁴		0.20 nM
c					
	sequence		note		
BimSAHB	Ac- ¹⁴⁶ EIWIAQELRXIGDXFNAYYA ¹⁶⁴ -NH ₂		X represents linked (S)-pentenylalanine residues		
BimLOCK	Ac- ¹⁴⁶ EIWIAQELRRIGDEFNAYYA ¹⁶⁴ -NH ₂		R154 and E158 linked by lactam bridge		
linear BimBH3	Ac- ¹⁴⁶ EIWIAQELRRIGDEFNAYYA ¹⁶⁴ -NH ₂				

(a) Competition experiments performed using a Biacore 3000 as previously described.²⁰ Values are presented as a K_i in nM, with SD ($n = 3$ independent experiments). (b) Direct affinity measurements for binding of linear BimBH3 and BimSAHB to Bcl-x_L and Mcl-1. Measurements were performed using a Biacore S51. Peptides were passed over a sensor chip to which either recombinant Bcl-x_L-GST or Mcl-1-GST fusion proteins had been coupled via an anti-GST antibody. Direct association and dissociation rates were measured at a range of concentrations from which K_D values were calculated. Unstapled BimSAHB refers to a BimSAHB peptide in which pentylalanine residues have not been linked. (c) Sequences for peptides.

intimate hydrophobic contact with the side-chain of Phe318, and one of the flanking α -disubstitution methyl groups contacts Gly262. Unexpectedly, enhanced helicity did not improve activity with regards to affinity for the pro-survival proteins or bioactivity, as measured by induction of cytochrome *c* release from mitochondria and cellular uptake. Our findings recapitulate earlier observations^{17,18} that stapling of peptides to enforce helicity does not necessarily impart enhanced binding affinity for target proteins and support the notion that interactions between the staple and target protein may be required for high affinity interactions in some circumstances.¹⁹ Thus, the design of stapled peptides should consider how the staple might interact with both the target *and* the rest of the peptide, and particularly in the latter case whether its introduction might disrupt otherwise stabilizing interactions.

In conclusion, we undertook an investigation of stapled peptides as part of our on going studies into the mechanism of the Bcl-2 family of proteins. We found that the addition of a hydrocarbon staple to the BimBH3 peptide, either via Grubbs metathesis or formation of an external amide bond, does not change the peptide-like properties of the molecules in a manner that makes them significantly more drug-like. While modification of peptides may yet prove beneficial for drug development, in this case, we describe that the addition of external constraints does not enhance either binding affinity or cell permeability.

METHODS

Circular Dichroism Measurements. Circular dichroism was used to measure the percentage of helical content for BimSAHB, BimLOCK, and an equivalent linear BimBH3 peptide. Spectra were performed on 50 μ M peptide solutions in 0.1 M potassium phosphate (pH 7) at 25 °C. Percent helicity was calculated as previously described.^{22,23}

Structure Determination. Bcl-x_L protein preparation, crystallization, and structure determination for both the Bcl-x_L:BimSAHB complex and the Bcl-x_L:BimLOCK complex were as previously

described for the Bcl-x_L:BimBH3 complex.¹⁴ Crystallographic statistics for the two complexes are reported in Supplementary Table 1.

Cytochrome *c* Release. Cells were pelleted and lysed in 0.025% (w/v) digitonin containing lysis buffer (20 mM Hepes, pH 7.2, 100 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, and 250 mM sucrose, supplemented with Complete Protease Inhibitor Cocktail from Roche). The crude lysates, containing mitochondria, were incubated with or without 1% (w/v) Triton-X100 or increasing concentrations of Bim peptides for 1 h at 30 °C, pelleted at 4000 rpm at 4 °C for 15 min, and the supernatant collected. The amount of released cytochrome *c* was determined by ELISA (R&D systems), according to the manufacturer's protocol.

Biacore Competition Assays. Biacore competition assays were performed as previously described.^{20,21} Pro-survival proteins (5 nM) (Bcl-2, Bcl-x_L, and Bcl-w prepared as described in ref 1, Mcl-1 prepared as described in ref 24, A1 prepared as described in ref 25) were incubated with increasing concentrations of BimBH3 peptides for 2 h in running buffer prior to injection onto a CM5 chip onto which either a wild-type 26-mer BimBH3 peptide or an inert BimBH3 mutant peptide (Bim4E) was immobilized.

Direct Affinity Measurements. Direct binding assays were performed with a Biacore S51 as previously described.⁹ Briefly, anti-GST antibody was immobilized to a CM5 chip using amine coupling. Recombinant GST-tagged Bcl-x_L or GST-tagged Mcl-1 (100 μ g mL⁻¹) was captured to the chip followed by injection of peptide at a variety of concentrations. Sensograms were generated by subtracting the binding response from that of a reference spot to which GST alone had been captured.

Killing Assays. MEFs were trypsinized, collected, and then washed twice in serum-free media followed by plating of cells (5 × 10⁴/well) in 50 μ L, exposed to BimSAHB (20 μ M) or vehicle in serum-free media for 2 h, and serum replacement (20% (v/v) serum in 50 μ L media) for an overall treatment duration as indicated.

Colony Survival Assays. Retroviruses encoding BH3-only proteins were transduced as described.²⁰ Infected MEFs (GFP(+) cells) were sorted by FACS and long-term assays of colony formation performed as described.²⁰

Molecular Modeling. The free energy of interaction between the side-chain groups on the BimBH3-peptide was calculated using the AUTODOCK function.¹⁵ In its original formulation, the AUTODOCK potential (model C) includes terms that represent the entropic

penalty for restriction of conformational freedom and desolvation of the ligand only. Here, we have included these two components for both molecular constituents and consequently reduced the contribution to the total free energy of interaction of each by half. The AMBER all-atom partial atomic charges²⁶ were used to calculate the electrostatic interaction energy. Hydrogen atoms were added to fill valencies using the UCSF Chimera package.²⁷

Detailed methods for peptide synthesis, structure determination, *K_d* calculations, and binding analysis are provided in the Supporting Information.

■ ASSOCIATED CONTENT

Supporting Information

BimSAHB peptide does not kill MEFs; reconstituted Bax^{-/-}Bak^{-/-} MEF cells express comparable levels of wild-type Bax and Bax K21E. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Accession Codes

Atomic coordinates and structure factors have been deposited in the Protein Data Bank (PDB ID codes, 2YQ6 for Bcl-xL:BimSAHB and 2YQ7 for Bcl-xL:BimLOCK).

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Notes

The authors declare the following competing financial interest(s): Genentech is a member of the Roche Group. Roche Pharma and Aileron Therapeutics have an independent collaboration developing stapled peptide technology. Between 2007 and 2010, the Walter and Eliza Hall Institute of Medical Research had a research collaboration agreement with Genentech and Abbott in the field of apoptosis, specifically the Bcl-2 protein family.

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Japanese Encephalitis Virus Core Protein Inhibits Stress Granule Formation through an Interaction with Caprin-1 and Facilitates Viral Propagation

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Stress granules (SGs) are cytoplasmic foci composed of stalled translation preinitiation complexes induced by environmental stress stimuli, including viral infection. Since viral propagation completely depends on the host translational machinery, many viruses have evolved to circumvent the induction of SGs or co-opt SG components. In this study, we found that expression of Japanese encephalitis virus (JEV) core protein inhibits SG formation. Caprin-1 was identified as a binding partner of the core protein by an affinity capture mass spectrometry analysis. Alanine scanning mutagenesis revealed that Lys⁹⁷ and Arg⁹⁸ in the α -helix of the JEV core protein play a crucial role in the interaction with Caprin-1. In cells infected with a mutant JEV in which Lys⁹⁷ and Arg⁹⁸ were replaced with alanines in the core protein, the inhibition of SG formation was abrogated, and viral propagation was impaired. Furthermore, the mutant JEV exhibited attenuated virulence in mice. These results suggest that the JEV core protein circumvents translational shutoff by inhibiting SG formation through an interaction with Caprin-1 and facilitates viral propagation *in vitro* and *in vivo*.

In eukaryotic cells, environmental stresses such as heat shock, oxidative stress, UV irradiation, and viral infection trigger a sudden translational arrest, leading to stress granule (SG) formation (1). SGs are cytoplasmic foci composed of stalled translation preinitiation complexes and are postulated to play a critical role in regulating mRNA metabolism during stress via so-called “mRNA triage” (2). The initiation of SG formation results from phosphorylation of eukaryotic translation initiation factor 2 α (eIF2 α) at Ser⁵¹ by various kinases, including protein kinase R (PKR), PKR-like endoplasmic reticulum kinase (PERK), general control non-repressed 2 (GCN2), and heme-regulated translation inhibitor (HRI), which are commonly activated by double-stranded RNA (dsRNA), endoplasmic reticulum (ER) stress, nutrient starvation, and oxidative stress, respectively. Phosphorylation of eIF2 α reduces the amount of eIF2-GTP-tRNA complex and inhibits translation initiation, leading to runoff of elongating ribosomes from mRNA transcripts and the accumulation of stalled translation preinitiation complexes. Thus, SGs are defined by the presence of components of translation initiation machinery, including 40S ribosome subunits, poly(A)-binding protein (PABP), eIF2, eIF3, eIF4A, eIF4E, eIF4G, and eIF5. Then, primary aggregation occurs through several RNA-binding proteins (RBPs), including T-cell intracellular antigen-1 (TIA-1), TIA-1-related protein 1 (TIAR), and Ras-Gap-SH3 domain-binding protein (G3BP). These RBPs are independently self-oligomerized with the stalled initiation factors and with other RBPs, such as USP10, hnRNP Q, cytoplasmic activation/proliferation-associated protein-1 (Caprin-1), and Staufen and with nucleated mRNA-protein complex (mRNP) aggregations (3, 4). SG assembly begins with the simultaneous formation of numerous small mRNP granules which then progressively fuse into larger and fewer structures, a process known as secondary aggregation (5). The aggregation of TIA-1 or TIAR is regulated by molecular chaperones, such as heat shock protein 70 (Hsp70) (3), whereas that of G3BP is controlled by its phosphor-

ylation at Ser¹⁴⁹ (4). SG formation and disassembly in response to cellular stresses are strictly regulated by multiple factors.

Viral infection can certainly be viewed as a stressor for cells, and SGs have been reported in some virus-infected cells. Since the propagation of viruses is completely reliant on the host translational machinery, stress-induced translational arrest plays an important role in host antiviral defense. To antagonize this host defense, most viruses have evolved to circumvent SG formation during infection. For example, poliovirus (PV) proteinase 3C cleaves G3BP, leading to effective SG dispersion and virus propagation (6). Influenza A virus nonstructural protein 1 (NS1) has been shown to inactivate PKR and prevent SG formation (7). In the case of human immunodeficiency virus 1 (HIV-1) infection, Staufen1 is recruited in ribonucleoproteins for encapsidation through interaction with the Gag protein to prevent SG formation (8). In contrast, some viruses employ alternative mechanisms of translation initiation and promote SG formation to limit cap-dependent translation of host mRNA (9, 10). In addition, vaccinia virus induces cytoplasmic “factories” in which viral translation, replication, and assembly take place. These factories include G3BP and Caprin-1 to promote transcription of viral mRNA (11).

Japanese encephalitis virus (JEV) belongs to the genus *Flavivirus* within the family *Flaviviridae*, which includes other mosquito-borne human pathogens, such as dengue virus (DENV), West Nile virus (WNV), and yellow fever virus, that frequently cause significant morbidity and mortality in mammals and birds (12). JEV has

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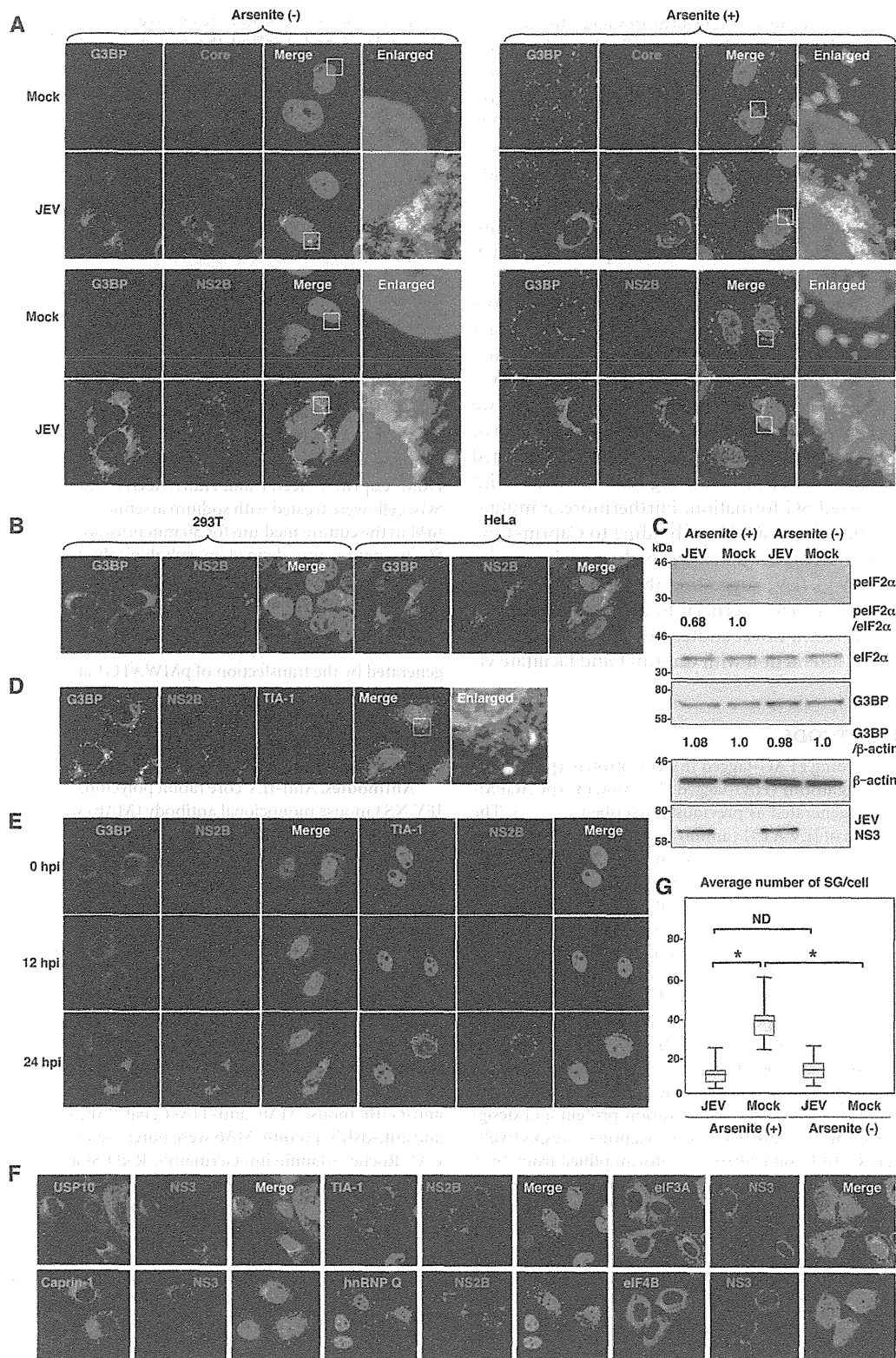


FIG 1 Dynamics of SG-associated factors during JEV infection. (A) Huh7 cells infected with JEV at an MOI of 0.5 were treated with or without 1.0 mM sodium arsenite for 30 min at 37°C, and the levels of expression of G3BP and JEV core protein/NS2B were determined at 24 h postinfection by immunofluorescence analysis with mouse anti-G3BP MAb and rabbit anti-core protein or anti-NS2B Pab, followed by AF488-conjugated anti-mouse IgG (Invitrogen) and AF594-conjugated anti-rabbit IgG, respectively. Cell nuclei were stained with DAPI (blue). (B) Cellular localizations of G3BP and JEV NS2B in 293T and HeLa cells infected with JEV were determined at 24 h postinfection by immunofluorescence analysis with mouse anti-G3BP MAb and rabbit anti-NS2B Pab, followed by AF488-conjugated anti-mouse IgG and AF594-conjugated anti-rabbit IgG, respectively. Cell nuclei were stained with DAPI (blue). (C) Phosphorylation of eIF2 α in cells prepared as described in panel A was determined by immunoblotting using the indicated antibodies. The band intensities were quantified by ImageJ

a single-stranded positive-sense RNA genome of approximately 11 kb. The genomic RNA carries a single large open reading frame, and a polyprotein translated from the genome is cleaved co- and posttranslationally by host and viral proteases to yield three structural proteins, the core, precursor membrane (PrM), and envelope (E) proteins, and seven nonstructural (NS) proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (13). PrM is further cleaved by the multibasic protease, furin, and matured to membrane (M) protein. The core, M, and E proteins are components of extracellular mature virus particles. NS proteins are not incorporated into particles and are thought to be involved in viral replication, which occurs in close association with ER-derived membranes (14). Previous reports have shown that WNV and DENV inhibit SG formation by sequestering TIA-1 and TIAR through specific interaction with viral RNA (15, 16). In addition, the membrane structure induced by WNV infection was suggested to prevent PKR activation and avoid induction of SG formation (17). In this study, we show that JEV core protein plays an important role in inhibition of SG formation. JEV core protein recruited several SG-associated proteins, including G3BP and USP10, through an interaction with Caprin-1 and suppressed SG formation. Furthermore, a mutant JEV carrying a core protein incapable of binding to Caprin-1 exhibited lower propagation *in vitro* and lower pathogenicity in mice than the wild-type (WT) JEV, suggesting that inhibition of SG formation by the core protein is crucial to antagonize host defense. These results reveal a novel strategy of JEV to inhibit SG formation through an interaction with Caprin-1 and facilitate viral propagation.

MATERIALS AND METHODS

Plasmids. Plasmids encoding FLAG-tagged JEV core protein (pCAGPM-FLAG-Core) and hemagglutinin (HA)-tagged JEV proteins (pCAGPM-HA-JEV proteins) were generated as previously described (18, 19). The cDNA of the core protein of JEV AT31 (amino acid residues 2 to 105) was amplified from the pCAGPM-FLAG-Core plasmid by PCR and cloned into pET21b (Novagen-Merck, Darmstadt, Germany) for expression in bacteria as a His-tagged protein and in pCAG-MCS2-FOS for expression in mammalian cells as a FLAG-One-STRIP (FOS)-tagged protein. The resulting plasmids were designated pET21b-Core-His and pCAG-Core-FOS, respectively. The cDNA of the core protein of DENV2 (amino acid residues 2 to 100) was amplified from the pCAG/FLAG-DEN2C-HA plasmid (19) by PCR and cloned into pCAGPM-N-FLAG. The cDNA of human Caprin-1 was amplified from 293T cells by reverse transcription-PCR (RT-PCR) and cloned into pCAGPM-N-HA (20) and pGEX 6P-1 (GE Healthcare, Buckinghamshire, United Kingdom) for expression in bacteria as a glutathione *S*-transferase (GST) fusion protein and designated pCAGPM-HA-Caprin-1 and pGEX-GST-Caprin-1, respectively. The cDNAs of human G3BP1 and USP10 were also amplified from 293T cells by RT-PCR and cloned into pCAGPM-N-HA. The nucleotide residues of the adenine at 384, adenine at 385, cytosine at 387, and guanine at

388 of the JEV genome in pMWATG1 were replaced with guanine, cytosine, guanine, and cytosine, respectively, by PCR-based mutagenesis to change Lys⁹⁷ and Arg⁹⁸ of the core protein to Ala, yielding pMWAT/KR9798A. The cDNA of the mutant core protein was also cloned into pCAGPM-N-FLAG and pET21b. To generate stable cell lines expressing *Aequorea coerulescens* green fluorescent protein (AcGFP)-fused Caprin-1, the cDNA of human Caprin-1 was amplified by RT-PCR and cloned into pAcGFP N1 (Clontech, Mountain View, CA), and the Caprin-1-AcGFP gene was subcloned into the lentiviral vector pCSII-EF-Rfa (21) and designated pCSII-EF-Caprin-1-AcGFP. All plasmids were confirmed by sequencing with an ABI Prism 3130 genetic analyzer (Applied Biosystems, Tokyo, Japan).

Cells and stress treatment. Mammalian cell lines, Vero (African green monkey kidney), 293T (human kidney), Huh7 (human hepatocellular carcinoma), and HeLa (human cervical carcinoma), were maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM) (Sigma, St. Louis, MO) supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, nonessential amino acids (Sigma), and 10% fetal bovine serum (FBS). The mosquito cell line C6/36 (*Aedes albopictus*) was grown in Leibovitz's L-15 medium with 10% FBS. Huh7 cells were transfected with a lentiviral vector expressing Caprin-1-AcGFP and AcGFP and designated Huh7/Caprin-1-AcGFP and Huh7/AcGFP, respectively. For induction of SGs, cells were treated with sodium arsenite at a final concentration of 1.0 mM in the culture medium for 30 min prior to fixation or lysis of the cells. SG formation was defined morphologically by immunostaining using anti-SG-related factor antibodies described below. Cell viability was determined by using CellTiter-Glo (Promega, Madison, WI) according to the manufacturer's instruction.

Viruses. The wild-type and 9798A mutant of the JEV AT31 strain were generated by the transfection of pMWATG1 and pMWAT/KR9798A, respectively, as described previously (22). Viral infectivity was determined by an immunostaining focus assay as described previously (20), and the results are expressed in focus-forming units (FFU). JEV and DENV serotype 2 New Guinea C strain were amplified in C6/36 cells.

Antibodies. Anti-JEV core rabbit polyclonal antibody (PAb) and anti-JEV NS3 mouse monoclonal antibody (MAb) were prepared as described previously (20, 23). Anti-JEV NS2B rabbit PAb was generated with synthetic peptides of JEV NS2B at Scrum, Inc. (Tokyo, Japan). Anti-DENV core protein rabbit PAb was prepared by using a GST-fused recombinant protein containing amino acid residues 2 to 100 of the DENV core protein. Anti-FLAG mouse MAb (M2) and rabbit PAb and anti- β -actin mouse MAb were purchased from Sigma. Anti-hnRNP Q mouse MAb (ab10687), anti-USP10 rabbit PAb (ab70895), and anti-eIF4B rabbit PAb (ab78916) were purchased from Abcam (Cambridge, United Kingdom). Anti-eIF2 α , anti-phospho-eIF2 α , and anti-eIF3A rabbit PABs were purchased from Cell Signaling Technology (Danvers, MA). Anti-HA mouse MAb (HA11), anti-HA rat MAb (3F10), anti-His mouse MAb, anti-GFP mouse MAb (JL-8), anti-JEV envelope protein mouse MAb (6B4A-10), anti-G3BP mouse MAb, anti-TIA-1 goat PAb, anti-Caprin-1 rabbit PAb, and anti-dsRNA mouse MAb were purchased from Covance (Richmond, CA), Roche (Mannheim, Germany), R&D Systems (Minneapolis, MN), Clontech, Chemicon (Temecula, CA), BD Biosciences (Franklin Lakes, NJ), Santa Cruz (Santa Cruz, CA), Proteintech (Chicago, IL), and Bio-

software (NIH, Bethesda, MD), and the relative levels for the indicated proteins are shown based on the level of the mock-infected cells. (D) Cellular localizations of G3BP, NS2B, and TIA-1 in Huh7 cells infected with JEV were determined at 24 h postinfection by immunofluorescence analysis with mouse anti-G3BP MAb, rabbit anti-NS2B PAb, and goat anti-TIA-1 PAb, followed by AF488-conjugated anti-mouse IgG, AF594-conjugated anti-rabbit IgG, and AF633-conjugated anti-goat IgG, respectively. Cell nuclei were stained with DAPI (gray). (E) Dynamics of G3BP and TIA-1 during JEV infection. Huh7 cells infected with JEV were immunostained at 0, 12, and 24 h postinfection (hpi) with mouse anti-G3BP MAb or goat anti-TIA-1 PAb and rabbit anti-NS2B PAb, followed by AF488-conjugated anti-mouse IgG or AF488-conjugated anti-goat IgG and AF594-conjugated anti-rabbit IgG, respectively. Cell nuclei were stained with DAPI (blue). (F) Cellular localization of SG-associated proteins (USP10, Caprin-1, TIA-1, hnRNP Q, eIF3A, and eIF4B) (green, AF488-conjugated secondary antibody) and JEV NS2B/NS3 (red, AF-594-conjugate secondary antibody) in Huh7 cells infected with JEV was determined by immunoblotting at 24 h postinfection. Cell nuclei were stained with DAPI (blue). (G) Numbers of G3BP-positive foci in 30 cells prepared as described in panel A were counted for each experimental condition. Lines, boxes, and error bars indicate the means, 25th to 75th percentiles, and 95th percentiles, respectively. The significance of differences between the means was determined by a Student's *t* test. *, $P < 0.01$; ND, no significant difference.

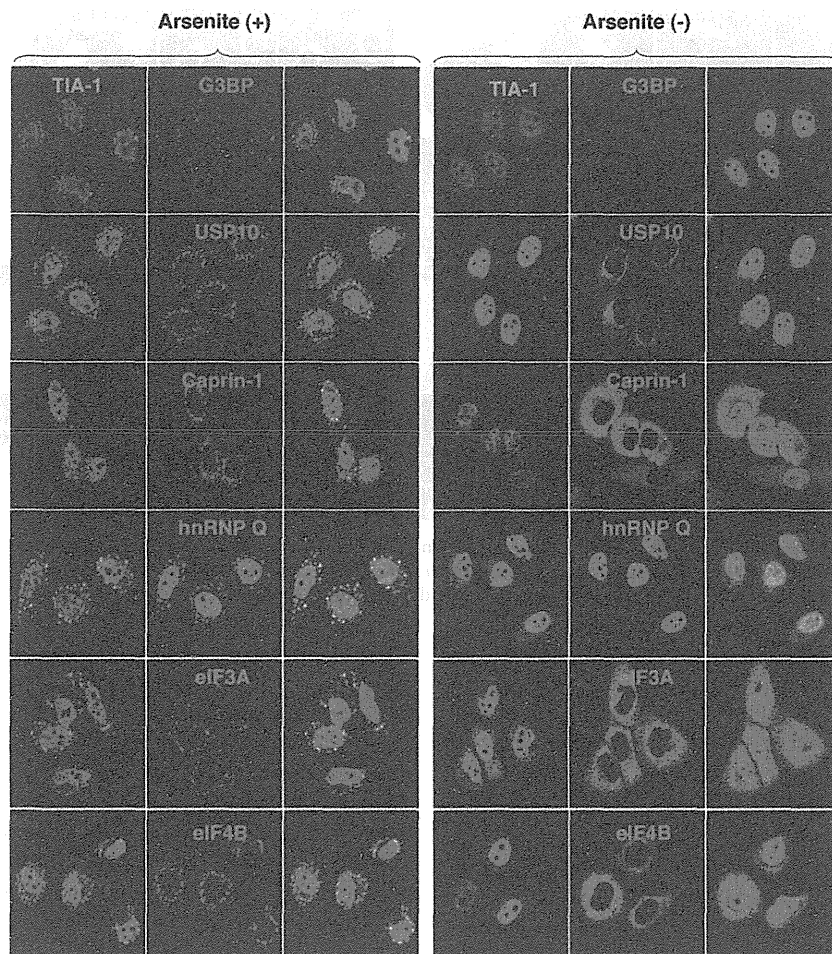


FIG 2 Each SG-associated factor forms SGs under oxidative stress. After treatment with 1.0 mM sodium arsenite for 30 min at 37°C, Huh7 cells were subjected to immunofluorescence analysis with the indicated primary antibodies, followed by AF488-conjugated anti-goat IgG and AF594-conjugated anti-mouse or rabbit IgG. Cell nuclei were stained with DAPI (blue).

center (Szirak, Hungary), respectively. Alexa Fluor (AF)-conjugated secondary antibodies were purchased from Invitrogen (Carlsbad, CA).

Immunofluorescence microscopy. Huh7 cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature. After cells were quenched for 10 min with PBS containing 50 mM ammonium chloride (NH_4Cl), they were permeabilized with 0.2% Triton X-100 in PBS for 10 min and blocked with PBS containing 2% bovine serum albumin (BSA) for 30 min at room temperature. The cells were then incubated with the antibodies indicated in the figure legends. Nuclei were stained with 4',6'-diamidino-2-phenylindole (DAPI). The samples were examined by a Fluoview FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan).

Transfection, immunoprecipitation, and immunoblotting. Plasmids were transfected into 293T or Huh7 cells by use of TransIT LT1 (Mirus, Madison, WI), and cells collected at 24 h posttransfection were subjected to immunostaining, immunoprecipitation, and/or immunoblotting as described previously (24). The immunoprecipitates were boiled in sodium dodecyl sulfate (SDS) sample buffer and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and incubated with the appropriate antibodies. The immune complexes were visualized with SuperSignal West Femto substrate (Thermo Scientific, Rockford, IL) and detected by use of an LAS-3000 image analyzer system (Fujifilm, Tokyo, Japan).

FOS-tagged purification and mass spectrometry. pCAG-Core-FOS or empty vector was transfected into 293T cells, harvested at 24 h posttransfection, washed with cold PBS, suspended in cell lysis buffer (20 mM Tris-HCl, pH 7.4, 135 mM NaCl, 1% Triton X-100, and protease inhibitor cocktail [Complete; Roche]), and centrifuged at $14,000 \times g$ for 20 min at 4°C. The supernatant was pulled down using 50 μl of STREP-Tactin Sepharose (IBA, Gottingen, Germany) equilibrated with cell lysis buffer for 2 h at 4°C. The affinity beads were washed three times with cell lysis buffer and suspended in $2 \times$ SDS-PAGE sample buffer. The proteins were subjected to SDS-PAGE, followed by Coomassie brilliant blue (CBB) staining using CBB Stain One (Nakalai Tesque, Kyoto, Japan). The gels were divided into 10 pieces, and each fraction was trypsinized and subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis to identify coimmunoprecipitated proteins. All of the proteins in gels were identified comprehensively, and the proteins detected in cells transfected with pCAG-Core-FOS but not in those with empty vector were regarded as candidates for binding partners of JEV core.

Gene silencing. A commercially available small interfering RNA (siRNA) pool targeting Caprin-1 (siGENOME SMARTpool, human Caprin1) and control nontargeting siRNA were purchased from Dharmacon (Buckinghamshire, United Kingdom) and transfected into 293T cells using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol.

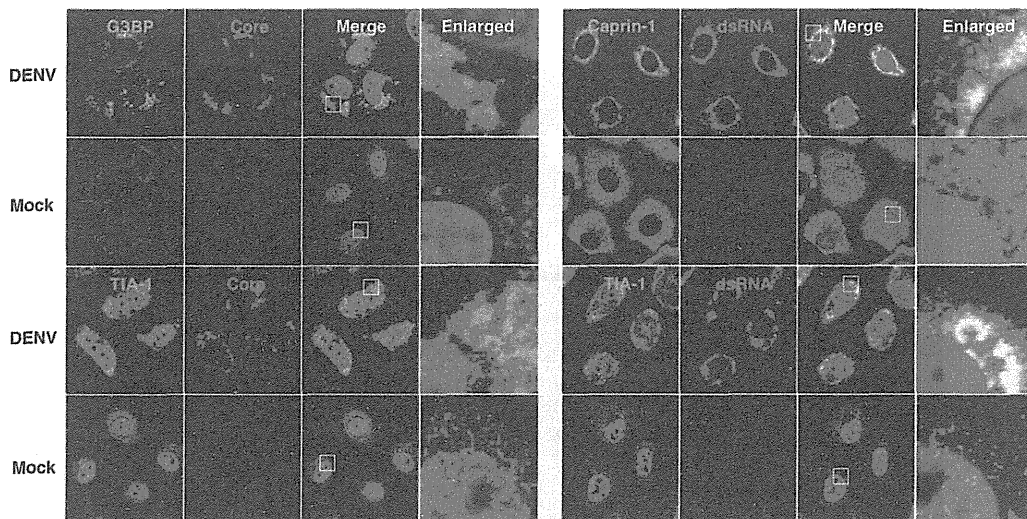


FIG 3 Subcellular localizations of the SG-associated proteins during DENV infection. Cellular localizations of G3BP, Caprin-1, and TIA-1 (green, AF488-conjugated secondary antibody) and viral components (core protein and dsRNA) (red, AF-594-conjugate secondary antibody) in Huh7 cells infected with DENV were determined by immunofluorescence analysis using the appropriate antibodies at 48 h postinfection. Cell nuclei were stained with DAPI (blue).

Preparation of recombinant proteins and GST pulldown assay. His-tagged JEV core protein (core-His) was purified as described in a previous report (25). Briefly, core-His was expressed in *Escherichia coli* (*E. coli*) Rosetta-gami 2(DE3) strain cells (Novagen-Merck) transformed with pET21b-Core-His (WT or 9798A). Bacteria grown to an optical density at 600 nm of 0.6 were induced with 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG), incubated for 5 h at 37°C with shaking, collected by centrifugation at $6,000 \times g$ for 10 min, lysed in 10 ml of bacteria lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and protease inhibitor cocktail [Complete; Roche]) by sonication on ice, and centrifuged at $10,000 \times g$ for 15 min. The supernatant containing core-His was subjected to ammonium sulfate fractionation, followed by cation exchange chromatography with a HiTrap SP column (GE Healthcare). The eluted core-His recombinant protein was dialyzed with 50 mM Tris-HCl buffer containing 150 mM NaCl at 4°C overnight. GST-fused Caprin-1 (GST-Caprin-1) was expressed in *E. coli* BL21(DE3) cells transformed with pGEX-GST-Caprin-1. Bacteria grown to an optical density at 600 nm of 1.0 were induced with 0.1 mM IPTG, incubated for 5 h at 25°C with shaking, collected by centrifugation at $6,000 \times g$ for 10 min, lysed in 10 ml of bacteria lysis buffer by sonication on ice, and centrifuged at $10,000 \times g$ for 15 min. The supernatant was mixed with 200 μ l of glutathione-Sepharose 4B beads (GE Healthcare) equilibrated with bacteria lysis buffer for 1 h at room temperature, and then the beads were washed five times with lysis buffer. Twenty micrograms of GST-Caprin-1 or GST was mixed with equal volumes of the purified core-His for 2 h at 4°C with gentle agitation. The beads were washed five times with bacteria lysis buffer and then suspended in SDS-PAGE sample buffer.

Mouse experiments. Experimental infections were approved by the Committee for Animal Experiment of RIMD, Osaka University (H19-2-0). Female ICR mice (3 weeks old) were purchased from CLEA Japan (Tokyo, Japan) and kept in specific pathogen-free environments. Groups of mice ($n = 10$) were intraperitoneally inoculated with 5×10^4 FFU (100 μ l) of the viruses. The mice were observed for 3 weeks after inoculation to determine survival rates. To examine viral growth in the brain, 5×10^4 FFU of the viruses were intraperitoneally administered to the groups of mice ($n = 3$). At 7 days postinfection, mice were euthanized, and the cerebrums were collected. The infectious titers in the homogenates of the cerebrums were determined in Vero cells as described above.

RESULTS

JEV infection confers resistance to SG induction. To examine the formation of SGs in cells infected with JEV, Huh7 cells were in-

fecting with JEV at a multiplicity of infection (MOI) of 0.5, and the expression of JEV proteins and an accepted marker for SGs, G3BP, was determined by immunofluorescence analysis at 24 h postinfection. G3BP was mainly accumulated in the perinuclear region and partially colocalized with the JEV core protein, while only partial colocalization with the NS2B protein was also observed (Fig. 1A, left). In addition, a few small G3BP-positive foci were scattered in the cytoplasm. This accumulation of G3BP was observed in not only Huh7 cells but also other cell lines, i.e., 293T and HeLa cells, infected with JEV (Fig. 1B). However, the expression level of G3BP in cells infected with JEV was comparable to that in mock-infected cells (Fig. 1C). To further investigate SG induction by JEV infection, expression of TIA-1, another SG marker, was examined. Although accumulation of TIA-1 in the perinuclear region was not observed, a few TIA-1-positive foci were observed in the JEV-infected cells and were colocalized with G3BP and JEV NS2B, indicating that SG foci were induced in cells infected with JEV (Fig. 1D). The accumulation of G3BP and the aggregation of TIA-1, indicating SG formation, appeared at 24 h postinfection in accord with the expression of viral proteins (Fig. 1E). We further examined the dynamics of other SG-associated factors in cells infected with JEV. Each factor formed clear SGs in cells treated with sodium arsenite, a potent SG inducer eliciting oxidative stress (Fig. 2). As shown in Fig. 1F, three distinct patterns of the subcellular localization of SG components were observed. USP10 and Caprin-1 were accumulated in the perinuclear region and also formed a few small foci scattered throughout the cytoplasm, as seen for G3BP; TIA-1 and hnRNP Q formed cytoplasmic foci but were not accumulated in the perinuclear region; and subcellular localization of eIF3A and eIF4B was not changed. The cytoplasmic foci were confirmed as SGs by immunofluorescence analyses using specific antibodies to SG-associated factors (data not shown). Taken together, these results indicate that JEV infection induces accumulation of several RBPs and formation of a few SGs.

It has been shown previously that infection with WNV or DENV confers resistance to SG formation induced by sodium arsenite (15). To determine the effect of JEV infection on the SG

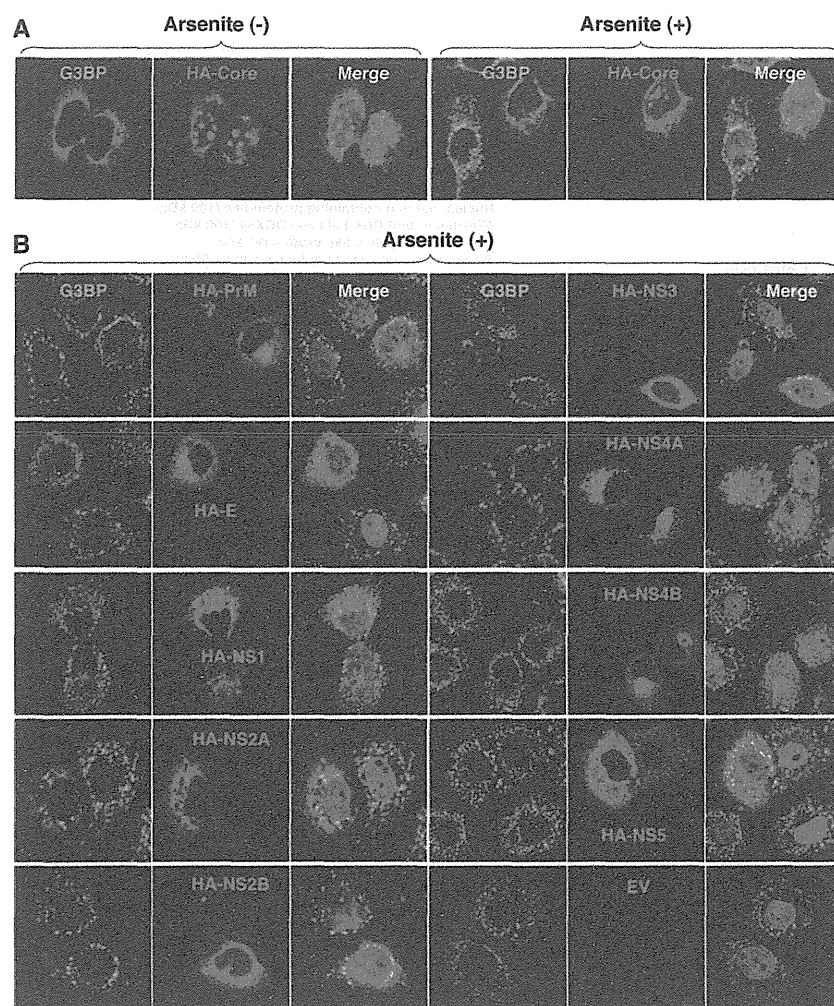


FIG 4 Inhibition of the arsenite-induced SG formation by the expression of JEV proteins. (A) Huh7 cells transfected with a plasmid, pCAGPM-HA-Core, were treated with or without 1.0 mM sodium arsenite for 30 min at 37°C, and the cellular localizations of G3BP and HA-Core were determined at 24 h posttransfection by immunofluorescence analysis with mouse anti-G3BP MAb and rat anti-HA MAb, followed by AF488-conjugated anti-mouse IgG and AF594-conjugated anti-rat IgG, respectively. Cell nuclei were stained with DAPI (blue). (B) Huh7 cells, which were separately transfected with a plasmid expressing an individual viral protein (pCAGPM-HA-JEV protein) as indicated in the figure, were treated with 1.0 mM sodium arsenite for 30 min at 37°C and subjected to an immunofluorescence assay using mouse anti-G3BP MAb and rat anti-HA MAb, followed by AF488-conjugated anti-mouse IgG and AF594-conjugated anti-rat IgG, respectively. Cell nuclei were stained with DAPI (blue).

formation induced by sodium arsenite, JEV-infected cells were treated with 0.5 mM sodium arsenite for 30 min at 24 h postinfection. Although many G3BP-positive foci were observed in mock-infected cells by the treatment with sodium arsenite, accumulation of G3BP in the perinuclear region was observed in the JEV-infected cells (Fig. 1A, right), and the numbers of G3BP-positive foci in the JEV-infected cells were less than those in the mock-infected cells (Fig. 1G). Although it has been reported that a significant reduction of the phosphorylation at Ser⁵¹ of eIF2 α in cells treated with arsenite was induced by infection with WNV (15), the phosphorylation of eIF2 α was slightly suppressed in the JEV-infected cells (Fig. 1C). Furthermore, while previous studies reported that Caprin-1 and TIA-1 were colocalized with dsRNA in cells infected with DENV (15, 26), no colocalization of G3BP or TIA-1 with the DENV core protein was observed in the present study (Fig. 3), suggesting that the mechanisms of the viral circumvention of SG formation in cells infected with JEV are different from those in cells infected with WNV and DENV.

JEV core protein suppresses SG formation induced by sodium arsenite. To elucidate the molecular mechanisms of suppression of SG formation induced by sodium arsenite during JEV infection, we tried to identify which viral protein(s) is responsible for the SG inhibition. Since G3BP was colocalized with JEV core protein, we first examined the involvement of the core protein in the perinuclear accumulation of G3BP and in the suppression of SG formation. The expression of JEV core protein alone induced the accumulation of G3BP in the perinuclear region (Fig. 4A, left panel) and suppressed sodium arsenite-induced SG formation (Fig. 4A, upper right cell in the right panel), similarly to JEV infection. In contrast, inhibition of SG formation induced by sodium arsenite was not observed in cells expressing other JEV proteins (Fig. 4B). These results suggest that JEV core protein is responsible for the circumvention of the SG formation observed in cells infected with JEV.

JEV core protein directly interacts with Caprin-1, an SG-associated cellular factor. Since JEV core protein was suggested to

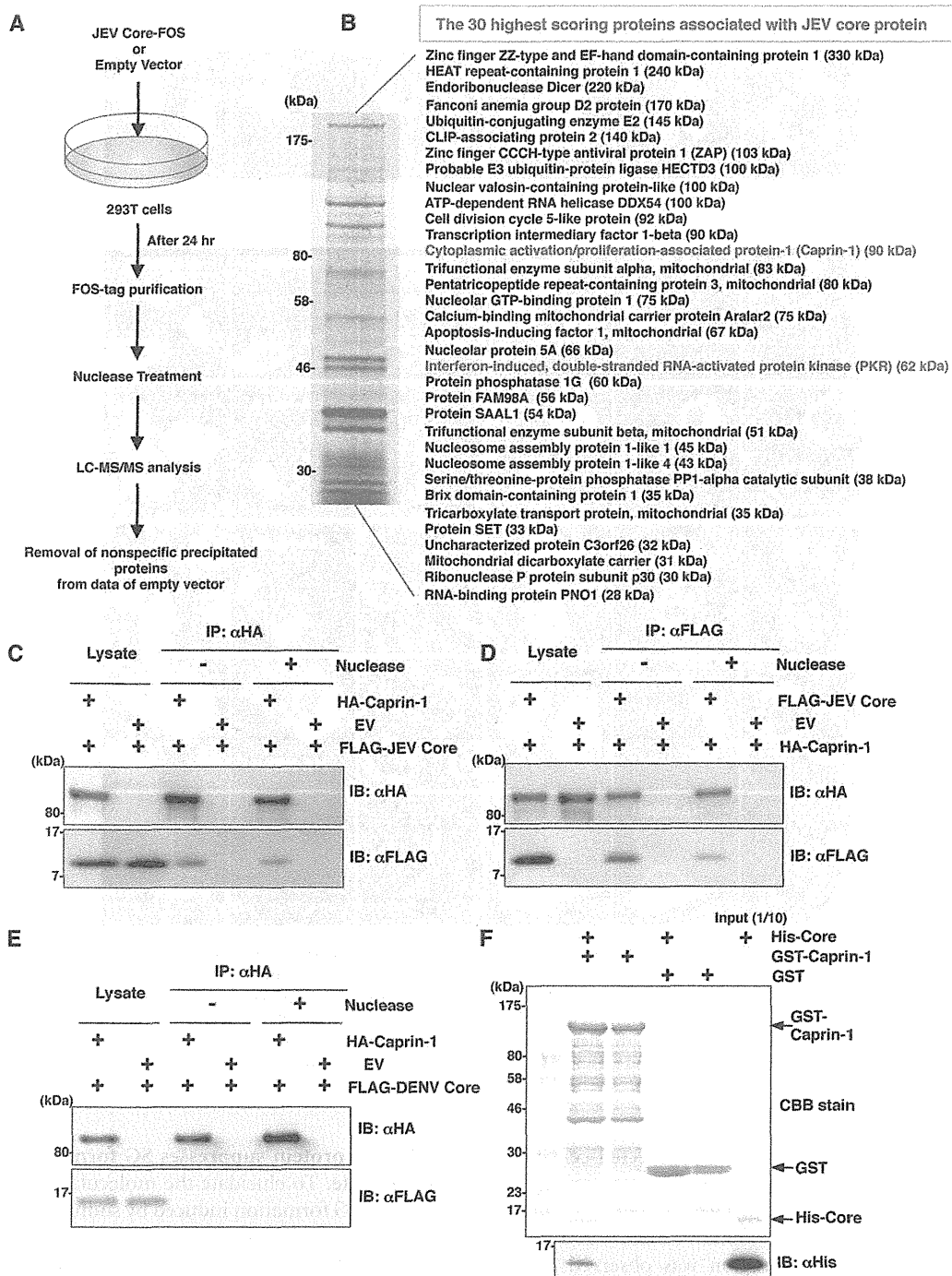


FIG 5 JEV core protein directly interacts with Caprin-1, an SG-associated cellular factor. (A) Identification of host cellular proteins associated with JEV core protein by FOS-tagged purification and LC-MS/MS analysis. Overview of the FOS-tagged purification of cellular proteins associated with JEV core protein. (B) The 30 candidate proteins as binding partners of JEV core protein exhibiting high scores are listed. PKR and Caprin-1 are indicated in red. (C and D) FLAG-JEV core protein and HA-Caprin-1 were coexpressed in 293T cells, and the cell lysates harvested at 24 h posttransfection were treated with or without micrococcal nuclease for 30 min at 37°C and immunoprecipitated (IP) with anti-HA (αHA) or anti-FLAG (αFLAG) antibody, as indicated. The precipitates were subjected to immunoblotting (IB) to detect coprecipitated counterparts. (E) FLAG-DENV core protein was coexpressed with HA-Caprin-1 in 293T cells, immunoprecipitated with anti-HA antibody, and immunoblotted with anti-HA or anti-FLAG antibody. (F) His-tagged JEV core protein was incubated with either GST-fused Caprin-1 or GST for 2 h at 4°C, and the precipitates obtained by GST pull-down assay were subjected to CBB staining and immunoblotting with anti-His antibody.

participate in the inhibition of SG formation, we tried to identify cellular factors associated with the core protein by LC-MS/MS analysis, as shown in Fig. 5A. Among the 30 factors with the best scores, two SG-associated proteins, PKR (Mascot search score,

206) and Caprin-1 (Mascot search score, 153), were identified as binding partners of JEV core protein (Fig. 5B). Although PABP1, hnRNP Q, Staufen, G3BP, and eIF4G were also identified, their scores were lower than those of PKR and Caprin-1. Because the

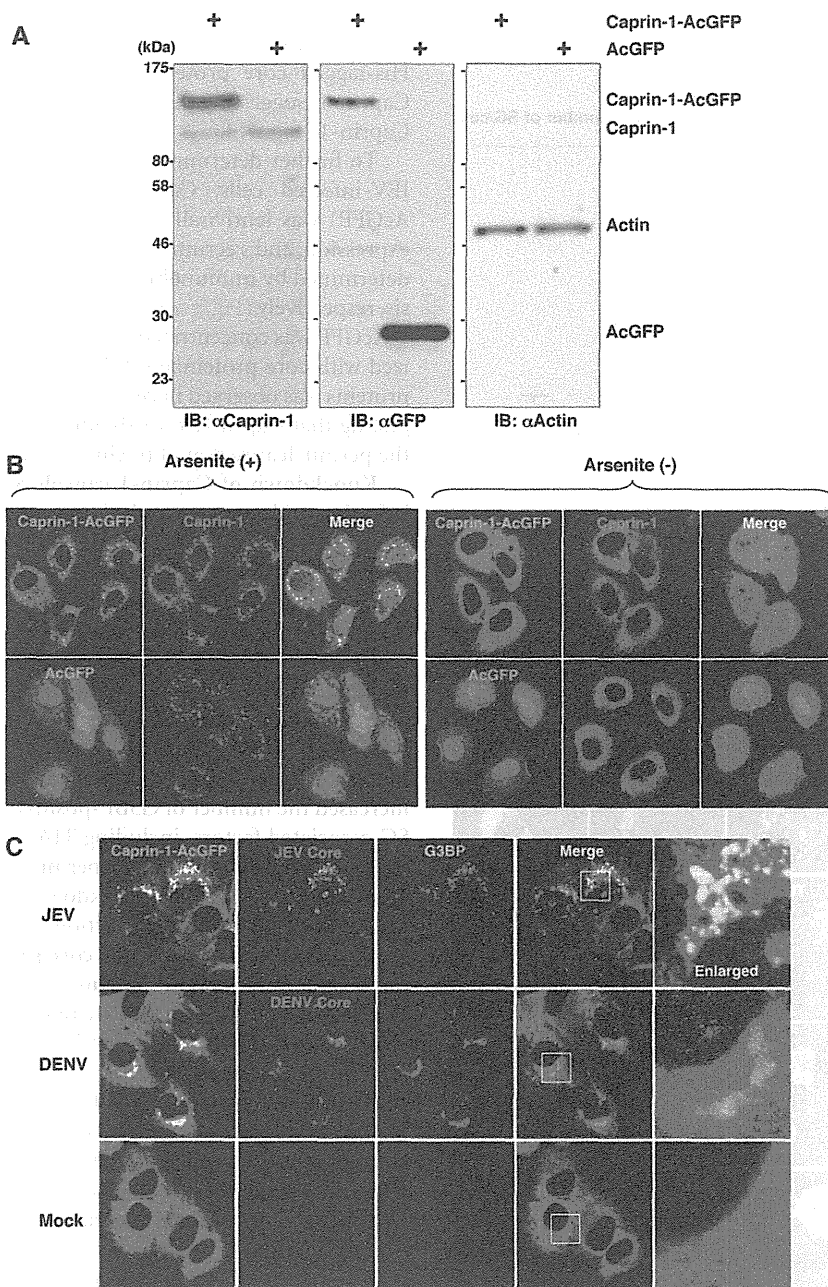


FIG 6 Caprin-1 is colocalized with the JEV core protein in the perinuclear region. (A) Expression of Caprin-1 fused with AcGFP (Caprin-1-AcGFP), Caprin-1, actin, or AcGFP in lentivirally transduced Huh7 cells was determined by immunoblotting using the appropriate antibodies. (B) Subcellular localization of Caprin-1-AcGFP or AcGFP (green) and endogenous Caprin-1 (red) in cells treated with/without 1.0 mM sodium arsenite for 30 min at 37°C was determined by immunofluorescence assay with rabbit anti-Caprin-1 PAb and AF594-conjugated anti-rabbit IgG. Cell nuclei were stained with DAPI (blue). (C) Huh7/Caprin-1-AcGFP cells were infected with either JEV or DENV at an MOI of 0.5, and the cellular localizations of JEV and DENV core (red) with Caprin-1-AcGFP and G3BP (blue) were determined at 24 h and 48 h postinfection, respectively. Cells were stained with mouse anti-G3BP MAb and rabbit anti-JEV or DENV core protein PAb, followed by AF633-conjugated anti-mouse IgG and AF594-conjugated anti-rabbit IgG, respectively, and examined by immunofluorescence analysis.

results shown in Fig. 1B suggest that the inhibition of SG formation takes place downstream of eIF2 α phosphorylation, we focused on Caprin-1 as a key factor involved in the inhibition of SG formation in cells infected with JEV. To confirm the specific interaction of JEV core protein with Caprin-1, FLAG-JEV core protein and HA-Caprin-1 were coexpressed and immunoprecipitated with anti-HA or anti-FLAG antibody in the presence or absence of

nuclease. FLAG-JEV core protein was coprecipitated with HA-Caprin-1 irrespective of nuclease treatment (Fig. 5C and D), suggesting that the interaction between JEV core protein and Caprin-1 is a protein-protein interaction. On the other hand, FLAG-DENV core protein was not coprecipitated with HA-Caprin-1 (Fig. 5E), indicating that the interaction with Caprin-1 was specific for JEV core protein. Next, the direct interaction be-

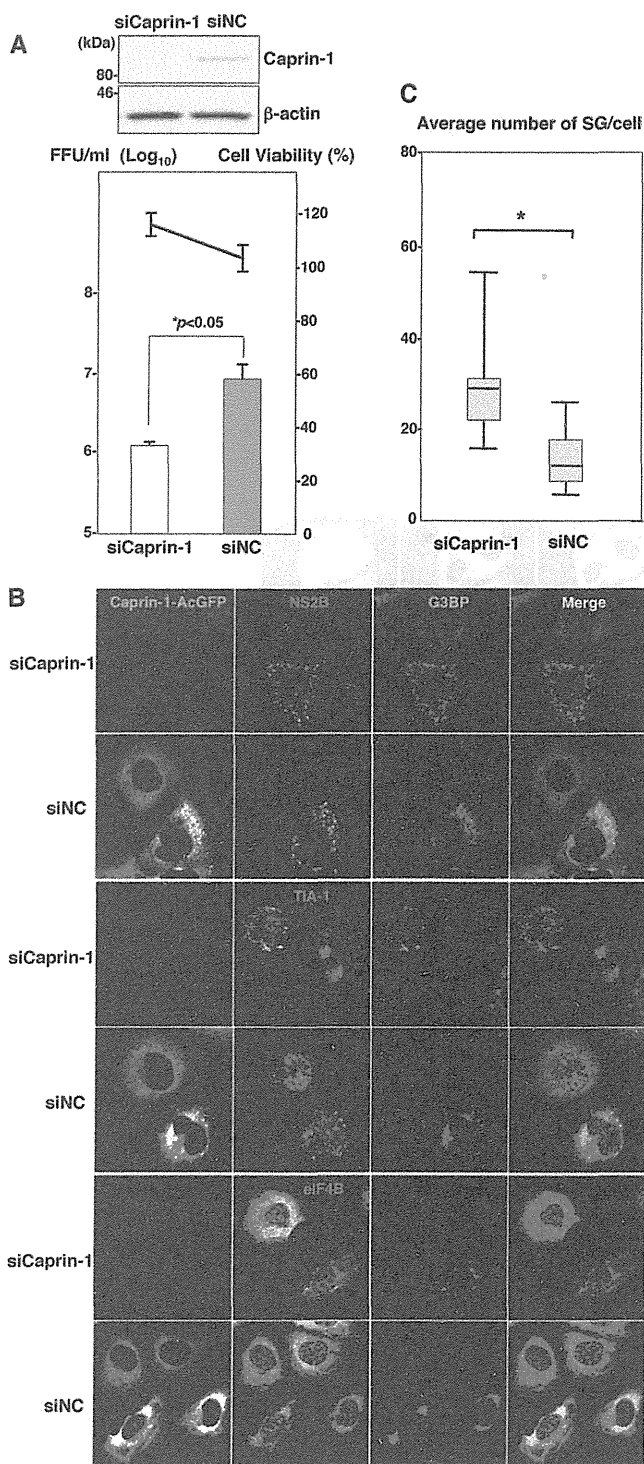


FIG 7 Knockdown of Caprin-1 cancels SG inhibition during JEV infection and suppresses viral propagation. (A) (Upper) The levels of expression of Caprin-1 in cells transfected with either siCaprin-1 or siNC was determined by immunoblotting using anti-Caprin-1 and anti-β-actin antibodies at 72 h posttransfection (top panel). At 48 h posttransfection with either siCaprin-1 or siNC, Huh7/Caprin-1-AcGFP cells were inoculated with JEV at an MOI of 0.5. At 24 h postinfection (72 h posttransfection), the infectious titers in the supernatants were determined by focus-forming assay in Vero cells (bottom panel, bar graph). Cell viability was determined at 72 h posttransfection and calculated as a percentage of the viability of cells treated with siNC (bottom panel, line graph). The results shown are from three independent assays, with the error bars representing the standard deviations. (B) At 48 h posttransfection

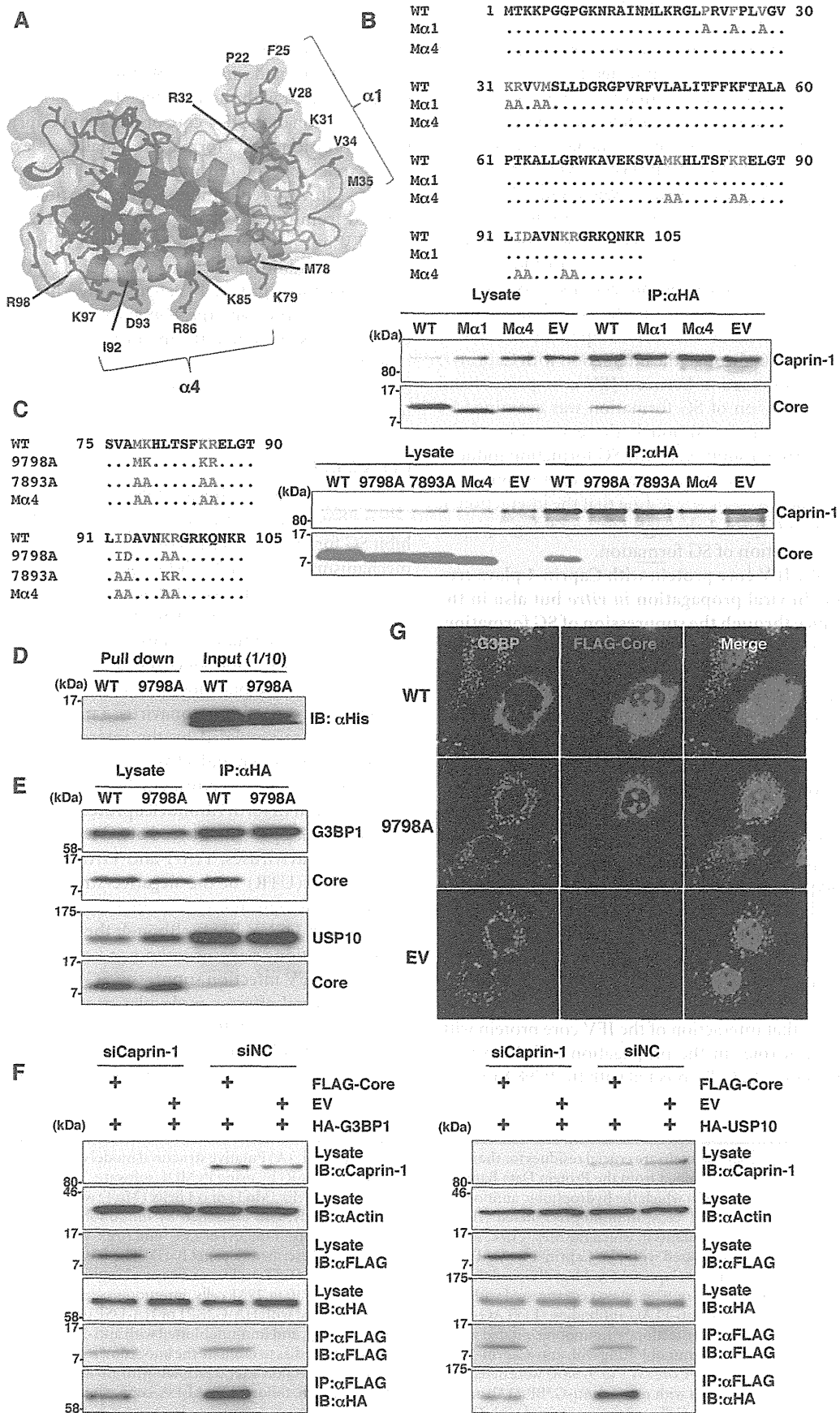
tween JEV core protein and Caprin-1 was examined by a GST-pulldown assay using purified proteins expressed in bacteria. The His-tagged core protein was coprecipitated with GST-tagged Caprin-1, suggesting that JEV core protein directly interacts with Caprin-1 (Fig. 5F).

To further determine the cellular localization of Caprin-1 in JEV-infected cells, Caprin-1 fused with AcGFP (Caprin-1-AcGFP) was lentivirally expressed in Huh7 cells. The levels of expression and recruitment of Caprin-1-AcGFP into SGs were determined by immunoblotting and immunofluorescence analysis, respectively (Fig. 6A and B). In cells infected with JEV, Caprin-1-AcGFP was concentrated in the perinuclear region and colocalized with core protein and G3BP, while no colocalization of the proteins was observed in cells infected with DENV (Fig. 6C), suggesting that Caprin-1 directly interacts with JEV core protein in the perinuclear region of the infected cells.

Knockdown of Caprin-1 cancels SG inhibition during JEV infection and suppresses viral propagation. To assess the biological significance of the interaction of JEV core protein with Caprin-1 in JEV propagation, the expression of Caprin-1 was suppressed by using Caprin-1-specific siRNAs (siCaprin-1). Transfection of siCaprin-1 efficiently and specifically knocked down the expression of Caprin-1 with a slight increase of cell viability and decreased the production of infectious particles in the culture supernatants of cells infected with JEV, in comparison with those treated with a control siRNA (siNC) (Fig. 7A). Furthermore, immunofluorescence analyses revealed that knockdown of Caprin-1 increased the number of G3BP-positive granules colocalized with SG-associated factors, including TIA-1 and eIF4B, and inhibited the G3BP concentration in the perinuclear region (Fig. 7B and C). These results suggest that knockdown of Caprin-1 suppresses JEV propagation through the induction of SG formation.

Lys⁹⁷ and Arg⁹⁸ in the JEV core protein are crucial residues for the interaction with Caprin-1. To determine amino acid residues of the core protein that are required for the interaction with Caprin-1, we constructed a putative model based on the structural information of the DENV core protein previously resolved by nuclear magnetic resonance (NMR) (27), as shown in Fig. 8A. Based on this model, we selected hydrophobic amino acids, which were located on the solvent-exposed side in the α1 and α4 helices, as amino acid residues responsible for the binding to host proteins. Amino acid substitutions in each of the α-helices shown in Fig. 8B were designed in the context of FLAG-Core (Mα1 and Mα4), and the interaction of FLAG-Core mutants with Caprin-1 was examined by immunoprecipitation analysis. WT and Mα1, but not Mα4, core proteins were immunoprecipitated with Caprin-1 (Fig. 8B). To determine the amino acids responsible for interaction with Caprin-1, further alanine substitutions were introduced in the α4 helix, and the interaction was examined by immunopre-

with either siCaprin-1 or siNC, Huh7/Caprin-1-AcGFP cells were inoculated with JEV at an MOI of 0.5. The cellular localizations of SG-associated factors and JEV NS2B were determined at 24 h postinfection (72 h posttransfection) by immunofluorescence analysis with mouse anti-G3BP MAb and rabbit anti-NS2B PAb, rabbit anti-eIF4B PAb, or goat anti-TIA-1 PAb, followed by AF633-conjugated anti-mouse IgG and AF594-conjugated anti-rabbit IgG or AF594-conjugated anti-goat IgG, respectively. (C) Numbers of G3BP-positive foci in 30 cells prepared as described in panel B were counted. Lines, boxes, and error bars indicate the means, 25th to 75th percentiles, and 95th percentiles, respectively. The significance of differences between the means was determined by a Student's *t* test. *, *P* < 0.01.



precipitation assay. As shown in Fig. 8C, double replacing both Lys⁹⁷ and Arg⁹⁸ with Ala (9798A) completely abrogated the interaction with Caprin-1. The importance of these two amino acids in the interaction with Caprin-1 was also confirmed by GST pulldown assay (Fig. 8D). These results indicate that Lys⁹⁷ and Arg⁹⁸ in the JEV core protein are crucial for the interaction with Caprin-1. Since G3BP has been reported to be one of the key molecules for SG formation and interacts with several SG component molecules including Caprin-1 and USP10 (28, 29), interactions of the core protein with SG components were examined by immunoprecipitation assay. The wild-type but not mutant 9798A core protein was associated with G3BP1 and USP10 (Fig. 8E). In addition, the knockdown of Caprin-1 weakened the interactions of core protein with G3BP1 or USP10 (Fig. 8F). These findings indicate that JEV core protein associates with several SG component molecules, such as G3BP1 and USP10, through the interaction with Caprin-1. Next, the role of the interaction between JEV core protein and Caprin-1 in the suppression of SG formation was examined by immunofluorescence analysis. Although the expression of the wild-type JEV core protein suppressed the SG formation induced by sodium arsenite treatment, as shown above, expression of the 9798A mutant did not (Fig. 8G), suggesting that the interaction of JEV core protein with Caprin-1 through Lys⁹⁷ and Arg⁹⁸ plays a crucial role in the inhibition of SG formation.

Interaction of the JEV core protein with Caprin-1 plays crucial roles not only in viral propagation *in vitro* but also in the pathogenesis in mice through the suppression of SG formation. To further examine the biological significance of the interaction between the JEV core protein and Caprin-1 in viral replication, we generated a mutant infectious cDNA clone (pMWJEAT/9798AA) of JEV encoding a mutant core protein deficient in the binding to Caprin-1 based on pMWJEAT. First, the cellular localization of the core protein in the 9798A mutant JEV-infected cells was examined by immunofluorescence analysis. The 9798A mutant core protein, as well as the wild-type core protein, was localized in the nucleus and the perinuclear region (Fig. 9A). However, the 9798A mutant core protein was not colocalized with Caprin-1, in contrast to the wild-type core protein. The sizes of infectious foci in Vero cells infected with the 9798A mutant were significantly smaller than those infected with the wild-type JEV (Fig. 9B). Furthermore, the infectious titers in C6/36 and Vero cells infected with the 9798A mutant were 6.1- and 12.6-fold lower than those infected with wild-type JEV at 48 h postinfection, respectively (Fig. 9C), suggesting that interaction of the JEV core protein with Caprin-1 plays crucial roles in the propagation of JEV in both insect and mammalian cells. Cells infected with the 9798A mutant

induced SGs containing both G3BP and Caprin-1, in contrast to the accumulation of G3BP in the perinuclear region observed in those infected with the wild-type JEV (Fig. 9D). The numbers of foci in cells infected with the 9798A mutant were higher than those in cells infected with the wild-type JEV (Fig. 9E), indicating that the interaction of the JEV core protein with Caprin-1 is crucial for the suppression of SG formation. Finally, we examined the biological relevance of the interaction of JEV core protein with Caprin-1 in viral replication *in vivo*. Infectious particles were recovered from the cerebrums of ICR mice inoculated with wild-type JEV but not from those inoculated with the 9798A mutant (Fig. 9F). In addition, all 10 mice had died by 12 days postinoculation with the wild-type JEV, while only 1 mouse had died at day 10 postinoculation with the 9798A mutant (Fig. 9G). Collectively, these results suggest that the interaction of JEV core protein with Caprin-1 plays crucial roles not only in viral replication *in vitro* but also in pathogenesis in mice through the suppression of SG formation.

DISCUSSION

Viruses are obligatory intracellular parasites, and their life cycles rely on host cellular functions. Many viruses have evolved to inhibit SG formation and thereby evade the host translation shutoff mechanism and facilitate viral replication (6, 30), while some viruses co-opt molecules regulating SG formation for viral replication (11, 31). The vaccinia virus subverts SG components to generate aggregates containing G3BP, Caprin-1, eIF4G, eIF4E, and mRNA of the virus, but not of the host, in order to stimulate viral translation (11). Replication, translation, and assembly of transmissible gastroenteritis coronavirus, a member of the *Coronaviridae* family, are regulated by the interaction of polypyrimidine tract-binding protein and TIA-1 with viral RNA (31). HIV-1 utilizes Staufen1, which is a principal component of SG, in the viral RNA selection to form ribonucleoproteins (RNPs) through interaction with Gag protein, instead of SG translation silencing (8). In the case of flaviviruses, TIA-1 and TIAR bind to the 3' untranslated region (UTR) of the negative-stranded RNA of WNV to facilitate viral replication (16), and G3BP1, Caprin-1, and USP10 interact with DENV RNA, although the biological significance of these interactions remains unknown (26). In this study, we have shown that JEV infection suppresses SG formation by the recruitment of several effector molecules promoting SG assembly, including G3BP and USP10, to the perinuclear region through the interaction of JEV core protein with Caprin-1. Furthermore, a mutant JEV carrying a core protein incapable of binding to

FIG 8 Lys⁹⁷ and Arg⁹⁸ in the JEV core protein are crucial residues for the interaction with Caprin-1. (A) Putative structural model of the core protein homodimer of JEV deduced from that of DENV obtained from the Protein Data Bank (accession number 1R6R) by using PyMOL software. The two α helices ($\alpha 1$ and $\alpha 4$) are indicated. (B) FLAG-Core mutants in which the hydrophobic amino acid residues in the $\alpha 1$ helix (M $\alpha 1$) or $\alpha 4$ helix (M $\alpha 4$) were replaced with alanine were coexpressed with HA-Caprin-1 in 293T cells, immunoprecipitated (IP) with anti-HA antibody, and examined by immunoblotting (IB) with anti-HA or anti-FLAG antibody. (C) FLAG-Core mutants in which the Met⁷⁸, Lys⁷⁹, Lys⁸⁵, Arg⁸⁶, Ile⁹², and Asp⁹³ (7893A) or Lys⁹⁷ and Arg⁹⁸ (9798A) in the $\alpha 4$ helix domain were replaced with alanine were coexpressed with HA-Caprin-1 in 293T cells and examined as described in panel B. (D) The His-tagged JEV core protein (WT or 9798A) was incubated with GST-fused Caprin-1 for 2 h at 4°C, and the precipitates obtained by GST pulldown assay were subjected to immunoblotting with anti-His antibody. (E) FLAG-Core (WT or 9798A) was coexpressed with HA-G3BP1 or HA-USP10 in 293T cells, immunoprecipitated with anti-HA antibody, and immunoblotted with anti-HA and anti-FLAG antibodies. (F) FLAG-JEV Core was coexpressed with HA-G3BP1 or HA-USP10 in 293T cells transfected with either siCaprin-1 or siNC at 72 h posttransfection, immunoprecipitated with anti-FLAG antibody, and immunoblotted with anti-HA and anti-FLAG antibodies. The cell lysates were also subjected to immunoblotting with anti-Caprin-1 and anti- β -actin antibodies to evaluate the knockdown efficiency of Caprin-1. (G) The cellular localizations of G3BP and FLAG-Core (WT or 9798A) were determined at 24 h posttransfection after treatment with 1.0 mM sodium arsenite for 30 min at 37°C by immunofluorescence analysis with mouse anti-G3BP MAb and rabbit anti-FLAG PAb, followed by AF488-conjugated anti-mouse IgG and AF594-conjugated anti-rabbit IgG, respectively. Cell nuclei were stained with DAPI (blue).

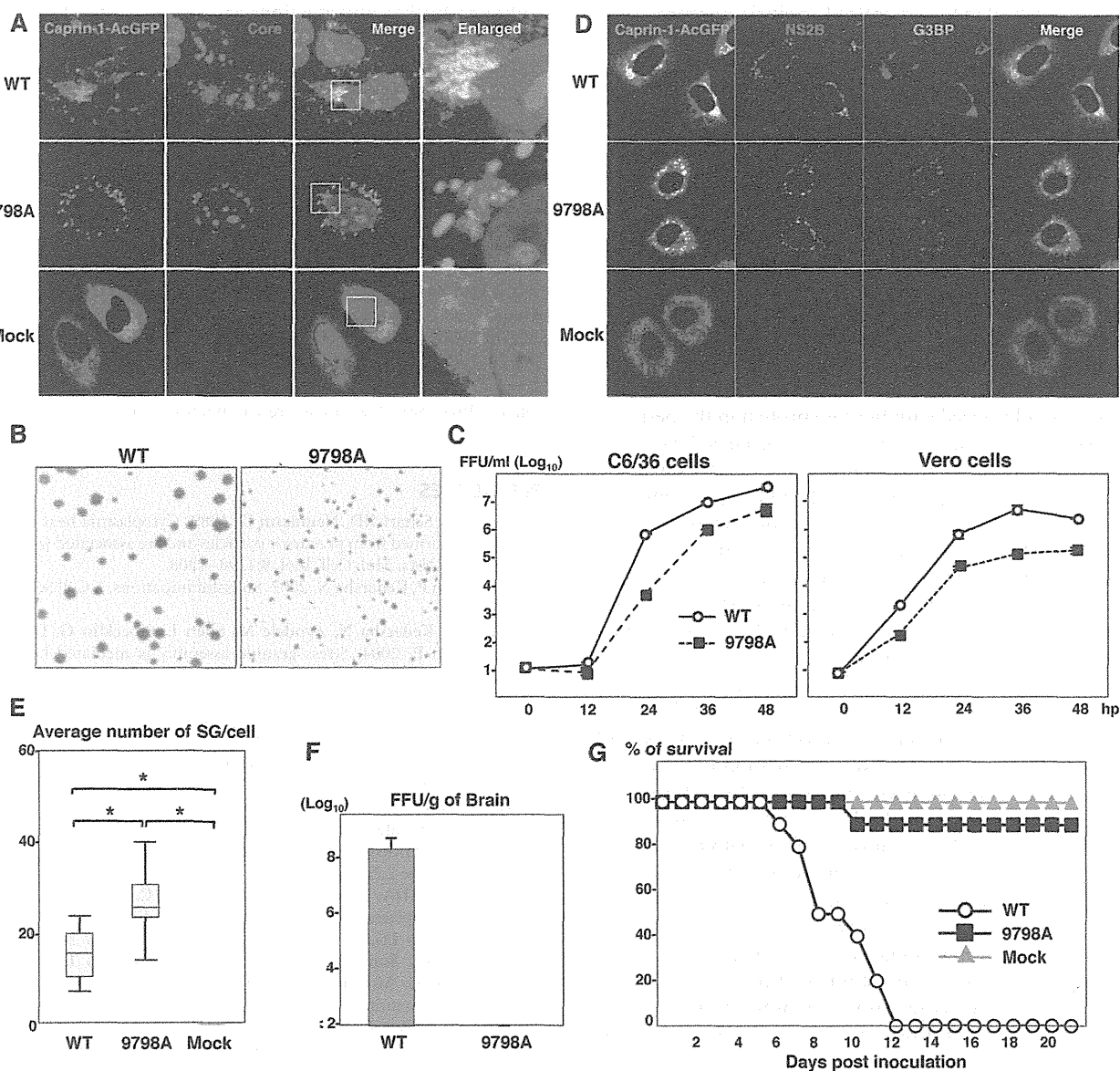


FIG 9 Interaction of JEV core protein with Caprin-1 plays crucial roles not only in viral replication *in vitro* but also in pathogenesis in mice through the suppression of SG formation. (A) Huh7/Caprin-1-AcGFP cells were infected with JEV (WT or 9798A mutant) at an MOI of 1.0, and the cellular localizations of Caprin-1-AcGFP and JEV core protein were determined at 24 h postinfection by immunofluorescence analysis with rabbit anti-core PAb and AF594-conjugated anti-rabbit IgG. Cell nuclei were stained with DAPI (blue). (B) Focus formation of JEV (WT or 9798A mutant) in Vero cells incubated in methylcellulose overlay medium at 48 h postinfection. The infectious foci were immunostained as described previously (20). (C) Growth kinetics of JEV (WT or 9798A mutant) in C6/36 and Vero cells infected at an MOI of 0.1. Infectious titers in the culture supernatants harvested at the indicated times were determined by focus-forming assays in Vero cells. Means of three experiments are indicated. (D) Huh7/Caprin-1-AcGFP cells were infected with either WT or 9798A at an MOI of 0.5, and cellular localizations of Caprin-1-AcGFP, G3BP (blue), and JEV NS2B (red) were determined at 24 h postinfection by immunofluorescence analysis with mouse anti-G3BP MAb and rabbit anti-NS2B PAb, followed by AF633-conjugated anti-mouse IgG and AF594-conjugated anti-rabbit IgG, respectively. (E) Numbers > of G3BP-positive foci in 30 cells prepared as described in panel D were counted. Lines, boxes, and error bars indicate the means, 25th to 75th percentiles, and 95th percentiles, respectively. The significance of differences between the means was determined by Student's *t* test. *, *P* < 0.01. (F) Infectious titers in the cerebrums of mice at 7 days postintra-peritoneal inoculation with 5×10^4 FFU/100 μ l of either WT or 9798A virus were determined in Vero cells. The means of titers in the homogenates of the cerebrums from three mice are indicated. The detection limit is 10^2 FFU/g of cerebrum. (G) Percentages of surviving mice (*n* = 10) after intraperitoneal inoculation with 5×10^4 FFU of either WT or 9798A virus. Mock, inoculation with DMEM.

Caprin-1 exhibited reduced replication *in vitro* and attenuated pathogenicity in mice.

G3BP is one of the key molecules involved in the SG aggregation process and self-oligomerizes in a phosphorylation-dependent manner to sequester mRNA in SGs (4). Therefore, G3BP knocked down cells (6) and G3BP knockout mouse embryonic

fibroblast cells are deficient in the SG formation. In addition, G3BP sequestration inhibits SG formation in response to arsenite treatment (32). Caprin-1, known as RNA granule protein 105 or p137 (33), also participates in SG formation through phosphorylation of eIF2 α (28) and is ubiquitously expressed in the cytoplasm. Caprin-1 regulates the transport and translation of mRNAs

of proteins involved in the synaptic plasticity in neurons (34) and cellular proliferation and migration in multiple cell types (28) through an interaction with G3BP. USP10, another SG-associated molecule, also interacts with G3BP and forms the G3BP/USP10 complex (29), suggesting that several SG-associated RBPs participate in the formation of a protein-protein network. In this study, the JEV core protein was shown to directly interact with Caprin-1, to sequester several key molecule complexes involved in SG formation to the perinuclear region in cells infected with JEV, and to facilitate viral propagation through the suppression of SG formation.

Flaviviruses replicate at a relatively low rate in comparison with most of the other positive-stranded RNA viruses, and thus rapid shutdown of host cellular protein synthesis would be deleterious for the viral life cycle. In cells infected with JEV, several SG components were colocalized with the core protein in the perinuclear region, while in those infected with WNV or DENV, SG components were accumulated in a replication complex composed of viral RNA and nonstructural proteins. In addition, the phosphorylation of eIF2 α induced by arsenite was completely canceled by the infection with WNV or DENV, whereas the suppression of the phosphorylation was limited in JEV infection (15). Incorporation of the nascent viral RNA into the membranous structure induced by viral nonstructural proteins prevents PKR activation and inhibits SG formation in cells infected with WNV (17). In cells infected with hepatitis C virus (HCV), which belongs to the genus *Hepacivirus* in the family *Flaviviridae*, induction of SG formation was observed in the early stage of infection, in contrast to the inhibition of the arsenite-induced SG formation in the late stage (35). Several SG components, such as G3BP1, PABP1, and ataxin-2, were colocalized with HCV core protein around lipid droplets (35), and G3BP1 was also associated with the NS5B protein and the 5' terminus of the minus-strand viral RNA (36) to mediate efficient viral replication. Collectively, these data suggest that flaviviruses have evolved to regulate cellular processes involved in SG formation through various strategies.

PKR is one of the interferon-stimulated genes and plays a crucial role in antiviral defense through phosphorylation of eIF2 α , which leads to host translational shutoff (37, 38). In the early stage of flavivirus infection, both positive- and negative-stranded RNAs transcribe at low levels, while genomic RNA predominantly synthesizes in the late stage of infection (39). It was shown that activation of PKR was suppressed (40) or only induced in the late stage of WNV infection (41) and impaired by the expression of HCV NS5A (42–44). Very recently, JEV NS2A was shown to suppress PKR activation through inhibition of dimerization of PKR in the early stage but not in the late stage of infection (45). In this study, we have shown that JEV core protein interacts with Caprin-1 and inhibits SG formation downstream of the phosphorylation of eIF2 α in the late stage of infection, suggesting that JEV has evolved to escape from host antiviral responses in the multiple stages of viral replication by using structural and nonstructural proteins.

The flavivirus core protein is a multifunctional protein involved in many aspects of the viral life cycle. In addition to the formation of viral nucleocapsid through the interaction with viral RNA (as a structural protein) (46), flavivirus core proteins interact with various host factors, such as B23 (47), Jab1 (48), hnRNP K (49), and hnRNP A2 (23), and regulate viral replication and/or modify the host cell environment (as a nonstructural protein).

Although further investigations are needed to clarify the precise mechanisms underlying the circumvention of SG formation through the interaction of JEV core protein with Caprin-1, leading to efficient propagation *in vitro* and pathogenicity in mice, these findings could help not only to provide new insight into strategies by which viruses escape host stress responses but also to develop novel antiviral agents for flavivirus infection.

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