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RNA binding property and RNA chaperone activity of dengue virus core protein and other viral RNA-interacting proteins

Wen-Li Pong [a], Zhi-Shun Huang [a], Pak-Guan Teoh [a,b], Chung-Chun Wang [a,c], Huey-Nan Wu [a,*]

[a] Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan
[b] Taiwan International Graduate Program, Graduate Institute of Life Sciences, National Defense Medical Center and Academia Sinica, Taipei, Taiwan
[c] Graduate Institute of Life Sciences, National Defense Medical Center, Taipei, Taiwan

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ABSTRACT

In this study we showed that the dengue virus (DENV) core protein forms a dimer with an α-helix-rich structure, binds RNA and facilitates the strand annealing process. To assess the RNA chaperone activity of this core protein and other dengue viral RNA-interacting proteins, such as NS3 helicase and NS5 proteins, we engineered *cis*- and *trans*-cleavage hammerhead ribozyme constructs carrying DENV genomic RNA elements. Our results indicate that DENV core protein facilitates typical hammerhead structure formation by acting as an RNA chaperone and DENV NS5 has a weak RNA chaperone activity, while DENV NS3 helicase failed to refold RNA with a complex secondary structure.

1. Introduction

Dengue virus (DENV), which belongs to the *Flavivirus* genus of the *Flaviviridae* family, is the most significant mosquito-borne human viral pathogen. There are four serotypes of DENV (types 1–4) identified so far [1]. The DENV genome consists of a 5′-capped single-stranded RNA with positive polarity ((+) RNA) of approximately 11 kb in length encoding three structural proteins (core, prM, and E) and seven non-structural proteins (NS1, 2A, 2B, 3, 4A, 4B, and 5). Structural proteins are the components of the virus particle, and most, if not all, non-structural proteins participate in viral replication [2,3]. DENV uses its capped genomic RNA to direct the translation of viral protein to produce more viral RNA progeny and virus particles. The 5′ and 3′ untranslated regions (UTR) flanking coding sequences in the genome contain key RNA elements that control viral translation and replication [4,5]. However, so far no viral RNA packaging signal has been identified. The DENV genomic RNA is assumed to fold into biologically relevant conformations and to switch between distinct conformations to accomplish its function at different stages of the viral life cycle. Hence, the genomic RNA and its RNA elements are most probably undergoing structural rearrangements facilitated by a virus-encoded protein with RNA chaperone activity.

The term RNA chaperone refers to proteins that aid in the process of RNA folding by preventing RNA misfolding and resolving misfolded RNA. They generally bind RNA non-specifically and cooperatively to facilitate RNA structural rearrangements in an ATP-independent manner [6]. The RNA chaperone property of a protein can be determined by strand annealing assay, strand displacement assay as well as its ability to rescue the formation of misfolded complex structures of a catalytic RNA, such as hammerhead ribozyme (HHRz) [7,8]. Some virus-encoded proteins are non-specific RNA chaperones, including core proteins of *Flaviviridae* and the nucleocapsid proteins of retroviruses, coronaviruses, and hepatitis delta virus [9–12]. Apart from their basic nature, no motif specific for RNA chaperones has been identified. Recently, it has been suggested that small, highly basic and flexible protein fragments are a hallmark of active RNA chaperone domains in viral core/nucleocapsid proteins [9,11].

Core proteins of all flaviviruses, including DENV, West Nile virus (WNV), Japanese encephalitis virus (JEV), and yellow fever virus (YFV) contain approximately 25% of basic amino acids and a conserved internal hydrophobic region, however they share very little sequence identity [13]. Recently, it has been suggested that DENV core protein possesses several features that fit the suggested characteristics of an RNA chaperone including small size and a high

* Corresponding author. Fax: +886 2 782 6085.
E-mail address: hnwu@gate.sinica.edu.tw (H.-N. Wu).

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proportion of basic amino acid residues. However, no direct evidence showing that DENV core protein assists RNA structural rearrangement exists so far.

Non-structural proteins are the components of the viral replicase complex which colocalize with cytoplasmic double-stranded replication intermediate RNA at the replication site [3]. Both NS3 and NS5 proteins interact with the RNA genome to exert their biological functions, and each protein has multiple enzymatic activities. NS3 helicase unwinds short dsRNA [14], whereas NS5 RNA-dependent RNA polymerase (RDRP) participates in viral RNA synthesis [15]. NS3 RNA triphosphatase (RTPase) together with NS5 guanylyl transferase (GTase) and methyl transferase (MTase) are for the 5′-capping of viral genomic RNA [14,16,17]. We hypothesized that NS3 and NS5 proteins play a role in chaperoning DENV genomic RNA to achieve a replication-competent conformation.

In this study, we investigated the RNA-interacting property of DENV core protein and analyzed the putative RNA chaperone activity of DENV RNA-interacting proteins including core protein, NS3 helicase, and NS5 protein. By using a newly engineered *trans*-cleaving hammerhead ribozyme construct in which DENV genomic RNA elements were incorporated, we showed that DENV core protein is a true RNA chaperone, while DENV NS5 has a weak RNA chaperone activity and DENV NS3 helicase fails to refold RNA with significant secondary structure.

2. Materials and methods

2.1. Expression vectors

Plasmid for the expression of DENV-3 (strain Philippines/H87/1956) core protein was constructed in a modified pET21b vector that had a stop codon upstream of the His-tag coding sequence. The full-length NS5 protein of DENV-2 strain PL046 was expressed as a SUMO-fusion protein from *Escherichia coli*. The helicase domain of the NS3 protein of DENV-2 strain PL046 was expressed as a His-tagged fusion protein as described [14]. The N-terminal domain of hepatitis delta antigen, NdAg, was expressed purified as described [12].

Additional materials and methods including protein expression and purification, CD spectroscopy, cross-linking, RNAs, electrophoretic mobility shift assay, strand annealing assay, hammerhead ribozyme reactions are listed in Supplementary data SD1.

3. Results and discussion

3.1. DENV core protein (CP) characterization

Recombinant DENV-3 core protein (referred to as CP from now on) was expressed and purified from *E. coli* to near homogeneity (Fig. 1A). Purification of this highly basic protein under native and high ionic strength conditions yielded a complex of CP with tightly associated *E. coli* nucleic acids as evidenced by the ratio of absorbance at 260 nm and 280 nm (Fig. 1B). Protein unfolding with increasing concentrations of urea (0–8 M) followed by the refolding with decreasing concentrations of urea (8–0 M) and elution led to CP that was highly purified from associated nucleic acids, as revealed by the significant decrease in the $A_{260/280}$ ratio (Fig. 1A and B). These results showed that CP has a high tendency to interact with nucleic acids and that the unfolding-refolding procedure with urea removed a large fraction of the associated nucleic acids from CP.

Far-UV circular dichroism (CD) measurements were next carried out to obtain insights into the structural features of CP. The double local ellipticity minima at 208 nm and 222 nm, which are characteristic of a typical α-helical protein, were detected with the non-refolded as well as the refolded forms of CP (Fig. 1C). Ellipticity changes at 222 nm upon heating showed a denaturing curve with a mid-point of thermal transition (T_m) at 60–65 °C for both forms of CP (Fig. 1D). These results showed that CP has an α-helix-rich structure similar to the structural features of DENV-2 and WNV core proteins [13,18], and suggested that the associated nucleic acids has a small effect on the folding and the stability of CP.

Cross-linking experiment was carried out to determine the oligomeric state of CP in solution. For both the non-refolded and the refolded forms of CP, the signal of the dimeric species increased with increasing glutaraldehyde concentration (Fig. 1E). The refolded form of the protein formed dimers more efficiently than the non-refolded form (Fig. 1E). It is likely that the associated nucleic acids of the non-refolded form protein perturbed the cross-linking reaction. The cross-linking study suggests that the native oligomeric state of CP is dimeric, similar to DENV-2 core protein [13].

3.2. CP–RNA interaction

CP of the non-refolded and the refolded forms were used for studying CP–RNA interaction. CP is rich in basic residues, about a quarter of the 100 amino acids are either lysines or arginines (Fig. S1), and its overall pi value is 22.7. To assess the RNA-binding ability of CP, we performed electrophoretic mobility shift assay (EMSA) using first the 3′-stem-loop of dengue genome, i.e., SL(+) (SD2), as the substrate RNA. Almost all SL(+) RNA (0.5 nM) formed a complex with CP (non-refolded form) at concentrations of 40 nM, 100 nM, and 400 nM under low-ionic strength conditions (0.1 M NaCl). The bound SL(+) RNA was retained in the gel well and no RNA–CP complex (RNP) of intermediate mobility was observed (Fig. 1F). This result demonstrated that a threshold amount of CP appears to be needed to effective aggregate this SL(+) RNA. To assess the RNA-binding specificity of CP, we replaced the SL(+) RNA in the binding assay with different DENV RNA fragments in the size range of 100–300 nt, which were derived from the 5′ or the 3′ non-coding region or the coding region of DENV genome. We found that each of these RNA fragments had a CP binding profile similar to that of SL(+) RNA (data not shown). These results showed that CP has a high affinity to RNA and possesses a broad RNA-binding specificity.

To evaluate the contribution of electrostatic interactions to the RNA-binding activity of the highly basic CP, the binding assay with SL(+) RNA was performed under conditions of increasing ionic strength (0.1–0.7 M NaCl). In the presence of 0.3 M, 0.5 M, and 0.7 M NaCl, complete formation of the large RNP was observed at 400 nM but not at lower concentrations of CP (non-refolded form); again, no RNP of intermediate mobility was detected under these reaction conditions (Fig. 1F). These results suggest that electrostatic interactions, which decease as the ionic strength increases, are important for the interaction of CP with RNA and the aggregation of RNA by CP.

Moreover, we observed no difference in RNA binding between the non-refolded and refolded forms of CP (data not shown). Therefore, the tightly associated nucleic acids did not saturate the RNA-binding capability or altered the RNA-binding activity of CP.

3.3. Strand annealing activity of CP

CP bound RNA with a broad specificity like many virus-encoded RNA chaperones. To assess the putative RNA chaperone activity of CP, its ability to enhance the annealing of a pair of short complementary RNAs, T41 and B16 (SD2), was first examined. CP (non-refolded form) and NdAg (the N-terminal domain of hepatitis delta antigen, a well-characterized RNA chaperone; ref 12) promoted the annealing of T41 and B16 RNAs (Fig. S2).

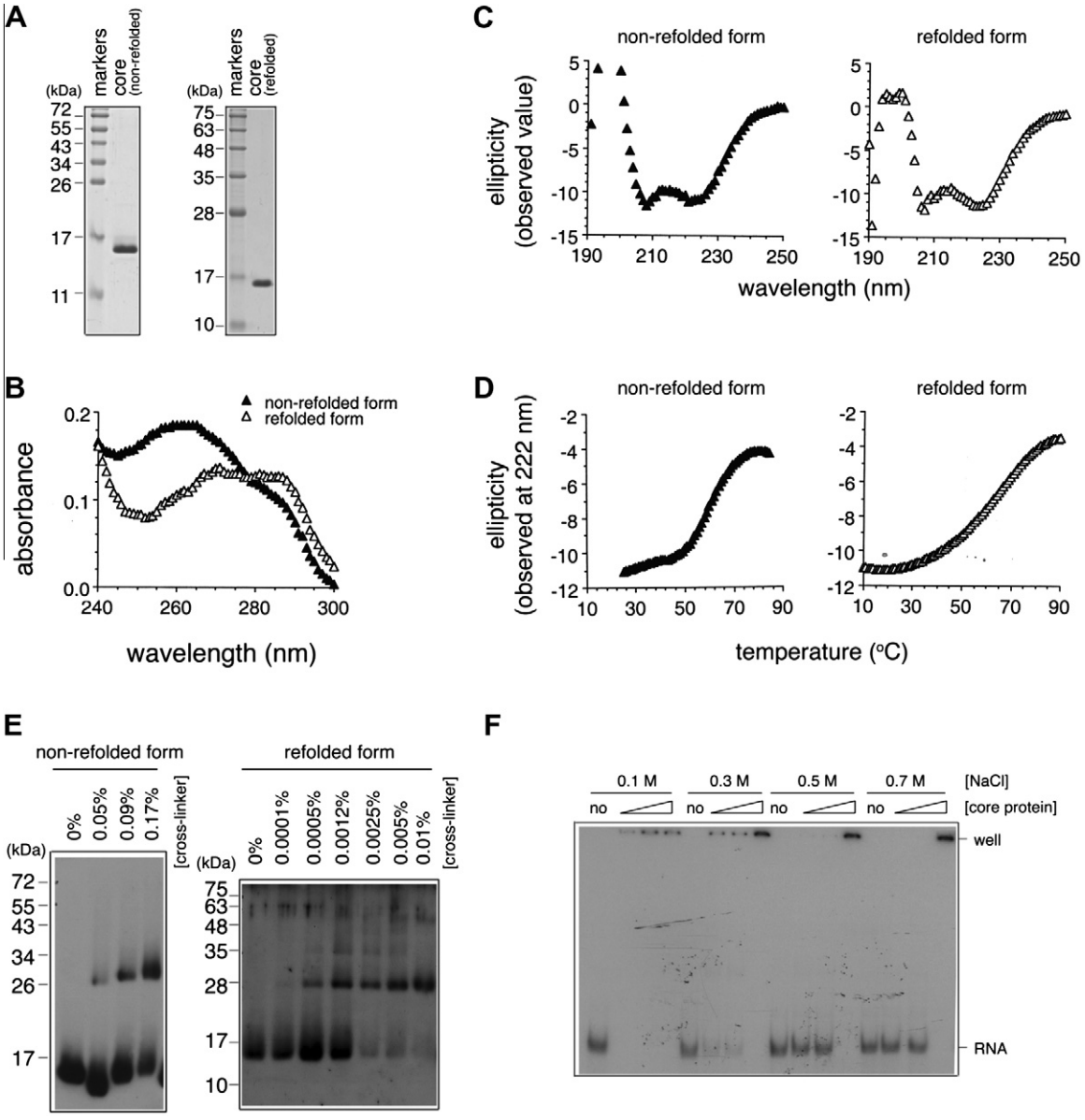

Fig. 1. Characterization of CP. (A) Coommassie blue staining pattern of purified CP. CP of the non-refolded form and the refolded form (2 μg each) was analyzed by 18% and 15% SDS–PAGE, respectively. (B) UV absorption. The ratio of absorbance of 260–280 nm was 1.55 for CP of the non-refolded form and 0.83 for CP of the refolded form. (C) CD spectra measured at 25 °C with 7.2 μM of CP of the non-refolded form in 10 mM potassium phosphate buffer (pH 7.0) containing 0.1 M KCl, or with 10 μM of CP of the refolded form in 5 mM HEPES buffer (pH 7.9) containing 0.1 M NaCl and 0.5% glycerol. (D) Melting curve. (E) Cross-linking. CP (1 μM) was treated with glutaraldehyde for 5 min. Products were resolved by 15% SDS–PAGE. (F) Analysis of the RNA binding by EMSA. CP (non-refolded form) at 0, 40, 100, or 400 nM was allowed to bind to 0.5 nM SL(+) RNA in the presence of indicated concentration of NaCl at 37 °C for 30 min. Reaction mixtures were resolved on a native 5% polyacrylamide gelrun at 4 °C. Well: gel well; RNA: unbound RNA.

Some RNA chaperones facilitate the restructuring of structured RNA in addition to promote strand annealing [8]. To monitor the ability of CP to mediate RNA refolding, we employed a pair of long and structured complementary RNAs SSL(+) and SSL(−) as substrate of the strand annealing assay. SSL(+) RNA was derived from the 3′ stem-and-loop of DENV genomic RNA, whereas SSL(−) RNA contained the antisense sequence of SSL(+) RNA (Fig. 2A). Same with NdAg, CP (non-refolded form) strongly promoted the formation of SSL(+)/(−) dsRNA, and the strand annealing process occurred rapidly in its presence (Fig. 2B and C). Overall, these studies showed that CP promotes the annealing of simple as well as structured complementary RNAs likely by acting as an RNA chaperone.

CP no longer stimulated SSL(+)/(−) dsRNA formation in the presence of $\geqslant$0.5 M NaCl, although the spontaneous, facilitator-independent dsRNA formation was promoted at elevated NaCl concentrations (Fig. 2D). The reduced RNA-binding affinity of CP at high ionic strengths could account for its loss of stimulatory activity on strand annealing in the presence of an elevated concentration of NaCl. This finding supports the idea that electrostatic interactions are important for CP–RNA interaction.

The fact that CP of the non-refolded form stimulated RNA duplex formation implied that the protein could exert its strand annealing activity in the presence of non-homologous RNA and/or DNA. Indeed, the formation of SSL(+)/(−) dsRNA by CP occurred in the presence of a 2-fold higher nucleotide concentration of cellular RNA or a 50-fold higher nucleotide concentration of tRNA (Fig. S3).

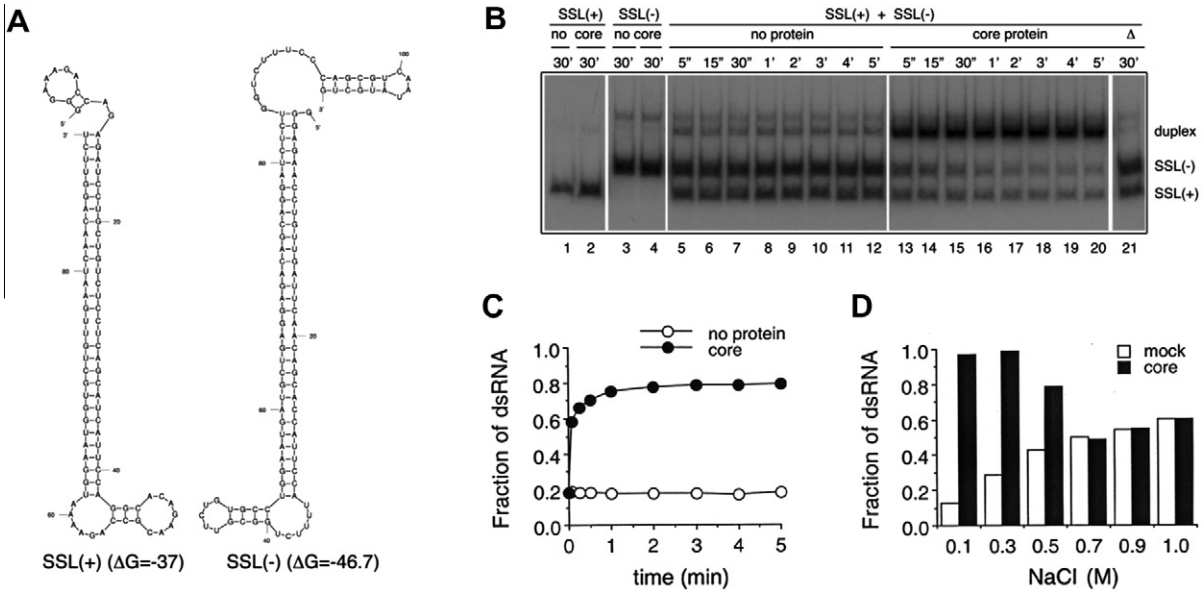

Fig. 2. Strand annealing assay. (A) Structure models of SSL(+) and SSL(−) RNAs with the lowest folding free energy [24]. (B,C) Representative gel showing dsRNA formation (B) and the quantitative data (C). Assays were performed with 0.5 nM of each RNA and 0 or 25 nM of CP (non-refolded form) at 37 °C "Δ" represents the heat-denatured reaction mixture. (D) The effect of ionic strength on the stimulatory activity of CP on dsRNA formation. Annealing reaction of 2 nM of each RNA by 100 nM CP (refolded form) was performed in presence of increasing concentrations of NaCl at 25 °C for 15 min. The reaction with mock protein served as the control.

3.4. RNA chaperone activity assay with a HHRz system

Hammerhead ribozyme (HHRz) contains a central core flanked by three helices. A *cis*-cleaving HHRz can be divided into two RNA strands and converted into a *trans*-acting construct in a way that the proper hybridization of the two RNA strands reconstitutes the HHRz structure [19]; the resulting cleaved strand acts as the substrate, while the other strand acts as the cognate *trans*-acting enzyme. HHRz-mediated RNA cleavage reactions have been used previously to assess the complex RNA remodeling events stimulated by RNA chaperones [7,11].

The long-range 5′–3′ RNA-RNA interactions in the DENV genome via the hybridization of two pairs of complementary sequences, the 5′ and 3′ UARs (upstream of AUG region) and the 5′ and 3′ CSs (conserved or complementary sequence), can lead to dengue genome cyclization, which is necessary for viral replication [4]. Here, we applied the genome cyclization feature of the DENV genome to engineer a *cis*-cleaving HHRz construct, SUBRZ (SD2 for sequence, Fig. S4A for predicted structure). The SUBRZ RNA resulted in *cis*-cleavage in the presence of magnesium ions (Fig. S4B). Thus, SUBRZ RNA by itself could form the catalytic active HHRz structure. To our knowledge, this is the first report that demonstrates the DENV 5′–3′ UTR interactions evidenced by the formation of an engineered HHRz structure.

The interaction of 5′ and 3′ UTRs in the DENV genome is believed to be relatively inefficient as the distance between the UTRs is nearly 10 kb. To investigate the interaction property of both UTRs, we modified the cis-cleaving SUBRZ construct to more closely mimic the genome-length conditions of becoming a *trans*-cleaving HHRz. Three substrate RNAs and two enzyme RNAs were made from the 5′ region and the 3′ region of SUBRZ, respectively. Three substrate RNAs differed in the sequence downstream of the 5′CS region and two enzyme RNAs differed in the sequence upstream of the 3′CS (Fig. 3A, SD2).

In theory, the hybridization of each substrate RNA with an enzyme RNA can reconcile the helices I and III of HHRz domain (Fig. 3B), and the substrate RNA can be cleaved site-specifically when magnesium ion is provided. For SUB1 and RZL RNAs, the SUB1 was not cleaved unless the two RNAs had been incubated with CP or NdAg (positive control) of 25–400 nM prior to the addition of $MgCl_2$ (Fig. 3C). Thus, CP, like NdAg, could increase the mutual accessibility of SUB1 and RZL RNAs for HHRz motif assembly. No *trans*-cleavage occurred when $MgCl_2$ was omitted or when SUB1 was replaced by its cleavage site substitution variant SUB1m (Fig. 3C). These control experiments indicated that the cleavage of substrate RNA is specific. CP and NdAg also facilitated *trans*-cleavage reactions of the other two substrate RNAs, SUB2 and SUB3, by the RZS enzyme RNA (Fig. 3E and F). Moreover, they promoted the hybridization of SUB3m and RZS RNAs (SUBm3 was a cleavage site mutant of SUB3, SD2) (Fig. S5). As judged by the trans-cleavage of each substrate RNA (Fig. 3E and F) and the formation RNA hybrid hybrid (Fig. S5), CP and NdAg could effectively promote strand annealing/hybrid formation when the nucleotide to protein ratio was in the range of 40–2 nucleotides per protein monomer.

We next examined whether CP still needed to be present for the cleavage reaction after it exerted its effect on facilitating the HHRz structure formation. To this end, the protein in the *trans*-cleavage reaction mixture was removed by extraction with phenol/chloroform before the cleavage reaction was initiated by the addition of MgCl2. The cleavage of substrate RNA occurred regardless of the removal of CP (Fig. 3D). Hence, the binding of CP to RNA was no longer required once two RNAs hybridized and adopted the catalytically active structure. This result indicated that CP acts as a *bona fide* RNA chaperone.

3.5. Analysis of NS5 and NS3H

NS5 and NS3 are two DENV non-structural proteins that interact with RNA to exert their biological functions. The C-terminal domain of NS5 is an RDRP that binds specifically to the 5′ stem-and-loop A (SLA) of DENV genome, and DENV genome circularization enables the translocation of NS5 from SLA to the 3′ terminus of the RNA genome to initiate the replication process [4]. NS3 is a component of the viral replicase complex [3], and the helicase domain of NS3 unwinds short 3′-tailed dsRNA of DENV and non-DENV sequences in an ATP hydrolysis-dependent manner [14].

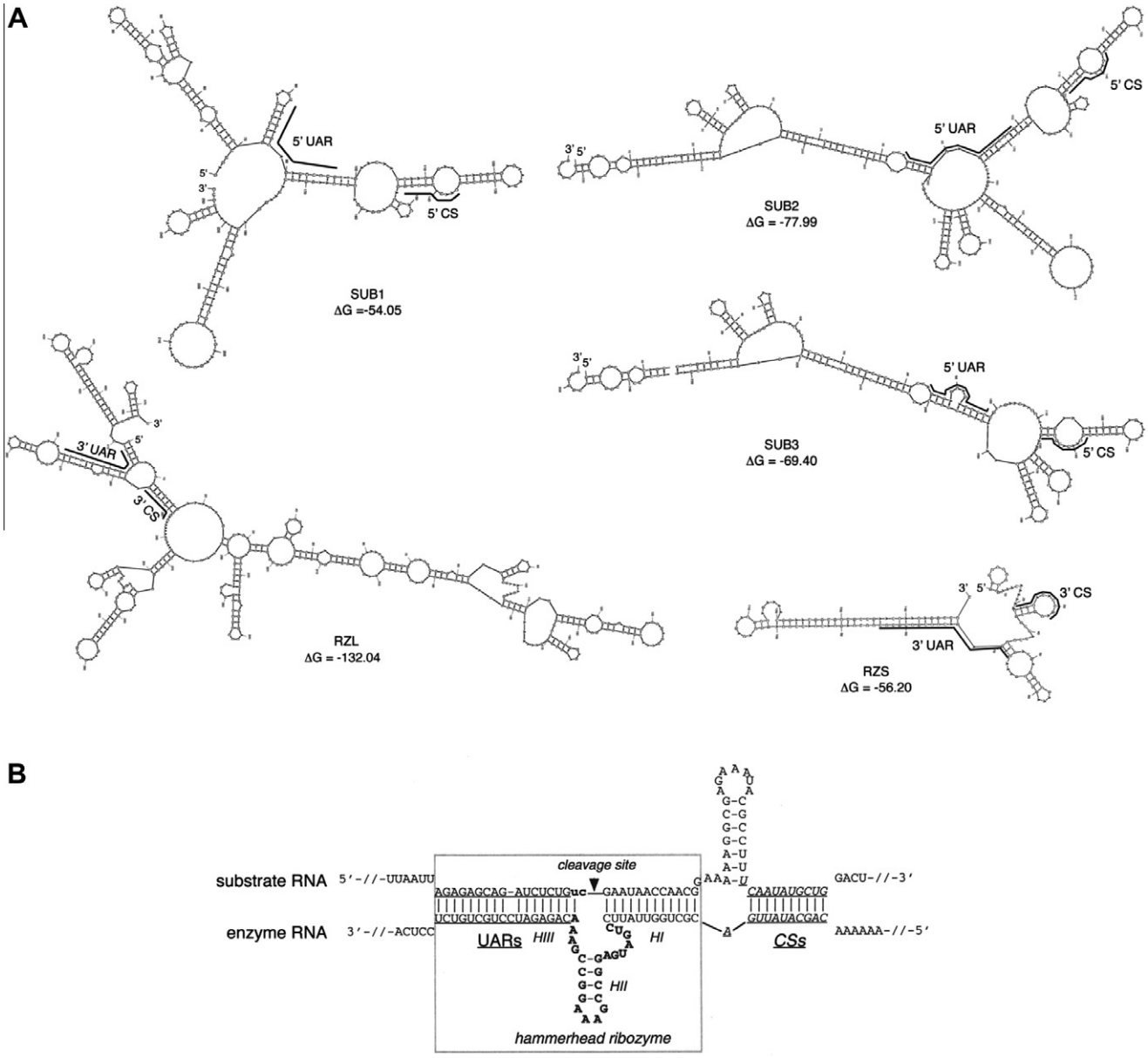

Fig. 3. Assays using the *trans*-acting HHRz systems. (A) Structure model of different RNAs [21]. The 5′–3′ UAR and 5′–3′ CS regions of DENV genome are indicated. (B) The reconstitution of HHRz motif (the boxed region). The three helical regions, the cleavage site (arrowhead), and the central core (in bold letters) of HHRz motif are indicated. (C) Representative gels showing the *trans*-cleavage reaction of SUB1 (0.1 nM) and RZL (0.5 nM) promoted by CP or NdAg. Reactions without $MgCl_2$ or with SUB1m are the controls. (D) Representative gels showing CP binding was not required for the *trans*-cleavage reaction. SUB1 (0.125 nM) and RZL (0.625 nM) were incubated with CP (400 nM) or with the mock protein plus 50 μg/ml BSA for 30 min. The samples were then extracted with phenol/chloroform (bottom panel) or not (top panel), and finally the cleavage reaction was conducted in the presence of $MgCl_2$. (E,F) Representative gels showing the trans-cleavage reaction of SUB2 or SUB3 (2 nM each) and RZS (2 nM) promoted by the indicated protein of 0, 25, 100, or 400 nM. The specific radioactivity of the RZS was one-fifth of the specific activity of the SUB2 or SUB3. All *trans*-cleavage reactions were performed at 37 °C in the presence of 10 mM $MgCl_2$ and detailed reaction conditions were summarized in SD1. RNAs were resolved on a 5% PA-7 M urea gel. 5′P and 3′P represent the 5′- and 3′-cleavage products, respectively.

Recombinant proteins corresponding to full-length NS5 protein (NS5) and the helicase domain of NS3 protein (NS3H) were prepared to analyze their putative RNA chaperone activities. The overall pI of NS5 and NS3H is 8.8 and 6.7, respectively. NS5 had a weak yet significant activity in facilitating the *trans*-cleavage of SUB2 and SUB3 RNAs by RZS RNA (Fig. 3E and F). We reproducibly observed that NS5 had a weak activity in promoting the hybridization of SUB3m and RZS RNAs (SUBm3 was a cleavage site mutant of SUB3, SD2) (Fig. S5). Moreover, the RDRP activity of NS5 did not contribute to RNA chaperone activity as a replication-defective mutant of NS5 displayed similar chaperone ability as the wild-type NS5 (data not shown). By contrast, NS3H did not promote the cleavage of SUB2 or SUB3 RNA by RZS RNA (Fig. 3E and F), and it failed to facilitate the SUBm3/RZS hybrid formation, independently of whether the annealing assay was carried out in the presence or absence of ATP (Fig. S5 and data not shown).

Besides the RDRP, GTase, and MTase activities [15–17], the contribution of NS5 in assisting the RNA secondary structure formation remained elusive. The lack of RNA chaperone activity of NS3 helicase indicates its major role as a helicase in RNA replication. Base on the finding of this study, we speculate that there is strong interaction between DENV genome and the replicase complex during RNA replication. NS3, a component of the replicase complex, unwinds the double-stranded RNA and works together with NS5 to provide RNA template for nucleotide incorporation. The formation of a stable SLA structure of 5′UTR and genome circularization

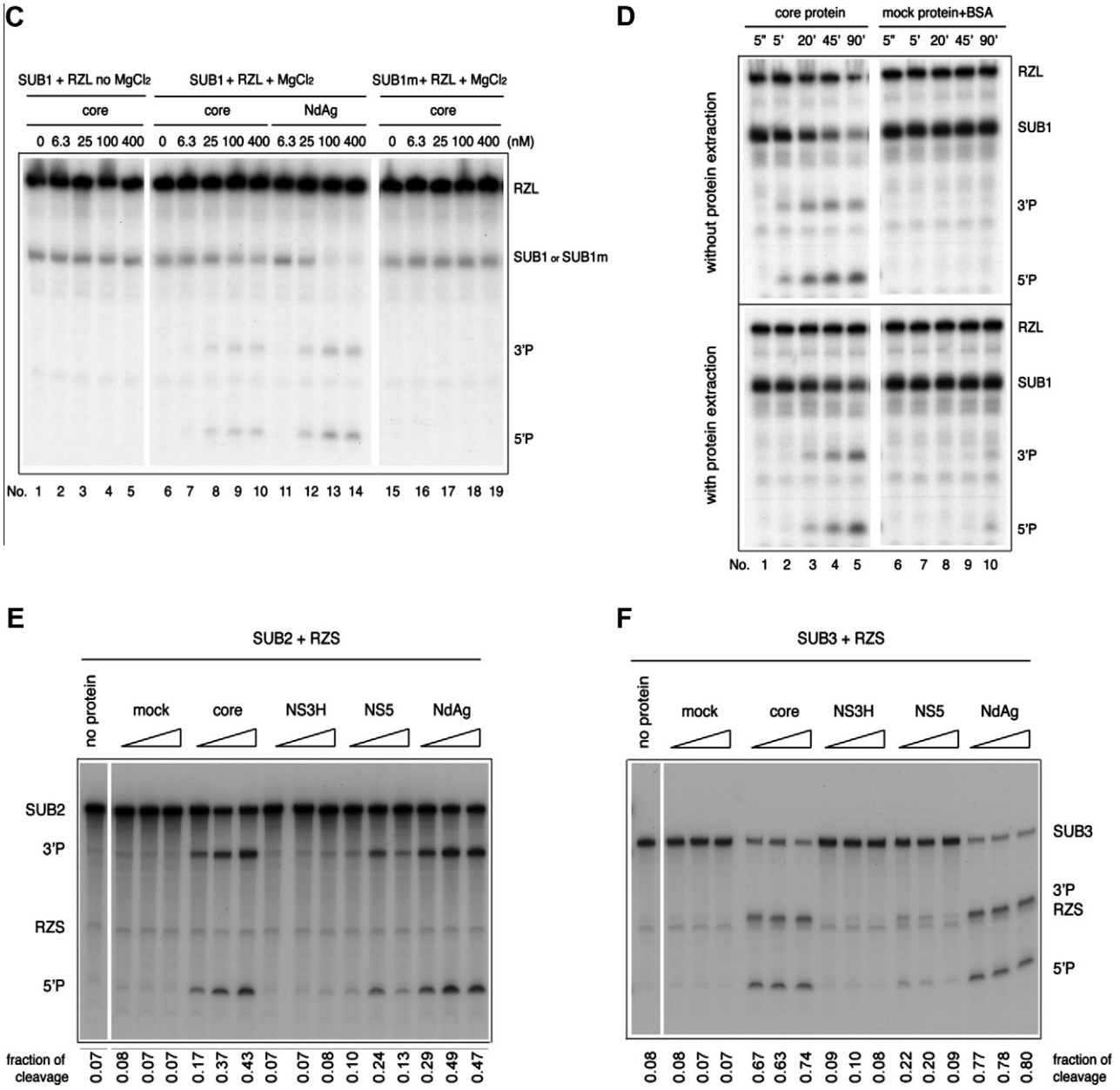

Fig. 3 (*continued*)

are essential for NS5 to exert its RDRP function [4]. Besides the limited chaperone ability of NS5 and its binding on 5′ SLA, it is speculated that the formation of the replicative form of circular genomic RNA requires concerted RNA-protein interactions among non-structural proteins as well as host membrane to have efficient RNA replication.

3.6. Biological implications

During virion assembly, binding of CP to viral genome forms a nucleocapsid followed by budding into the ER lumen for virion envelopment. Since CP does not display specificity of RNA binding, it could either bind to (+) or (−) strand of viral RNA. The (−) strand exists in the (+)/(−) replication-intermediate form [20]; thus, the (+) strand is more accessible to CP for nucleocapsid formation. Contributions to the RNA-binding ability of the core protein by the basic domains at both ends [21] and the membrane-associated hydrophobic cleft formed by the internal hydrophobic region suggest a model for nucleocapsid formation [13]. The nucleocapsid within the dengue virion lacks a well-ordered structure and no details pertaining to the interactions between the core protein and RNA or envelope proteins have been reported [22,23]. Furthermore, the vesicle packets and convoluted membranes within the membranous web structures, which are induced upon virus infection, are the sites for viral RNA replication [3]; hence, they provide micro environments for both replication and virion formation events to overcome the non-specific RNA binding feature of CP. Since these three tested viral RNA-interacting proteins are targeting the same RNA in carrying out their functions, it is conceivable that there are regulations of RNA affinity among CP, NS3, and NS5 in maintaining the balance among different events in the viral life cycle.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.06.038.

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