



Research Article

The nonstructural protein 2C of Coxsackie B virus has RNA helicase and chaperoning activities

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ABSTRACT

RNA-remodeling proteins, including RNA helicases and chaperones, play vital roles in the remodeling of structured RNAs. During viral replication, viruses require RNA-remodeling proteins to facilitate proper folding and/or re-folding the viral RNA elements. Coxsackieviruses B3 (CVB3) and Coxsackieviruses B5 (CVB5), belonging to the genus *Enterovirus* in the family *Picornaviridae*, have been reported to cause various infectious diseases such as hand-foot-and-mouth disease, aseptic meningitis, and viral myocarditis. However, little is known about whether CVB3 and CVB5 encode any RNA remodeling proteins. In this study, we showed that 2C proteins of CVB3 and CVB5 contained the conserved SF3 helicase A, B, and C motifs, and functioned not only as RNA helicase that unwound RNA helix bidirectionally in an NTP-dependent manner, but also as RNA chaperone that remodeled structured RNAs and facilitated RNA strand annealing independently of NTP. In addition, we determined that the NTPase activity and RNA helicase activity of 2C proteins of CVB3 and CVB5 were dependent on the presence of divalent metallic ions. Our findings demonstrate that 2C proteins of CVBs possess RNA-remodeling activity and underline the functional importance of 2C protein in the life cycle of CVBs.

1. Introduction

Coxsackieviruses (CVs) belong to the genus *Enterovirus* in the family *Picornaviridae* and are one of the most prevalent gastrointestinal infectious agents globally (Pond et al., 2005). Basing on the differences in pathogenicity (Crowell and Landau, 1997), CVs are subdivided into 23 serotypes of Coxsackie A virus (CVA 1–22, 24) and 6 serotypes of Coxsackie B virus (CVB 1–6). Among CVBs, CVB3 and CVB5 are the most prominent serotypes and can cause a variety of infectious diseases, including viral myocarditis (Babb et al., 1961; Zhang et al., 1997), aseptic meningitis (Lee et al., 2007; Wong et al., 2011; Tao et al., 2012; Zhang et al., 2016) and hand-foot-and-mouth disease (Gao et al., 2018; Han et al., 2019). There is currently no effective therapy and vaccine available for the diseases caused by CVB3 and CVB5.

CVB genome comprises a linear positive-sense, single-stranded RNA (+ssRNA) of around 7.4 kb in length, encoding an opening reading frame (ORF) that is flanked by untranslated regions (UTR) at both 5' and 3' ends. The ORF consists of three main genomic regions known as P1–P3,

which encodes viral capsid proteins (VP1, VP2, VP3 and VP4) and non-structural proteins (2A, 2B, 2C and 3A, 3B, 3C, 3D) (Fig. 1A).

During viral replication, the *cis*-acting RNA elements within the genomes of RNA viruses must be folded into the correct tertiary structures to be functional (Jarmoskaite and Russell, 2014). However, RNAs are easily folded into wrong and relatively stable intermediate states. To help RNA molecules function properly, viruses have evolved to encode proteins bearing RNA-remodeling activities, such as RNA helicases and RNA chaperones, to rescue RNAs trapped in misfolded states and allow reformation of RNA structures (Bleichert and Baserga, 2007; Russell, 2008; Jarmoskaite and Russell, 2014). In addition, the replicative intermediate double-stranded RNAs (dsRNAs) produced during viral RNA replication must be timely unwound to ensure the replicative efficiency. This process requires viral RNA remodeling protein to unwind dsRNAs, thereby enabling the recycling of viral RNA templates for further rounds of viral RNA synthesis. RNA remodeling proteins can be classified into two major classes including RNA helicase and RNA chaperone. The former one can unwind dsRNA

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concentration of inorganic phosphate was determined by matching the A620 value in a standard curve of A620 value versus known standard phosphate concentrations. The results were obtained from averages of three independent replicate experiments.

2.4. Preparation of oligonucleotide helix substrates

The RNA helix substrates were composed of two complementary nucleic acid strands: one with a short hexachloro fluorescein (HEX) label at the 5' end, and the other with no label. The labeled RNA strands were synthesized by TaKaRa (Dalian, China), while the unlabeled strands were produced by *in vitro* transcription using T7 RNA polymerase (Promega, Madison, WI, USA). The posttranscriptional RNA purification was performed with Poly-Gel RNA extraction kit (Omega Bio-Tek, Guangzhou, China) according to the manufacturer's protocol. The labeled RNA strands and the unlabeled RNA strands were mixed in the ratio of 1:1, and were added to a 10- μ L reaction mixture containing 25 mmol/L HEPES-KOH (pH 8.0) and 25 mmol/L NaCl. Subsequently, the reaction mixture underwent an annealing process, which was specifically to heat the reaction mixture to 95 °C for 5 min, followed by a gradual cooling to 25 °C to produce helical duplexes. Two RNA helix substrates with both 5'- and 3'-protrusions were annealed with RNA1 and RNA2. The 3'-protruded RNA helix and 5'-protruded RNA helix were produced by annealing RNA1 with RNA3 and RNA4, respectively. [Supplementary Table S2](#) shows all the oligonucleotides that have been used.

2.5. Nucleic acid helix-unwinding assays

The standard helix-unwinding assay was as according to a previous research ([Shu et al., 2019](#)) with minor modifications. Briefly, unless mentioned otherwise, 20 pmol of recombinant protein and 0.1 pmol of HEX-labeled helix substrates were added to the standard reaction mixture containing 50 mmol/L HEPES-KOH (pH 8.0), 2 mmol/L MgCl₂, 5 mmol/L ATP, 50 mmol/L NaCl and 20 U RNasin (Promega, Madison, WI, USA). After a 60 min incubation at 37 °C, the reaction was terminated with 10 \times loading buffer [100 mmol/L Tris-HCl, 50% glycerol, 1% SDS, and bromophenol blue (pH 7.5)]. The mixtures were separated by electrophoresis on 15% native-PAGE gels, and the gels were scanned with a Typhoon 9500 imager (GE Healthcare, Piscataway, NJ, USA).

2.6. RNA strand annealing activity assay

The RNA hybridization assay was conducted as described ([Gebhard et al., 2012](#)). The reaction mixture was prepared containing 50 mmol/L HEPES-KOH (pH 8.0), 40 mmol/L KCl, 20 U of RNasin and 2 mmol/L dithiothreitol. Subsequently, 0.1 pmol HEX-labeled stem-loop-structured RNA strand, 0.1 pmol unlabeled stem-loop-structured RNA strand and the indicated amount of protein were added to the reaction mixture, followed by an incubation at 37 °C for 30 min. Annealing reactions were terminated by adding 10 \times ending buffer (1.2 mg/mL proteinase K, 1.0% sodium dodecyl sulphate) to the mixture. The reaction products adding 10 \times loading buffer were resolved on 15% native PAGE gels, followed by scanning with a Typhoon 9500 imager (GE Healthcare, Piscataway, NJ, USA).

3. Results

3.1. The 2C proteins of CVB3 and CVB5 have NTPase activity

The amino acid sequences of 2C proteins encoded by CVB3 and CVB5 were aligned with those of other reported SF3 viral helicases, including EV71 2C, CVA16 2C, norovirus NS3 and human papillomavirus 11 (HPV11) E1 ([Lin et al., 2002](#)). The comparison of amino acid sequences showed that 2C proteins of CVB3 and CVB5 contained the conserved SF3 helicase A, B, and C motifs ([Fig. 1B](#)), implying that CVB 2C putatively has helicase activity. To test this possibility, we expressed and purified 2C

proteins of CVB3 and CVB5 as the fusion protein with MBP at its N-terminus via baculovirus expression system. Subsequently, we conducted a classical NTPase reaction assay, which was to incubate CVB3 2C and CVB5 2C with different NTPs (ATP, UTP, CTP, or GTP) under standard reaction condition containing MgCl₂ ([Shu et al., 2019](#)). The NTP hydrolysis was assessed using a colorimetric technique that sensitively detected released inorganic phosphate. Our results showed that 2C proteins of both CVB3 and CVB5 exhibited the hydrolyzing activities for all four NTPs, with a preference for ATP and GTP ([Fig. 2A](#)).

We further investigated the NTP hydrolyzing activity of both CVB3 2C and CVB5 2C under a variety of conditions, including increasing ATP concentrations and different divalent metallic ions. As shown in [Fig. 2B](#), both 2C proteins could hydrolyze ATP in a dose-dependent manner within the range from 0 to 4 mmol/L, while the ATPase activity was reduced when the concentration of 2C proteins exceeded 4 mmol/L. In addition, our data suggested that 2.5 mmol/L Mg²⁺, Mn²⁺, or Ca²⁺ could support the ATPase activity of 2C proteins, and their efficiencies were as follows: Mg²⁺ > Mn²⁺ > Ca²⁺ ([Fig. 2C](#)). Both CVB3 2C and CVB5 2C had the optimal ATPase activity in the presence of 2 mmol/L Mg²⁺, whereas higher concentrations than 2 mmol/L of Mg²⁺ showed some inhibitory effect on the ATPase activity ([Fig. 2D](#)). Together, our findings indicate that 2C proteins of CVB3 and CVB5 possess the NTPase activity dependently of divalent metallic ions.

3.2. The 2C proteins of CVB3 and CVB5 have the RNA helix-unwinding activity

After determining that 2C proteins of CVB3 and CVB5 have NTPase activity, we then examined their RNA helix-unwinding activity. A standard RNA/RNA helix (R*/R) substrate was constructed with single-stranded protrusions at both 5' end and 3' end by annealing a short HEX-labeled nucleic acid and a long non-labeled nucleic acid as reported ([Fig. 3A](#)) ([Shu et al., 2019](#)). The helix-unwinding assay was performed by incubating MBP-fusion CVB3 2C or CVB5 2C with the RNA helix substrate in the optimal biochemical reaction condition containing ATP and MgCl₂. The HEX-labeled substrate strands were then separated by gel electrophoresis. The reaction mixture with MBP alone were used as a negative control, while the reaction mixture with MBP-fusion EV71 2C, a well-characterized viral RNA helicase, was used as a positive control ([Fig. 3B](#), lanes 3 and 6). Our data showed that CVB3 2C and CVB5 2C efficiently unwound the HEX-labeled RNA strand from the RNA helix substrate. In addition, we found that each type of NTPs alone could support RNA helix unwinding activity of CVB3 2C and CVB5 2C ([Fig. 3C](#) and [D](#)). Together, our findings indicate that both CVB3 2C and CVB5 2C have the RNA helicase activity.

3.3. The RNA helicase activity of CVB3 2C and CVB5 2C is dependent on the presence of divalent metallic ions

After determining the helix unwinding activity of CVB3 2C and CVB5 2C, we seek to examine whether the RNA helix unwinding activity of CVB3 2C and CVB5 2C requires divalent metallic ions like other known SF3 viral RNA helicases ([Xia et al., 2015](#); [Li et al., 2018](#)). Thus, recombinant MBP-fusion CVB3 2C and CVB5 2C were used to react with the standard RNA helix substrate ([Fig. 3A](#)) under different reaction conditions. The 2C proteins of CVB3 and CVB5 showed the most robust RNA helix unwinding activity in the presence of Mg²⁺ or Mn²⁺, and these 2C proteins exhibited some RNA unwinding activity in the presence of Zn²⁺. Ca²⁺, however, barely showed any contribution in terms of RNA unwinding activity ([Fig. 4A](#) and [B](#)). Moreover, Mg²⁺ could promote both CVB3 2C and CVB5 2C in RNA unwinding in a dose-dependent manner within the range from 0 to 2.5 mmol/L ([Fig. 4C](#) and [D](#)). Therefore, our results showed that the RNA helicase activity of CVB 2C proteins was dependent upon the presence of certain divalent metallic ions such as Mg²⁺ and Mn²⁺.

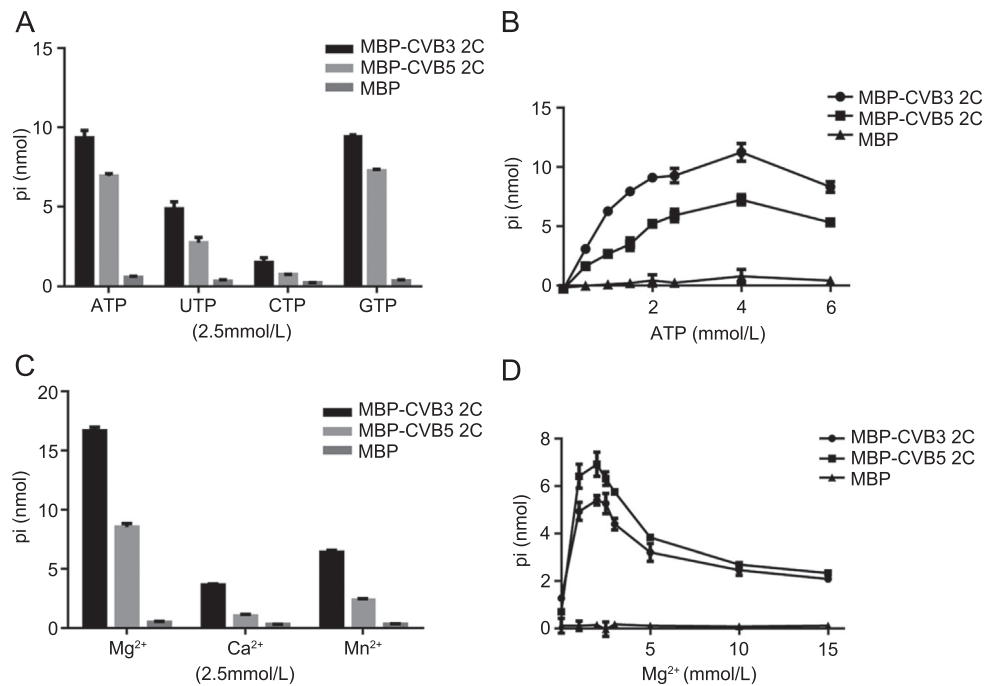


Fig. 2. CVB3 2C and CVB5 2C have NTPase activity. **A** 20 pmol of MBP-CVB3 2C, MBP-CVB5 2C or MBP alone was reacted with the indicated NTPs (2.5 mmol/L) under the standard NTPase reaction condition containing Mg^{2+} . The NTPase activity of MBP-CVB3 2C, MBP-CVB5 2C or MBP was measured at nanomoles of released inorganic phosphate by using a sensitive colorimetric assay. **B–D** The NTPase activity of MBP-CVB3 2C or MBP-CVB5 2C or MBP was determined at the indicated concentrations of ATP (**B**), at the indicated divalent metal ions (2.5 mmol/L) (**C**), or at the indicated concentrations of Mg^{2+} (**D**). MBP alone was used as a negative control. The error bars represent standard deviation (SD) values from three individual experiments. Pi on the Y axis represents the phosphate released by NTP hydrolysis. CVB, Coxsackie B virus; MBP, maltose-binding protein.

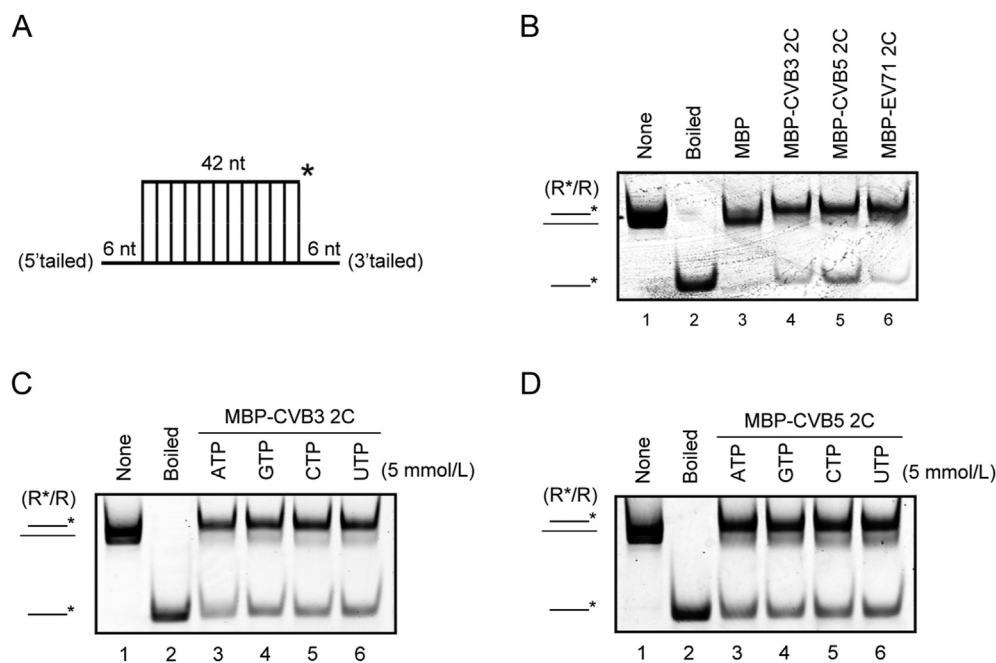


Fig. 3. CVB3 2C and CVB5 2C have RNA helix-unwinding activity. **A** The standard RNA/RNA helix (R^*/R substrate) consists of two complementary RNA strands. The 42-nt RNA strand is with a HEX label at the 5' end. The 54-nt RNA strand is with 6-nt protrusions at both 5' end and 3' end. **B** The R^*/R substrate (0.1 pmol) was incubated with each indicated protein (20 pmol). Lane 1 (nonboiled reaction mixture) and lane 3 (reaction mixture with MBP alone) were used as negative controls. Lane 2 (boiled reaction mixture) and lane 6 (reaction mixture with MBP-EV71 2C ATPase) were used as positive controls. **C, D** R^*/R substrate (0.1 pmol) was incubated with MBP-CVB3 2C or MBP-CVB5 2C (20 pmol) in the presence of each kind of NTP (5 mmol/L). Lane 1 (nonboiled reaction mixture) was used as a negative control, and lane 2 (boiled reaction mixture) was used as a positive control. The asterisks indicate the HEX-labeled strands. CVB, Coxsackie B virus; MBP, maltose-binding protein; HEX, hexachloro fluorescein.

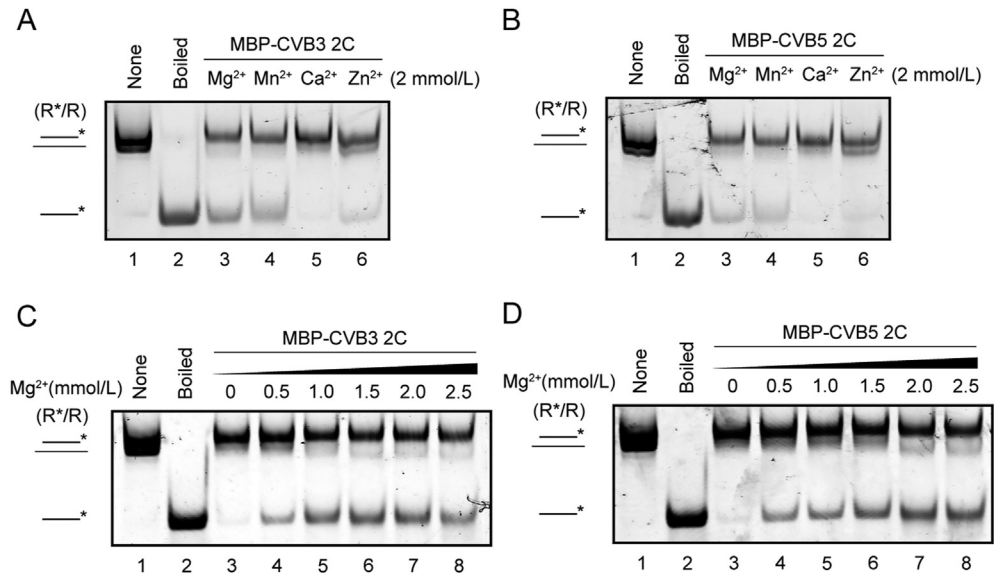


Fig. 4. The RNA helicase activity of CVB3 2C and CVB5 2C is dependent on divalent metallic ions. **A, B** R*/R substrate (0.1 pmol) was incubated with MBP-CVB3 2C or MBP-CVB5 2C (20 pmol) in the presence of each indicated divalent metal ions (2 mmol/L for each). **C, D** R*/R substrate (0.1 pmol) was incubated with MBP-CVB3 2C or MBP-CVB5 2C (20 pmol) under the condition with increasing concentrations of Mg²⁺. Lane 1 (nonboiled reaction mixture) was used as a negative control, and lane 2 (boiled reaction mixture) was used as a positive control. The asterisks indicate the HEX-labeled strands. CVB, Coxsackie B virus; MBP, maltose-binding protein; HEX, hexachloro fluorescein.

3.4. The 2C proteins of CVB3 and CVB5 unwind RNA helix directionality

The helix-unwinding directionality is one of the principal characteristics for helicase. To examine the unwinding directionality of the 2C proteins from CVB3 and CVB5, we constructed two different RNA helix substrates, containing single-strand protrusion at either 3' end or 5' end as previously described (Shu et al., 2019) (Fig. 5A and B). Subsequently, these substrates were used for the helix-unwinding experiments by incubation with CVB3 2C or CVB5 2C. The directionality of the unwinding activity of 2Cs could be assessed via examining whether it can unwind the 3'- or 5'-protruded helix or both. As shown in Fig. 5C–F, both 3'-tailed and 5'-tailed RNA helix were released in the presence of CVB3 2C or CVB5 2C, indicating that these two 2C proteins were able to unwind RNA helix in both directions. Usually, a given RNA helicase exerts its helix-unwinding activity in one directionality. However, our data show that CVB 2C proteins can unwind helix bidirectionally, which lead to the speculation that CVB 2C proteins may have some other activity along with helicase activity.

3.5. The 2C proteins of CVB3 and CVB5 have NTP-independent RNA chaperoning activity

The requirement for ATP or other NTPs is a basic feature of a classic RNA helicase. Therefore, we seek to examine whether the RNA helix-unwinding activity of 2C proteins is dependent on the presence of ATP. To this end, CVB3 2C and CVB5 2C were used to react with the standard RNA helix substrate with increasing concentrations of ATP (from 0 to 5 mmol/L). We showed that the presence of ATP could promote the helix-unwinding by CVB3 2C and CVB5 2C dose-dependent manner. Of note, we noticed that RNA helix substrates could be still unwound by CVB3 2C or CVB5 2C in the absence of ATP (Fig. 6A and B, lane 3). To confirm this, AMP-PNP (adenyl-lyl-imidodiphosphate), a non-hydrolysable analog of ATP that is incapable of being hydrolyzed and can block the NTPase activity, was introduced. We found that CVB3 2C and CVB5 2C could unwind the RNA helix even in the presence of AMP-PNP (Fig. 6C and D), indicating that 2C proteins of CVB3 and CVB5 display NTP-independent RNA helix unwinding activity. Considering that activity of RNA chaperone is NTP-independent, our findings suggest

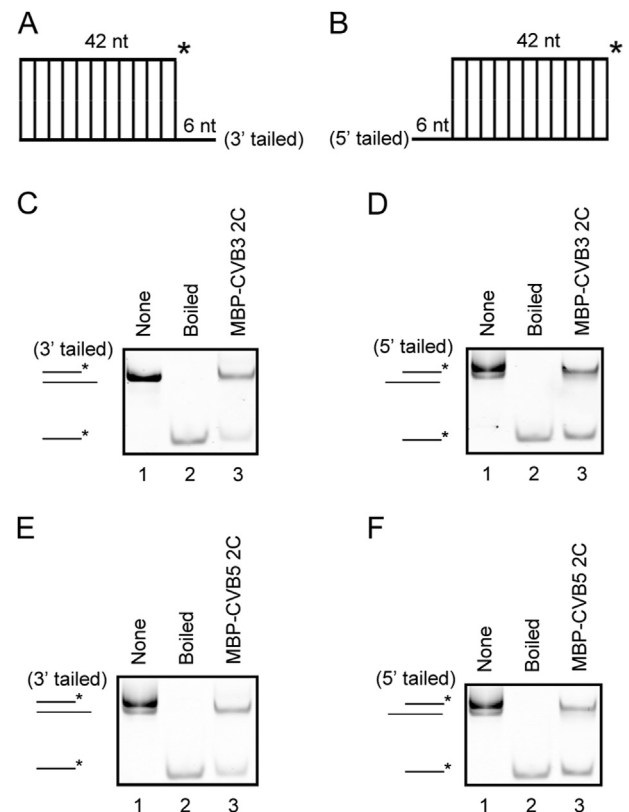


Fig. 5. CVB3 2C and CVB5 2C can unwind the RNA helix in a bidirectional manner. **A, B** The RNA/RNA helix (R*/R substrate) consists of two complementary RNA strands. The 42-nt RNA strand is with a HEX label at the 5' end. The 48-nt RNA strand is with a 3' single-strand protrusion (A) or a 5' single-strand protrusion (B). **C–F** MBP-CVB3 2C or MBP-CVB5 2C (20 pmol) was incubated with 0.1 pmol 3'-tailed (C, E) or 0.1 pmol 5'-tailed (D, F) RNA helix substrate. Lane 1 (nonboiled reaction mixture) was used as a negative control, and lane 2 (boiled reaction mixture) was used as a positive control. The asterisks indicate the HEX-labeled strands. CVB, Coxsackie B virus; HEX, hexachloro fluorescein; MBP, maltose-binding protein.

that 2C proteins of CVB3 and CVB5 also contain NTP-independent RNA-chaperoning activity.

RNA chaperones are commonly considered to participate in destabilizing misfolded RNA structures to assist annealing more stable RNA structures (Russell, 2008). To confirm the RNA chaperoning activity of 2C proteins of CVB3 and CVB5, we synthesized HEX-labeled single stranded RNA substrates and its non-labeled complementary RNA strand as described (Xia et al., 2015). The two strands can spontaneously form a defined stem-loop secondary structure (Fig. 6E). The two strands were

mixed at equal ratio in the presence or absence of CVB3 2C or CVB5 2C, respectively, and the hybridization of the two strands was examined via using electrophoretic mobility shift assays. Of note, ATP or other NTPs were not added in these reactions. We found that a significant rise in RNA hybrids level was observed via increasing the concentrations of CVB3 2C or CVB5 2C (Fig. 6F and G), although a small amount of single stranded RNA substrates could spontaneously form RNA strand hybrids in the absence of 2C protein (Fig. 6F and G, lane 3). In addition, we examined the amount of RNA strand hybrids under different incubation time,

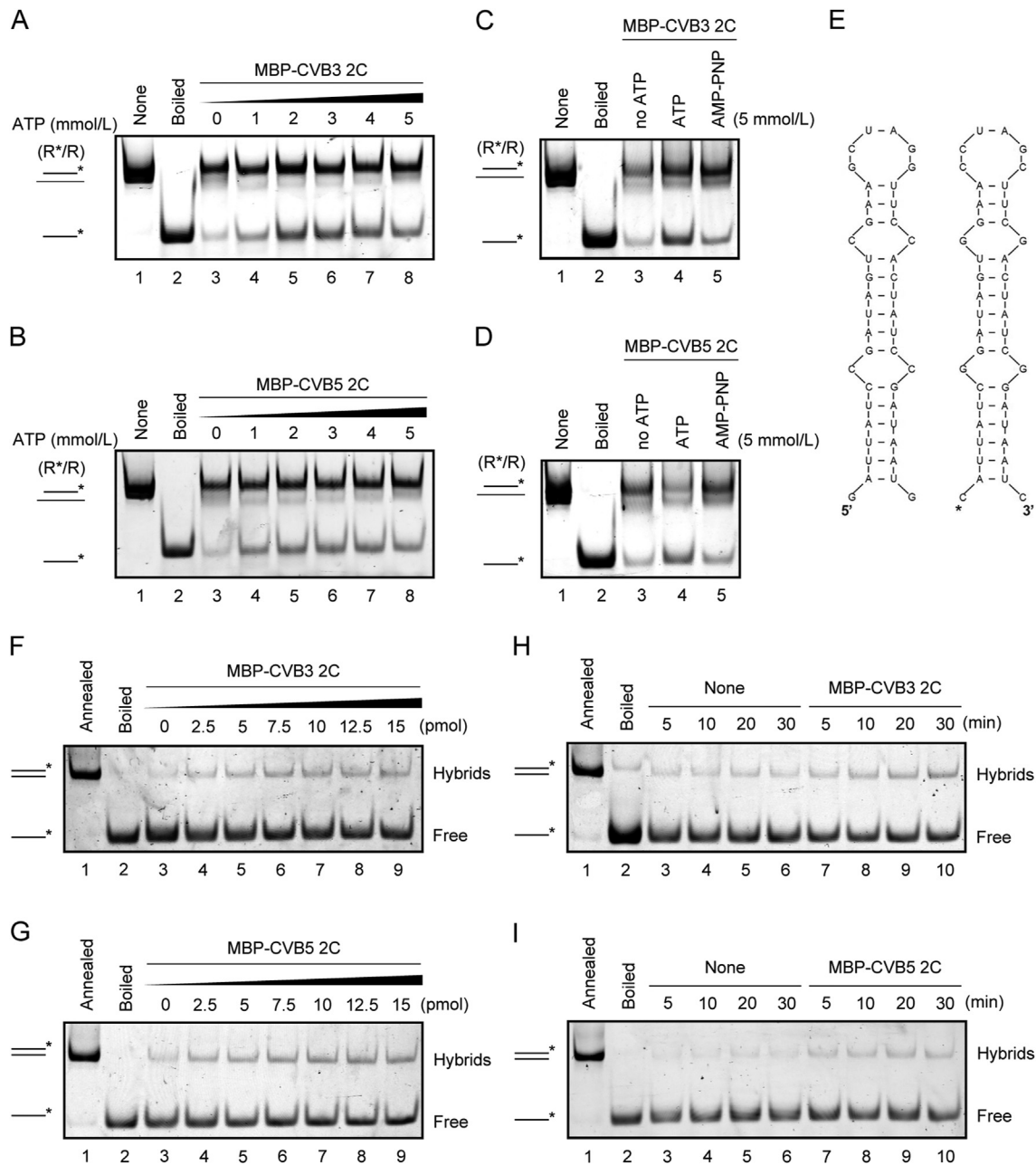


Fig. 6. CVB3 2C and CVB5 2C have RNA-chaperoning activity to destabilize structured RNA strands and promote annealing. **A, B** The standard RNA helix (R*/R) substrate (0.1 pmol) was incubated with 20 pmol MBP-CVB3 2C (**A**) or MBP-CVB5 2C (**B**) under increasing concentration of ATP as indicated. **C, D** R*/R substrate (0.1 pmol) was reacted with 20 pmol MBP-CVB3 2C (**C**) or MBP-CVB5 2C (**D**) in the absence or presence of 5 mmol/L ATP or ATP analog (AMP-PNP) as indicated. Lane 1 (nonboiled reaction mixture) was used as a negative control, and lane 2 (boiled reaction mixture) was used as a positive control. Lane 3, reaction mixture in the absence of ATP; lane 4, reaction mixture in the presence of 5 mmol/L ATP; lane 5, reaction mixture in the presence of 5 mmol/L AMP-PNP. **E** Two complementary 42-nt RNA strands with stem loop structures were illustrated in this diagram. The asterisk indicates the HEX-labeled strand. **F, G** The two complementary 42-nt RNA strands were mixed 1:1 (0.1 pmol each) and incubated with the indicated amounts of MBP-CVB3 2C (**F**) or MBP-CVB5 2C (**G**). **H, I** The hybridization assay was conducted in the absence (lane 3–lane 6) or presence (lane 7–lane 10) of 20 pmol MBP-CVB3 2C (**H**) or MBP-CVB5 2C (**I**) for the indicated reaction times. **F–I** Lane 1 (the pre-annealed strands) was used as a positive control, and lane 2 (the boiled strands) was used as a negative control. The dsRNA hybrids and free strands ssRNA are indicated. CVB, Coxsackie B virus; MBP, maltose-binding protein; HEX, hexachloro fluorescein; dsRNA, double-stranded RNA; ssRNA, single-stranded RNA.

ranging from 5 min to 30 min. As shown in Fig. 6H and I, the addition of CVB3 2C or CVB5 2C indeed further stimulated RNA hybridization at each time-point. Together, our findings showed that 2C proteins of CVB3 and CVB5 could destabilize the structured RNA strands and facilitate the reformation of a more stable RNA structure, supporting that 2C proteins of CVB3 and CVB5 contained an NTP-independent RNA chaperoning activity.

4. Discussion

RNA remodeling proteins, including RNA helicases and RNA chaperones, are generally believed to play important roles in viral life cycles. In this study, we uncover that 2C proteins of CVB3 and CVB5 have NTP-dependent RNA helicase activity that can unwind RNA duplexes, as well as NTP-independent RNA-chaperoning activity that can remodel structured RNA molecules. In addition, previous studies have shown that EV71 2C and CVA16 2C exhibit both RNA helicase and RNA chaperone activities (Xia et al., 2015). Given that 2C proteins of CVB3, CVB5, EV71 and CVA16 share the conserved SF3 signature motifs and possess the RNA-remodeling activity, our findings suggest that using 2C as the RNA remodeling protein should be a common strategy for enteroviruses during their viral life cycles.

RNA remodeling proteins are essential for viral RNA replication and/or transcription since they can unwind dsRNA intermediates and facilitate viral RNA templates recycling for more efficient RNA replication/transcription. Our previous works demonstrate that EV71 2C and norovirus NS3 can promote RNA-dependent RNA polymerase (RdRP)-mediated viral RNA replication/transcription *in vitro* (Xia et al., 2015; Li et al., 2018). Moreover, our previous study has also shown that a peptide-based drug targeting EV71 2C can block its RNA helicase activity and inhibit EV71 replication in cells and in mice (Fang et al., 2021). Together with our current finding that 2C proteins of CVBs also exert the RNA helicase and chaperoning activities, these findings highlight the pivotal roles of the RNA remodeling activities during enteroviral life cycle.

RNA helicases and RNA chaperones are the two distinct types of RNA remodeling proteins. The fundamental difference between these two proteins is that RNA helicase requires the participation of NTP to function, while the activity of RNA chaperone is NTP-independent. Interestingly, we found that 2Cs of CVB3 and CVB5 functioned as both RNA helicase and RNA chaperone, consistent with the previous studies that EV71 2C and norovirus NS3 possessed both RNA helicase and chaperoning activities. It is still unclear that how to coordinate these two functions in the same protein. A plausible explanation is that NTP-independent RNA annealing activity occurs in the process of NTP-dependent RNA unwinding to establish a steady state between RNA unwinding and annealing (Gebhard et al., 2012).

5. Conclusions

Together, the findings in this work provide the demonstration of the RNA helicase and chaperoning activities associated with 2C proteins encoded by CVB3 and CVB5, which shed lights on the important roles of RNA-remodeling protein in the viral life cycles of CVBs.

Data availability

All data included in this study are available upon request by contact with the corresponding author.

Ethics statement

This study does not contain any studies with human or animal subjects performed by any of the authors.

Author contributions

Ziyu Chen: investigation, methodology, validation, data curation. Xiaobei Xiong: investigation, validation, data curation, writing-original draft. Yiyang Li: investigation. Muhan Huang: validation. Yujie Ren: data curation. Di Wu: software. Yang Qiu: writing-original draft. Mingzhou Chen: supervision, project administration. Ting Shu: writing-original draft, project administration. Xi Zhou: supervision, writing-original draft, project administration.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.virs.2022.05.004>.

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