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A phage RNA-binding protein binds to a non-cognate structured RNA and stabilizes its core structure

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ABSTRACT

Recent studies suggest that some RNA-binding proteins facilitate the folding of non-cognate RNAs. Here, we report that bacteriophage MS2 coat protein (MS2 CP) bound and promoted the catalytic activity of *Candida* group I ribozyme. Cloning of the MS2-bound RNA segments showed that this protein primarily interacts with the P5ab–P5 structure. Ultraviolet cross-linking and the T1 footprinting assay further showed that MS2 binding stabilized tertiary interactions, including the conserved L9–P5 interaction, and led to a more compact core structure. This mechanism is similar to that of the yeast mitochondrial tyrosyl-tRNA synthetase on other group I introns, suggesting that different RNA-binding proteins may use common mechanisms to support RNA structures.

RNA-binding proteins (RBPs) constitute a major class of proteins exerting critical functions in all living organisms [1,2]. It is believed that functional RNA molecules always associate with proteins in living cells, because there is no evidence that unbound RNA exists *in vivo* [3]. The interaction of several RBPs with structural RNAs has been shown to modulate RNA folding properties [4–6].

Group I introns are large ribozymes among these structural RNAs, and fold into defined tertiary structures required for their own splicing [7]. Assembly of the conserved P4–P6 and P3–P9 domains forms the ribozyme core, while highly variable peripheral elements are present to stabilize the core structure via long-range interactions [8,9]. Because their ribozyme activity provides a direct read-out for the native folds, group I introns have been widely used as a model for studying RNA folding mechanisms.

Self-splicing of group I introns *in vitro* in the absence of protein factors is much slower and less efficient than *in vivo* splicing, due to the thermodynamic and kinetic challenges that they encounter [10]. Early studies were limited to yeast mitochondrial proteins that, when mutated, resulted in splicing deficiency phenotypes. These studies led to the discovery of Cbp2 protein which captures the native-like structure of the group I intron bI5, and of Cyt-18 protein, which stabilizes the tertiary structure of the ribozyme by binding to the P4–P6 domain [4,11]. These two proteins are RNA-binding proteins with a general feature of binding directly to RNA and therefore stabilizing the RNA tertiary folds. Interestingly, CYT 18 also stimulates the activity of several non-cognate group I introns [12], questioning the specificity of the action of RNA-binding proteins.

MS2 CP tightly binds to a small RNA hairpin in its genomic RNA with a dissociation constant of 39 nM [13]. The binding represses translation of the early replicase gene and identifies the RNA for future packaging into virus particles [14]. In addition, MS2 CP binds to a number of non-native RNA hairpins produced by *in vitro* SELEX [15]. *Candida* group I ribozyme is a self-splicing intron located in the 26S rRNA gene of the pathogenic yeast *Candida albicans*. The folding and structure of this ribozyme have been extensively studied recently [16–18]. This intron contains 13 base-paired structures, this study sought to assess whether MS2 CP non-specifically interacts with this ribozyme.

Materials and methods

RNA and protein preparation. Preparation and radioactive labeling of the *Candida* group I intron ribozyme RNAs were performed as described [17]. MBP and MS2–MBP fusion protein were expressed and purified [19].

Analysis of the ribozyme activity. The purified RNAs randomly labeled by incorporation of [α-^{32}P]GTP during *in vitro* transcription was denatured and equilibrated as previously described [17]. The equilibrated RNA was incubated in the folding buffer containing 2 mM or 6 mM $MgCl_2$ and 1 μM of the indicated proteins at 4 °C for 15 min. Hydrolysis reactions were initiated by incubation at 37 °C for 90 min in the presence or in the absence of 2 μg/μl protease K, then stopped and analyzed as described previously [17]. To determine the 3′ hydrolysis kinetics, time-dependent accumulation

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of the hydrolysis products was determined by removal and analysis of reaction aliquots at the indicated rime points.

RNA electrophoretic mobility shift assay (EMSA). The internally labeled Ca.L-11 ribozyme RNA [17] was denatured and then incubated with the indicated concentrations of purified proteins in 10 μl folding buffer (6 mM Mg^{2+}) at 4 °C for 15 min as described above, except that 0.2 μg/μl tRNA and 5 μg/μl heparin were included. The samples were then fractionated on 5% native polyacrylamide gels [18].

In this study, all denature and native PAGE gels were analyzed using the variable scanner Typhoon 9200 (Amersham-Pharmacia Biotech). All data were plotted using the GraphPad Prism 4.0 program (www.graphpad.com).

Protein-protected RNA cloning method (PRC) to identify the MS2-binding site on Ca.L-11 ribozyme RNA. The method is detailed in Supplementary Data. In brief, the ribonucleoprotein (RNP) complex containing Ca.L-11 RNA and the MS2 fusion protein was formed; the unprotected RNA segments were removed by RNaseA to produce miniRNP. The miniRNP band was recovered from 5% native polyacrylamide gels, and the small RNAs in miniRNP were then cloned.

Results

MS2 CP binds to the Candida group I ribozyme

The binding capacity of MS2 CP with the *Candida* ribozyme was investigated by gel retardation assay, showing that the level of the MS2–ribozyme complex increased with the concentrations of MS2 protein (Fig. 1A). Quantification of three independent sets of experiments resulted in a K_d value of 610 ± 130 nM (Fig. 1B). Consistently, a K_d value of 226 ± 30 nM was obtained using fluorescence spectrum assay (Supplementary Figure S1). These data suggested that MS2 CP binds to ribozyme RNA at an affinity much lower than that of the native RNA substrate, while is comparable to some aptamer RNA of this protein [13,14]. The fluorescence data also suggest that one *Candida* ribozyme molecule was bound by one MS2 protein molecule (Supplementary Figure S1).

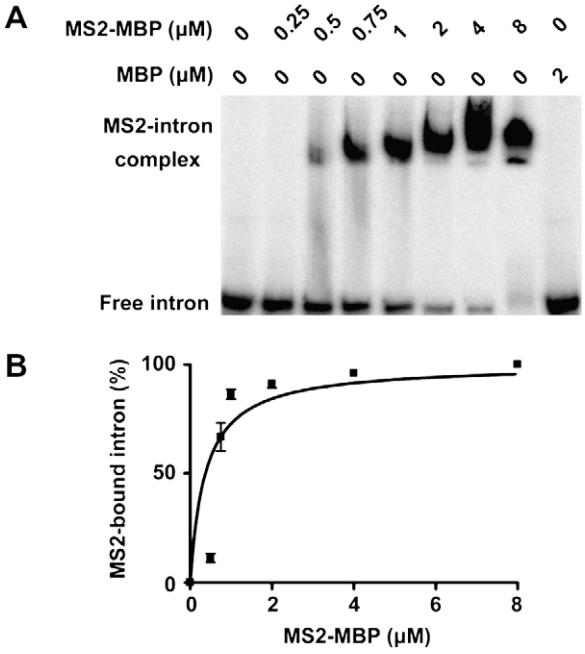

Fig. 1. Gel-shift analysis of MS2 binding to the ribozyme. (A) Varying concentrations of the purified MS2 CP and Ca.L-11 ribozyme RNA were incubated in 40 mM Tris–HCl, pH 7.9, 6 mM $MgCl_2$, 10 mM DTT, 10 mM NaCl, 2 mM spermidine, 0.2 μg/μl tRNA, and 5 μg/μl heparin for 15 min at 4 °C. The reactions were then analyzed by native gel electrophoresis. (B) The fraction of the labeled ribozyme bound to MS2 (Y) at each protein concentration was obtained from all three independent gel-shift experiments, which was then plotted against the MS2 concentrations (X) by fitting to the standard one-site binding equation $Y = 100 * X/(K_d + X)$. The bound fraction (Y) was the ratio of the radioactivity associated with the shifted band to total radioactivity in each lane. A K_d of 0.611 ± 0.138 μM was obtained.

MBP alone did not interact with the ribozyme RNA (Fig. 1A), supporting the conclusion that MS2–MBP fusion protein binds to the ribozyme using the RNA-binding function of the MS2 CP.

MS2 CP binding significantly promotes the ribozyme activity

To examine whether MS2 binding affects the ribozyme activity, the 3′ hydrolysis reaction of the *Candida* ribozyme was performed. It was shown that 1 μM of MS2 CP enhanced the ribozyme hydrolysis, while MBP protein alone showed no appreciable effect (Fig. 2A and B). A greater stimulation by MS2 CP was observed at 2 mM than at 6 mM Mg^{2+}, suggesting that it stabilizes a functionally important structure similar as magnesium does. Protease K treatment almost completely abolished MS2-stimulated ribozyme activity (Fig. 2A and B), supporting a conclusion that the physical association of MS2 CP with the ribozyme is required for its promotion of the ribozyme activity.

Interestingly, MS2 CP did not alter the observed rate constant of the ribozyme catalysis, but significantly increased the maximal fraction of the catalytic active ribozyme (Fig. 2C–E), suggesting that binding of MS2 CP converts a larger fraction of the ribozyme to the active structure.

The similar effect of MS2 CP on the catalytic activity of two other eukaryotic group I introns from *Tetrahymena thermophila* (Tth.L1925) and *Pneumocystis carinii* (Pca.L1921) was also evident (Supplementary Figure S2), suggesting a general effect of MS2 CP on the group I ribozyme function, presumably reflecting a similar alteration of the ribozyme structure.

MS2 CP primarily interacts with the P5ab–P5 structure of the Candida ribozyme

Because *Candida* group I ribozyme contains no typical RNA-binding motif of MS2 CP, we were very interested in addressing where this protein binds on the group I intron. A new method called PRC was developed to identify the direct-binding site of MS2 CP on the ribozyme RNA. Interestingly, all the RNA sequences bound by MS2 CP were located in the P5ab–P5 structure (Fig. 3A). The shortest sequence was mapped to 135A to 151C, while the longest one was from sites 131A to 200C of the ribozyme RNA. It is particularly interesting to notice that the unpaired A139–A140 in J5/5a was protected from RNase A cleavage in all six clones. Because RNase A specifically cleaves the single-stranded RNA, the exclusive protection of J5/5a and P5ab harboring a number of unpaired nucleotides readily suggests that MS2 primarily binds to J5/5a–P5ab structure and protects it from RNase A digestion.

MS2 binding stabilizes the L9/P5 tertiary interaction and the core structure

We then addressed how MS2 binding globally affects the ribozyme structure. The T1 ribonuclease footprinting experiment was performed as described in Supplementary Data. Consistent with the view that MS2 primarily interacts with P5a, T1 protection was clearly evident at G183–186, a G-rich region in the P5a helix, in the presence of MS2 CP but not in the presence of the control protein (Fig. 4A). Strikingly, G286/287 in the J8/7 junction, G256/G257 in the P3 helix, and G245–247 in the P7 helix became strongly resistant to T1 cleavage in the presence of MS2 CP (Fig. 4A). A weak protective effect was also observed at G308 in