Biochemical and Biophysical Research Communications 432 (2013) 281–286

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Convergent evolution of two different random RNAs for specific interaction with methionyl-tRNA synthetase

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ARTICLE INFO

Article history:
Received 29 January 2013
Available online 8 February 2013

Keywords:
Aminoacyl-tRNA synthetase
In vitro evolution
Protein–RNA interaction
SELEX
tRNA

ABSTRACT

Aminoacyl-tRNA synthetases (ARSs) recognize a specific sequence or structural characteristics of their cognate tRNAs. To contribute to the understanding how these recognition sites were selected, we generated two different RNA libraries containing either 42mer or 70mer random sequence and used them to select RNA aptamers that specifically bound to methionyl-tRNA synthetase (MRS) of *Mycobacterium tuberculosis*. The aptamer pools selected from the two RNA libraries showed strong binding affinity and selectivity to *M. tuberculosis* MRS compared to that of the homologous *Escherichia coli* MRS. The RNA aptamers selected from the two completely unrelated RNA pools shared the octamer sequence including CAU and the anticodon sequence of $tRNA^{Met}$. The secondary structure prediction suggested that the octamer motif in the selected aptamers would form a loop similar to the anticodon loop of $tRNA^{Met}$. The results suggest that the RNA loop containing CAU triplet could selected as a major recognition site for MRS during evolution more or less regarding, and also showed that species-specific ARS inhibitors can be obtained by *in vitro* evolution.

1. Introduction

As aminoacyl-tRNA synthetases ligate specific amino acids to their cognate tRNAs, the correct recognition of tRNAs by these enzymes determines the fidelity of protein synthesis. To maintain the specificity of the interaction between two molecules, ARSs developed an idiosyncratic pattern of tRNA recognition, and the specificity is thought to result from adaptive co-evolution of the enzyme and its cognate tRNA [1–4]. However, it is not understood how the tRNA recognition sites have been selected during evolution.

Interestingly, only limited tRNA sites have been used for specific recognition by ARSs and they are usually localized in the acceptor stem and anticodon loop. For instance, alanyl-tRNA synthetase recognizes the G3-U70 base pair and A73 discriminator nucleotide of its cognate tRNA [5–7]. In contrast, methionyl-tRNA synthetase interacts with the $tRNA^{Met}$ anticodon [8,9]. Although ARSs are grouped into two classes based on their structural features [10], the tRNA recognition pattern does not appear to be related to this classification [11,12], as both class I methionyl- and glutaminyl-tRNA [13], and class II aspartyl-tRNA synthetases [14] specifically interact with the anticodon of their cognate tRNAs. In addition, these enzymes use different structural domains for interacting with specific anticodons. While the two β-barrels in the C-terminal region of glutaminyl-tRNA synthetase are involved in the interaction with the $tRNA^{Gln}$ anticodon of [13], the α-helix and loop in the C-terminal domain of methionyl-tRNA synthetase is used for the interaction with the cognate anticodon [8]. In contrast, the N-terminal five stranded β-barrels of aspartyl-tRNA synthetase are used for anticodon recognition [14]. These data suggest that there should be many different ways for ARSs to specifically interact with the anticodon loop of tRNA.

In this study, we used two random RNA libraries of different size and sequence context to explore the structural motifs that are important for the specific interaction with methionyl-tRNA synthetase (MRS) isolated from a pathogenic microorganism, *Mycobaterium tuberculosis*. MRS consists of two distinct domains; the N-terminal domain is involved in the catalysis and acceptor stem interaction [15–17], whereas the C-terminal domain is responsible for the interaction with the CAU anticodon, which is the major recognition site [18]. This domain contains a helix and loop in which a highly conserved tryptophan is located. This tryptophan is critical for the specific interaction with the $tRNA^{Met}$ anticodon [8,19]. We have previously isolated the MRS structural gene from *M. tuberculosis* [20]. Its overall sequence and structure are homologous to the *Escherichia coli* MRS with 42% similarity and the enzyme cross-reacts with *E. coli* $tRNA^{Met}$ [20]. Nevertheless, we selected RNA molecules that could distinguish the two

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http://dx.doi.org/10.1016/j.bbrc.2013.01.116

homologous MRSs by *in vitro* evolution and their sequence characteristics were analyzed.

2. Materials and methods

2.1. Preparation of methionyl-tRNA synthetase

M. tuberculosis and *E. coli* MRSs were expressed as a glutathione *S*-transferase (GST) fusion protein in *E. coli*. The structural genes for *M. tuberculosis* and *E. coli* MRSs were cloned into the *Bam*HI-*Eco*RI sites of pGEX4T-2 and the *Eco*RI-*Xho*I sites of pGEX4T-1, respectively. The fusion protein was purified by affinity chromatography using glutathione-Sepharose 4B (Amersham Pharmacia Biotech) following the manufacturer's instructions. The purity of the GST-fused MRS was determined by SDS gel electrophoresis (data not shown).

2.2. Preparation of random sequence RNA libraries

The DNA templates were designed to generate RNA libraries containing 42mer and 70mer random sequences. The random 70mer sequence was flanked by the fixed sequences containing the T7 promoter and the *Sac*I site at the 5′ end and the *Bam*HI site at the 3′ end. The random 42mer template was also designed to contain the *Hind*III site and T7 promoter at the 5′ end and the *Bam*HI site at the 3′ end. Each of the oligonucleotides was made with double-stranded DNA and Klenow fragments.

Each of the RNA libraries was generated from the DNA templates by *in vitro* transcription. The reaction included 1–2 μg of the template DNAs in 8 mM Tris–HCl, pH 7.9, 1.2 mM $MgCl_2$, 0.4 mM spermidine and 2 mM NaCl containing 8.3 mM of each NTP in 20 μl, and was incubated in the presence of T7 polymerase (40 units, Ribomix, Promega) at 37 °C for 3 h. The template DNA was removed by the treatment of RNase-free DNase I (Promega) at 37 °C for 15 min. The resulting RNAs were separated from the reaction mixture by phenol extraction and a G-50 quick spin column (Boehringer Mannheim) followed by precipitation with ethanol. The quality of the RNA preparation was determined by running the sample on a denaturing polyacrylamide gel.

2.3. Selection of RNA aptamers

Different amounts of the purified *M. tuberculosis* MRS fused to GST (GST-MRS) or GST were added to 0.1 ml glutathione conjugated to Sepharose 4B and the beads were packed into a Spin-X column (Corning). The columns were then pre-equilibrated with a binding buffer containing 4 mM HEPES, pH 8.0, 80 mM ammonium acetate, 2 mM magnesium acetate, 0.002% NP-40 and 1% glycerol. The original RNA libraries (10 μg) were heated at 75 °C for 5 min, cooled down and then passed through the GST column to remove the RNA molecules that bound to GST. The eluted RNAs were loaded to the GST-MRS column. The RNAs that were not bound to GST-MRS were washed off with the binding buffer. The RNAs bound to GST-MRS were eluted with 15 mM reduced glutathione, and the RNAs were precipitated with ethanol. The precipitated RNAs were used as a template to generate cDNAs. Reverse transcription was initiated by adding avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim, 25 units) to the reaction buffer containing 50 mM Tris–HCl, pH 8.5, 8 mM $MgCl_2$, 30 mM KCl and 1 mM DTT and incubated at 42 °C for 1 h. The cDNA was mixed with the specific primers (forward, 5′-CACTATAGGGGAGCTCGG-3′; reverse, 5′-AAGGATCCTCTGCAAAGC-3′) and amplified by polymerase chain reaction (PCR) with Taq polymerase in 10 mM Tris–HCl, pH 8.3, 50 mM KCl and 0.1 mg/ml gelatin. The amplified DNAs were first separated from the reaction mixture by phenol extraction and then purified using PCR product purification kit (Boehringer Mannheim). The amount of DNA obtained was determined by spectrophotometry at 260 nm and used for *in vitro* transcription. For the analysis of the aptamer sequences, the amplified 70mers were directly ligated into pGEM-T vector (Promega), and the 42mers were cut with *Hind*III and *Bam*HI and cloned to pBluescript II KS(+) (Stratagene). The plasmids were transformed into *E. coli* DH5α and re-isolated to determine the sequences of the inserted aptamers. The secondary structures of the selected RNA aptamers were predicted using the Mfold program [21].

2.4. Filter binding assay and determination of binding affinity

The radioactively labeled RNA aptamers were prepared by *in vitro* transcription at 37 °C for 90 min with T7 RNA polymerase in a reaction buffer containing 0.5 mM (A, G, U)TP, 12 μM CTP, [α-^{32}P] CTP 10 μCi (Amersham Pharmacia Biotech) in the presence of RNase inhibitor (RNasin 20 units, Promega). After removing DNA with RNase-free DNase I, the remaining RNAs were separated by phenol extraction and ethanol precipitation. The radioactive RNAs (0.75 nM) were mixed with different amounts of GST or MRS-GST of *M. tuberculosis* and *E. coli* in binding buffer as described above and incubated at 37 °C for 15 min. Then, the mixtures were loaded on a Hybond-C nitrocellulose membrane using a 96-well vacuum manifold filtration apparatus. The unbound RNAs were removed by vacuum suction and the filters were washed with binding buffer. The RNAs retained in the filter were quantified using a phosphor image analyzer (FLA-3000, Fuji). The data were used to make a Scatchard plot with $r = [\text{RNA-MRS}]/[\text{RNA}]_{\text{total}}$ on the *x*-axis and $r/[\text{MRS}]_{\text{total}}$ on the *y*-axis. The dissociation constant was determined from the slope of the plot.

2.5. Aminoacylation assay

The aminoacylation reaction of the *M. tuberculosis* or *E. coli* MRS was carried out in 20 mM HEPES buffer, pH 7.5, 0.1 mM EDTA, 150 mM NH_4Cl, 100 μg/ml BSA, 2 mM ATP, 4 mM $MgCl_2$, 20 μM [^{35}S] methionine, 4 μM *E. coli* total tRNA (Sigma). To determine the effect of the RNA aptamers on aminoacylation activity, the indicated amounts of RNAs were added to the aminoacylation reaction mixture containing 30 nM *M. tuberculosis* MRS or 3 nM *E. coli* MRS. The reaction was sampled at specific time intervals and spotted onto 3MM filter paper pre-soaked with 10% trichloroacetic acid containing 1 mM methionine. The filters were washed with cold 5% trichloroacetic acid containing 1 mM methionine and then with 95% ethanol. The amount of charged tRNA was determined by counting radioactivity on the dried filters using a liquid scintillation counter (Wallac 1409 DSA).

3. Results

3.1. Selection of RNA aptamers

Two different random 42mer and 70mer RNA libraries were used for the *in vitro* selection experiments. The DNA template used to prepare the random 70mer RNAs contained a T7 promoter sequence and *Sac*I site at the 5′ end and *Bam*HI site at the 3′ end (Supplementary Fig. 1). *In vitro* transcription of this template generated a 111mer (random 70mer flanked by the fixed sequences of 21mer and 20mer at the 5′ and 3′ ends, respectively). Another template for the random 42mer RNA contained *Hind*III site and the T7 promoter at the 5′ end and the *Bam*HI site at the 3′ end. The *in vitro* transcript from this template produced the 96mer containing the random