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Ribosomal protein S19 binds to its own mRNA with reduced affinity in Diamond-Blackfan anemia

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ABSTRACT

Heterozygous mutations in the ribosomal protein S19 (*RPS19*) gene are associated with Diamond-Blackfan anemia (DBA). The mechanism by which RPS19 mediates anemia are still unclear, as well as the regulation of RPS19 expression. We show herein that RPS19 binds specifically to the 5' untranslated region of its own mRNA with an equilibrium binding constant (K_D) of 4.1 ± 1.9 nM. We investigated the mRNA binding properties of two mutant RPS19 proteins (W52R and R62W) identified in DBA patients. We observed a significant increase in K_D for both proteins (16.1 ± 2.1 and 14.5 ± 4.9 nM, respectively), indicating a reduced RNA binding capability ($p<0.05$). We suggest that the binding of RPS19 to its mRNA has a regulatory function and hypothesize that the weaker RNA binding of mutant rRPS19 may have implications for the pathophysiological mechanisms in DBA.

Introduction

Ribosomal protein S19 (RPS19) belongs to the small 40S subunit of the ribosome, where it presumably binds 18S rRNA [1]. In humans, heterozygous mutations in the *RPS19* gene have been identified in approximately 25% of patients with Diamond-Blackfan anemia (DBA), a congenital bone marrow failure syndrome characterized by a decrease or absence of erythroid precursors [2–4]. RPS19 is highly conserved among species [5] and missense mutations associated with DBA were recently located using the 3-dimensional structure of an archaeal Rps19 protein [6]. The precise mechanism by which mutations in the ubiquitously expressed RPS19 give rise to the tissue specific phenotype observed in DBA is still unclear [7]. It has been suggested that RPS19 is a target for proteins interacting with the ribosome, or as a factor for the recruitment and translation of particular mRNAs [8,9]. However, it is believed that perturbed ribosomal biosynthesis and impaired ribosomal function are important contributing mechanisms in DBA [2,10]. Indeed, RPS19 is required for ribosomal RNA maturation [11,12], suggesting that RPS19 may have RNA binding properties. Protein–RNA binding may also serve as a way to regulate the levels of ribosomal proteins through the interaction with specific mRNAs. Ribosomal proteins are required in stoichiometric amounts for subunit assembly and reduced amounts of one component involved in this process will be rate limiting [13,14]. Several ribosomal proteins have been shown to regulate their expression levels through binding to their own mRNA. For example, human ribosomal proteins L30, S13 and S26 interact with their respective mRNA in a feedback mechanism that regulates splicing and ribosomal protein levels [15–17].

We show herein that rRPS19 binds to the 5'UTR of its own mRNA. We also show that two DBA missense mutations introduced into rRPS19 impair this binding. From our results we suggest that this protein–mRNA interaction has a regulatory function on RPS19 protein levels.

Materials and methods

Recombinant proteins

Recombinant RPS19 (rRPS19) was prepared as described [18]. rRPS19[W52R] and rRPS19[R62W] were kindly provided by T. Bünder.

Synthesis of RNA substrates

RNA substrates (wild-type 5'UTRs: LONG, SHORT and TOP; 5'UTR mutants: MUT1, MUT2 and MUT3; scrambled control, SCR) were synthesized by *in vitro* transcription, using T7-RNA polymerase (USB) and PCR products made in standard PCR reactions with Platinum-*Taq*-

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DNA Polymerase (GE Healthcare) amplified from human genomic DNA or using two partially complementary primers specific for the construct to be synthesized (sequences available upon request). *In vitro* transcribed RNA was 5'-end labeled using γ-^{32}P -ATP (Perkin Elmer) and T4-polynucleotide kinase (Fermentas) following manufacturer's recommendations.

PAGE analysis/RNA substrate refolding/secondary structure prediction

5'-end labeled RNA substrates were refolded in buffer GS (30 mM Tris/Cl, pH 7.7, 10 mM $MgCl_2$, 1 mM EDTA, 200 mM KCl, 15% Glycerol) by heating 5 min to 70 °C and subsequent cool-down for 30 min at room temperature. Refolded RNA was kept on ice until further use. Approximately 0.2 pmol of refolded RNA was analyzed on a 10% denaturing (8 M Urea) or 10% native polyacrylamide gel (Acrylamide/Bisacrylamide 19:1). Gels were dried and RNA visualized by phosphoimager analysis. Secondary structure predictions were performed using the mfold v3.1 web server at http://www.bioinfo.rpi.edu/applications/mfold [19].

Electrophoretic mobility shift assay

Approximately 0.6 pmol 5'-end labeled substrate RNA was refolded and subsequently incubated for 15 min at room temperature with increasing amounts of rRPS19 (5 to 180 nM) in Buffer GS (30 mM Tris/Cl, pH 7.7, 10 mM $MgCl_2$, 1 mM EDTA, 200 mM KCl, 15% Glycerol) supplemented with 0.7 μg/μl BSA, 26.7 ng/μl yeast RNA (Ambion), 2.7 ng/μl polycytidylic acid (Poly(C) RNA, GE Healthcare). Mock incubations were run in parallel without any protein or adding BSA. Complexes and free RNA were separated on a native 4% polyacrylamide gel containing 2% glycerol. Dried gels were analyzed by phosphoimager analysis on a BAS 1800 II Bio-Imager (Fujifilm).

Filter binding assays

Refolded 5'-end labeled substrate RNA (increasing from 0 to 200 nM) was incubated with 80 nM recombinant protein in buffer GS supplemented with 0.7 μg/μl BSA, 26.7 ng/μl yeast RNA, 2.7 ng/μl poly(C) RNA for 15 min at room temperature. Reactions were applied to a nitrocellulose filter mounted on a vacuum manifold and equilibrated in GS buffer without glycerol and washed three times with 0.75 ml ice-cold buffer. Nitrocellulose filters were dried and retained RNA determined by Cherenkov counting. The equilibrium binding constant (K_D) was obtained by plotting experimental data, and subsequently analyzing the data with non-linear regression (Origin7.0® software, OriginLab Corporation) using the first order binding equation

$$[\text{Complex}] = \frac{[\text{Protein}] * [\text{Substrate}]}{K_D + [\text{Substrate}]}.$$

Obtained K_D values for the different mutants were tested for significant difference using student's two-tailed *t*-test.

Conservation analysis, alignment and 3D-structure visualization

Conservation of the first exon of human *RPS19* was analyzed using the UCSC genome browser (http://www.genome.ucsc.edu/). Sequences of the RNA substrates were aligned using BioEdit Sequence Alignment Editor v7.0.5.3. The 3D structure model of RPS19 was generated using ViewerLite v5.0 and PDB file 2v7f (http://www.rcsb.org/pdb/home/home.do) [6].

Results

RPS19 binds to the 5'UTR of its mRNA

Recent studies have shown that the first exon of *RPS19* is almost completely conserved among vertebrates suggesting a functional importance (Fig. 1A, [5]). The first exon is non-coding and comprises the 5' untranslated region only, as the ATG-start codon is located at the immediate beginning of the second exon. Several exon 1 (i.e. 5'UTR) variants have been identified spanning 35 to 372 nucleotides all of which are continuous with genomic DNA (NCBI database at htpp://www.ncbi.nih.gov/ and the UCSC genome browser at http://www.genome.ucsc.edu/; Fig. 1A). The shortest and capped 5'UTR variant identified in the databases spans 35 nucleotides and contains a so-called 5'terminal oligopyrimidine (5'TOP) sequence [20].

We investigated binding of RPS19 to the 5'UTR sequence of its own mRNA by incubating increasing amounts of recombinant RPS19 (rRPS19) with three distinct 5'-end labeled RNA substrates followed by an electrophoretic mobility shift assay (data not shown). These substrates correspond to accession numbers BC018616 (375 nt 5'UTR including AUG, termed LONG), BC000023 (72 nt 5'UTR including AUG, termed SHORT) and D28389 (38 nt 5'UTR including AUG, termed TOP), respectively (see Fig. 1). We observed binding of rRPS19 to all three 5'UTR variants. As the TOP sequence is present in all three variants (Fig. 1), we reasoned that binding is dependent on this sequence. Therefore, we focused our subsequent analysis on the TOP variant in order to characterize the binding in more detail.

rRPS19 binds to the TOP RNA substrate in a concentration dependent manner, indicating that rRPS19 has RNA binding properties (Fig. 2A). We then used filter binding assays to quantify the RNA binding of rRPS19 and to clarify whether the observed binding is RNA-sequence specific. The equilibrium binding constant (K_D) was measured by incubating rRPS19 with increasing amounts of TOP RNA substrate and the binding constant was determined to $K_D[\text{TOP}] = 4.1 \pm 1.9$ nM (Fig. 2B, Table 1).

Attempts to map the rRPS19 binding site on the RNA substrate by enzymatic structure probing gave inconsistent results (data not shown). Therefore, we investigated the structural conformation of the wild-type TOP RNA substrate by native polyacrylamide gel electrophoresis (PAGE). The wild-type TOP RNA substrate appears as multiple bands upon non-denaturing gel electrophoresis suggesting that the RNA is structurally heterogeneous after refolding (Fig. 3A, B). This may also explain why structure probing was inconsistent.

We next introduced distinct nucleotide substitutions into the TOP RNA sequence that presumably alter or destabilize the secondary structure of the TOP RNA substrate as predicted by mfold (Fig. 3A–C). We obtained one distinct structure with similar folding energy for each of the mutant substrates whereas two possible structures were obtained for the wild-type TOP substrate (Fig. 3C). No secondary structure was obtained for the scrambled RNA substrate used as a negative control. We also analyzed the effects of the introduced mutations on the secondary structure by native PAGE (Fig. 3B). All TOP mutant substrates are of equal size but display distinct electrophoretic mobility in a native gel. The mutant RNA variants migrate as single bands, indicating that the introduced mutations lock the substrates into distinct conformations (Fig. 3B–C). This supports the secondary structure prediction of the mutant RNA substrates using mfold (Fig. 3C) [19].

We then measured the equilibrium binding constant (K_D) of the interactions between rRPS19 and all mutant RNA variants. A strong interaction was observed between rRPS19 and the variants MUT1 and MUT2, whereas MUT3 and the scrambled variant showed linear relationships indicating unspecific binding (Fig. 4, Table 1). Taken together, the results show that rRPS19 binds specifically to the 5'UTR of its own mRNA and to a sequence corresponding to nt −30 to −5