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The key function of a conserved and modified rRNA residue in the ribosomal response to the nascent peptide

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 29 June 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. Let me first of all apologise for the exceptionally long delay in getting back to you with a decision. However, two of the referees were not able to return their reports a quickly as initially expected, and, in fact, despite multiple reminders, one of the referees still has not sent us his/her report. As the other two reports are in fair agreement I am taking a decision on your manuscript now, based on the two enclosed reports, in order to save you from yet further unnecessary loss of time. We will forward you the third report once we have received it.

Your manuscript has now finally been seen by two referees whose comments are shown below. You will see that the referees are generally positive about your work and that they would support its ultimate publication in The EMBO Journal. I would thus like to invite you to prepare a revised manuscript in which you need to address the points put forward by referee 1 in an adequate manner.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The paper by Mankin et al. addresses a very exciting question of how nascent peptides that promote stalling are recognized by the ribosome. Here the authors identify nucleotide A2503 in 23S rRNA as potential sensor of stalling. The lack of methylation of A2503 results in impaired stalling (although the effect should be quantified, see below) and the mutation abolished stalling in a number of cases. Interestingly, two groups of stalling constructs were identified, one on which stalling was abolished by mutations of A2503 and A2062, and the second which tolerated the mutations. This result is truly fascinating, because it argues against a uniform mechanism of stalling, which is entirely new. What is less convincing is the proposed relay mechanism involving position 2504 and 2451, for which no direct evidence is presented. The experiments are elegant and technically sound. I would be in favor of publication in EMBO J if the conclusions concerning the relay mechanism and the role of methylation are stated in a more careful way.

Specific comments:

1. The effect of A2503 methylation should be quantified, e.g. using toeprinting analysis as shown in Fig. 4. This is particularly important, given that the effect on erythromycin sensitivity is only 4-fold (p. 9). Also the data shown in Fig. 4 should be presented in a quantitative way, i.e. as bar graphs with standard deviations, in order to judge the magnitude of the effect.

2. P. 9, Kd = 0.18 μM. How was the value measured?

3. Why could the A2503G mutant support cell growth, whereas other mutants of A2503 not? The effect of different substitutions on stalling seems very similar.

4. P. 12 "Having verified that the isolated wt and mutant ribosomes are active in in vitro protein synthesis and able to bind erythromycin". Please provide the data and the Kd values for erythromycin.

5. If A2503 and A2602 are the part of the common sensory pathway, the double mutant should show synthetic phenotype. Can this be tested?

6. P. 18. The mechanism for the sensing pathway is speculative. It is fine for the discussion but it should be clear from the text that this is a hypothesis only.

Minor points:

p. 5, "...Similarly, the walls of the NPET are formed primarily of the rRNA residues." This is overstated. The most important regions, i.e. the constriction site or the sensing site at the end of the tunnel, are composed of proteins, L4, L22, and L23, respectively.

p. 18, Refs to Nissen et al., and Bogdanow et al. are not meaningful, because the mechanism of peptide bond formation proposed Nissen (and the work by Bogdanov which was based on that crystal structure) turned out to be wrong. More recent structural work is recommended (Schmeing et al. 2005 and the related biochemical work, mutagenesis, and MD simulations which lead to the present view of how the peptidyl transferase works).

Referee #2 (Remarks to the Author):

The paper by Vazquez-Laslop et al. analyzes in detail the role of a specific base, A2503, of the E. coli 23S rRNA of the large ribosomal subunit. In particular, its role in inducing antibiotic resistance and in stalling of nascent polypeptide chains in the ribosome has been studied.

A2503 is one of the few post-transcriptionally modified bases in the ribosomal RNA, and the authors can show that this modification modulates the ribosomal response to nascent chains: in the absence of the modification the stalling induced translation of the ermC resistance gene is significantly decreased. Moreover, mutation of the A2503 to G (in contrast to neighboring bases) also abolished the induction of translation as shown by elegant in vivo reporter assays. Further analysis of a variety of stalling nascent chains such as additional erm regulators , SecM and TnaC by toeprinting assays revealed a very specific dependence of the different peptides on A2503. This dependence correlated with the dependence on another well characterized residue, A2062, indicating a concerted action in relaying regulatory signals to the peptidyl transferase center (PTC).

The paper is highly interesting and informative. It elegantly addresses the mechanistic question of how the signal of stalled nascent peptides is allosterically relayed to the ribosomal PTC in order to induce translational stalling. A2503 is clearly shown to play an important role together with A2062 for a subset of peptides and, in addition, the significance of its posttranscriptional modification is demonstrated.

The interesting conclusions are well supported by the data and first mechanistical insights are provided regarding the allosteric regulation of the PTC by nascent polypeptide chains. Taken together, the paper addresses a core question of translational regulation and identifies a new key component of the ribosome. Without any further points I support publication in its current from.

Additional Correspondence - 3rd referee report 30 June 2010

Please find below the third report on your manuscript. Referee 3 is also positive regarding publication of your work here and has no further comments.

I am looking forward to receiving your revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #3 (Remarks to the Author):

This manuscript is revealing mechanistic details of how ribosomal RNA bases control the stalling of ribosome complexes in nascent peptide specific ways. It is, to my understanding, an interesting work of high technical quality.

1st Revision - authors' response 30 June 2010

Referee 1.

"...the conclusions concerning the relay mechanism and the role of methylation [should be] stated in a more careful way."

We have modified the text (pp. 2, 6, 14, 15, 16, 17, 18, 20) to emphasize that the proposed relay mechanism and possible role of methylation in the ribosome-nascent peptide interactions are only hypothetical.

Other specific comments:

1. The effect of A2503 methylation should be quantified, e.g. using toeprinting analysis as shown in Fig. 4. This is particularly important, given that the effect on erythromycin sensitivity is only 4-fold (p. 9).

We did try to use toeprinting to examine the effect of A2503 modification on the in vitro stalling, but the difference in the intensity of the toeprinting band compared to wild type was too weak to be conclusive. This is not surprising. As the reviewer notice, the lack of modification had only modest (although significant) effect on erythromycin sensitivity. Yet, the drug-sensitivity assay is expected to be more sensitive than toeprinting because even one ribosome stalled at the leader ORF can lead to repeated rounds of translation of the resistance gene. The weak effect of the A2503 modification on drug sensitivity (or toe-printing signal, for that matter) is compatible with healthy phenotype of the rlmN(-) cells and is in line with our proposal that modification of A2503 only modulates interactions of the ribosome with the nascent peptide, but is not absolutely critical for it.

Also the data shown in Fig. 4 should be presented in a quantitative way, i.e. as bar graphs with standard deviations, in order to judge the magnitude of the effect.

Following the reviewer suggestion, we have quantified the gels shown in Figure 4 and present a bar diagram in the revised Figure 4. Unfortunately, these experiments, which utilize commercial 'delta-ribosome' cell-free translation system are prohibitively expensive to be repeated multiple times with a number of templates. Therefore, adding standard deviations to the data values is impossible. However, the qualitatively parallel effects of both tested mutations observed with different templates could be viewed as an internal control that the general trend represented by the bar diagrams is accurate.

2. P. 9, Kd = 0.18± 0.03μM. How was the value measured?

The details of the antibiotic binding experiments are presented in the Supplemental Information section of the manuscript. We added the corresponding reference to the main text (p.9).

3. Why could the A2503G mutant support cell growth, whereas other mutants of A2503 not? The effect of different substitutions on stalling seems very similar.

We can only speculate why some mutants with changes at the conserved rRNA position are viable and the others are not. Change of A to G is arguably a less dramatic change than the A to U or A to C transversions; the latter may have a more dramatic effect upon the overall structure of the tunnel possibly incompatible with efficient translation. In our previous studies we found that some highly deleterious mutations had a strong effect upon ribosome assembly whereas other mutations at the same position may have only mild effect on cell growth. This could be also the case with the A2503U and/or A2503C mutants. We feel, however, that discussion of the lethal mutations would divert the attention of the reader from the main point we are trying to make.

4. P. 12 "Having verified that the isolated wt and mutant ribosomes are active in in vitro protein synthesis and able to bind erythromycin". Please provide the data and the Kd values for erythromycin.

Both wild type and A2503G mutant ribosomes readily translated a reference protein (DHFR) in cell-free system; in the revised manuscript, we added Figure S3 to the Supplementary Information section in order to illustrate this point. We did not directly

compare affinities of the wild type and A2503G mutant ribosomes for erythromycin. The similar MICs of the drug with wild type and mutant cells (Supplementary Table S2), however, argue that the affinities should be comparable. We have slightly modified the text (p.12) to make this point more clear. In addition, we want to point out that stalling of the wild type and mutant ribosomes at the ermBL and ermDL templates (Figure 4) clearly shows that the concentration of erythromycin (50 uM) in the toeprinting experiments is sufficient to saturate ribosome binding even if minor differences in affinity existed.

5. If A2503 and A2602 are the part of the common sensory pathway, the double mutant should show synthetic phenotype. Can this be tested?

The double mutants with mutations at A2503 and A2062 were lethal. This may indicate that either single mutation only partly disrupted the sensing/signaling pathway and the double mutant completely interrupted it (the 'synthetic phenotype') or that alterations in ribosome structure or assembly were too severe in the double mutant (see response to question 3 above). Because of the uncertainty of the interpretation of the lethality of the double mutants, we chose to avoid discussing them in the paper.

6. P. 18. The mechanism for the sensing pathway is speculative. It is fine for the discussion but it should be clear from the text that this is a hypothesis only.

We have toned down the discussion of the sensing pathway mechanism and made clear that this is only a hypothesis (pp. 2, 6, 14, 15, 16, 17, 18, 20)

Minor points of Referee 1:

p. 5, "...Similarly, the walls of the NPET are formed primarily of the rRNA residues." This is overstated. The most important regions, i.e. the constriction site or the sensing site at the end of the tunnel, are composed of proteins, L4, L22, and L23, respectively.
In the revised version of the paper we mention the proteins of the exit tunnel wall on p.3.

p. 18, Refs to Nissen et al., and Bogdanow et al. are not meaningful, because the mechanism of peptide bond formation proposed Nissen (and the work by Bogdanov which was based on that crystal structure) turned out to be wrong.

We mention these references in the context of the proximity of A2451 to the Asite amino acid, rather than A2451 being a player in catalysis. This aspect of the original findings of Nissen et al., has not been revised by subsequent studies. However, following the reviewer suggestion, we added the reference to the paper of Schmeing et al.

Referees 2 and 3 did not have any concerns

2nd Editorial Decision 06 July 2010

Thank you for sending us your revised manuscript. Our original referee 1 has now seen it again, and you will be pleased to learn that in his/her view you have addressed all criticisms in a satisfactory manner. The paper will now be publishable in The EMBO Journal and you will receive a formal acceptance letter shortly.

Thank you very much again for considering our journal for publication of your work.

Yours sincerely,

Editor
The EMBO Journal

Referee 1

The authors have made adequate changes and answered my question. The work will be of great interest for the readers of EMBO J.

