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Review of Alena Zíková PhD Thesis "Functional and structural analysis of mitochondrial RNA binding proteins in procyclic *Trypanosoma brucei*"

PhD thesis of Alena Zíková is easily readable and it was a pleasure to review it. The thesis contains besides common introduction and discussion one manuscript prepared for submission and 3 papers already published in journals Molecular and Biochemical Parasitology, Journal of Biological Chemistry and Cell. Most of the work has already been peer-reviewed really strongly and publishing in Cell is already very high valuations of Alena's work. These facts indicate that the reviewed thesis is without any doubts above standard. In this situation it is probably not very big surprise that I could not find anything for real criticism. At the end of this review I am mentioning two imprecisions in the text. But even they could be questionable and do not decrease rate of this work. Therefore, I will have mostly questions regarding issues not entirely clear to me and some general questions, since I am interested to know Alena's opinion.

Firstly two questions about some assertions in the text.
Page 13: "Isolation of distinct complexes that perform either deletion or insertion (of U) … … raised new questions, such as what is responsible for the specific recognition of the editing site and which domains of which proteins contribute to the discrimination between deletion and insertion editing?"
Are really proteins responsible for this discrimination? Should not have been directly gRNA (as a part of „accurate" editosome) responsible for decision „to delete" or „to add" uridines?

Page 80
It was shown that arginine residues of MRP proteins are not methylated. However, it is not clear to me whether these two proteins show signs of any other type of posttranslational modification?

MRP1 protein was originally purified without MRP2. Do you think that MRP1 is able to perform some functions without its partner? Do you think that

in *T. brucei* MRP1 could have more functions (some independent from MRP2) than in *L. tarentoale* or *C. fasciculata*?

Very complex phenotype of MRP1/MRP2 knock-downs cannot be explained by only one function of these proteins. Do you have planes for studying those hidden functions of MRP1 and MRP2 proteins?

"The MRP1/MRP2 complex and its interaction with the editosome." Your results manifest that MRP1/MRP2 complex is probably not obligatory part of editosome otherwise their RNA interference should affected all edited mRNAs. I have impression that immunoprecipitation of RNA editing activities with MRP1/MRP2 complex could be just a side effect of immunoprecipitation of edited mRNA that is in the same time linked with MRP1/MRP2 complex and editosome. According this view could be possible that these two complexes are not associated together at all. Could be this idea right or I've missed something?

There is several hypothesis about "Possible roles for the MRP1/MRP2 complex" built up on coimmunoprecipitation some proteins with Ltp26/Ltp28 complex of *L. tarentoale*. Has been the association of these proteins characterized by similar way as was done with Tb42 protein? Only in that case would make sense to speculate about involvement of MRP1/MRP2 in DNA metabolism. I've found more feasible idea about putative role of the MRP1/MRP2 complex as a transcription factor. Possible connection of trypanosomatids with plant kingdom is not new idea and structural motives based on crystal structure are more valid parameter than presence of certain protein in immunoprecipitated package.

Inaccuracies:
On page 59
„ ...high molecular weight bands correspond to oligomers of the MRP proteins and are composed almost exclusively of these two proteins, as confirmed by MS analysis and Western blot with specific antibodies …"
Western blot analysis can only confirm whether protein is present or not but cannot say anything about presence/absence of another proteins.

In the thesis Alena several times use to put RNA editing and RNA processing to contradiction.
I took only one example from page 87:
„Because never-edited transcripts were also affected by the lost of MRP1/MRP2 proteins, a role in RNA processing as well as in RNA editing has been proposed."

RNA editing is unique fenomen but it is still kind of RNA processing, so this expressions are not exactly correct. (However I am not sure if it is worthy to waste time to find more proper formulations.)

Finally, it is my pleasure to state that the presented PhD thesis is qualitatively and quantitatively excellent. Consequently, I deliver an opinion completely favorable to the defense of this thesis to obtain the rank of Doctor Philosophy - PhD.

Assoc. Prof. Anton Horváth, PhD.

Review of the Ph. D. thesis by Alena Zikova, Ceske Budejovice, October 10, 2006

The thesis submitted by Ms. Zikova describes the structural and functional analyses of the MRP complex in *Trypanosoma brucei*. This is a very interesting research carried out at a high technical level and the thesis is very well written. In particular, I would like to mention the brilliant introduction which describes the trypanosomes' molecular biology, biochemistry and reverse genetics. I would highly recommend this section to any graduate student as an example of how introductions should be written for a thesis. This part combines the breadth and depth of coverage with a very elegant writing style and a perfect English. I suggest that this part be published as a mini-review in an appropriate journal.

The experimental part consists of three published works and one manuscript in preparation. No technical concerns are raised by the published works. However, I would like to criticize the general interpretation of the data. I find it difficult to agree with the often repeated statement (in the thesis and during the presentation) that the results demonstrate a functional role of the MRP protein complex in RNA editing. This view had originated from the initial discovery of the MRP1 protein as a guide RNA binding protein. This was a result obtained in vitro and it did not necessarily apply to the in vivo function. This work had been done in the other laboratory and is not a part of this thesis. However, this view has been adopted in this thesis, although the results obtained by Ms. Zikova, especially her analysis of the RNAi-mediated silencing of the MRP1 and MRP2 functions, make this initial hypothesis unnecessary. My view is that the effects observed may be explained exclusively by proposing that the MRP proteins function as factors selectively regulating the stability of several RNA species (some edited, some unedited). Albeit this function is presented in the thesis, it is listed as secondary, with the function in RNA editing still regarded as primary. The real function remains an open question until the new data are obtained (e.g. the impact of the MRP silencing on polyadenylation).

Two additional critical comments are technical. They refer to the last chapter of the thesis (the manuscript in preparation). First, the composition of the isolated TAP-tagged MRP complexes needs an additional investigation. It is difficult to reconcile the observed multiplicity of the polypeptide bands with the proposed simplicity of the composition. In particular, additional evidence is needed to support the interpretation of the higher molecular bands as MRP1/2 aggregates. Second, the analysis of the MRP-promoted gRNA-mRNA annealing needs to be repeated using a different assay, because the assay chosen by the applicant is prone to artifacts and requires additional controls. I recommend to use RNAse protection assays, which proved to be effective in the similar analyses of the *Leishmania* proteins performed elsewhere.

Overall, these criticisms do not diminish the great value of the work. In my opinion, the thesis is very strong and Ms. Zikova deserves to be awarded a Ph.D. degree.

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Amsterdam, 03-10-2006

Dear Alena,

After having read your proposed thesis work, performed as a PhD student of the Laboratory of molecular biology of protists in Ceske Budejovice, I am happy to state that, in my view, the work is more than acceptable to grant you a PhD.

I congratulate you and your supervisor, Julius Lukes, with the amount and quality of the work as illustrated by the fact that 3 articles (one by The Journal of Biological Chemistry, one by Molecular & Biochemical Parasitology, and one by Cell!) have already been accepted by these important biochemical journals and that at least one other will (soon?) follow. On a personal note, I might add that publishing an article in Cell is one of my personal ambitions that probably will never be fulfilled and that on this occasion I am filled with a (healthy) sense of jealousy.....

Having stated this, however, of course I still have some remarks and questions regarding the content of the thesis. I will focus on scientific considerations here (some minor remarks concerning slight language errors in the manuscript I can discuss with you if you so desire). As usual I will concentrate on the work presented in the thesis most close to my own interests. Let me stress that not all these questions have to be dealt with!

Regarding the Introduction:

A. Under heading 2.3.2 the correlation between extensive poly-A tails (up to 200 nt) and stability of edited mRNAs on the one hand, and small poly-A tails (about 20 nt) and lability of unedited mRNAs on the other hand, is discussed. This is described in terms of: 'effect of polyadenylation on the stability'. This implies that editing and further polyadenylation are seen as linked. This model would leave 'never-edited' mRNAs out in the cold, however. Thus, presumably, the model can not be correct or is at least too simple. Is it not more likely that e.g. the length of the A tail is a measure of the activity of a 3' exonuclease (busy with breaking down this class of mRNAs)?

B. A related question: is anything known about the distribution of the lengths op the poly-A tails of these 3 classes (never-, un- and edited) of mRNAs?

C. On page 80 of the introduction it is stated that peptides of MRP1 and MRP2 were checked for single arginine methylation by mass spectrometric analysis, that more that 50% of sequence coverage was obtained and no indications for methylation were found. It is then concluded that the proteins appear not to be methylated. This is a very dangerous conclusion:
1. More than 50% is far from a complete analysis.

2. If these results were obtained with tryptic digestions I am sure the fact that trypsin does not recognise methylated arginine was taken into account.
3. Most importantly, argenine is the most important residue when it comes to the ionisation efficiency of peptides (whether by MALDI or ESI). Tryptic peptides with a carboxy-terminal R on average are much better detected than tryptic peptides with a carboxy-terminal K, for instance. The methylations could be present in abundance, but such peptides could be impaired in their ionisation efficiency.

Regarding the The Journal of Biological Chemistry article:

None.

Regarding the Molecular & Biochemical Parasitology article:

A. As you know, there has been a long controversy about the existence of complex I in procyclics. First let me again stress that the existence of mitochondrial genes and nuclearly encoded subunits for NADH dehydrogenase in themselves do not prove they are used in procyclics to form a classical complex I as sometimes is implied. They seem to be used in the short stumpy bloodstream forms and this could be the only reason for their presence in the respective genomes.

You again state that the presence of the genuine complex I (*i.e. the complex in which all or most of the mt encoded proteins are present*) in procyclics has been demonstrated (in reference 10 = Horvath *et. al.* (2005) Downregulation of the nuclear-encoded subunits of the complexes III and IV disrupts their respective complexes but not complex I in procyclic *Trypanosoma brucei*. Mol. Microbiol. **58,** 116-130). I must say that I am still not completely convinced by the arguments and experimental findings. Here are some of my reasons for this:

1.The observed editing patterns do not seem to correlate with the expression of complex I in procyclics.

2. In *C. fasciculata* (Speijer *et. al.* (1997) Characterization of the respiratory chain from cultured *Crithidia fasciculata*. Mol. Biochem. Parasitol. **85**, 171-186) we find no trace of complex I while we can nicely demonstrate the presence of *all* the other respiratory chain complexes. This strain has of course been cultured over an extensive period, but still demonstrates that respiration starting with complex II could also be the normal pathway in *T.brucei* procyclics.

3. We do not see anything resembling a complex I with even a few different subunits in the neighbourhood of complex IV in all the different BNE gels of figure 2, as predicted by the comparison of histochemical staining in figure 3A and B. While figure 3A is quite convincing, 3B is not. To see whether the knockdowns of MRP1/MRP2 individually or in combination really impair the staining due to NADH dehydrogenase activity, the BNE (it

is *blue* native, is it not?) has to be shown *before and after* the staining due to NADH dehydrogenase activity. But even if this shows convincingly that this staining is impaired it still does not tell us that any of the mt encoded proteins form part of the enzyme complex responsible for the staining (which could still be non-classical, especially in the light of the pleiotropic effects of these knock downs).

4. It is stated that the histochemical staining in figure 3B at about 650 kD must be that of the 'real' complex I and can not be of an alternative NADH dehydrogenase activity. Its very small size makes this unlikely (though it is not impossible). But why is this staining not due to a totally unrelated NADH dehydrogenase activity? In table 1 you show that all the knockdowns have no significant effect on the NADH dehydrogenase activity, and you explain this (in my opinion correctly) in the discussion by allowing for alternative NADH dehydrogenase activities. There seems to be no hard evidence that the 650 kD band is not one of them.

5. Even the argument that the possible reduction in 650 kD activity correlates with the claimed down-regulation of never-edited ND 4 and 5 mRNAs in these knock downs would still be far from a proof for the claim that this is the authentic complex I. And then there is the the claimed *upregulation* of edited ND 7 mRNA in these knock downs.

I think this controversy has still not been resolved yet.

B. Would you not be more certain about the relative contribution of cytochrome mediated oxidation (COX) and alternative oxidation (TAO) in the different knock downs by also reversing the order: starting with inhibition by SHAM, followed by inhibition with KCN ? It seems such a nice control.

C. How can you explain that the reduction of mitochondrial membrane potential is much stronger in the MRP1 knockdown than in both the MRP2 and the double knockdown (especially in the light of the well proven observation that both proteins become unstable if their heterotetrameric partner is missing)?

Regarding the Cell article:

First of all my compliments for a well written, interesting and enlightening paper; this does not mean that I have no questions, however.

A. It is always stated that gRNAs have a general conserved secondary structure (stemloop I -intervening sequence-stemloop II and the U tail; with stemloop I representing the anchor sequence and stemloop II representing part of the informational domain). I have always had trouble believing this. First of all, do the always differing requirements of the different anchors and informational domains not exclude the possibility of this general structure? Secondly, especially with stemloop I, it seems obvious that these kinds of thermodynamic free energy gains are not physiologically relevant (in the case gND7-506: 2 A-U basepairs and a single G-C basepair?). I do believe in the importance of the

heterotetrameric MRP1/MRP2 complex in keeping the anchor sequence in an unpaired conformation and reducing electrostatic repulsion between RNAs which so nicely fits with the crystallographic structure obtained. However, the possible intramolecular basepairing involving the anchor sequence that the complex prevents is probably different with different gRNAs. What do you think?

B. Why was the crystallographic structure of the heterotetrameric MRP1/MRP2 complex in association with the gND7-506 gRNA obtained with a gRNA without a U tail? The U tail is the only part that all the gRNAs have in common. Couldn't you get crystals?

C. In the article it is stated that the MRPs have no significant interaction with the oligo(U) tail. In *Crithidia fasciculata* we identified the MRP1/2 homologues by crosslinking them to oligo(U) and purified them (amongst others) with the aid of a poly(U) sepharose column (Leegwater *et. al.* (1995) Identification by UV cross-linking of oligo(U)-binding proteins in mitochondria of the insect trypanosomatid *Crithidia fasciculata*. Eur. J. Biochem. **227**, 780-786 and Blom *et. al.* (2001) Cloning and characterization of two guide RNA-binding proteins from *Crithidia fasciculata*: gBP27, a novel protein, and gBP29, the orthologue of *Trypanosoma brucei* gBP21. Nucleic Acid Res. **29**, 2950-2962). How would you explain these discrepancies?

Regarding the manuscript for The Journal of Molecular and Cellular Biology:

A. First a technical remark: protein bands from SDS PAGE gels were excised and subjected to tryptic digestion. The resulting peptides were only subjected to LC-ms/ms. Why not first test 10 % of the eluted peptides of a specific band on a MALDI-TOF (my personal favorite, a classical peptide mass fingerprint) to get a quick and easy protein identification? You can always use the remaining 90 % for specific generation of ms/ms data. There is even the danger of losing specific peptides on the LC and ESI has other ionisation preferences. In conclusion: these are complementary techniques and I think you should always use them both.

B. A comparison of figure 6A and 6C is not possible and you also do not discuss the annealing efficencies of the non-cognate gRNA-mRNA pairs by the heterotetrameric MRP1/MRP2 complex in any detail. Question: are the 'cognate' annealing efficencies higher than the 'non-cognate' annealing efficencies or are they the same in the presence of 'our favorite complex' (I would expect 'cognate' to do better, if 'misediting' is to be kept at a minimum)?

C. I thank you for the possibiliy to make an evolutionary observation (to end my list of remarks and questions). You state that ' .....mistake-prone mechanism may in fact generate protein diversity,...' and speculate whether this is the reason the kinetoplastids retained RNA editing. I think this is highly unlikely. Practically all changes in an organism are for the worse (most 'new' proteins are 'less good' or even positively 'bad'; consider also the existence of quality control and rapid breakdown of misfolded proteins). Evolution is a revolutionary, individual organisms are conservatives (although not of the

'Tory' variety, I hope). An evolutionary explanation for the presence of RNA editing in these organisms must be found elsewhere, I think.

With kind regards and sincere compliments for the work presented,

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