Final Report

NKFI-OTKA-identifier: 84008

Uracil-DNA in Holometabola insects and in human cells: physiological role in DNA-damage repair and in signaling

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1 Quantitative Measures on Publications

1.1 Articles in international peer-reviewed journals (in English)

Based on the experiments and research work funded by the present grant, we have published 24 articles to date in international peer-reviewed journals of high esteem. These articles all contain an acknowledgement of the NKFI-OTKA funding with the grant number 84008. The sum of the impact factors of these 24 publications is 122.96. In addition, also containing acknowledgment of the NKFI-OTKA 84008 grant, three (3) additional manuscripts from our laboratory are under revision following favorable editorial and referee opinions on the first submission. Moreover, three (3) other manuscripts have been submitted during the last month. Finally, and also importantyl with regard to publication activities, there are nine (9) other manuscripts under progress in our laboratory at the stage where the required experimental work is already done and we are compiling the text and the final figures for these studies. It will be my pleasure to provide the reviewers of this final report with additional information on these studies in case such information is needed.

The 24 published articles describe in depth the details of our research work during the present NKFI-OTKA grant. In this final report, my aim is to provide a summary on the most important results. These articles report on the result directly related to the original aims of our research proposal, however, during our research, we have also discovered several additional intriguing aspects and have successfully followed some of these (cf section 2.3 of this final report).

Among the 24 published articles, in my opinion, the following five studies are the most important:

Gergely Rona, Ildiko Scheer, Kinga Nagy, Hajnalka L Palinkas, Gergely Tihanyi, Mate Borsos, Angela Bekesi, Beata G Vertessy:
Detection of uracil within DNA using a sensitive labeling method for in vitro and cellular applications,
NUCLEIC ACIDS RES 2016 Feb 18;44(3):e28. doi: 10.1093/nar/gkv977. Epub 2015 Oct 1.,
(IF 9.112)

Hirmondó R, Szabó JE, Nyíri K, Tarjányi S, Dobrotka P, Tóth J, Vértessy BG: ***Cross-species inhibition of dUTPase via the Staphylococcal Stl protein perturbs dNTP pool and colony formation in Mycobacterium***, DNA REPAIR 30: 21-27, 2015
(IF 3.111)

Judit E Szabó, Veronika Németh, Veronika Papp-Kádár, Kinga Nyíri, Ibolya Leveles, Ábris Á Bendes, Imre Zagyva, Gergely Róna, Hajnalka Pálinkás, Balázs Besztercei, Olivér Ozohanics, Károly Vékey, Károly Liliom, Judit Tóth, Beáta G Vértessy:
Highly potent dUTPase inhibition by a bacterial repressor protein reveals a novel mechanism for gene expression control, NUCLEIC ACIDS RES 42: (19) 11912-11920, 2014
(IF 8.808)

Muha V, Horváth A, Békési A, Pukáncsik M, Hodoscsek B, Merényi G, Róna G, Batki J, Kiss I, Jankovics F, Vilmos P, Erdélyi M, Vértessy BG.: ***Uracil-containing DNA in Drosophila: stability, stage-specific accumulation, and developmental involvement.***,
PLos Genet, 2012;8(6):e1002738. doi: 10.1371/journal.pgen.1002738. Epub 2012 Jun 7.
(IF 8.690)

I Pecsi, R Hirmondo, AC Brown, A Lopata, T Parish, BG Vertessy, J Tóth:
The dUTPase enzyme is essential in Mycobacterium smegmatis,
PloS one 7 (5), e37461, 2012
(IF 3.730)

I wish to underline also the fact that in the 24 published articles, the contribution from my laboratory is of key significance – in 18 out of the 24, I am corresponding author.

1.2 PhD dissertations

During the research work funded by the present NKFI-OTKA project, seven PhD dissertations were also defended based on the results obtained during our reserch, as listed below (cf data on www.doktori.hu, supervisors Beáta Vértessy, Judit Tóth):

Ildikó Pécsi, cum laude (PI Tóth)
Mária Pukáncsik, cum laude (PI Vértessy)
András Horváth, summa cum laude (PI Vértessy)
Gergely Róna, summa cum laude (PI Vértessy)
Gergely Nándor Nagy, summa cum laude (PI Vértessy)
Judit E. Szabó, cum laude (PI Tóth)
Rita Hírmondó, summa cum laude (PI Tóth)

(details on other forms of involvement of young researchers are to be found on the relevant section of our webpage http://www.biostruct.org/index.php/research/dissertations).

2 Selected major results

2.1 Results concerning Specific Aim 1 of the project NKFI-OTKA 84008

Specific Aim 1: Critical investigation of the hypothesis that development in Holometabola is associated with synthesis and degradation of uracil-DNA

During our research, we have investigated in details development of the following Holometabola insects: Drosophila melanogaster, Drosophila virilis, Tribolium castaneum. (We have also initiated developmental studies on Apis mellifera – these results will be published later).

Our most significant discovery related to this aim was the finding that level of the uracil contentr in genomic DNA shows a developmental pattern, in negative correlation with the level of the dUTPase enzyme in D. melanogaster (publications Muha et al, Plos Genetics 2012 and Horvath et al, Fly 2013). The background in this topic is provided by the generally accepted observation that base-excision repair and control of nucleotide pools safe-guard against permanent uracil accumulation in DNA relying on two key enzymes: uracil–DNA glycosylase and dUTPase. Lack of the major uracil–DNA glycosylase UNG gene from the fruit fly genome and dUTPase from fruit fly larvae prompted the hypotheses that i) uracil may accumulate in Drosophila genomic DNA where it may be well tolerated, and ii) this accumulation may affect development. We have shown that i) Drosophila melanogaster tolerates high levels of uracil in DNA; ii) such DNA is correctly interpreted in cell culture and embryo; and iii) under physiological spatio-temporal control, DNA from fruit fly larvae, pupae, and imago contain greatly elevated levels of uracil (200–2,000 uracil/million bases). These pioneering data in quantitative levels of uracil moieties in DNA were determined using our novel real-time PCR–based assay). Uracil is accumulated in genomic DNA of larval tissues during larval development, whereas DNA from imaginal tissues contains much less uracil. Upon pupation and metamorphosis, uracil content in DNA is significantly decreased. Based on these results, we proposed that the observed developmental pattern of uracil–DNA is due to the lack of the key repair enzyme UNG from the Drosophila genome together with down-regulation of dUTPase in larval tissues. In agreement with this suggestion, we have shown that dUTPase silencing increases the uracil content in DNA of imaginal tissues and induces strong lethality at the early pupal stages, indicating that tolerance of highly uracil-substituted DNA is also stage-specific. Silencing of dUTPase perturbs the physiological pattern of uracil–DNA accumulation in Drosophila and leads to a strongly lethal phenotype in early pupal stages. The observed lethal phenotype is linked to DNA breaks (both single strand breaks and double strand breaks are proposed to occur as suggested by TUNEL and pH2AX staining on Drosophila tissues). Our results suggested a novel role of uracil-containing DNA in Drosophila development and metamorphosis and presented a novel example for developmental effects of dUTPase silencing in multicellular eukaryotes. Importantly, we have also shown by genome analysis the lack of the UNG gene in all available genomes of other Holometabola insects, indicating a potentially general tolerance and developmental role of uracil–DNA in this evolutionary clade.

During the analysis of the further Holometabola species, we have realized the need for a potentially high-throughput assay for uracil levels in DNA. To realize such an assay, we have designed a highly specific molecular tool for recognition of uracil moities in DNA. This uracil-sensor was constructed such as to promote not just in vitro, but also in situ detection.

Our main interest in the occurrence of uracil in DNA had a dual aspect: on the one hand, we have been interested in the developmental control of the level of uracil-DNA in insects, and on the other hand, we have realized that large number of anti-cancer chemotherapeutic drugs

interfere with thymidylate biosynsthesis. Such treatments are expected to induce an elevation of uracil levels in DNA in the well-responding patients. It would be of high significance to be able to follow the response rate at the molecular level by directly determining uracil levels in DNA. Also, it is now widely accepted that uracil-DNA constitutes be a physiologically important DNA element in diverse systems from specific phages to antibody maturation. In these systems again, a versatile and high-throughput method for determination of uracil-DNA would be of high significance to gain quantitative and qualitative information on uracil levels in DNA both in vitro and in situ, especially since current techniques does not allow in situ cellular detection. Such investigations, however, were hampered by the lack of a uracil-DNA antibody.

In the present project, starting from a catalytically inactive uracil-DNA glycosylase protein, we have designed several uracil sensor fusion proteins. The designed constructs can be applied as molecular recognition tools that can be detected with conventional antibodies in dot-blot applications and may also serve as in situ uracil-DNA sensors in cellular techniques. Our method was verified on numerous prokaryotic and eukaryotic cellular systems. The method is easy to use and can be applied in a high-throughput manner. It does not require expensive equipment or complex know-how, facilitating its easy implementation in any basic molecular biology laboratory. Elevated genomic uracil levels from cells of diverse genetic backgrounds and/or treated with different drugs can be demonstrated also in situ, within the cell (publication Rona et al, Nucleic Acids Res 2015).

We have also performed studies to discover if the lethal phenotype of depletion of dUTPase from D. melanogaster can be rescued by simultaneous silencing of other base-excision repair proteins in the organism. It was necessary to employ a full knock-out using the recently developed efficient CRISPR-Cas9 system to evaluate these aspects, since double silencing raised some perturbing issues. We now have obtained data showing that Thd1 (the Drosophila homologue of TDG) silencing provides rescue for the dUTPase knock-out D. melanogaster. A manuscript based on these observations will be submitted later this year.

2.2 Results concerning Specific Aim 2 of the project NKFI-OTKA 84008

Specific Aim 2 Investigation of the role of dUTPase in uracil-DNA metabolism in human cell lines

Regarding this aim, we have constructed dut -/- knock-out cell lines in the HCT116 cell line using Zn-finger nuclease (ZFN) technology. The HCT116 cell line is deficient in mismatch repair and hence perturbation of dUTPase and uracil-DN glycosylase in this cell line in comparison with its isogenic mismatch repair proficient cell line offers a unique opportunity to look into potential connections between base-excision repair and mismatch repair pathways. To achieve the dUTPase knock-outs, first we had to insert a rescue copy of the dUTPase gene at a genomic location different from the site of the endogenous dUTPase gene. We selected an AAVS1 site on chromosome 19 and using ZFN technology, we inserted the rescue copies of dUTPase between loxP sequences. We have succeeded in selecting single cell clones where the rescue copy was inserted and then used again the ZFN technology to remove the endogenous dUTPase gene from this cell line. We have verified the success of our experiments by clone selections and sequencing. Using the cell line clones where the endogenous dUTPase gene is knocked-out on both alleles, we employ tamoxiphen-induced Cre recombinase action in the nucleus to cut out the rescue copy of the dUTPase gene. The resulting KO cell lines are investigated for different phenotypic characteristics.

During the construction of these cell lines we met several hard challenges, which we could now overcome. Our present data indicate that knock-out of dUTPases is fully lethal. It is also evident that the cells with reduced dUTPase content became much sensitive to fluoro-deoxyuridine, a drug commonly used in anti-cancer chemotherapy. We have also determined the uracil-DNA levels of these cells. We found that in the dUTPase KO cells, the genomic level of uracil show a dramatic increase upon inhibition of the cellular uracil-DNA glycosylase with its specific inhibitor protein (UGI).

These results form the basis of an additional manuscript that will be submitted later this year.

2.3 Other major results, linked to the topic of the NKFI-OTKA 84008 grant

Other published results concern the role of uracil-DNA metabolism in Mycobacteria, which served as a simple cellular model for our assays. We have shown that the cellular dUTP pools, the dUTP/dTTP ratio and uracil-DNA levels are under the control of dUTPase (Pecsi et al. 2012, Plos One and Hirmondo et al, 2015, DNA Repair)

During our studies on Drosophila virilis, we have also discovered an unusual protein architecture for the dUTPase enzyme. In this organism, interestingly, genome annotations predicted a rather unique arrangement for dUTPase: three copies of the monomeric dUTPase gene segments are joined together so that the resulting predicted protein product is a pseudo-heterotrimer in which the three dUTPase domains are only slightly different (87.76% identity) and are connected through linker peptide regions. This predicted protein possesses only one nuclear localization signal (NLS), and has an estimated molecular mass of ~ 55.7 kDa. The finding that in this case, only one NLS is attached to the dUTPase protein and only this single isoform of dUTPase is present in the organism was in contrast with other eukaryotes, eg D. melanogaster or human, where there are two isoforms, one of these nuclear, while the other is cytoplasmic (or mitochondrial). In the usual arrangement, the nuclear isoform contains one NLS/subunit, i.e. three NLSs in one trimer, whereas the other isoform, generated by alternative splicing, does not contain any NLSs. We wished to understand the physiological consequences of the unusual protein architecture in D. virilis. Although the interaction of individual NLSs with the karyopherin importin-alpha protein, responsible for nuclear import cargo proteins, has been well characterized, the question of how multiple NLSs of oligomeric cargo proteins affect their trafficking has been less frequently addressed in adequate detail. Using the D. virilis dUTPase as a fully relevant physiologically occurring model protein, we showed that NLS copy number influences the efficiency of nuclear import in both insect and mammalian cell lines, as well as in D. melanogaster and D. virilis tissues. Biophysical data indicated that NLS copy number determines the stoichiometry of complexation between importin-a and dUTPases. The main conclusion of our study is that, in D. virilis, a single dUTPase isoform efficiently reproduces the cellular dUTPase distribution pattern that requires two isoforms in D. melanogaster. During these studies, an additional related and highly intriguing issue was also discovered, Namely, we have detected a mechanism that controls the availability of dUTPase within cellular organelles 8nucleus vs cytoplasm) during the cell cycle. Starting from this observation, we have performed an in-depth proteomic study to understand how phosphorylation adjacent to the nuclear localizations signal governs the pattern of nucleo-cytoplasmic trafficking during the cell cycle (publications Horvath et al 2015 FEBS J, Rona et al 2013, Acta Cryst. D, Rona et al, 2014 Cell Cycle, Christie et al, 2015, J Mol Biol).