Molecular biological investigations of *Drosophila* and human enzymes involved in uracil-DNA metabolism

Thesis of PhD dissertation

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Introduction

The uracil base is a common and naturally appearing pyrimidine derivative that is normally found only in RNA molecule as a constituent for nucleic acid polymers. Uracil is able to build up U:A base pair with adenine through two hydrogen bonds. However, the non-canonical base uracil may be present in small quantities in DNA molecule as well. The uracil base in DNA may arise as results of spontaneous hydrolytic deamination of cytosine, through incorporation of dUMP during DNA replication and enzymatic deamination of cytosine. On one hand, deamination of cytosine in double stranded DNA molecule results in U:G mispairs that have highly mutagenic potential leading to C > T base transitions. On the other hand, introducing dUMP instead of dTMP into DNA during replication results in U:A mispairs that are not directly mutagenic, however, are more likely to cause cytotoxic effects or perturb the replication and the transcriptional process. There is evidence that uracil can be introduced into immunoglobulin genes of B cells in an enzymatic manner by acting of activation-induced cytidine deaminase (AID). The existence of uracil base in immunoglobulin genes is involved in a mechanism of antibody diversification through somatic hypermutation and also contributes to the class switch recombination process during maturation of B cells.

Two different types of conserved enzymes are considered to be responsible for keeping DNA free from uracil. One of these is the deoxyuridine triphosphate nucleotidohydrolase (dUTPase) enzyme that performs the hydrolysis of dUTP into dUMP and pyrophosphate. The enzyme simultaneously removes dUTP components from the deoxyuridine biosynthetic pathway and provides substrate (dUMP) for the de novo synthesis of thymidylate. Thus, dUTPase has a dual essential role in preserving DNA integrity and in maintenance of the normal homeostatic function of the cell. In rare cases, in spite of dUTPase prevention, dUMP may still be incorporated into DNA. Also, uracil may arise in the double stranded DNA molecule by cytosine deamination. In these cases, the other type of conserved enzymes involved in uracil metabolism termed uracil specific DNA glycosylases (UNG, SMUG1, TDG and MBD4) are considered to be responsible for removing the uracil from both G:U and also from A:U mispairs in DNA. Uracil specific DNA glycosylases are the prominent members of the base-excision repair mechanism that is responsible for the proper correction of the problem of uracil presence in DNA.
Adequate nucleocytoplasmic transport of enzymes involved in uracil-DNA metabolism is of primary importance for maintenance of the normal homeostatic function of the cell. The proteins described above function in the nuclear compartment and may possess different types of nuclear localization signals (NLS). NLS is a basic requirement for nuclear proteins to be transported into the nuclear compartment by importin molecules. Decreased activity of dUTPase may lead to expansion of the intracellular dUTP pool resulting in uracil-substitutions in DNA. High level of uracil in DNA overloads the capacity of uracil-directed base-excision repair mechanism. The repair system constantly reincorporates uracil and finally the repair pathway gets transformed into a suicide cycle resulting in multiple DNA gaps and breaks. The DNA damages induce chromosomal fragmentation and consequently thymine-less cell death. The latter type of apoptosis can also be induced by blocking the de novo pathway of dTTP synthesis. For instance, the mechanism of action of 5-fluoro-2’-deoxyuridine monophosphate (FdUMP), one of the active metabolites of the anticancer drug 5-fluorouracil (5FU) is to bind to and inhibit thymidylate synthase (TYMS) in cells. TYMS processes the rate-limiting step of converting dUMP to deoxythymidine monophosphate (dTMP). Besides dUTPase, dCMP deaminase (DCTD) is also considered to produce dUMP as a dTTP precursor. Furthermore, dTMP can also be produced via the salvage pathway by acting of thymidine kinase (TK). Subsequent phosphorylation of dTMP by thymidylate kinase (TYMK) results in deoxythymidine diphosphate (dTDP) which is converted to dTTP in the further step catalyzed by deoxynucleotide diphosphate kinase (dNDPK). The 5FU and 5-fluoro-2’-deoxyuridine (FUdR) compounds, which are frequently used in anticancer chemotherapy, may also incorporate into DNA and RNA resulting in cell death. Inhibition of TYMS by fluoropyrimidines and downregulation of the dUTPase gene (DUT) by RNA interference (RNAi) at the same time would be able to reduce dramatically the level of dTTP precursors in cells. Those kinds of inhibitions of thymidylate metabolism may accelerate the apoptotic event of malignant tumor cells.
Aims

1.) In the first part of my doctoral thesis I aimed to identify and characterize NLS sequences for Drosophila melanogaster proteins involved in uracil-DNA metabolism. Four such major proteins have been identified to date:

- The newly discovered uracil-DNA degrading factor (UDE), which recognizes uracil-substituted DNA and promotes its degradation
- dUTPase, which is responsible for prevention of uracil incorporation into DNA
- Two DNA glycosylases, thymine-DNA glycosylase (TDG) and the single-strand-specific monofunctional uracil-DNA glycosylase 1 (SMUG1) homolog protein.

The major objectives of my work were the followings:

- First, I wished to assess and identify putative NLS signals within the primary protein structure of the aforementioned proteins using the PSORTII in silico method.
- Having identified the putative NLS signals of proteins I wished to perform a cellular experiment to investigate the subcellular distribution pattern of wild type UDE and dUTPase isoforms in insect and human cells.
- My further objectives were the molecular characterization of the putative NLS segments of UDE and dUTPase proteins to identify the essential residues and peptides which are responsible and critical for the proper and adequate NLS function of the sequences.
- I also wished to carry out my experimental examinations using fluorescent reporter constructs in Sf9 (insect) and in HeLa (human) cell lines. The subcellular localization of fluorescent reporters was detected by fluorescent microscopy method.

2/a) In the second part of my doctoral thesis I studied the process of deoxyuridylate and thymidylate metabolism in stable human cell lines. We aimed to estimate the molecular and cellular events within thymidylate metabolism as responses to dUTPase and TYMK gene silencing in HeLa cells.

- Our major objectives were to achieve an efficient gene silencing of dUTPase and TYMK enzymes using a Tetracyclin regulated vector-based RNAi system. First, we wanted to repress either the DUT or the TYMK genes independently. Second, we aimed to downregulate both enzymes simultaneously.
Our further objectives were to select and generate stable HeLa shRNA cell cultures in which the consequences of gene silencing could be investigated by molecular biology and cell biology methods.

With respect to the importance of DUT, TYMK, TK1, DCTD and TYMS enzymes in the de novo and salvage pathways of thymidylate synthesis, I aimed to characterize gene expression patterns of these enzymes in the stable shRNA cell lines using quantitative mRNA measurement.

My further aim was to perform a phenotypic analysis of the stable shRNA cell lines in order to estimate the cytotoxic effects of fluoropyrimidines (5FU and F UdR) and topoisomerase inhibitor (VP-16) on cells.

2/b) With respect to the high importance of thymidylate synthesis in DNA integrity and normal homeostatic function, my further aim was to establish a transgenic human cell line in which the phenomenon of the thymineless cell death caused by dUMP incorporation can be followed by phenotypic analysis.

- My major objective was to generate a cell line in which the constitutive and stable expression and nuclear localization of UDE protein could be provided. The uracil-DNA degrading activity of UDE may sensitize cells against agents (F UdR and folate-analogue s) blocking dTTP production and inducing dUTP/dTTP imbalance.
- We wished to establish the transgenic cell line by using HEK293 human cells and a HIV-vector based lentiviral gene transfer method.
- After successfully gene transfer the transfected cells can be sorted for producing a homogeneous cell population. The transgenic cell line would be a potential model system in a further experiment to study the mechanism of thymineless cell death induced by fluoropyrimidines and the contribution of uracil-DNA degrading factor.

**Methods**

- Sequence analysis by *in silico* methods
- Establishment of fluorescent reporter constructs by molecular cloning
- Transient transfection of insect and human cells
- Cell fixing and DAPI staining
- Fluorescent and confocal microscopy
Establishment of shRNA vector constructs by molecular cloning
Stable transfection of human cell line
RNA isolation and reverse transcription
Quantitative PCR (Q-PCR)
Immunoblot analysis
Determination of dTTP and dUTP pool of cells
Cytotoxic assay
Establishment of lentiviral vector construct
Lentiviral gene transfer
Flow cytometry (FACS and cell sorting)

Results and Conclusions

1.) Results obtained and conclusions drawn with respect to the molecular characterization of NLS signals of Drosophila proteins involved in uracil-DNA metabolism:

- The in silico sequence analysis predicted two putative NLS sequences for the uracil-DNA degrading factor, one for dUTPase and a large number of NLS segments for TDG glycosylase. For the SMUG1 homologue protein no nuclear targeting signal could be predicted by the used method. In this doctoral work only the UDE and the dUTPase putative NLS segments were examined by experimental methods using fluorescent reporter constructs in in vitro cell lines.

- According to the prediction, the flexible C-terminal region of the UDE protein possesses two NLS segments spaced by several residues (NLS1: \(320^{PEKRKQE}326\) and NLS2: \(347^{PKRKKKR}353\)). Extensive deletion and point mutation studies revealed that the monopartite classical NLS2 segment, which has a close homology to the SV40 T-ag NLS, is sufficient for exclusive nuclear localization of the protein by itself. Further mutation screen showed that the segment has an extremely high nuclear targeting potential and mutation tolerance owing to its high number of basic residues in a row. The \(350^{KKKR}353\) segment of the basic context seemed to be critical for establishing the selective and exclusive nuclear localization of the protein.

- Based on in silico prediction the Drosophila dUTPase protein possesses a non-classical monopartite NLS segment \((10^{PAAKKMK}16^{ID})\) consisting of non-polar and basic residues in a sequence. The identified unusual segment is homologous to the sequence of human c-Myc protein NLS and is located on the N-terminus of the protein. According to the results of molecular studies conducted in insect cells, the
existence of the predicted NLS segment seemed to be critical for the nuclear distribution of the physiologically occurring long isoform of dUTPase. The physiologically occurring short isoform of dUTPase that lacks the first fourteen residues of N-terminus seemed to remain in the cytoplasm.

- Extensive deletion studies performed in the predicted dUTPase NLS sequence revealed the importance of presence of non-polar (\textsuperscript{10}PAA\textsuperscript{12} and \textsuperscript{17}ID\textsuperscript{18}) and basic residues (\textsuperscript{13}KKMK\textsuperscript{16}) for exclusive nuclear localization of the long dUTPase isoform.
- In a comparison the different types of NLS sequences described above, it can be concluded that the nuclear targeting potential depends on the high proportion of basic residues and the presence of non-polar residues flanked to a basic core of NLS stretches.

2/a) Results obtained and conclusions drawn with respect to the RNAi investigations of human enzymes involved in deoxyuridylate and thymidylate metabolism:

- In our system we could achieve a robust RNAi silencing of the DUT gene: according to immunoblot results, the level of dUTPase protein was under detection limit in the case of the dUTPase silenced cell line as compared to control cells, indicating an efficient silencing effect of shRNA vector constructs on DUT gene. Q-PCR quantitations of mRNA levels of the DUT gene correlated positively to protein levels detected by immunoblot in silenced cell line: dUTPase expression level was significantly reduced by 17-fold decrease.
- According to the mRNA quantitations of genes involved in thymidylate metabolism, it could be concluded that effective silencing of dUTPase led to a significant 3.2-fold increment of the TYMK gene expression. Furthermore, a significantly 2-fold elevation was also observed for the mRNA level of TK1 gene. No noticeable up-regulation could be observed for the TYMS enzyme. Elevated level of TYMK could provide a sufficient amount of dTDP for dTTP synthesis. The results suggested that TYMK and TK1, but perhaps not TYMS, are important enzymes involved in reconstitution of thymidylate biosynthesis pathway in our cells.
- Phenotypic analysis (cytotoxic assay) results revealed that repression of DUT gene by RNAi increases the sensitivity of cells for both 5FU and F UdR agents, with a much more increased sensitization for F UdR. The repressed cell line was ~6-fold more
sensitive to 5FU and ~40-fold more sensitive to FUdR, relative to control cells. Results indicated that following 5FU uptake into the cells, the drug is more likely to be metabolized through the uridylylate metabolism rather than via of deoxynucleosylate synthetic pathway.

- Down-regulation of the TYMK gene on its own did not alter the expression patterns of genes involved in thymidylate biosynthesis and did even not induce any drastic sensitization to fluoropyrimidines.
- The simultaneous silencing of the DUT and the TYMK genes induced an elevated expression level of the TK1 gene. Surprisingly, the simultaneous downregulation of the aforementioned genes caused less sensitivity for cells towards fluoropyrimidines as compared to the DUT silencing on its own. However, both the TYMK and the TK1 are considered to be enzymes involved in phosphorylation of diverse prodrugs into biochemical active metabolites of fluoropyrimidines. Thus, the drug-resistance against fluoropyrimidines could be increased by reducing TYMK expression level to physiological level in the simultaneous silencing cell line.
- It can be concluded that efficient silencing of dUTPase gene could perturb normal homeostatic function of cells treated with fluoropyrimidines.

2/b) Generation of UDE expressing transgenic human cell line:
- Using lentiviral transduction we could generate the $\text{HEK UDE}^{\text{WT}}$-eGFP/500 transgenic and cell-sorted cell line in which the expression of the UDE fused with eGFP reporter was constitutive and stable.
- During the establishment of the cell line, I observed a tendency of decreased amount of UDE$^{\text{WT}}$-eGFP transprotein. In order to produce a homogenous cell population, the UDE$^{\text{WT}}$-eGFP transprotein expressing cells were cell-sorted several times. Owing to repeated cell-sorting events the cells seemed to retain and stable express the transprotein. Confocal microscopy observation revealed that the UDE$^{\text{WT}}$-eGFP transprotein did locate exclusively in the nuclear compartment.
Publications

Relating publications:

1. Merényi G, Kónya E and Vértessey BG


3. Kovari J., Barabas, O., Merenyi, G., Zagyva, I., Vértessey, B. G. *dUTPase mRNA silencing triggers apoptosis in cancer cells* 31st FEBS Congress, Molecules in Health & Disease, Isztambul, Turkey, 24-29. 06. 2006., poster presentation


Other relating publication: