FUNCTIONAL INVESTIGATION OF AN RNA BINDING PROTEIN WITH

METHYL-TRANSFERASE DOMAIN

Ph.D. Thesis

by

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1. INTRODUCTION

RNA-protein interactions direct a diverse variety of cellular processes, which range from transcriptional regulation to targeted translation of proteins. Hence, to discover new proteins with a specific affinity to RNA molecules and find out their specific function will expand our understanding on a wide range of cellular functions. Following this statement, a screen was developed to search *Drosophila* proteome for RNA binding proteins and an unknown protein with RNA binding property was retrieved (Udvardy et al.). In this work we aimed to characterize this new *Drosophila* protein and its mammalian homologue.

The retrieved protein had well conserved homologues in different species and is represented with many ESTs (expressed sequence tags) in databases suggesting an important role in cellular function. In the beginning of our work none of the homologues had an identified function. However during our investigation, other research groups revealed certain aspects of the functional properties of this new class of proteins.

1.1. Theoretical Background

Although once RNA was thought to be a passive genetic blueprint, now it is known as a key player in a wide variety of cellular processes. An RNA molecule conforms into double helices of base-complementary strands with a combination of deviations from plain helix such as hairpins, stem-loops, symmetrical or asymmetric interior loops, bulge loops, purine-purine mispairs, GU or wobble pairs and pseudoknots (1,2). The combination of helical stems separated with the above non-helical motifs, provide RNA molecules the ability to form intricate three-dimensional architectures comparable to the complexity of protein folds. RNA binding proteins target these three dimensional arrangements for recognition. As a result, a great diversity in RNA and RNA binding structural motifs is observed.

1.1.1. RNA Binding Proteins

RNA-binding proteins have a modular structure and contain RNA binding domains of 70-150 amino acids that mediate RNA recognition. There are three major classes of eukaryotic RNA-binding domains:

1. The RNA recognition motif (RRM),
2. K-homology domain (KH),
3. Double stranded RNA binding domain (dsRBD).

In addition to these RNA binding domains, there are many other less common motifs and domains to recognize RNAs.

1.1.2. RNA Editing

Several phenomena are included under the title RNA editing; these include nucleotide modifications (19-21) and post-transcriptional insertions and/or deletions (22,23), sometimes involving the participation of a guide RNA. The phenomenon of RNA-guided nucleotide modification is the conversion of a nucleotide in a precursor RNA to another form by an RNA-protein complex. The ribonucleoproteins (RNPs) that mediate these reactions include a small nucleolar RNA (snoRNA) that provides the guide function through base pairing with the substrate and a set of proteins, one of which catalyzes the modification reaction. Two common modifications exist, formation of 2'-O-methylated (Nm) nucleosides and conversion of uridine to pseudo (Ψ) uridine. These modifications are mediated by two large, heterogeneous populations of RNPs that are modification type-specific and site-specific (21).

1.1.3. Spliceosomes

Splicing of precursor messenger RNAs is an important and ubiquitous type of gene regulation in metazoans. Splicing joins the coding sequences called exons by removing the intervening noncoding sequences, introns, from primary transcripts (25).

The intron is cleaved from the exons at the 5´ and 3´ ends, called the splice sites. These sequence elements must be recognized by the spliceosome, a multi-unit complex of proteins and RNA. The RNA components are small nuclear RNAs, U1, U2, U4, U5 and U6, assembled into ribonucleoprotein particles (snRNPs) (26).

1.1.4. Transcriptional Trans-activation and TAT

Less frequently RNAs are involved directly in transcription modulation as well. One example of this is TAT, the transcriptional transactivation protein of HIV. The Tat-TAR ribonucleoprotein (RNP) is the essential switch that controls transcription from the HIV-1 long terminal repeats (LTR). The transactivator (Tat) binds to the trans-acting response element (TAR) found at the 5' end of viral transcripts, as a hairpin formation (29).

1.2. Aims of the thesis

In this work we aimed to characterize a newly identified protein with RNA binding ability. A screen was designed to detect RNA binding proteins in *Drosophila* proteome by testing their affinity against the TAR RNA of Human Immunodeficiency Virus (Udvardy et al.). A previously unidentified protein was retrieved from this screen and named Drosophila TAT Like (DTL). Surprisingly, proteins related to DTL can be identified in a wide range of organisms. From yeast to human they share well-conserved boxes of amino acids characteristic for RNA

methyltransferases. The presence of large number of ESTs in databases indicates that the genes encoding these proteins are intensively expressed as well.

The general aims of the study were:

- To identify homologues of DTL by performing similarity searches against the known sequences found in databases,

- By using the data obtained from similarity searches:
  1. to determine conserved regions which will point out functional regions or motifs of DTL related proteins,
  2. to find EST (expressed sequence tags) sequences belonging to mammalian homologues of DTL, to work in parallel with *Drosophila* sequences,

- To test whether the conserved motifs or domains that are found are functional,

- To characterize the expression pattern and the possible isoforms of DTL and its mammalian homologues. For this purpose we raised specific polyclonal antibodies, and we analyzed protein samples from different tissues and compartments of the cell, by Western blotting and immunolocalization,

- To find partner proteins of DTL and its mammalian homologues and determine the nature of interacting structures, by using *in vitro* or *in vivo* techniques like yeast two hybrid screen and immunolocalization,

- To attribute a functional property for the DTL related proteins in cellular events, by siRNA knock down in established cell lines.

2. MATERIALS AND METHODS

2.1. Yeast two-hybrid: pACT human spleen MATCHMAKER cDNA library (Clontech) was screened using pBTM-PIMTC as bait as described by the manufacturer (Clontech). Briefly: *S. cerevisiae* strain L40 transformed with pBTM-PIMTC was transformed with the cDNA library and positive candidate colonies were selected on minimal synthetic dropout medium. Colonies grown in the absence of histidine were tested for their ß-galactosidase activity. From His positive clones plasmids were recovered and sequenced.

2.2. In vitro protein binding assays: WAIT-1 protein was expressed in *Escherichia coli* BL21 as glutathione S-transferase (GST) fusion proteins and affinity purified on glutathione-Sepharose

gel (Amersham Pharmacia Biotech). For in vitro interactions, glutathione-Sepharose bead suspension was mixed with bacterial cell lysate pGEX-PIMTCdGST in phosphate-buffered salin, containing 1% TritonX100 and 1mM PMSF and incubated at room temperature. After extensive washing aliquots of the affinity beads were mixed with lysate of bacterial cells expressing PIMT-C and incubated for 2 hrs at room temperature. The beads were washed extensively and boiled in 50 µl of SDS sample buffer. GST protein alone was processed parallel as negative control. Eluted proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

2.3. Nuclear and Cytoplasmic Protein Fractionation of the Cells: Cultured HeLa cells were washed twice with PBS, scraped, harvested and resuspended in ice cold buffer A (10mM HEPES pH 7.9, 10mM KCl, 0.5mM DTT, 0.5 mM PMSF). Following incubation on ice for 15min, 25 l 10% NP40 was added, vortexed for 10 sec, and nuclear fraction was pelleted by centrifugation at 3000 rpm for 5 min. Supernatant was kept as cytoplasmic fraction. Pellet was resuspended in 200 µl buffer B (0.42M KCl, 20mM Tris pH 7.8, 1.5mM MgCl2, 10% sucrose, 2mM DTT, 0.5 mM PMSF), incubated on ice for 30min, and sonicated for 15 sec.

2.4. Immunolocalization: HeLa cells were fixed on coverslips with 4% paraformaldehyde in PBS for 20min at room temperature. After washing, cells were permeabilized with 0.3% Triton-X-100 for 20 min. Cells were incubated with rat polyclonal anti-tubulin (YOL1/34, Sera Labs, Sussex, UK), and rabbit polyclonal anti-PIMT antibodies in 1:200 dilution for 2h at 23°C. TRITC (tetramethyl rhodamine isothiocyanate) or FITC (fluorescein isothiocyanate)-labelled secondary antibodies (Sigma) were used as appropriate for the isotype of the primary antibodies. Nuclei were stained with DAPI (4', 6-diamidino-2phenylindole HCl) (100ng/ml) and cells were mounted with Citifluor (Ted Pella Inc. CA, USA). Control experiments in the absence of fluorescent primary antibody or replacing the primary antibody with pre-immunserum were performed for all the immunolocalization experiments. Colchicine treatment was performed by incubating the cells on coverslips with 10µl per ml colchicine in DME media for 17 hours at 37°C.

2.5. Flow cytometry

HeLa cells were trypsinized and washed twice. After the last wash, the cells were fixed and permeabilized with ice cold 70% ethanol on ice for 20 min. Cells were centrifuged at 3000 rpm for 5 min and resuspended in staining buffer (0.1% sodium citrate, 0.1% TritonX100, 10µg/ml Propidium Iodide (PI) and 10µg/ml RNase) for 30 min. PI is a double stranded nucleic acid binding fluorochrome that intercalates in the double-helix. Ribonuclease-A was used to

eliminate the staining of double-stranded RNA. Measurements were evaluated by WinMDI and Cylcred software and represented as the percentage of the cells in G1, S and G2 phases.

2.6. Synthesis of siRNAs

SiRNAs were designed and synthesized according to the criteria and procedure published by Donzé et al. (36). The following oligo nucleotides were used for the synthesis of siRNAs specific to PIMT mRNA:

T7 promoter complementary oligonucleotide 5'-TAATACGACTCACTATAG-3'; PIMT nucleotides 626-630 relative to the initial methionine: sense, 5'-TGGTGAACTTGAAACAGAAAACTATAGTGAGTCGTATTA-3'; antisense, 5'-ATGTTTTCTGTTTCAAGTTCACTATAGTGAGTCGTATTA-3'.

3. RESULTS

3.1. Drosophila TAT Like protein (DTL)

3.1.1. *dtl* gene encodes an RNA binding protein with a methyltransfearse domain

In a screen cDNA fragments of a protein with RNA binding specificity similar to those of Human Immunodeficiency Virus (HIV) TAT transactivator protein are isolated by Udvardy et.al. (personal communication)

Since in this screen, TAR (Transactivation Response RNA element) RNA of HIV was used as a bait the cDNA fragment representing a so far unknown gene designated as *dtl* (Drosophila Tat Like) gene was isolated. The transcript of *dtl* had an interesting structure having two open reading frames (ORFs).

To identify functional domains through conserved domains of homologous proteins, database search was performed. This search revealed that DTLdown had several homologues in different species extending from yeast to human. The most conserved motifs found in similar methylase domains were a 9-amino acids methyltransferase Motif I (VVDAFCGVG), a 7-amino acids Motif II (KADVVFL), and an S-adenosyl-L-methionine interacting region.

3.1.2. DTL localizes in the cytoplasm in the Schneider S2 cell line

Affinity purified polyclonal antibodies specific to DTLdown were used to determine the localization of the DTL protein in *Drosophila* S2 cell line. S2 cells were fractionated into their cytoplasmic and nuclear components and blotted. Western blots showed that both isoforms of DTL were cytoplasmic proteins. The same antibodies were used to immunolocalize DTL in S2 cells. To simplify perception mouse anti-tubulin antibody was used together with rabbit anti-DTL

antibodies. Double immunolocalization revealed that DTL was co-localized with microtubule cytoskeleton.

3.2. Mammalian homologue of DTL

3.2.1. Different isoforms of mammalian DTL exist

DTL was recovered through its interaction with a mammalian virus RNA. This is why to characterize mammalian homologue of DTL might support and clarify certain aspects of DTL. Since the amino acid similarities shared by DTL and its relatives were located in the C-terminal half of the homologues, a murine EST sequence with a high similarity to the C-terminal of DTL was purchased.

To facilitate *in vitro* studies polyclonal antibodies were raised against the 14 AA peptide: **EIPNSPHATEVEIK** , which corresponds to the amino acids 44 to 57 of the purchased EST sequence. Recently, mammalian homologues have been identified as PIMT (PRIP interacting protein with methyl transferase domain). To prevent confusion of terminology, mammalian homologue of DTL will be named as PIMT and the EST sequence PIMT-C, because it also codes the whole C-terminal of PIMT protein (amino acids 555-852).

To determine the tissue distribution of PIMT (mammalian DTL) protein, rat tissues were screened by Western blot analysis. Immunoblots developed by anti-PIMT antibody showed three specific bands. A 55kDa protein was detected in all tissue samples examined, a 90-kDa protein was observed only in brain and testicular homogenates, whereas a much smaller, 30kDa protein was observed only in kidney homogenate.

3.2.2. 55-kDa isoform of PIMT is a cytoplasmic protein that co-localizes with microtubule cytoskeleton

In HeLa cells, the anti-PIMT antibody recognized a single 55-kDa form of PIMT. This shorter isoform was named as PIMT55. To determine the cellular localization of PIMT55, HeLa cells were fractionated into cytoplasmic and nuclear fractions and analyzed by Western blots. It was seen that the PIMT55 was a cytoplasmic protein as its *Drosophila* homologue DTL. In HeLa cells PIMT was immunolocalized in the cytoplasm showing a distribution similar to that of the microtubule cytoskeleton as in case of DTL.

3.2.3. C-terminal region of PIMT interacts with WAIT-1

In order to identify interacting partners of PIMT, a yeast two hybrid screen was carried out using the PIMT-C as a bait against a human hematopoietic cDNA library. The strongest interactions were detected with cDNA clones encoding WAIT-1, which was recovered in several

independent clones. WAIT-1 is a mammalian homologue of mouse Eed and Drosophila Extra Sex Combs (ESC) proteins.

3.2.4. SiRNA knock down of PIMT in HeLa cells delays cell proliferation

To have more insights about the function of PIMT, PIMT protein was transiently knocked down by using siRNAs specific to PIMT mRNA. siRNAs specific to PIMT and specific to GFP sequences were selected and synthesized according to the criteria and procedure of Donze et al. (36).

This analysis showed that in siRNA treated samples the transition to the next phase of cell cycle was delayed in comparison to the controls. According to the results at 24-hrs the number cells in G1 phase were higher than control. At 40 hrs, S phase cells, and at 48hrs G2/M phase was higher.

3.2.5. PIMT55 expression increases before S phase

Since the absence of PIMT55 altered cell cycle progressing, it would be that at different stages of cell cycle PIMT55 levels might also change. To check this idea HeLa cells were treated in different ways to arrest the cell cycle at different stages . Cells were serum starved to block in early G1, hydroxyurea treated to block in G1/S phase boundary, and finally colchicine treated to block in G2 phase. Equal amount of protein from each sample was resolved with SDS-PAGE and blotted with PIMT specific antibodies. The results revealed that there was an increase in the PIMT levels in S phase in comparison to other phases.

4. DISCUSSION

4.1. DTL is the product of down stream ORF

DTL is encoded by an interesting transcript containing two ORFs. With one base shift towards the 5´-end of the first stop codon the second frame encodes a highly conserved protein among many different species.

The specific polyclonal antibodies that were raised against the down stream ORF were affinity purified to improve their specificity and DTL presence was checked in Schneider S2 cells by Western blotting. There were two specific bands recognized by DTLdown antibodies, one with the size of ~60 kDa, and a second with the size of ~120 kDa. The 60-kDa protein should be a translation product from an internal ribosomal entry site, and the 120-kDa protein is a posttranslational modification of this 60-kDa protein.

4.2. Homologues of DTL

Our data base search revealed several homologues of DTL in different species. In the beginning of our investigation, there was not any identified DTL related protein. Mammalian homologues were represented only by EST sequences, which have similarity to the conserved C-terminal of DTL.

Zhu et al. (2001) isolated a nuclear receptor co-activator-interacting protein, designated PIMT, from a human liver cDNA library by using the co-activator peroxisome proliferator-activated receptor-interacting protein (PRIP) as bait in a yeast two-hybrid screen. PIMT (PRIP-interacting protein with methyltransferase domain) cDNA encodes an 852-amino acid protein containing a 9-amino acid methyltransferase motif I (VVDAFCGVG) and an invariant segment (GXXGXXI) found in K-homology motifs of many RNA-binding proteins. Immunofluorescence studies showed that the 92 kDa PIMT protein and PRIP proteins are colocalized in the nucleus. PIMT binds S-adenosyl-L-methionine, the methyl donor for the methyltransfer reaction, and it also binds RNA, suggesting that it is an RNA methyltransferase (38).

Furthermore in 2002 Mouaikel et. al. identified the yeast homologue of DTL as TGS1p. TGS1p was found to hypermethylate the 5' cap structures of a subset of snoRNAs and localizes to the Cajal bodies in the nucleus as well as in the cytoplasm though the nature of this cytoplasmic localization was not precise (41). Human TGS1 protein (hTGS1), or as it was named earlier PIMT, was also shown to share the same characteristics as the yeast homologue (42).

4.3. Three different isoforms of PIMT exist in different tissues

According to our results from western blots performed on different rat tissues samples, different isoforms PIMT/hTGS1 could be detected. A 55 kDa protein was ubiquitously expressed in all the samples, a 90 kDa protein as described in Zhu et. al. work could be found only in brain and heart samples, and a 30 kDa protein was only in kidney sample. Furthermore, in HeLa cell line, which was used in the majority of experiments, the 55-kDa isoform was the main isoform detected.

PIMT gene contains more than 13 exons according to Zhu et. al. (38). This suggests that alternatively spliced variations of PIMT mRNA may result in different isoforms of PIMT protein. The presence of shorter cDNA sequences related to PIMT in database also supports the idea that the alternatively spliced versions of *PIMT/hTGS1* gene exist.

4.4. DTL and 55-kDa isoform of PIMT are cytoplamsic, microtubule cytoskeleton associated proteins

Western blots of cytoplasmic and nuclear fractions of insect and mammalian cell lines showed that DTL and PIMT55, the 55-kDa isoform of PIMT localize in the cytoplasm.

Immunolocalization experiments added to this fact the coexistence of DTL and PIMT together with microtubule cytoskeleton. In the light of our results, we can speculate that the nuclear localization of PIMT/hTGS1 may be restricted to the 90-kDa isoform, while 55-kDa isoform is a cytoplasmic protein. However it is obvious that a major property of this family that is the cytoskelatal localization, was omitted in the publications mentioned above.

4.5. Mammalian DTL interacts with WAIT-1

BLAST searches and amino acid sequence multi-alignments showed that DTL related proteins are highly conserved through their C-terminal end. Therefore, we focused our attention to this region for further analyses. We performed a yeast two-hybrid screen using C-terminal of PIMT/hTGS1 as bait against a human hematopoietic cDNA library. This screen demonstrated the interaction between PIMT/hTGS1 and WAIT-1 (NP_003788) proteins. Using deleted versions of the bait/prey constructs or non-specific constructs further supported this interaction. With GST pull down assays we proved the interaction between human DTL and WAIT-1 proteins *in vitro*.

WAIT-1 is a mammalian homologue of mouse Eed (Embryonic Ectoderm Development) and *Drosophila* ESC (Extra Sex Combs) proteins (39). These two proteins belong to the polycomb group of proteins, that act by altering the accessibility of DNA to factors required for gene transcription (43).

4.6 Function of DTL

siRNA knock down of PIMT55 in HeLa cells revealed that absence of PIMT55 interfered with cell cycle progression. Moreover, protein samples enriched in different cell cycle stages showed that PIMT55 levels increase in S phase. This observation is another proof of the relation between PIMT55 and cell cycle. Previously it was shown that knock down of *C. elegans* homologue of PIMT (T08G11.4) resulted in abnormal spindle orientation, slow growth and larval lethality (47).

There are eukaryotic proteins that have been identified as splicing factors and independently as essential for cell cycle progression (48). Previously it was shown that in yeast, tubulin pre-mRNA splicing and cell cycle control is linked to each other (49,50).

Therefore delayed cell cycle progression can be explained by the fact that reduction of the PIMT/hTGS1 levels should also reduce the rate of sn(o)RNA maturation, and this in its turn should disable the proper splicing of mRNAs and editing of rRNA.

However, abnormal spindle orientation that is observed in C. elegans, can be indicator of a more elaborate function of PIMT/hTGS1 than just efficient splicing of mRNAs (including tubulin mRNA) or ribosomal RNA maturation. PIMT55 colocalization with microtubules and its

interaction with WAIT-1 suggest a potential pathway for signals from cell-cell contact and cell-matrix interaction to influence cytoskeletal structure in higher eukaryotes. There is evidence that spindle orientation correction is related with cortex interactions (namely with actin structures) of the very short astral microtubules (51). Probably, through astral microtubule-mediated delivery of a signal from the periphery i.e. integrin or actin cytoskeleton, has a role on this function.

On the other hand evidence suggests that the bulk of the mRNAs in the cytoplasm, are cytoskeleton-bound (52). This indicates that there are probably a number of so-called "general" RNA-binding proteins within eukaryotic cells that mediate the interaction between a broad range of mRNAs and the cytoskeleton. 55-kDa version of PIMT/hTGS1 may be one of these general RNA-binding cytoskeleton-associated proteins. Its uniform distribution throughout of microtubule cytoskleton rather supports this possibility.

5. CONCLUSIONS

- In *Drosophila* Schneider S2 cells two isoforms of DTL exists. 60 kDa isoform is translated from an internal ribosomal entry site, and 120 kDa protein is a post-translational modified version of the 60 kDa protein. Both isoforms of DTL are cytoplasmic proteins and are colocalized with microtubule cytoskeleton.

- Mammalian homologue of DTL has three isoforms translated in rat tissues: 90 kDa isofrom is translated in brain and heart tissues; 55 kDa isoform is ubiquitously translated in all tissues examined; 30 kDa isoform is only found in kidney tissue samples.

- 55 kDa isoform of the mammalian DTL is the major product in human HeLa cells. 55 kDa protein is a cytoplasmic protein and colocolizes with microtubule cytoskeleton as DTL.

- Mammalian DTL interacts with WAIT-1 protein through its carboxyl-terminal. β-propellar structure of WAIT-1 formed by the WD repeats is essential for this interaction.

- SiRNA inhibition of mammalian DTL interferes with proper cell cycle progression in HeLa cells.

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7. PUBLICATIONS

"Different isoforms of PRIP-interacting protein with methyltransferase domain/trimethylguanosine synthase localizes to the cytoplasm and nucleus"
Izzet **Enünlü**, Gábor Pápai, Imre Cserpán, Andor Udvardy, Kuan-Teh Jeang and Imre Boros
Biochemical and Biophysical Research Communications, 2003 Vol. 309, No. 1, 44-51

"Two different *Drosophila* ADA2 homologues are present in distinct GCN5 histone acetyltransferase-containing complexes"
Selen Muratoglu, Sofia Georgieva, Gábor Pápai, Elisabeth Scheer, Izzet Enünlü, Orbán Komonyi, Imre Cserpán, Lubov Lebedeva, Elena Nabirochkina, Andor Udvardy, Laszlo Tora, and Imre Boros
Molecular and Cellular Biology 2003. 23: 306-321

Poster presentations

Investigation of a complex genetic system in Drosophila
Pápai Gábor, Selen Muratoglu, Izzet Enünlü, Komonyi Orbán, Tóth Zsolt, Cserpán Imre,Udvardy Andor, Boros Imre
Meeting of the Hungarian Biochemistry Society 2002, Keszthely, Hungary

Drosophila Tat like protein human homologue
Izzet Enünlü, Pápai Gábor, Udvardy Andor, Kuan-The Jeang, Boros Imre
Meeting of the Hungarian Biochemistry Society 2000, Sopron, Hungary

Analysing of novel *Drosophila* genes participating in transcription regulation
Selen Muratoglu, Komonyi Orbán, Tóth Zsolt, Izzet Enünlü, Maróy Péter, Udvardy Andor, Boros Imre
Meeting of the Hungarian Biochemistry Society 2000, Sopron, Hungary

Drosophila mutation, which leads to round chromosomes (and where else?)
Komonyi Orbán, Pápai Gábor, Izzet Enünlü, Tóth Zsolt, Selen Muratoglu, Udvardy Andor, Boros Imre
Straub Days, Biological Research Center 2002, Szeged, Hungary