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Biochemical and Biophysical Research Communications 309 (2003) 44–51
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Different isoforms of PRIP-interacting protein with methyltransferase domain/trimethylguanosine synthase localizes to the cytoplasm and nucleus

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Received 24 July 2003

Abstract

A protein family including the recently identified PIMT/Tgs1 (PRIP-interacting protein with methyltransferase domain/trimethylguanosine synthase) was identified by searching databases for homologues of a newly identified *Drosophila* protein with RNA-binding activity and methyltransferase domain. Antibodies raised against a short peptide of the mammalian homologue show a 90-kDa isoform expressed specifically in rat brain and testis and a 55-kDa form expressed ubiquitously. In HeLa cells, the larger isoform of the protein is nuclear and associated with a 600-kDa complex, while the smaller isoform is mainly cytoplasmic and co-localizes to the tubulin network. Inhibition of PIMT/Tgs1 expression by siRNA in HeLa cells resulted in an increase in the percentage of cells in G2/M phases. In yeast two-hybrid and in vitro GST pull down experiments, the conserved C-terminal region of PIMT/Tgs1 interacted with the WD domain containing EED/WAIT-1 that acts as a polycomb-type repressor in the nucleus and also binds to integrins in the cytoplasm. Our experiments, together with earlier data, indicate that isoforms of the PIMT/Tgs1 protein with an RNA methyltransferase domain function both in the nucleus and in the cytoplasm and associate with both elements of the cytoskeletal network and nuclear factors known to be involved in gene regulation.

Keywords: PIMT; Tgs1; WAIT-1; DTL; Methyltransferase; Cytoskeleton; Yeast two-hybrid

The RNA-binding nuclear protein PIMT (PRIP-interacting protein with methyltransferase domain) was identified recently by Zhu et al. [1] in yeast two-hybrid screen on the basis of its interaction with the nuclear receptor coactivator PRIP (peroxisome proliferator-activated receptor-(PPAR)-interacting protein). PIMT was shown to localize to the nucleus and to interact with a variety of transcriptional coactivators such as CBP, p300, and PBP. In transient gene expression studies PIMT enhanced PBP-mediated transcriptional activity of PPARγ and PRIP and acted as a repressor of CBP/p300-mediated transactivation of PPARγ [1,2]. More recently the yeast homologue of PIMT, and subsequently the human protein itself, has been identified again, this time by Mouaikel et al. [3] as Tgs1 (trimethylguanosine synthase), the methyltransferase responsible for the hypermethylation of the 5′ m^7G cap structure of snRNAs and snoRNAs. Yeast Tgs1 was isolated also in a two-hybrid screen using as bait the C-terminal domain of SmBp, one of the core proteins that assemble around snRNAs during spliceosome biogenesis. Tgs1 was shown to localize to the nucleus, specifically to the Cajal bodies associated with the nucleolus [4]. However, earlier data also established that the methyltransferase that hypermethylates mammalian snRNAs is a cytoplasmic protein, since in mammals the association of Sm core proteins with snRNAs occurs in

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doi:10.1016/S0006-291X(03)01514-6

the cytoplasm. The methyl-2,2,7-guanosine cap structure together with the Sm core complex provides signals for nuclear import of the assembled core U-snRNPs [5]. Independently, we identified PIMT/Tgs1 as the mammalian homologue of a *Drosophila* RNA-binding protein, DTL which we isolated based on its ability to bind to the HIV TAR RNA. In this paper, we describe isoforms and an interacting partner of the mammalian homologue of DTL. For clarity, we refer to this protein as PIMT/Tgs1.

Human PIMT/Tgs1 mRNA has coding capacity for an 852 amino acid polypeptide with an RNA-binding motif on the N-terminal and an *S*-adenosyl-L-methionine (SAM)-binding domain on the C-terminal region. Although the C-terminal region of PIMT/Tgs1 binds CBP/p300 and PBP specifically, it appears that the SAM-binding domain is not necessary for its activator role through PRIP [1,2]. On the other hand, the evolutionary conserved C-terminal third of the protein carries the RNA methyltransferase activity. The functionally active yeast snRNA cap hypermethylase, Tgs1 is a 315 aa polypeptide, and the identity between the yeast protein and the C-terminal region of the human protein is 38% [3]. Interestingly, the region found to be responsible for RNA binding in PIMT has no homology region in yeast Tgs1.

In this study, we used antibody specific for PIMT/Tgs1 to show that in rat tissues and also in HeLa extracts different PIMT/Tgs1 isoforms are detectable. One isoform present in HeLa nuclear extracts is associated with a high molecular weight complex, while a smaller cytoplasmic isoform co-localizes with microtubules. We also demonstrate that down-regulation of PIMT/Tgs1 protein expression by siRNA inhibition resulted in an accumulation of HeLa cells in G2/M phase. Furthermore, we describe PIMT/Tgs1 as an interacting partner of WAIT-1 (WD repeat protein associating with integrin cytoplasmic tails), a human WD repeat protein, which has been shown to be present both in the nucleus and cytoplasm [6].

Materials and methods

Plasmid constructs. A partial cDNA of PIMT/Tgs1 (PIMT-C) coding for amino acids 555–852 was obtained as a 1500-bp EST sequence (GenBank Accession No.: AA174934). Following addition of *Eco*RI and *Bam*HI sites to the ends, this fragment was cloned into the BTM116 vector in frame with the DNA-binding region of LexA gene to generate pBTM-PIMTC plasmid. For in vitro binding experiments, an *Eco*RI–*Bgl*II fragment coding for PIMT-C was also cloned into the pGEX-4T-2 plasmid so as to generate pGEX-PIMTCGST which directs the expression of a GST fusion protein. To generate pGEX-PIMTCdGST, the region coding for GST was removed from the pGEX-PIMTC with *Bam*HI–*Bal*I digestion, then filled in, and religated. The pACT–WAIT-1 clone was recovered in yeast two-hybrid screen of human spleen cDNA library using pBTM–PIMTC as a bait. pACT–WAIT-1, which carries the complete wait1 cDNA (GenBank Accession No.: NM_003797) was used to prepare the constructs pACT–WAIT-1dc and pACT–WAIT-1dn by partial and complete *Nco*I digestion, which removed the carboxyl- and amino-terminal region of WAIT-1, respectively. To obtain pACT–WAIT-1dwd and dwd2, pACT–WAIT-1 was linearized first with *Xho*I and *Bsa*BI, respectively, then the *Xho*I digested DNA was further treated by BAL31 nuclease, and both DNAs were filled in with Klenow and religated. To construct GST–WAIT-1 fusion protein, the wait1 cDNA was inserted as a 1500-bp *Eco*RI–*Xho*I fragment into pGEX-4T-2.

Yeast two-hybrid. A human spleen MATCHMAKER cDNA library cloned in the pACT vector (Clontech) was screened using pBTM-PIMTC as bait as described by the manufacturer (Clontech). Briefly, cells of *Saccharomyces cerevisiae* strain L40 transformed with pBTM-PIMTC were 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 β-galactosidase activity. From His^+, $β\text{-}gal^+$ clones plasmids were recovered and sequenced.

In vitro protein binding assays. GST–WAIT-1 fusion protein was expressed in *Escherichia coli* BL21 and affinity purified on glutathione–Sepharose gel as recommended (Amersham–Parmacia Biotech). For in vitro interactions, glutathione–Sepharose bead suspension was mixed with GST–WAIT-1 and lysate of bacterial cells expressing PIMT-C in phosphate-buffered saline, containing 1% Triton X-100, 1 mM PMSF, and incubated for 2 h at room temperature. After extensive wash the beads were boiled in SDS sample buffer. Eluted proteins were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblot. GST protein alone was processed parallel as negative control.

Preparation of protein samples. Rat tissues samples were homogenized in buffer containing 10 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF and used immediately or frozen in liquid nitrogen and kept at −80 °C until further use. For cell extract preparation, cultured HeLa cells were washed twice with PBS, scraped, harvested, and resuspended in ice-cold buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.5 mM DTT, and 0.5 mM PMSF). Following incubation on ice for 15 min, 10% NP40 was added, vortexed for 10 s, and nuclear fraction was pelleted by centrifugation at 3000 rpm for 5 min. Supernatant was kept as cytoplasmic fraction. Pellet was resuspended in buffer B (0.42 M KCl, 20 mM Tris, pH 7.8, 1.5 mM $MgCl_2$, 10% sucrose, 2 mM DTT, and 0.5 mM PMSF), incubated on ice for 30 min, and sonicated for 15 s. Nuclear extracts were fractionated on Superose column using a Pharmacia ATKA FPLC system.

Antibody. PIMT/Tgs1-specific antibodies were raised in rabbits injected with the synthetic peptide EIPNSPHAETEVEIK (Spring Valley Laboratories). Anti-PIMT antiserum was collected and further purified by affinity chromatography. To prepare affinity matrix the GST fusion PIMT-C protein was purified on glutathione–Sepharose beads (Amersham–Pharmacia) according to Pharmacia manual and the purified protein was bound to CnBr-activated Sepharose (Amersham–Pharmacia). Anti-sera was added to a 1:20 dilution and incubated at room temperature for 3–4 h. After extensive washing, bound antibody was eluted with 100 mM glycine/HCl, pH 2.5, and immediately neutralized with the addition of 1:10 of the elution volume 1 M Tris, pH 8.0.

For immunoblots protein samples in SDS loading buffer were resolved by SDS–PAGE and blotted onto nitrocellulose according to standard protocols. Anti-PIMT/Tgs1 antibodies or preimmune sera from the same rabbit were used in 1:5000 dilution. Secondary antibody was anti-rabbit IgG1 coupled to peroxidase (1:7000) (DAKO). Immunocomplexes were visualized by chemiluminescence. For competition assay, immunoblots were performed by adding purified GST–PIMTC protein bound to glutathione together with the primary antibody. For immunocytochemistry, HeLa cells were cultured on coverslips in DMEM supplemented with 10% fetal calf serum. Cells were fixed on coverslips with 4% paraformaldehyde in PBS for 20 min 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/Tgs1 antibodies in 1:200 dilution for 2 h at 23 °C. TRITC (tetramethyl rhodamine isothiocyanate) or FITC (fluorescein isothiocyanate)-labeled secondary antibodies (Sigma) were used as appropriate for the isotype of the primary antibodies. Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole HCl) (100 ng/ml) and cells were mounted with Citofluor (Ted Pella, CA). Control experiments in the absence of fluorescent primary antibody or using pre-immunoserum instead of the primary antibody were performed for each immunolocalization experiments. Colchicine treatment was performed by incubating the cells on coverslips with 10 μl per ml colchicine in DME media for 17 h at 37 °C. Cytological analyses were done using a Nikon Eclipse TE300 fluorescence microscope equipped with SPOT RT CCD camera (Diagnostic Instruments, MI). To reach the maximum possible magnification and resolution, the majority of the images were captured using oil-immersion Plan Apo 60×/1.40 objective. Saved pictures were colored in RGB format.

RNA interference experiments were done as described by Donzé and Picard [7]. PIMT/Tgs1 specific siRNAs were transcribed by T7 RNA polymerase using the oligonucleotide-templates: si1s: ATGAATC CTGTGATGTCTCCTCTATAGTGAGTCGTATTA and si1as: ATG AGGAGACATCACAGGATTCTATAGTGAGTCGTATTA. Oligonucleotide templates to synthesize GFP-specific siRNA were as described [7]. For silencing the endogenous PIMT/Tgs1 gene, siRNA (2μ) and carrier plasmid DNA were transfected into HeLa cells grown on 6-well plates by Lipofectamine following the standard protocol. For silencing GFP expression pEGFP was co-transfected with siRNA. Efficiency of transfection was estimated by counting and FACS analysis of GFP positive cells. For flow cytometry, HeLa cells were trypsinized and washed twice in PBS. Cells were fixed and permeabilized in ice-cold ethanol (70%) on ice for 20 min. After brief centrifugation cells were stained with propidium iodide (10 μl/ml in 0.1% sodium citrate, 0.1% Triton X-100, and 10 μg/ml RNase) for 30 min. Following several washes in PBS, cells were sorted and the data analyzed by WinMDI and Cylcred software.

Results

DTL and PIMT/Tgs1 are related proteins with SAM-binding domain

Using the HIV TAR sequence as target in a bacterial in vivo screen similar to those described by Harada et al. [8], we isolated a number of *Drosophila* cDNA fragments encoding putative RNA-binding proteins. Since one of the identified *Drosophila* proteins showed limited resemblance to both the RNA binding and core region of HIV Tat, we designated it DTL (*Drosophila* Tat-like) and initiated studies to uncover the possible function of this protein. In addition to the RNA-binding motif DTL also contains well-conserved amino acid blocks characteristic for RNA methyltransferases. Since the function of DTL was completely unknown, we conducted in silico searches to identify homologues in other organisms. These searches recovered a number of putative protein sequences, each characterized by the presence of SAM-binding domain (Fig. 1). Extending from yeast to human, these putative proteins share conserved boxes of amino acids characteristic of RNA methyltransferases. Not only are these protein-coding genes preserved, but the presence of large number of ESTs in databases indicates that they are extensively expressed as well. Since the amino acid similarities shared by DTL and its relatives are located in the C-terminal half of the protein, we focused our attention on this region of the mammalian homologue. First, we isolated a partial cDNA clone, expressed a truncated version of the protein in bacteria, and raised polyclonal antibodies against the EIPNS-PHAETEVEIK peptide sequence, located at the methyltransferase homology region. The specificity of the raised antibody was tested on the bacterially expressed truncated and GST–PIMT fusion proteins by Western blots (data not shown). Through antibody titration and competition experiments (Fig. 2A), we established that the raised antibody specifically recognized the C-terminal part of the human homologue of DTL. Interestingly, polyclonal antibody raised against the C-terminal half of the *Drosophila* protein also interacted specifically with the mammalian protein. While these studies were in progress, the identification of the mammalian homologue of DTL as PIMT and Tgs1 was reported by two groups independently [1,3]. Therefore, for clarity we will refer to this, protein as PIMT/Tgs1.

Different isoforms of PIMT/Tgs1 are expressed in rat tissues

In order to determine the tissue distribution of PIMT/Tgs1 protein tissue homogenates were prepared from different rat tissues and equal amounts of protein samples were resolved on SDS–PAGE and analyzed on Western blots. Immunoblots developed by anti-PIMT/Tgs1 antibody showed a 55-kDa protein in all tissue samples examined, while a 90-kDa protein was observed only in brain and testicular homogenates. Moreover, in some preparations a 30-kDa protein interacting specifically with the antibody was detected in kidney samples. Probing the filter with pre-immune sera collected from the same rabbit before immunization did not reveal any non-specifically interacting protein (Fig. 2B). We also wanted to analyze the cellular localization of PIMT/Tgs1 and because of the high degree of similarity between the mouse and human protein for this we chose HeLa cells. First, by Western blots we established that in HeLa cells too, different isoforms of PIMT/Tgs1 can be detected. Subsequently, nuclear and cytoplasmic extracts of HeLa cells were prepared and analyzed by Western blots (Fig. 2C). These experiments revealed that a smaller, 55-kDa isoform of PIMT/Tgs1 is localized mainly in the cytoplasm of HeLa cells. In contrast with that, a larger isoform is found in much smaller quantities almost exclusively in the nuclear fractions. Further fractionation of HeLa nuclear extracts on Superose column suggested that this latter form of the protein is part of 600 kDa complex (Fig. 2D).

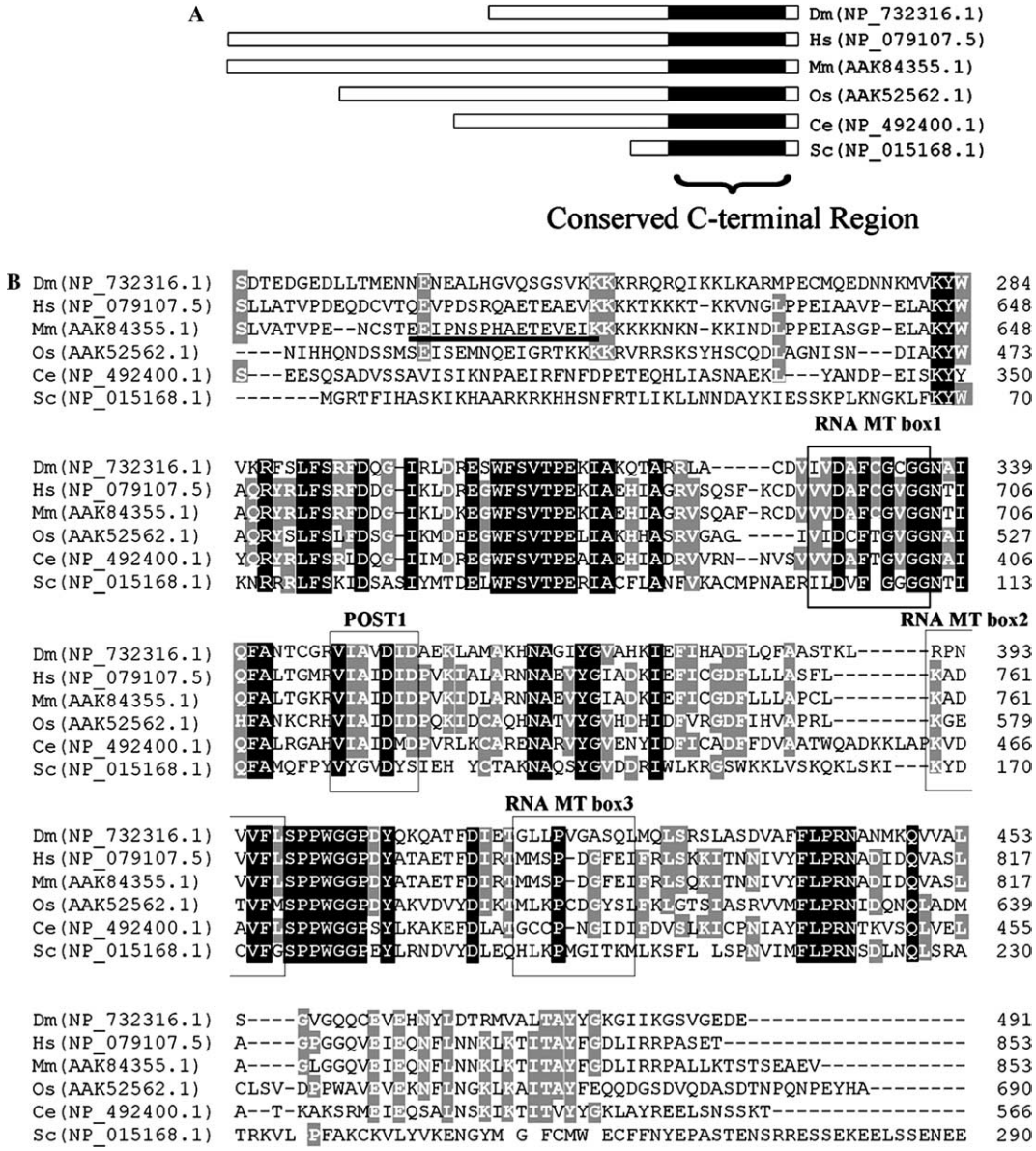

Fig. 1. Alignment of DTL-related putative protein sequences from different organisms. (A) Schematic structure of DTL homologues. Amino acid sequences of DTL homologues in various species were extracted from the GenBank, EMBL, DDBJ, and Genome Sequencing Center databases by iterative searches using the standard BLAST program and DTL as query. The amino acid one-letter code is used. The various DTL-related sequences are as follows: *Drosophila melanogaster* (Dm) DTL (CG31241-PA) (Accession No.: NP_732316.1), *Homo sapiens* (Hs) PIMT (Accession No.: NP_079107.5), *Mus musculus* (Mm) (Accession No.: AAK84355.1), *Oryza sativa* (Os) (Accession No.: AAK52562.1), *Caenorhabditis elegans* (Ce) T08G11.4.p (Accession No.: NP_492400.1), and *Saccharomyces cerevisiae* (Sc) Ypl157wp (Accession No.: NP_015168.1). (B) Conserved C-terminal regions of DTL homologues. Multiple alignment was performed with Multalin program. Residues conserved amongst all DTL homologues have a black background, residues conserved in 4 out of the 6 of the homologues have a light gray background. The highly conserved RNA methyltransferase (RNA MT) domains and *S*-adenosyl-L-methionine interacting region (POST1) are boxed. The sequence of oligopeptide used for immunization to generate PIMT/Tgs1 specific antibody is underlined.

Fifty-five kilodalton isoform of PIMT/Tgs1 localized to the cytoskeleton

The cytoplasmic localization of the 55-kDa PIMT/Tgs1 protein was a surprising result. Hence, we wished to investigate the cellular localization of PIMT/Tgs1 with another method. To achieve this we visualized the distribution of PIMT/Tgs1 in HeLa cells by immunohistochemistry. The antiserum used in these experiments was affinity purified against bacterially expressed PIMT/Tgs1. In fixed HeLa cells, PIMT/Tgs1 was detected in the cytoplasm showing a distribution similar to that of the microtubule cytoskeleton (Fig. 3). Double immunolocalization experiments with anti-tubulin and PIMT/

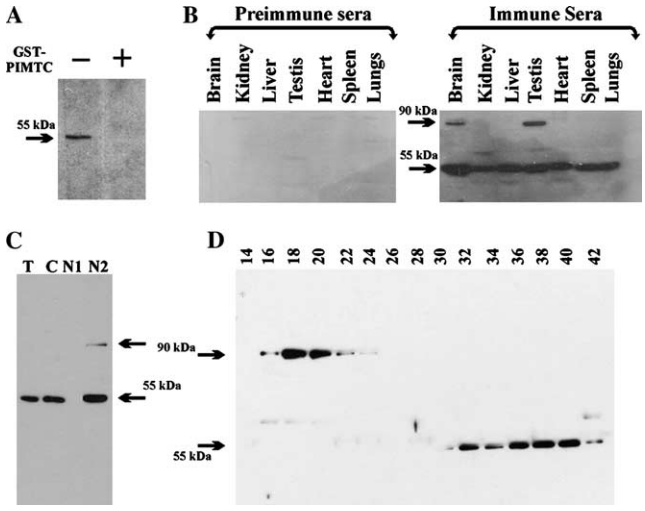

Fig. 2. Different PIMT/Tgs1 isoforms are expressed in rat tissues and in HeLa cells. (A) Competition experiments with bacterially expressed, purified GST–PIMTC protein to demonstrate the specificity of anti-PIMT/Tgs1 antibody. HeLa total cell extracts were resolved on 10% SDS–PAGE and blotted with anti-PIMT/Tgs1 polyclonal sera. On line "+" PIMT/Tgs1 anti-sera were preincubated with Sepharose–glutathione-bound purified GST–PIMTC as described in Materials and methods. (B) A 55-kDa PIMT/Tgs1 protein is expressed ubiquitously in rat. Protein extracts of different rat tissues as indicated were resolved on 10% SDS–PAGE and blotted with anti-PIMT/Tgs1 antibody (Immune) or with pre-immune (Preimmune) sera taken from the same rabbit before immunization. Arrow indicates a ubiquitously expressed 55 kDa protein interacting specifically with PIMT/Tgs1 antibody. A 90-kDa form present in brain and testis samples is also indicated. (C) PIMT55 localized to the cytoplasm in HeLa cells. Total cell (t), cytoplasmic (c), and nuclear (n1 and n2) extracts of HeLa cells were separated on 12% SDS–PAGE and blotted with anti-PIMT/Tgs1 antibody. On lane n2, a concentrated nuclear extract was separated. (D) Nuclear extract of HeLa cells was separated on Superose gel filtration column. Aliquots of every second fractions were electrophoresed in 8% SDS–PAGE and blotted with anti-PIMT/Tgs1 antibody. Calibration of the columns (not shown) indicated that fractions 16–20 containing the peak of the 90-kDa PIMT/Tgs1 protein correspond to the elution of 600 kDa complexes.

Tgs1 antibodies also indicated overlapping patterns. To further prove that this pattern was identical to the one shown by the microtubule cytoskeleton, cells were treated with the microtubule depolymerizing agent, colchicines, for 17 h before the addition of primary antibodies. This resulted in the disruption of the pattern formed by both the anti-tubulin and anti-PIMT antibodies (data not shown). Thus, from these experiments we concluded that the 55-kDa isoform of PIMT/Tgs1 colocalizes with the microtubule cytoskeleton.

Inhibition of PIMT/Tgs1 protein expression results in block in G2

Dtl is an essential gene in *Drosophila* (our unpublished observation), while in yeast Tgs1 mutation results in cold sensitive splicing defect [3]. Since there are no data available on the effect of PIMT/Tgs1 mutation in mammalian cells, we wanted to test the effect of the inhibition of PIMT/Tgs1 protein production in HeLa cells. To achieve this, we used the siRNA approach to downregulate transiently PIMT/Tgs1 expression. Double-stranded siRNAs targeted against PIMT/Tgs1 were synthesized by T7 RNA polymerize as described by Donzé and Picard [7]. Oligonucleotides were designed so as to target specifically the conserved regions of PIMT/Tgs1. Annealed siRNAs were introduced into HeLa cells by Lipofectamine, and cells were harvested 24 and 40 h later for FACS analysis to determine the fraction of cells in different stages of the cell cycle. In order to estimate the efficiency of transfection and also that of siRNA inhibition, a plasmid construct directing GFP expression (pEGFP-N1, Clontech) and GFP-specific siRNAs was used in parallel experiments. By cotransfecting pEGFP-N1 and GFP-specific siRNA, we established that the RNA inhibition in the transfected cells reached almost 100%; however, the transfection efficiency in our experiments was routinely 30–50%. The level of expressed PIMT/Tgs1 protein as estimated by immunoblot analysis in PIMT/Tgs1 siRNA transfected cell cultures was approximately 50% of the level observed in parallel samples transfected with GFP-specific siRNA. Based on the estimated efficiencies of siRNA inhibition and transfection, we concluded that the residual PIMT/Tgs1 protein observed by immunoblot originated from the untransfected cells (Fig. 4A). FACS analysis of PIMT/Tgs1 siRNA transfected samples indicated that downregulation of PIMT/Tgs1 protein level resulted in a twofold increase in the proportion of HeLa cells in G2/M phase (Fig. 4B). By contrast, control samples transfected only with the GFP-specific siRNA samples showed no significant change in the proportion of cells at different phases of the cell cycle when compared to mock transfected cells.

PIMT interacts with WAIT-1 via its C-terminal region

In order to identify interacting partners of PIMT/Tgs1, we carried out yeast two-hybrid screen using the C-terminal region (amino acids 555–852) of mouse PIMT/Tgs1 protein as a bait against a human hematopoietic cDNA library. Mouse PIMT/Tgs1 and human PIMT/Tgs1 have 83% identity and 87% similarity in this region. The strongest interactions were detected with cDNA clones encoding WAIT-1, which we recovered in several independent plasmids. Negative controls with unrelated cDNA clones indicated that the PIMT–WAIT-1 interaction was specific.

To identify interacting region(s) of WAIT and PIMT/Tgs1, we created a series of mutations in the WAIT-1 and PIMT/Tgs1 cDNA. The tested WAIT-1 constructs included WAIT-1dc, a carboxyl-terminal deletion which removed all WD40 domains; WAIT-1dn, an amino terminal deletion that partially removed the 1st WD40

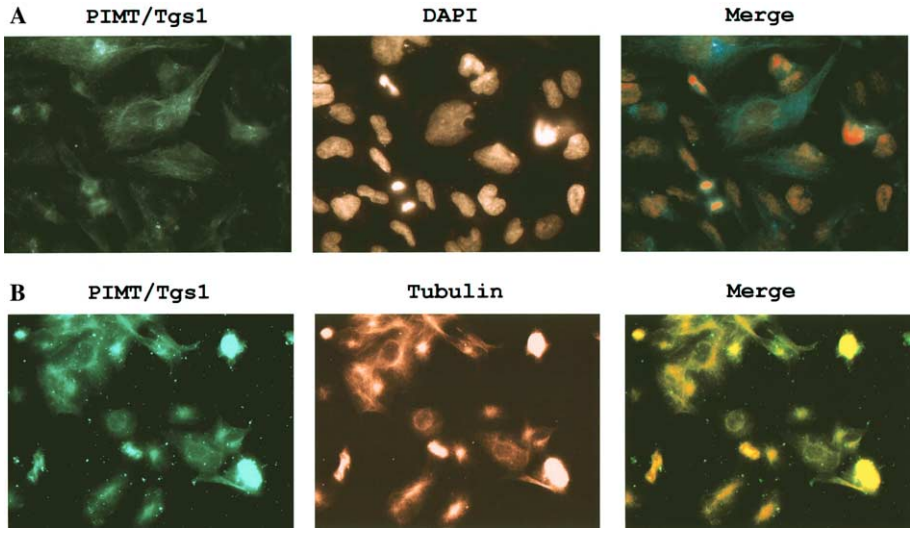

Fig. 3. Detection of PIMT protein in HeLa cells by immunohistochemistry. (A) Anti-PIMT antibody affinity purified on GST–PIMTC matrix was used to detect PIMT/Tgs1 protein in HeLa cells. PIMT/Tgs1 staining, DAPI staining of DNA, and merged pictures are as indicated. (B) PIMT/Tgs1 colocalizes with tubulin in the cytoplasm. Immunostaining of PIMT/Tgs1, tubulin, and the merged image is shown.

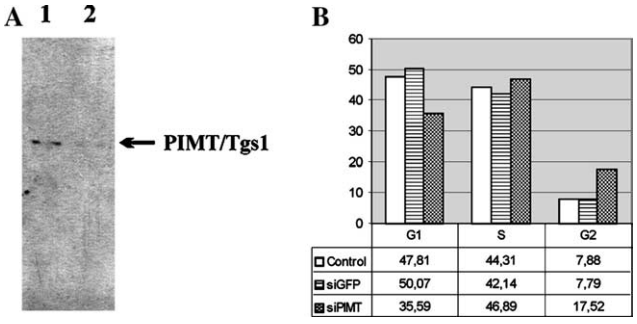

Fig. 4. Downregulation of PIMT/Tgs1 protein interferes with cell cycle progression. (A) Western blot of HeLa total extract prepared from cells treated with siRNA specific for GFP (1) or PIMT/Tgs1 (2). The Western blot was developed with anti-PIMT/Tgs1 antibody. (B) Distribution of untreated, GFP-specific, and PIMT/Tgs1-specific siRNA treated HeLa cells in different stages of cell cycle. Cells were collected 48 h following transfection stained with propidium iodide as described in Materials and methods and analyzed for DNA content. The graph shows percentage of cells in the indicated stage of cell cycle.

domain; WAIT-1dwd2, a deletion that removed the entire 3rd WD40 domain and partially the 1st and 4th; and WAIT-1dwd, which partially deleted the 5th WD40 domain. All mutations generated in WAIT-1 gave negative results in β-galactosidase assays. From these results, we concluded that any mutation which disturbed the propeller β shape of WAIT-1 also abolished its interaction with PIMT/Tgs1. On the other hand, by constructing deletions in PIMT/Tgs1 we localized the WAIT-1 interacting region of PIMT/Tgs1 to within a 91 amino acid-long region between amino acids 555 and 641 (Fig. 5A).

To obtain further experimental support for PIMT/Tgs1-WAIT-1 interaction, we carried out GST pull-down experiments. For this, WAIT-1 was expressed as a GST fusion protein and mixed with bacterial extract prepared from cells which express the C-terminal region of PIMT/Tgs1 (amino acids 555–852). The resulting complexes were bound to glutathion–Sepharose, washed extensively, eluted, and analyzed on Western blots. As shown in Fig. 5B, a significant amount of PIMT/Tgs1 was retained on GST–WAIT-1 containing matrixes, while the controls (matrix alone and GST-bound matrix) did not significantly bind PIMT/Tgs1. Thus, the GST pull-down experiment provided further support to the observed interaction between WAIT-1 and the C-terminal of PIMT/Tgs1 as detected by our yeast two-hybrid screen.

Discussion

By in silico searches, we identified in a wide spectrum of organisms putative homologues of a newly identified *Drosophila* protein with RNA and SAM-binding domains. The large number of EST sequences corresponding to these proteins indicate that their mRNAs are expressed throughout evolution. Each of the putative proteins is characterized by the presence of a SAM-binding domain and other motifs with resemblance to RNA methyltransferases. The human homologue of DTL has been identified recently by two laboratories as PIMT and Tgs1, a nuclear receptor coactivator interacting partner and a methyltransferase responsible for the hypermethylation of the cap of small non-messenger RNAs, respectively [1–4]. In addition, the *Caenorhabditis elegans* homologue T08G11.4.p when inactivated by siRNA was reported to result in mitotic spindle

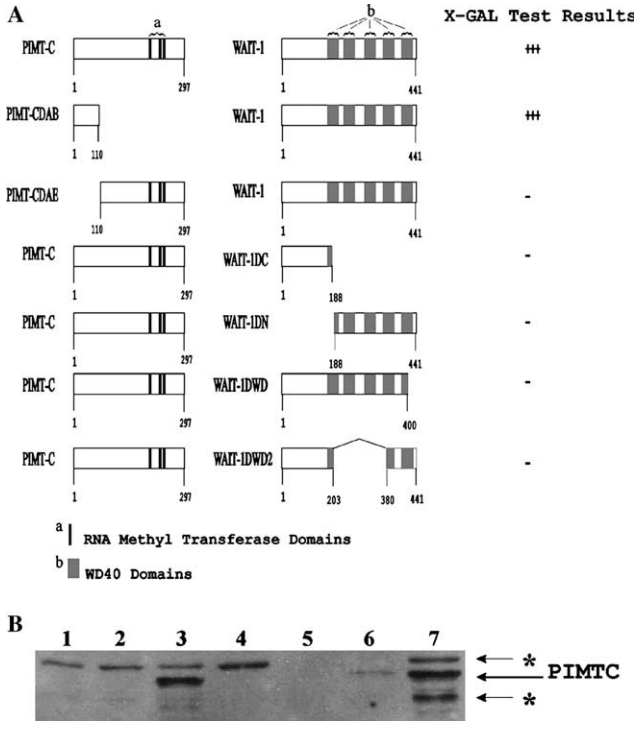

Fig. 5. PIMT/Tgs1 interacts with WAIT-1 both in yeast two-hybrid and in vitro pull down experiments. (A) Mapping of the interaction regions of PIMT/Tgs1 and WAIT-1. The schematic structure of PIMT/Tgs1 and WAIT-1 proteins. The RNA methylase, SAM-binding domains, and the WD repeats, respectively, are depicted. The amino acid regions encoded by the used plasmid constructs are labeled. On the right side, β-galactosidase activity units measured in liquid cultures of yeast cells co-expressing the two plasmids are shown. (B) In vitro interaction between GST–WAIT-1 and PIMT/Tgs1. Western blot developed with PIMT/Tgs1 specific antibody. Lanes 1, 2, and 3 contain control bacterial cell extracts used in the pull down experiment: 1, GST; 2, GST–WAIT-1; and 3, PIMTC. Lanes 4 and 7 contain proteins eluted from GST–WAIT-1 affinity matrix which was incubated with PBS (4) or with PIMTC (7). Lanes 5 and 6 are controls for nonspecific binding of PIMTC to the matrix. Retention of PIMTC protein on Sepharose beads (lane 5) and on GST–Sepharose matrix (lane 6). PIMTC and a bacterial protein interacting non-specifically are shown by arrows.

misalignment, slow growth, and larval lethality [9]. The proposed diverse roles of PIMT/TGS1 suggest that identical or different forms of the protein are functioning in different compartments of the cell. In order to learn more about these various forms of protein, we isolated a partial cDNA of the mouse homologue of PIMT/TGS1, obtained specific antibodies against the protein, and studied its localization and interaction with other factors.

Rabbit polyclonal antibody generated against an internal peptide in the C-terminal region of PIMT/TGS1 detected different isoforms of PIMT/Tgs1 in different rat tissues. We detected a 90-kDa protein in brain and testicular tissues. In contrast, a shorter protein of 55-kDa was ubiquitously found in all tissues examined. Moreover, in some experiments an even shorter protein was seen in kidney samples. This prompted us to speculate that different isoforms of PIMT/Tgs1 might have different tissue distribution. Indeed, separation of HeLa extracts showed that the 55-kDa isoform was mainly a cytoplasmic protein, while a larger form was exclusively in the nuclear fraction. The simplest explanation for the existence of different forms of PIMT/Tgs1 would be that the smaller forms resulted from nonspecific degradation of the larger one(s). However, the sharp distribution of the different forms between the nuclear and cytoplasmic fractions makes this explanation unlikely and suggests that the 55- and 90-kDa forms are genuine isoforms of PIMT/TGS1. Whether the 30-kDa protein observed in some samples is a degradation product of larger precursors remains to be clarified.

Our results on the different isoforms and divergent localizations of PIMT seemingly contradict data published by Zhu et al. [1] who identified PIMT as a 95-kDa nuclear protein. However, several arguments can be put forward to resolve the discrepancies. First, Zhu et al. calculated the molecular mass of PIMT based on the amino acid sequence deduced from the cloned cDNA; second, the expression of PIMT as a 95-kDa protein was verified in an in vitro translation system; third, to demonstrate the cellular localization of PIMT these authors expressed the protein in transiently transfected Cos cells, with a FLAG epitope attached to its C-terminal. In light of these, it could well be that the 55-kDA isoform of PIMT/Tgs1 we described here was not identified earlier. On the other hand, it may be that full length PIMT/Tgs1 is expressed at a much lower level than its smaller isoform which is found in abundance, docked onto microtubules. Our finding that the higher molecular weight form of the protein is present in the nucleus of HeLa cells in much smaller quantity than its 55 kDa cytoplasmic counterpart supports this notion. Antibodies raised against the C-terminal RRPALLKTSTSEAEV peptide of PIMT/TGS1 also interact specifically with a 55-kDa protein present in the cytoplasmic fraction of different cell lines (K.T. Jeang, data not shown). The function and cellular distribution of Tgs1 as described by Mouaikel et al. [3,4] is in good agreement with the existence of the different isoforms of the protein we propose here. These authors pointed out that the human Tgs1 gene has complex structure which could well result in several alternatively spliced products of the protein, and they also noted, although not explicitly discussed, that in their immunolocalization experiments some signal was detected in the cytoplasm. Furthermore, the notion that hypermethylation of the cap structure of mammalian snRNAs takes place in the cytoplasm is well documented [5]. Consequently, it is conceivable that one isoform of PIMT/TGS1 plays a role in transcriptional regulation and RNA maturation in the nucleus, while a shorter isoform with the methyltransferase domain is involved in snRNA hypermethylation in the cytoplasm. The cytoplasmic localization of PIMT55 is not controversial in light of its

interaction with WAIT-1. WAIT-1 is associated with the cytoplasmic tail of integrins [6]. β7-integrins play an important role in physiological functions and pathological alterations of the immune system [10]. The cytoplasmic tails of the β7 subunit are critical for integrin function and several interactors of integrin cytoplasmic tails have been described, which may either link integrins to the cytoskeleton or may exert regulatory functions for integrin adhesion and signaling. The human WAIT-1 and murine EED (embryonic ectoderm development) are identical proteins, each containing five sequence motifs found in WD repeats [11]. Members of the WD repeat protein family have been found to be involved in major cellular processes such as transport, signaling, regulation of gene expression, and cell division [12]. In accordance with this, EED and WAIT-1 have been implicated in different processes. The murine EED was proposed to function as a transcriptional regulator of homeobox genes based on developmental abnormalities of mutant mice [13,14]. Similarly, the related *Drosophila* protein ESC belongs to the Polycomb group proteins and is involved in maintaining the repressed state of silenced genes [15,16]. This shows that EED/WAIT-1 by itself interacts with different types of proteins and may localize to the nucleus but may stay in the cytoplasm as well. Based on the observed interaction of PIMT/Tgs1 with WAIT-1 and its co-localization with microtubules, we suggest that proteins with RNA methyltransferase domain like PIMT/Tgs1 and its relatives participate in cellular processes both in the nucleus and the cytoplasm, linking gene regulation and cell-growth, and/or cell cycle regulation. In support of this notion, we observed that inhibition of PIMT/TGS function in higher eukaryotes interfered severely with cell cycle progression. Tgs1 mutations in *C. elegans* and *Drosophila* result in larval lethality ([9] and our unpublished data) and downregulation of PIMT/Tgs1 in mammalian cells disturbed cell cycle progression.

Acknowledgments

We thank Katalin Ökrös for her expert technical help. This work was supported by the Hungarian Science Fund (OTKA Grant No. T29939) and Hungarian Ministry of Education (Grant No. FKFP0060/2000).

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