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Human polypyrimidine tract-binding protein interacts with mitochondrial tRNA^{Thr} in the cytosol

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ABSTRACT

Human polypyrimidine tract-binding protein PTB is a multifunctional RNA-binding protein with four RNA recognition motifs (RRM1 to RRM4). PTB is a nucleocytoplasmic shuttle protein that functions as a key regulator of alternative pre-mRNA splicing in the nucleoplasm and promotes internal ribosome entry site-mediated translation initiation of viral and cellular mRNAs in the cytoplasm. Here, we demonstrate that PTB and its paralogs, nPTB and ROD1, specifically interact with mitochondrial (mt) tRNA^{Thr} both in human and mouse cells. *In vivo* and *in vitro* RNA-binding experiments demonstrate that PTB forms a direct interaction with the T-loop and the D-stem-loop of mt tRNA^{Thr} using its N-terminal RRM1 and RRM2 motifs. RNA sequencing and cell fractionation experiments show that PTB associates with correctly processed and internally modified, mature mt tRNA^{Thr} in the cytoplasm outside of mitochondria. Consistent with this, PTB activity is not required for mt tRNA^{Thr} biogenesis or for correct mitochondrial protein synthesis. PTB association with mt tRNA^{Thr} is largely increased upon induction of apoptosis, arguing for a potential role of the mt tRNA^{Thr}/PTB complex in apoptosis. Our results lend strong support to the recently emerging conception that human mt tRNAs can participate in novel cytoplasmic processes independent from mitochondrial protein synthesis.

INTRODUCTION

Polypyrimidine tract-binding protein (PTB or PTBP1) is an abundant multifunctional RNA-binding protein implicated in various aspects of cellular mRNA metabolism, including pre-mRNA splicing and polyadenylation, mRNA export, stability and translation initiation (1,2). PTB carries four RNA recognition motifs (RRM1 to RRM4) with distinct RNA-binding properties (3,4). Human PTB has two paralogs, nPTB (PTBP2) and ROD1 (PTBP3), which in contrast to the generally expressed PTB, accumulate in a fairly tissue-restricted manner. nPTB shows the most efficient accumulation in brain, muscle and testis, while ROD1 is preferentially expressed in hematopoietic cells (5,6).

PTB is a nucleocytoplasmic shuttle protein with predominant nucleoplasmic accumulation (7,8). The major function of nuclear PTB is in controlling alternative exon selection during pre-mRNA splicing (1,9). Through binding to CU-rich intronic or exonic pre-mRNA sequences, PTB induces exon skipping or less frequently, exon inclusion depending on the actual sequence context (10–12). PTB has been also proposed to modulate pre-mRNA 3′ end processing and polyadenylation upon binding to 3′ UTR sequences (13). In the cytoplasm, PTB has a well-documented role in promoting internal ribosome entry site (IRES)-mediated mRNA translation initiation (14). PTB is considered to be a general IRES trans-acting factor that binds to specific structures in the 5′ untranslated region (UTR) of mRNAs and promotes recruitment of the translation initiation machinery to IRESs (15). PTB promotes IRES-mediated translation of both viral and cellular mRNAs under stress conditions, including viral infection and apoptosis, which inhibit cap-dependent translation initiation (2). Through binding to the 3′ UTR of mRNAs, PTB can control the cytoplasmic stability and localization of mRNAs (16–18) or it can regulate mRNA translation through promoting or suppressing microRNA (miRNA) binding (19–21).

Mitochondria are essential membrane-bound cytoplasmic organelles which produce cellular ATP by oxidative phosphorylation and control intrinsic apoptosis. The human mitochondrial genome encodes 13 mitochondrial proteins dedicated to oxidative phosphorylation, two mitochondrial rRNAs (12S and 16S) and the minimal set of 22 mitochondrial tRNAs (mt tRNAs) necessary and sufficient for mitochondrial protein synthesis (22). In other eukaryotes, the mitochondrial genomes frequently lack a few or sometimes most tRNA genes. Moreover, the mitochondrial DNAs of certain species are even completely devoid of tRNA genes. In these cases, mitochondrial protein synthesis is supported by nuclear-encoded tRNAs imported from

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the cytoplasm (reviewed in (23,24). Interestingly, it has been found that human cytoplasmic (cyt) $tRNA^{Gln}$ and yeast cyt $tRNA^{Lys}$ are efficiently imported into the mitochondria, albeit both human and yeast mitochondria encode the complete set of mt tRNAs required for mitochondrial protein synthesis (25–27). This suggests that the molecular mechanism supporting mitochondrial importation of cyt tRNAs is preserved during evolution.

Although the major function of mt tRNAs is in mitochondrial protein synthesis, recent observations have raised the intriguing possibility that mt tRNAs may play novel, highly unexpected roles in the cytoplasm. First, human argonaute-2 (Ago2) protein, a key component of the RNA-induced silencing complex (RISC), has been reported to specifically interact with mt $tRNA^{Met}$ exported into the cytoplasm (28). More recently, human mt tRNAs have been demonstrated to participate in controlling apoptosis in the cytosol (29). Intrinsic apoptotic signals promote mitochondrial membrane permeabilization to release mitochondrial pro-apoptotic proteins, including the apoptosome activator cytochrome *c* (Cyt *c*) (30). It has been found that mt tRNAs bind to Cyt *c* and block its interaction with the cytoplasmic protease activating factor 1 (Apaf-1) in order to inhibit Apaf-1-mediated caspase activation and apoptosis (29). Thus, mt tRNAs act as negative regulators of apoptosis (31).

Here, we demonstrate that human polypyrimidine tract-binding proteins PTB, nPTB and ROD1 bind with great specificity and efficiency to mt $tRNA^{Thr}$ in various human and mouse cell lines. Through utilizing its RRM1 and RRM2 domains, PTB forms a direct interaction with the T-loop and the D-stem-loop regions of mt $tRNA^{Thr}$. We also demonstrate that PTB and mt $tRNA^{Thr}$ interaction occurs in the cytoplasm outside of mitochondria and it is increased upon induction of apoptosis. Thus, besides providing support to the emerging view that mt tRNAs may possess novel cytoplasmic functions, our results also point to a possible role of PTB and mt $tRNA^{Thr}$ association in controlling intrinsic apoptosis.

MATERIALS AND METHODS

General procedures, oligonucleotides and cell cultures

Unless stated otherwise, standard laboratory procedures were used for manipulating DNA, RNA, oligonucleotides and proteins (32). Oligonucleotides listed in Supplementary Data were synthesized by Eurofins MWG. Human HeLa, HEK293, MRC5 and fibroblast cells and mouse NIH 3T3 cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (Invitrogen). Apoptosis was induced by treatment of HeLa cells with 1 μM staurosporine (Sigma) for 4 h and caspase activity was inhibited with 60 μM of z-DEVD-FMK (Santa Cruz) added 1 h before staurosporine administration. Expression plasmids (jetPRIME, Ozyme), siRNAs and *in vitro* transcribed mt tRNAs (Lipofectamine 2000, Invitrogen) were transfected into HeLa cells by using the indicated transfection reagents as recommended by the suppliers.

Plasmid construction

To generate pPTB, pPTBd1, pPTBd2, pPTBd3, pPTBd4, pPTBd5, pPTBd3*m* pPTBd3*b,* pPTBd3*m*+*b*, pnPTB and pROD1 expression constructs, the entire coding regions or appropriate fragments of the coding regions of human PTB, nPTB and ROD1 genes were PCR-amplified using wild-type and mutant PTB and codon optimized nPTB and ROD1 cDNA templates (33). The amplified fragments were inserted into the NotI–BamHI (PTB and ROD1) or into the NotI–KpnI sites (nPTB) of the p3xFLAG expression plasmid (Sigma) by using PCR-introduced restriction sites. The identity of the expression plasmids was verified by sequence analysis.

Immunoprecipitation and protein analyses

Extract preparation and immunoprecipitation (IP) have been performed essentially as described before (34). Briefly, about 9×10^6 cells were suspended in 500 μl of ice-cold NET-150 buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.05% Nonidet P40) and sonicated 5×30 s with 30 s intervals with a Bioruptor Plus Sonicator (Diagenode) at high setting. Cell homogenates were clarified by centrifugation at 16 000 × g for 10 min. For IP, 15 μl of packed FLAG MS2 beads (Sigma) or 20 μl of packed protein G agarose beads (Millipore) coupled with 5 μl of mouse monoclonal BB7 PTB antibody (provided by Drs C. Smith and D. Black) were incubated with 500 μl of cell extract for 1 h at 4°C with gentle agitation. Beads were washed 3 times with NET-150 buffer. A monoclonal antibody against human PTB (ThermoFisher Scientific, 32-4800) that cross-reacts with rodent PTBs was used for IP of mouse PTB. *In vivo* RNA–protein cross-linking and IP was performed according to (35), except that the RNA IP (RIPA) and wash buffers contained 0.2% SDS. For western blot analysis, the following antibodies were used in the indicated dilutions: mouse monoclonal BB7 PTB (1:1000), rabbit nPTB (1:500) (provided by Dr D. Black), FLAG HRP (1:100,000) (Sigma), rabbit Erp72 (1:1000) (Euromedex), mouse tubulin (1:1000) (Sigma), mouse HSP60 (1:500) (Sigma), mouse TOM20/clone 29 (1:1000) (BD Biosciences), rabbit PARP (1:1000) (Abcam), mouse hnRNPA1 (1:1000) (Santa Cruz), mouse ATP5A1 (1:1000) (Invitrogen) and mouse cytochrome *c* (1:5000) (DB Pharmigen).

RNA analyses

HeLa cellular RNAs were purified by phenol–chloroform extraction. After IP reactions, RNAs were recovered from the beads by proteinase K treatment followed by phenol–chloroform extraction. For northern blot analysis, purified RNAs were size-fractionated on a 6% denaturing polyacrylamide gel and electroblotted onto a Hybond-N nylon membrane (GE Healthcare). The immobilized RNAs were probed with 5′-terminally labeled sequence-specific oligodeoxynucleotides. RNA 3′ end labeling with [5′-^{32}P] pCp and T4 RNA ligase (Thermo Scientific) and direct chemical sequencing of RNA have been described (36). RNA 3′ end race experiments have been performed as described before (37).

Recombinant PTB protein purification

The bacterial expression vector pHis-PTBpQE9 (provided by Dr C. Smith) encoded a truncated version of human PTB lacking its N-terminal 54 amino acids and carrying a 6xHis tag. *E. coli* M15 cells harboring pHis-PTBpQE9 were grown until OD_{600} reached 0.6–0.8 and expression was induced by treatment with 1 mM IPTG for 2 h. Cells were collected and lysed in 300 mM NaCl, 50 mM NaH_2PO_4 (pH 8.0) in the presence of EDTA-free protease inhibitor cocktail (Roche). The expressed His-tagged PTB was affinity-selected by His-Pur Ni-NTA Resin (Thermo Scientific), eluted with 250 mM and 350 mM imidazole in 300 mM NaCl, 50 mM NaH_2PO_4 (pH 8.0) supplemented with EDTA-free protease inhibitor cocktail (Roche) and dialyzed against buffer E (10 mM HEPES pH 7.9, 3 mM $MgCl_2$, 5% glycerol, 1 mM DTT). The purified His-PTB was at least 80% pure as verified by coomassie staining.

In vitro RNA transcription

DNA templates for *in vitro* synthesis of internally labeled or cold wild-type and mutant mt $tRNA^{Thr}$, mt $tRNA^{Asp}$ and cyt $tRNA^{Thr}$ or a 78 nt-long intronic fragment of the c-src pre-mRNA carrying PTB binding site (3) were generated by PCR amplification by using appropriate primers incorporating the T7 promoter. *In vitro* RNA synthesis was performed with T7 RNA polymerase (Promega) in the presence or absence of 50 μCi of [α-^{32}P]-CTP (specific activity: 30–40 Ci/mmol). The RNA products were purified on a 6% sequencing gel.

Electrophoretic mobility shift assay

One fmol of internally [α-^{32}P]-CTP labeled RNA was incubated with 22.5, 45, 150 and 450 nM of recombinant His-PTB in 10 mM HEPES, pH 7.9, 3 mM $MgCl_2$, 5% glycerol, 1 mM DTT, 100 mM KCl, 100 ng/μl BSA (Sigma), 25 ng/μl rRNA (Roche), 5.5 ng/μl heparin (Sigma) at 30°C for 30 min. RNA–protein complexes were analyzed on a 1.5 mm thick 5% native polyacrylamide gel in 0.5 x TBE.

In vitro RNA label transfer to HeLa PTB

HeLa cell lysates corresponding to ∼80 μg of cellular proteins were incubated with 100 fmol of internally labeled mt $tRNA^{Thr}$ or cyt $tRNA^{Thr}$ in 200 μl of reconstitution buffer (100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT and 0.2 mM PMSF, 20 mM HEPES, pH 7.9) for 10 min at 30°C. After incubation with 50 μg of *E. coli* tRNA for 5 min, the mixtures were irradiated with UV light of 254 nm wavelength for 5 min from 8 cm distance using a BIO-LINK® photocross-linker apparatus (Fisher Bioblock). RNAs were degraded by treatment with 25 μg of RNase A at 37°C for 30 min. After IP, PTB was analyzed by SDS-PAGE, electroblotted onto a nitrocellulose membrane (Hybond-C Extra, GE Healthcare), and the RNA label transfer was monitored by autoradiography.

Metabolic labeling of nascent mitochondrial protein synthesis

HeLa cells were grown in a six-well plate and incubated in methionine/cysteine/glutamine-free DMEM (Life Technologies) supplemented with 10% fetal calf serum and 1× GlutaMAX™ supplement (Life Technologies) for 30 min. To block cytoplasmic translation, emetine (Sigma, 100 μg/ml final concentration) was added 5 min before administration of 300 μCi of $^{35}S^{35}S$ Cysteine/Methionine Easy tag Express labeling mixture (Perkin Elmer). After 1 h of *in vivo* protein labeling, the radioactive medium was replaced by normal DMEM and the cells were incubated for additional 10 min. Cells were washed twice in PBS and in NET-150 buffer. Proteins were quantitated by Bradford assay and equal amounts of proteins were separated (without boiling) on a 15–20% gradient SDS polyacrylamide gel and labeled mitochondrial proteins were visualized by autoradiography.

Cell fractionation

To isolate nuclei, HeLa cells were incubated in ten packed-cell volume of ice-cold MB buffer (210 mM mannitol, 70 mM sucrose, 1 mM EDTA and 10 mM HEPES, pH 7.9) for 10 min and disrupted by 25 strokes in a Dounce homogenizer (potter B). Nuclei were collected by centrifugation at 400 × g for 5 min. The crude nuclear pellet was resuspended in MB buffer and the Dounce homogenization (10 strokes) and centrifugation steps were repeated. To obtain cytoplasmic fraction, HeLa cells incubated in MB buffer as described above were disrupted by 5 Dounce strokes and the homogenate was clarified by two repeated centrifugation steps at 400 × g for 5 min. The supernatant was considered as crude cytoplasmic fraction. Centrifugation of the cytoplasmic fraction at 20 000 × g for 10 min resulted in the mitochondrial (pellet) and cytosolic (supernatant) fractions. For extract preparation, HeLa cells, the nuclear and mitochondrial fractions of HeLa cells were resuspended in NET-150 buffer, homogenised by sonication and clarified by centrifugation as described above.

RESULTS

Human polypyrimidine tract-binding proteins PTB, nPTB and ROD1 interact with mt $tRNA^{Thr}$

To detect putative PTB-associated RNAs, human PTB was immunoprecipitated from a HeLa cell extract by using a PTB-specific monoclonal antibody and recovery of PTB was confirmed by western blotting (Figure 1A, upper panel). RNAs co-precipitated with PTB were 3′ end-labeled with [5′-^{32}P]pCp and T4 RNA ligase and analyzed on a 6% sequencing gel (lower panel). In addition to a ∼165 nt-long weakly labeled RNA that likely corresponds to the U1 spliceosomal snRNA, a known target RNA of PTB (38), autoradiography detected an intensively labeled ∼70 nt-long RNA (lane 1) that was missing from the control mock IP reaction (lane 2). To confirm that PTB specifically interacts with the newly detected RNA, Flag- (FL)-tagged versions of PTB and its nPTB and ROD1 paralogs which are preferentially expressed in neuronal and hematopoietic cells, respectively, were transiently expressed in HeLa cells. The accumulating epitope-tagged FL-PTB, FL-nPTB and FL-ROD1 proteins were immunoprecipitated with an anti-Flag antibody (Figure 1B, upper panel). The co-precipitated RNAs were 3′ end-labeled and fractionated on a 6% sequencing gel (lower panel). Like endogenous

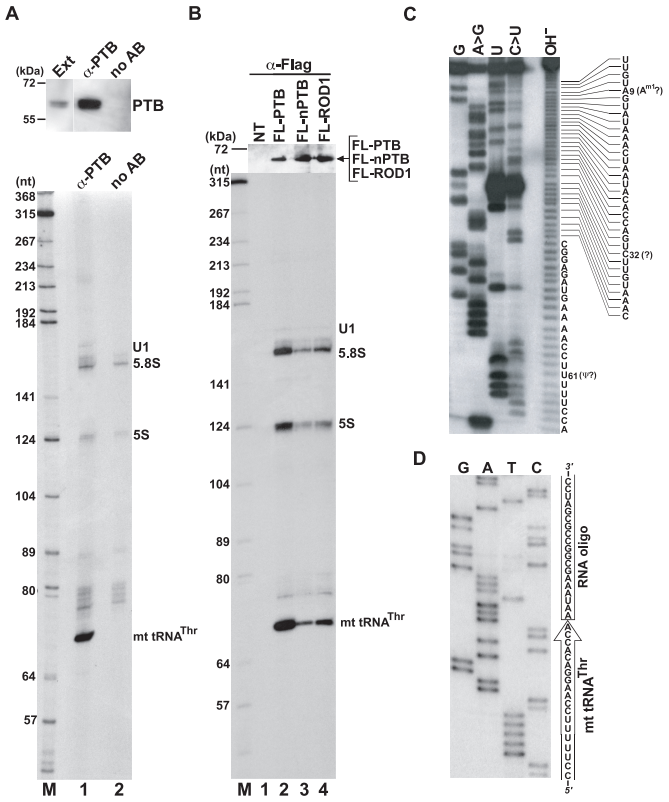

Figure 1. Human polypyrimidine tract-binding proteins PTB, nPTB and ROD1 interact with mt tRNA^{Thr}. (**A**) Detection of a novel PTB-associated RNA. RNAs co-immunoprecipitated with PTB from a HeLa extract (Ext) were 3′ end-labeled and analyzed on a 6% sequencing gel (lower panel). The 5S and 5.8S rRNAs, the U1 snRNA and mt tRNA^{Thr} are indicated. IP of PTB was confirmed by western blot analysis (upper panel). Mock IP performed without antibody (lanes no AB) and molecular size markers (M) are also shown. (**B**) RNAs co-precipitated with transiently expressed Flag-tagged PTB, nPTB and ROD1 proteins. IP was monitored by western blot analysis with anti-Flag antibody (α-Flag). NT, non-transfected cells. Other details are identical to panel A. (**C**) RNA chemical sequencing. HeLa mt tRNA^{Thr} co-immunoprecipitated with PTB was 3′ end-labeled, purified on a denaturing gel and subjected to chemical sequencing. RNA fragments were fractionated on an 8% sequencing gel. The nucleotide sequence of mt tRNA^{Thr} is indicated and numbered according to (54). A9 may correspond to 1-methyl-adenosyl (40), while U61 may be a pseudouridine, since it shows no hydrazine reactivity. The nature of the highly reactive C32 residue is unknown. Lane OH$^-$, hydrolysis ladder. (**D**) Determination of the 3′-terminal sequence of PTB-associated mt tRNA^{Thr} by 3′ end race. Sequences of the RNA tag and the 3′ end region of mt tRNA^{Thr} are indicated.

HeLa PTB, the ectopically expressed FL-PTB, FL-nPTB and FL-ROD1 proteins also interacted with a ∼70 nt-long RNA (lanes 2, 3 and 4).

To determine its identity, the ∼70 nt-long PTB-associated end-labeled RNA was extracted from the gel and subjected to direct chemical sequencing (Figure 1C). Surprisingly, the obtained partial sequence was identical to the human mt tRNA^{Thr} from position U5 to A67. The immediate 3′-terminal sequence was determined through ligation of an oligoribonucleotide tag to the RNA 3′ end followed by RT-PCR. Sequencing of the obtained cDNA confirmed that the 3′ end of PTB-associated mt tRNA^{Thr} was correctly processed and it carried the common CCA terminal nucleotides of mature tRNAs (Figure 1D). Mammalian mt tRNAs, including tRNA^{Thr}, also carry several post-transcriptionally added covalent nucleoside modifications (39). Chemical sequencing of PTB-associated mt tRNA^{Thr} detected three ribonucleosides which showed extremely high (C32) or no (A9 and U61) reactivity with base-specific sequencing reagents, suggesting that these nucleosides are post-transcriptionally modified (Figure 1C, and legends to Figure 1C). In fact, bovine mt tRNA^{Thr} has been reported to carry modified bases at least at positions A9 and C32 (40). Thus, we propose that the fraction of human mt tRNA^{Thr} that co-purifies with PTB is structurally indistinguishable from canonical mt tRNA^{Thr} participating in mitochondrial translation.

Human PTB, nPTB and ROD1 specifically associate with mt tRNA^{Thr}

IP of transiently expressed FL-PTB, FL-nPTB and FL-ROD1 recovered a HeLa RNA that co-migrated with mt tRNA^{Thr} associated with PTB (see Figure 1A and B). Indeed, northern blot analysis confirmed that the 70 nt-long HeLa RNA co-purified with ectopically expressed Flag-tagged PTB, nPTB and ROD1 corresponded to mt tRNA^{Thr} (Figure 2A, lanes 6, 7 and 8). IP of PTB from human HEK293, MRC5 and primary fibroblast cell extracts and northern blot analysis of the co-purified RNAs demonstrated that interaction of PTB and mt tRNA^{Thr} is not confined to HeLa cells (Figure 2B, lanes 3, 6 and 9). Moreover, PTB IP from a mouse 3T3 fibroblast cell extract also recovered mouse mt tRNA^{Thr}, indicating that PTB-mt tRNA^{Thr} interaction is conserved in rodents (lane 12). In contrast to mt tRNA^{Thr}, the mouse U1 snRNA showed a less obvious PTB association. The significance of the observed co-precipitation of 5S and 5.8S rRNAs with PTB, FL-PTB, FL-nPTB and FL-ROD1 remains uncertain (Figure 1A and B), but it might reflect the function of polypyrimidine tract-binding proteins played in translation regulation (see Introduction section).

RNA 3′ end labeling experiments identified mt tRNA^{Thr} as the major small RNA associated with HeLa endogenous PTB and transiently expressed FL-PTB, FL-nPTB and FL-ROD1 (Figure 1A and B). To confirm the specificity of PTB-mt tRNA^{Thr} interaction, RNAs co-immunopurified with PTB were analyzed by northern blotting with oligonucleotide probes specific for mt tRNAs and the cytoplasmic (cyt) tRNA^{Thr} (Figure 2C). Apart from mt tRNA^{Thr}, neither cyt tRNA^{Thr} nor the tested mt tRNAs were detected in the pellet of PTB IP, corroborating the notion that PTB interacts with mt tRNA^{Thr} with high specificity. Thus, we concluded that human polypyrimidine tract-binding proteins PTB, nPTB and ROD1 specifically associate with mt tRNA^{Thr}.

PTB directly interacts with mt tRNA^{Thr} in living cells

To exclude the formal possibility that PTB binds mt tRNA^{Thr} released from mitochondria damaged during extract preparation, we assayed the interaction of PTB and mt tRNA^{Thr} in living cells by *in vivo* RNA–protein cross-linking studies. Unfortunately, the available PTB antibodies failed to sustain PTB-binding under stringent IP condi-

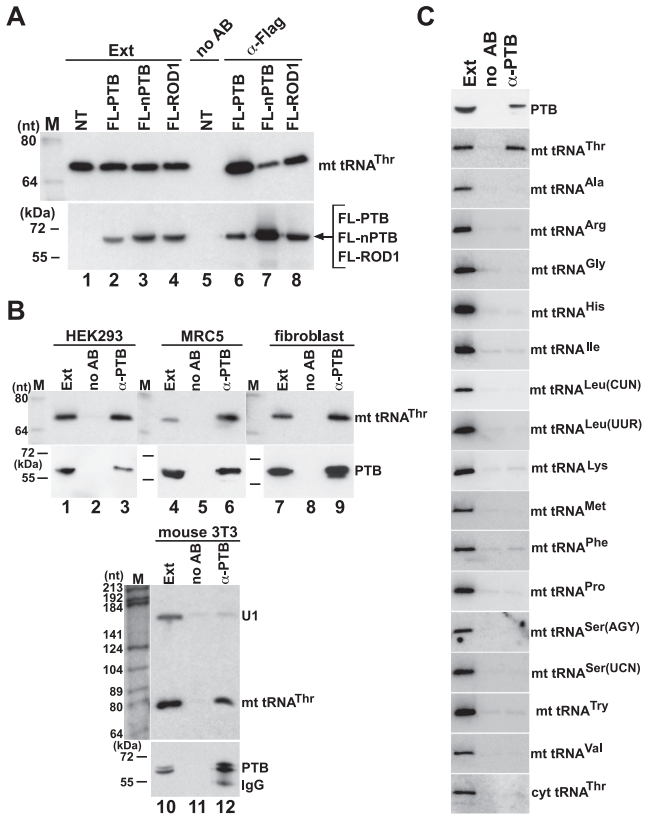

Figure 2. Human polypyrimidine tract-binding proteins specifically interact with mt tRNA^{Thr}. (**A**) Human nPTB and ROD1 interact with mt tRNA^{Thr}. Transiently expressed Flag-tagged PTB, nPTB and ROD1 were immunoprecipitated from HeLa cell extracts (Ext) with anti-Flag antibody (α-Flag). IP was confirmed by western blot analysis with anti-Flag antibody (lower panel) and co-precipitation of mt tRNA^{Thr} was monitored by northern blotting (upper panel). (**B**) Conservation of PTB and mt tRNA^{Thr} interaction in human and mouse cells. PTB was immunoprecipitated from extracts prepared from human HEK293, MRC5, primary fibroblast and mouse NIH 3T3 fibroblast cells. Co-precipitation of mt tRNA^{Thr} was monitored by northern blotting. (**C**) Northern blot analysis of RNA co-immunoprecipitated with HeLa PTB. PTB IP was confirmed by western blotting (upper panel). The oligonucleotide probes are indicated.

tions applied to destroy non-covalent RNA–protein interactions (data not shown). Therefore, Flag-tagged PTB was transiently expressed in HeLa cells. Western blot analysis showed that the ectopically expressed FL-PTB protein accumulated under the level of endogenous PTB (Figure 3A, upper panel). In order to fix *in vivo* RNA–protein interactions, the transfected cells were treated with formaldehyde before extract preparation (35). The formaldehyde treatment largely reduced the levels of soluble FL-PTB, PTB and U1 snRNA in the cell extract, but had no effect on mt tRNA^{Thr} and mt tRNA^{Lys} extraction (lane 3 and data not shown). Nevertheless, IP of FL-PTB from the cross-linked extract under harsh conditions efficiently recovered mt tRNA^{Thr}, but it failed to precipitate mt tRNA^{Lys} (lane 6). As expected, neither mt tRNA^{Thr} nor mt tRNA^{Lys} co-purified with FL-PTB immonoprecipitated from the untreated control extract under stringent wash conditions (lane 5), confirming the notion that PTB and mt tRNA^{Thr} specifically and directly interact in HeLa cells. The U1 spliceosomal snRNA showed a very weak, if any, *in vivo* cross-linking with FL-PTB (lane 6), suggesting that only a marginal fraction of cellular U1 associates with PTB (38).

To further characterize the interaction of mt tRNA^{Thr} with PTB, mt tRNA^{Thr} and mt tRNA^{Asp} were synthesized *in vitro* and they were transfected into HeLa cells (Figure 3B). After 24 h of incubation, cell extracts were prepared and accumulation of the transfected mt tRNAs was monitored by northern blot analysis (upper panel). In mt tRNA^{Thr}-transfected cells, in addition to the endogenous mt tRNA^{Thr} another mt tRNA^{Thr} with slightly retarded electrophoretic mobility was detected (lanes 4 and 7). Since this low mobility mt tRNA^{Thr} was missing from the non-transfected extract (lane 1), we concluded that it corresponded to the *in vitro* transcribed mt tRNA^{Thr}. We believe that the absence of post-transcriptionally added charged modification(s), for example, a *N6*-threonyl-carbamoyl-adenosine found in bovine mt tRNA^{Thr} (40), might be responsible for the reduced electrophoretic mobility of the *in vitro* synthesized tRNA^{Thr}. Nevertheless, IP of PTB efficiently recovered both the endogenous and the *in vitro* synthesized transfected mt tRNA^{Thr} (lanes 6 and 9). In contrast, neither the endogenous nor the *in vitro* produced transfected mt tRNA^{Asp} was pulled down by the anti-PTB antibody (lanes 12 and 15). These results demonstrated that *in vitro* transcribed mt tRNA^{Thr} is highly stable in transfected HeLa cells and it binds to PTB with high efficiency and specificity.

In vivo covalent cross-linking of HeLa mt tRNA^{Thr} with transiently expressed FL-PTB strongly supported the idea that PTB and mt tRNA^{Thr} form a direct interaction in the cell (Figure 3A). To further demonstrate that PTB binds directly to mt tRNA^{Thr}, we performed *in vitro* RNA–protein ultraviolet (UV) cross-linking studies. HeLa extracts were incubated with *in vitro* synthesized internally ^{32}P-labeled mt tRNA^{Thr} or as a negative control, with cyt tRNA^{Thr} and subjected to UV irradiation (Figure 3C). The cross-linked extracts were treated with RNase and proteins radiolabeled by covalently linked residual ribonucleotides were fractionated by SDS-PAGE and visualized by autoradiography (lower panel, lanes 1 and 2). IP of PTB from the extract reacted with mt tRNA^{Thr} revealed a label transfer to PTB (lane 6). In contrast, PTB recovered from the cyt tRNA^{Thr}-treated extract showed no radioactivity (lane 4), confirming that PTB directly interacts with mt tRNA^{Thr}. IP of PTB was confirmed by western blot analysis (upper panel).

Next, we found that pre-incubation of *in vitro* synthesized ^{32}P-labeled mt tRNA^{Thr} with increasing amounts of recombinant PTB resulted in a concentration-dependent RNA mobility retardation on a native polyacrylamide gel (Figure 3D, lanes 1–5, shifts 1 and 2). Highly similar RNA gel retardation profile was observed when the extensively characterized PTB substrate RNA, c-src, was incubated with increasing amounts of PTB (3,41) (lanes 8–12). On the other hand, PTB failed to alter the gel electophoretic mobility of cyt tRNA^{Thr} (lanes 15–19). Finally, inclusion of anti-PTB antibody into the gel retardation reaction caused a supershift of the pre-formed PTB/mt tRNA^{Thr} and PTB/c-src complexes, confirming that PTB formed a specific interaction with these RNAs (lanes 7 and 14).

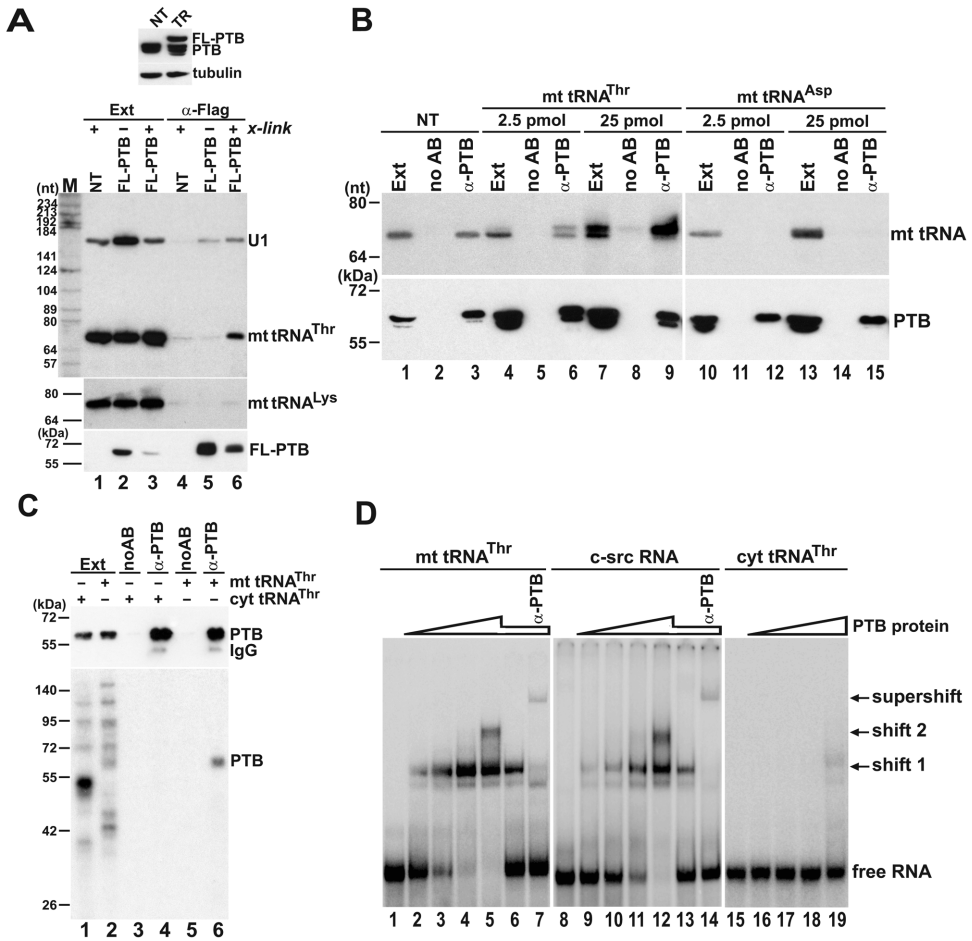

Figure 3. PTB forms a direct interaction with mt tRNA^{Thr} in HeLa cells. (**A**) *In vivo* RNA–protein cross-linking. Accumulation of endogenous PTB and transiently expressed FL-PTB in transfected (FL-PTB) and non-transfected (NT) HeLa cells was monitored by western blotting with an anti-PTB antibody (upper panel). Tubulin, loading control. Cells were *in vivo* cross-linked (x-link) with formaldehyde as indicted above the lanes. After extract (Ext) preparation, FL-PTB was immunoprecipitated under stringent conditions. IP of FL-PTB was confirmed by western blotting with anti-Flag antibody and co-precipitation of mt tRNA^{Thr} and U1 snRNA were tested by northern. (**B**) PTB association with *in vitro* synthesized mt tRNA^{Thr} introduced into HeLa cells. PTB was immunoprecipitated from extracts (Ext) prepared from HeLa cells non-transfected (NT) or transfected with the indicated amounts of *in vitro* transcribed mt tRNA^{Thr} and mt tRNA^{Asp} (lower panel). Co-IP of tRNAs was monitored by northern blotting (upper panel). (**C**) Label transfer from mt tRNA^{Thr} to PTB. *In vitro* transcribed internally labeled mt tRNA^{Thr} and cyt tRNA^{Thr} were incubated with HeLa cell extracts. After UV irradiation, RNAs were hydrolyzed by RNase treatment and PTB was immunoprecipitated and analyzed on an SDS gel. PTB IP was confirmed by western blotting (upper panel) and label transfer was visualized by autoradiography (lower panel). (**D**) Gel shift analysis. *In vitro* transcribed internally labeled mt tRNA^{Thr}, c-src, and cyt tRNA^{Thr} RNAs (1 fmol) were incubated without or with increasing amounts of recombinant PTB (0.2, 0.4, 1.5 and 4 pmol) with or without anti-PTB antibody (3). The RNA–protein complexes were fractionated on a 5% native gel.

The RRM1 and RRM2 domains of PTB bind to the T-loop and D-stem-loop of mt tRNA^{Thr}

To determine the elements of mt tRNA^{Thr} required for PTB binding, *in vitro* synthesized internally labeled mutant mt tRNA^{Thr} transcripts were transfected into HeLa cells (Figure 4A). The introduced nucleotide alterations were not expected to alter the overall structures of the mutant tRNAs. About 24 h after RNA transfection, cell extracts were prepared and PTB was immunoprecipitaded and analyzed by western blotting (lower panel). Co-IP of the labeled tRNA transcripts was monitored by autoradiography after size fractionation on a denaturing gel (upper panel). Similar to the wild-type mt tRNA^{Thr} transcript (lane 1), all mutant tRNAs proved to be stable in transfected HeLa cells (lanes 4, 7, 10, 13, 16, 19 and 22). Alteration of the antisense-loop (*ASL*), the acceptor-stem (*ACS*), the T-stem (*TS*) and the antisense-stem (*ASS*) nucleotides did not influence PTB binding (lanes 12, 15, 21 and 24). Modification of the D-loop (*DL*), the D-stem (*DS*) and the T-loop (*TL*) sequences, however, fully abolished PTB association with the mutant tRNAs (lanes 6, 9 and 18). We concluded that the D-stem-loop and the T-loop regions coordinate PTB recruitment to mt tRNA^{Thr}.

Human PTB carries four RNA recognition motifs (RRM1 to RRM4) with distinct RNA structural preferences (3) (Figure 4B). To define protein elements supporting PTB interaction with mt tRNA^{Thr}, a series of terminally truncated flag-tagged PTB proteins (FL-PTBd1 to FL-PTBd5) were transiently expressed in HeLa cells and their association with mt tRNA^{Thr} was monitored by co-IP experiments. IP of the FL-PTBd1, FL-PTBd2 and FL-

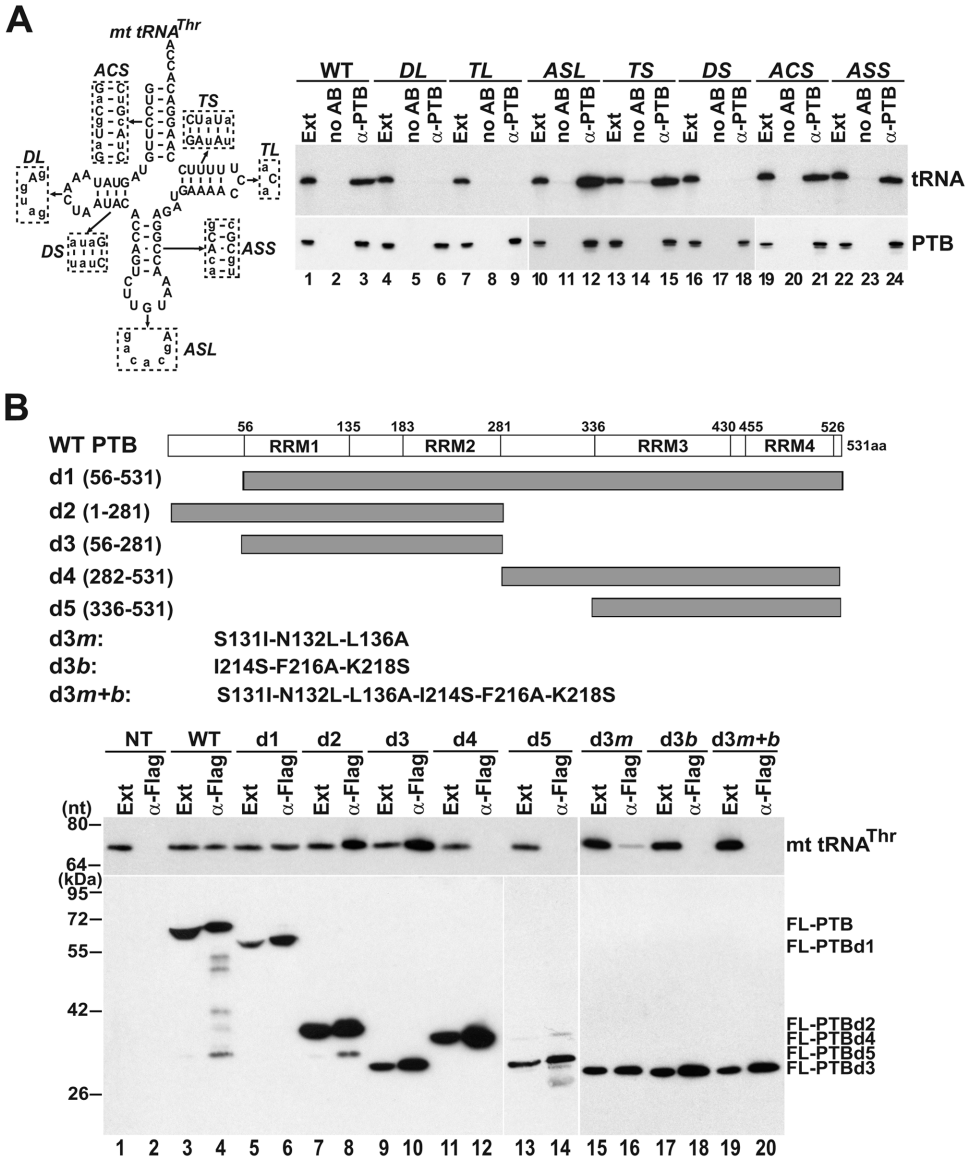

Figure 4. RNA and protein elements directing interaction of PTB and mt tRNA^{Thr}. (**A**) The D-stem-loop and T-loop nucleotides of mt tRNA^{Thr} are essential for PTB binding. Internally labeled WT and mutant mt tRNA^{Thr} transcripts were transfected into HeLa cells. After 24 h of incubation, cell extracts (Ext) were prepared, PTB was immunoprecipitated and analyzed by western blotting (lower panel). Co-IP of mt tRNA^{Thr} transcripts was analyzed by fractionation on a 6% sequencing gel. The structure of wild-type mt tRNA^{Thr} and the nucleotide alterations (lower case letters) in the acceptor-stem (*ACS*), antisense-stem (*ASS*), D-stem (DS), T-stem (TS), D-loop (DL), antisense-loop (ASL) and T-loop (TL) of mutant mt tRNA^{Thr} transcripts are shown. Lanes no AB, control IPs without antibody. (**B**) *In vivo* interaction of mutant PTB proteins with mt tRNA^{Thr}. Schematic structure of human PTB and positions of the RNA recognition motifs (RRM1 to RRM4) are indicated. Flag-tagged terminally truncated PTB proteins (FL-PTBd1 to FL-PTBd5) were transiently expressed in HeLa cells and immunoprecipitated with an anti-Flag antibody. The amino acid alterations carried by the *m*, *b* and *m*+*b* mutants of FL-PTBd3 are indicated. The IP reactions were analyzed by western (lower panel) and northern (upper panel) blotting.

PTBd3 truncated proteins, each encompassing RRM1 and RRM2, efficiently recovered mt tRNA^{Thr} (lanes 6, 8 and 10). In contrast, IP of FL-PTBd4 and FL-PTBd5 lacking RRM1 and RRM2 failed to recover detectable amounts of mt tRNA^{Thr} (lanes 12 and 14), demonstrating that the RRM1 and RRM2 domains of PTB are required and are sufficient to form a specific interaction with mt tRNA^{Thr}.

Since the RRM1 and RRM2 domains alone showed very weak accumulation in transfected cells (data not shown), point mutations known to abolish the picornaviral IRES and FAS mRNA binding capacity of the RRM1 (*m,* S131I-N132L-L136A) and RRM2 (*b*, I214S-F216A-K218S) domains of PTB were introduced into the efficiently accumulating FL-PTBd3 truncated protein (42,43). Alteration of RRM1 or RRM2 largely inhibited or fully abolished the interactions of the expressed FL-PTBd3*m* and FL-PTBd3*b* proteins with mt tRNA^{Thr}, respectively (lanes 16 and 18). As expected, the double mutant FL-PTBd3*m*+*b* protein also failed to bind mt tRNA^{Thr} (lane 20), confirming that the RRM1 and RRM2 domains of PTB are required for efficient *in vivo* binding of mt tRNA^{Thr}. We propose that the

RRM1 and RRM2 domains of PTB recognize mt tRNA^{Thr} in a cooperative and interdependent manner.

PTB interacts with mt tRNA^{Thr} in the cytoplasm outside of mitochondria

PTB is an abundant nucleoplasmic protein which, although has a significant cytoplasmic accumulation, has never been reported to reside within mitochondria (44). In contrast, synthesis, processing and function of human mt tRNAs have been strictly confined to the mitochondria. To solve this apparent contradiction, we attempted to determine where PTB and mt tRNA^{Thr} interaction takes place in the cell. First, HeLa cells were fractionated into nuclear and cytoplasmic fractions (Figure 5A). To confirm the correctness of cell fractionation, we determined the distribution of several nuclear (U2 snRNA and hnRNP A1) and cytoplasmic (12S mt rRNA, mt tRNA^{Lys}, ERp72, tubulin, TOM20 and HSP60) marker RNAs and proteins (lanes 2 and 3). As expected, the majority of PTB was found in the nuclear fraction, while mt tRNA^{Thr} showed a cytoplasmic accumulation. IP of PTB from the cytoplasmic fraction efficiently recovered mt tRNA^{Thr} (lane 7), demonstrating that the mt tRNA^{Thr}/PTB complex accumulates predominantly, if not exclusively, in the cytoplasm.

To decide whether cytoplasmic PTB associates with mt tRNA^{Thr} within or outside of mitochondria, the HeLa cytoplasmic fraction was further sub-fractionated by centrifugation at 20,000 x g for 10 min (Figure 5B). The pellet was considered as crude mitochondrial fraction, while the post-mitochondrial supernatant was regarded as crude cytosolic fraction. As expected, mt tRNA^{Thr} was found predominantly in the mitochondrial pellet together with the 12S mt rRNA and the TOM20, ATP5A1 and Cyt *c* mitochondrial marker proteins (lane 3), and the cytoplasmic soluble fraction contained only a small amount of mt tRNA^{Thr} that was apparent after longer exposure (lane 2, and data not shown). In contrast to mt tRNA^{Thr}, PTB was found exclusively in the crude cytosolic fraction together with the 7SL signal recognition particle RNA and tubulin (lane 2). The ERp72 endoplasmatic reticulum (ER) marker protein appeared also in the mitochondrial fraction, probably because ER structures are physically connected to mitochondria. In the next step, PTB was immunodepleted from the total cytoplasmic, the crude cytosolic and mitochondrial extracts. To readily detect co-IP of mt tRNA^{Thr} with PTB, we used fifty times more extract for PTB IP than analyzed on lanes 1–3. As expected, IP of PTB from the mitochondrial fraction failed to recover detectable amounts of PTB and mt tRNA^{Thr} (lane 9), but pulling down PTB from the cytosolic fraction recovered mt tRNA^{Thr} (lane 7), indicating that PTB interacts with a small fraction of mt tRNA^{Thr} that is present in the cytosol outside of the mitochondrion. In conclusion, the results of our cell fractionation, *in vivo* cross-linking and *in vitro* RNA binding experiments together provide strong support to the notion that PTB directly interacts with mt tRNA^{Thr} in the cytoplasm outside of mitochondria.

Next, we tested the formal possibility that some step(s) of post-transcriptional mt tRNA^{Thr} maturation take(s) place in the cytosol outside of mitochondria and it requires transient PTB interaction. To this end, we investigated the ac-

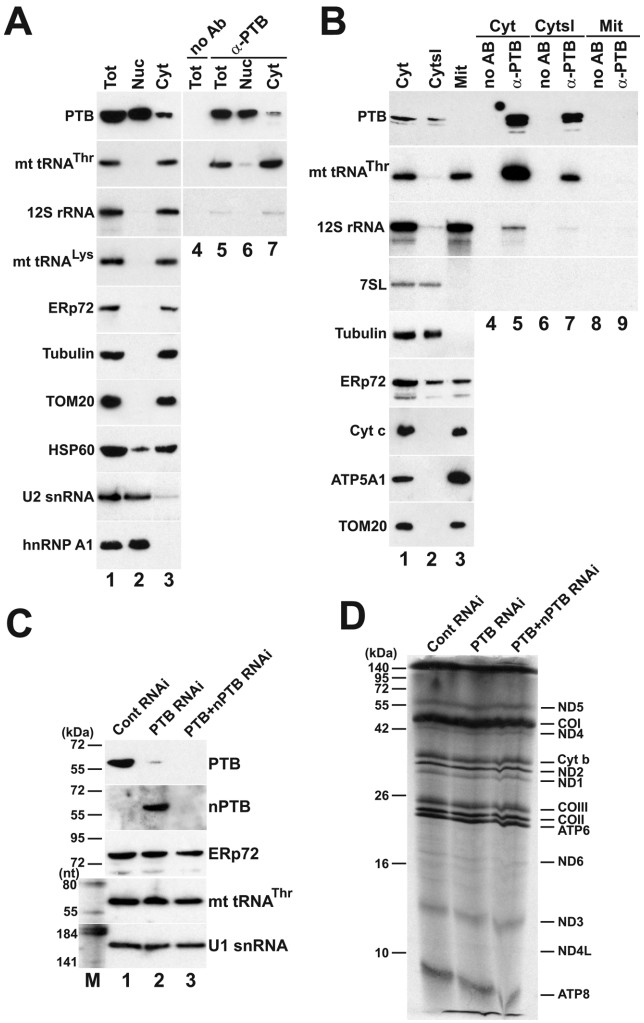

Figure 5. Subcellular localization of the PTB/mt tRNA^{Thr} complex. (**A**) PTB and mt tRNA^{Thr} interact in the cytoplasm. PTB was immunoprecipitated from extracts prepared from HeLa cells (Tot) or from the nuclear (Nuc) or cytoplasmic (Cyt) fractions of HeLa cells. IP of PTB and co-precipitation of mt tRNA^{Thr} and 12S rRNA were measured by western and northern blot analyses. Distribution of cytoplasmic (12S rRNA, mt tRNA^{Lys}, ERp72, tubulin, TOM20 and HSP60) and nuclear (U2 snRNA and hnRNP A1) markers is shown. (**B**) PTB interacts with mt tRNA^{Thr} outside of mitochondria. The cytoplasmic fraction of HeLa cells was further fractionated into crude mitochondrial (Mit) and cytosolic (Cytsl) fractions. One fiftieth of the extracts were used to determine the distribution of PTB, mt tRNA^{Thr} and several mitochondrial (12S rRNA, Cyt c, ATP5A1 and TOM20) and cytosolic (7SL RNA, tubulin, ERp72) markers (lanes 1–3). From the remaining cytoplasmic, cytosolic and mitochondrial extracts, PTB was immunoprecipitated and co-precipitation of mt tRNA^{Thr}, 12S rRNA and 7SL RNA was measured (lanes 4, 7 and 9). Lanes no AB, control IPs without antibody. (**C**) PTB is not required for mt tRNA^{Thr} expression. Accumulation of mt tRNA^{Thr} was measured in HeLa cells treated with control, PTB-specific or PTB- and nPTB-specific interfering RNAs. PTB and nPTB accumulation was measured by western blotting. ERp72 and U1 snRNA were used as loading controls. (**D**) PTB activity is not required for mitochondrial translation. Pulse chase labeling of mitochondrial proteins in control, PTB-depleted and PTB and nPTB-codepleted HeLa cells. Proteins are indicated according to (55). Cytoplasmic translation was arrested by emetine treatment.

cumulation of mature-sized mt tRNA^{Thr} in HeLa cells depleted of PTB (Figure 5C). As reported before, suppression of PTB accumulation by RNA interference (RNAi) induced expression of the neuronal nPTB (lane 2) (45). Since nPTB can take over PTB functions, both PTB and nPTB were depleted from HeLa cells by RNAi (lane 3). However, northern blot analysis demonstrated that neither PTB depletion nor PTB and nPTB co-depletion had a detectable effect on the accumulation of mt tRNA^{Thr} (lanes 2 and 3). Moreover, *in vivo* protein labeling studies performed with a mixture of ^{35}S-labeled methionine and cysteine in HeLa cells with arrested cytoplasmic translation failed to detect any alteration in the synthesis of nascent mitochondrial proteins in PTB- and PTB+nPTB-depleted cells (Figure 5D). Based on these observations, we concluded that the cytosolic interaction of mt tRNA^{Thr} with PTB is not connected to the biogenesis and mitochondrial function of mt tRNA^{Thr}.

PTB shows an increased association with mt tRNA^{Thr} in apoptotic cells

Mammalian mt tRNAs, together with cyt tRNAs, have been reported to inhibit apoptosis through binding to and inactivating the Apaf-1 apoptosome activator Cyt c (29). Therefore, we examined the *in vivo* interaction of PTB with mt tRNA^{Thr} in apoptotic HeLa cells (Figure 6A). Upon induction of apoptosis in HeLa cells by staurosporine administration, we observed caspase-mediated PARP-1 cleavage that is a characteristic hallmark of apoptotic cells (lanes 2–4) (46). As reported before, proteolytic cleavage of PTB by caspase-3 was also observed in staurosporine-treated cells (lanes 2–4) (47). During apoptotic progression, the level of mt tRNA^{Thr} did not change in the cell extracts used for PTB IP (lanes 1–4). However, contrary to the fact that caspase cleavage gradually diminished intact PTB during apoptosis (lanes 1–4), the remaining PTB showed an augmented association with mt tRNA^{Thr} (lanes 6–9).

To test whether the increased PTB association with mt tRNA^{Thr} observed in apoptotic cells is a consequence of excessive caspase-mediated protein degradation, HeLa cells were treated with caspase inhibitor (z-DEVD-FMK) before inducing apoptosis by staurosporine (Figure 6B). Preincubation of HeLa cells with z-DEVD-FMK largely inhibited the proteolytic cleavage of both PARP-1 and PTB (lane 4), but it had no influence on elevated PTB and mt tRNA^{Thr} association (lane 9). This indicates that augmented mt tRNA^{Thr}/PTB complex formation is a characteristic feature of apoptotic cells, it is not linked to cellular protein degradation and it is apparent already in the early stage of apoptosis. These results point to the intriguing possibility that cytoplasmic interaction of PTB with mt RNA^{Thr} may be linked to some aspect of the complex process controlling apoptosis.

DISCUSSION

It has been firmly believed for long time that biogenesis and cellular function of mt tRNAs are strictly confined to the mitochondria. Recent reports, however, raised the possibility that in human cells, especially under some stress conditions, e.g. apoptosis, mt tRNAs can accumulate in the cytosol where they participate in cellular processes unrelated to mitochondrial protein synthesis (28,29). This study provides further support to the emerging view that human mt tRNAs possess unorthodox cytoplasmic functions. Searching for RNAs interacting with human HeLa PTB identified mt tRNA^{Thr} as a major cellular RNA associated with PTB (Figure 1). We demonstrated that in addition to the generally expressed PTB, its two tissue-specific paralogs, nPTB and ROD1, can also interact with mt tRNA^{Thr} (Figures 1B and 2A). Finally, we showed that the PTB-mt tRNA^{Thr} interaction is conserved in various human and mouse cell lines, providing strong support for the functional significance of the observed association of PTB and mt tRNA^{Thr} (Figure 2B).

The functional diversity of PTB is supported by the flexible RNA-binding specificity of the four RNA-binding domains (RRM1 to RRM4) of the protein (9). RRM domains are present in a large number of RNA-binding proteins and they adopt a characteristic β_1-α_1-β_2-β_3-α_2-β_4 topology in which the two α-helices are packed on an antiparallel four stranded β-sheet (48). Typically, the aromatic side chains of conserved tyrosine and phenylalanine residues in the β_1 (RNP2) and β_3 (RNP1) strands form stacking interactions with RNA bases or they intercalate between two ribose rings. The four RRM domains of PTB also have the canonical β-α-β-β-α-β fold, but they are rather atypical in their lack of aromatic amino acids. *In vitro* and *in vivo* RNA binding experiments demonstrated that PTB forms a specific and direct interaction with mt tRNA^{Thr} (Figures 1–4). The human mt tRNA^{Thr} carries a bipartite PTB binding element composed of the D-stem-loop region and the short T-loop of the tRNA (Figure 4A). While the RRM3 and RRM4 domains of PTB are dispensable for mt tRNA^{Thr} binding, the N-terminal portion of the protein encompassing only the RRM1 and RRM2 domains and their linker region contains all the elements which are sufficient to form a specific and efficient interaction with mt tRNA^{Thr} (Figure 4B). The RRM1 and RRM2 domains of PTB seem to function in a strictly interdependent manner in mt tRNA^{Thr} binding. Disruption of the RNA-binding capacity of either the RRM1 or the RRM2 domain through introducing point mutations into their RNA-binding surfaces abolishes the mt tRNA^{Thr} binding capacity of PTB. Since nucleotide alterations introduced into the D-stem-loop region or into the T-loop of mt tRNA^{Thr} can fully abolish PTB binding, we propose that the RRM1 and RRM2 domains of PTB interact with this two regions of mt tRNA^{Thr} in an interdependent fashion. A very similar RRM1- and RRM2-dependent RNA recognition mechanism was described for PTB binding to the U1 spliceosomal snRNA during splicing repression (38). While each RRM of PTB can independently interact with single-stranded CU-rich target sequences with slight sequence preferences, it seems that cooperative RNA binding by the RRM1 and RRM2 domains provides a more complex and specific RNA recognition capacity for PTB (3). The flexible linker regions separating RRM1 from RRM2 and RRM2 from RRM3/RRM4 may support a highly flexible, substrate RNA-dependent folding of the N-terminal RRM1-RRM2 region, independently from the structurally more rigid C-terminal RRM3-RRM4 region (49–51). In hematopoietic cells, alternative

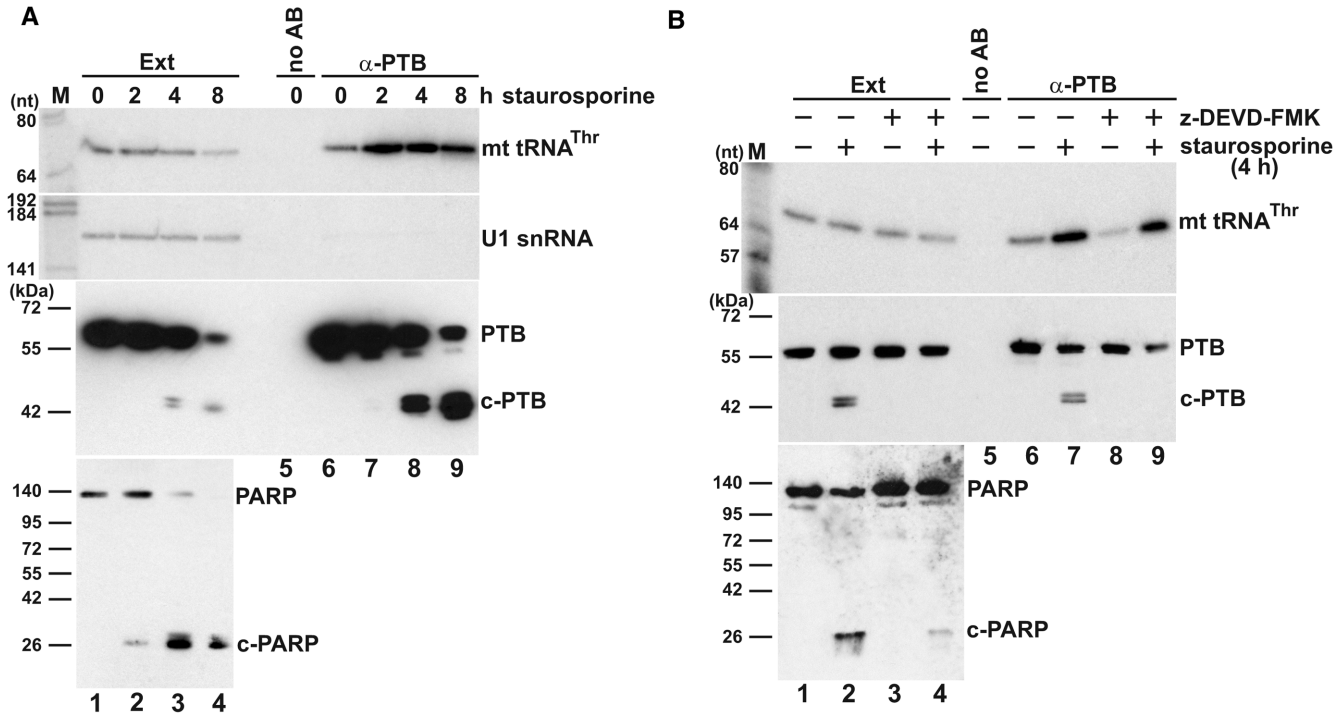

Figure 6. Increased PTB association with mt tRNA^{Thr} in apoptotic cells. (**A**) Interaction of PTB and mt tRNA^{Thr} during apoptosis. PTB was immunoprecipitated from extracts (Ext) prepared from HeLa cells treated with staurosporine as indicated. Accumulation of PTB and PARP-1 (lanes 1–4) and IP of PTB (lanes 6–9) was monitored by western blotting. Caspase-cleaved PTB (c-PTB) and PARP1 (c-PARP) are indicated. Co-IP of mt tRNA^{Thr} and U1 snRNA was measured by northern blotting. (**B**) Caspase inhibition in apoptotic cells has no effect on PTB and mt tRNA^{Thr} association. PTB was immunoprecipitated from extracts prepared from HeLa cells treated with z-DEVD-FMK and staurosporine as indicated. Other details are identical to panel A.

pre-mRNA splicing and translation initiation events generate various N-terminally truncated ROD1 isoforms which lack nuclear localization signals (52). The shortened ROD1 isoforms accumulate in the cytoplasm, but they are not expected to efficiently interact with mt tRNA^{Thr}, because they also lack part or most of the RRM1 domain. The truncated ROD1 proteins with their differential RNA-binding capacity may participate in cytoplasmic processes specific to hematopoietic cells.

Previous efforts aimed to determine the list of nuclear-encoded mitochondrial proteins failed to detect PTB as a potential mitochondrial protein (44). In accordance with this, our cell fractionation experiments demonstrated that PTB interacts with mt tRNA^{Thr} in the cytoplasm, indicating that a fraction of mt tRNA^{Thr} resides outside of the mitochondria (Figure 5). Induction of apoptosis alters mitochondrial membrane permeability and might result in formation of membrane lesions (53), that may explain the appearance of mt tRNAs in the cytoplasm during apoptosis (29). However, it remains unclear how mt tRNA^{Met} (28) and mt tRNA^{Thr} (this study) accumulate in the cytoplasm, whether they are actively exported by a yet unknown mitochondrial RNA transport mechanism or they are passively released, for example, from damaged mitochondria. In this context it is noteworthy that human mitochondria, while encode the complete set of mt tRNAs required for mitochondrial protein synthesis, possess the capacity to import tRNAs from the cytoplasm, indicating that the mitochondrial membrane is 'permeable' for tRNAs (see Introduction).

Although genome wide analysis identified a large number of PTB binding sites in the human transcriptome (11), our results strongly suggest that mt tRNA^{Thr} is the most abundant single cellular RNA associated with human PTB (Figure 1). Characterization of the PTB-associated fraction of mt tRNA^{Thr} revealed that PTB interacts with correctly processed mature mt tRNA^{Thr}. Consistent with this, PTB activity is dispensable both for mt tRNA^{Thr} expression and mitochondrial protein synthesis, suggesting that mt tRNA^{Thr} accumulating in the cytoplasm and interacting with PTB has a novel cytoplasmic function (Figure 5C and D). The increased accumulation of PTB/mt tRNA^{Thr} complex in apoptotic cells points to a possible functional connection between apoptosis and PTB and mt tRNA^{Thr} association. Interestingly, in apoptotic cells where cap-dependent translation is compromised, PTB is known to promote IRES-dependent translation of key regulatory proteins controlling apoptosis, including Apaf-1, p53, p58/Cdk11, c-myc and VEGF (reviewed in 1), raising the possibility that mt tRNA^{Thr} might modulate the translational stimulatory capacity of PTB. Unfortunately, we failed to reduce significantly the cytoplasmic level of mt tRNA^{Thr} by using RNAi (our unpublished results). On the other hand, increasing of the concentration of mt tRNA^{Thr} in the cytoplasm of apoptotic or control HeLa cells by transfection of *in vitro* transcribed mt tRNA^{Thr} had no significant effect on the translation of a dicistronic luciferase reporter mRNA placed under

the control of the PTB-dependent c-myc or rhinoviral IRES (data not shown).

Finally, our more recent preliminary results strongly support that mt $tRNA^{Met}$ and mt $tRNA^{Thr}$ are not the only mt tRNAs accumulating in the cytoplasm of human cells. We have found that all mt tRNAs are present in the cytosol of HeLa cells and they seem to interact with cytoplasmic RNA-binding proteins (Jády and Kiss, unpublished observations), supporting the fascinating idea that mt tRNAs have a more complex cytoplasmic function than anticipated before.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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