tRNA-controlled Nuclear Import of a Human tRNA Synthetase**

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Background: Many tRNA synthetases were found in the nucleus of eukaryotic cells where translation does not occur. Results: Nuclear localization of human TyrRS is dependent on a dual-function motif also used for tRNA binding. Conclusion: The cognate tRNA controls nuclear localization of human TyrRS.

Significance: The tRNA-controlled regulation coordinates nuclear import of a human tRNA synthetase with the demands of protein synthesis in the cytoplasm.

Aminoacyl-tRNA synthetases, essential components of the cytoplasmic translation apparatus, also have nuclear functions that continue to be elucidated. However, little is known about how the distribution between cytoplasmic and nuclear compartments is controlled. Using a combination of methods, here we showed that human tyrosyl-tRNA synthetase (TyrRS) distributes to the nucleus and that the nuclear import of human TyrRS is regulated by its cognate tRNA\(^{\text{Tyr}}\). We identified a hexapeptide motif in the anticodon recognition domain that is critical for nuclear import of the synthetase. Remarkably, this nuclear localization signal (NLS) sequence motif is also important for interacting with tRNA\(^{\text{Tyr}}\). As a consequence, mutational alteration of the hexapeptide simultaneously attenuated aminoaacilation and nuclear localization. Because the NLS is sterically blocked when the cognate tRNA is bound to TyrRS, we hypothesized that the nuclear distribution of TyrRS is regulated by tRNA\(^{\text{Tyr}}\). This expectation was confirmed by RNAi knockdown of tRNA\(^{\text{Tyr}}\) expression, which led to robust nuclear import of TyrRS. Further bioinformatics analysis showed that to have nuclear import of TyrRS directly controlled by tRNA\(^{\text{Tyr}}\) in higher organisms, the NLS of lower eukaryotes was abandoned, whereas the new NLS was evolved from an anticodon-binding hexapeptide motif. Thus, higher organisms developed a strategy to make tRNA a regulator of the nuclear trafficking of its cognate synthetase. The design in principle should coordinate nuclear import of a tRNA synthetase with the demands of protein synthesis in the cytoplasm.

Aminoacyl-tRNA synthetases catalyze a two-step aminoacylation reaction to link amino acids with their cognate tRNAs to provide amino acid building blocks for cytoplasmic ribosomal protein synthesis (1, 2). Interestingly, many tRNA synthetases were also found in the nucleus of eukaryotic cells where translation does not occur (3, 4). At least in some organisms, the nuclear distributions of tRNA synthetase are involved with an aminoaacilation-dependent quality control mechanism to ensure nuclear export of mature and functional tRNAs (5–7). In addition to tRNA proofreading, nuclear functions of tRNA synthetases include regulation of transcription and ribosomal RNA synthesis (8, 9). However, little is known about how the distribution of synthetases between cytoplasmic and nuclear compartments is controlled.

Tyrosyl-tRNA synthetase (TyrRS)\(^2\) is a class I tRNA synthetase with a Rossmann fold-based catalytic domain that contains the active site where the two-step aminoaacilation reaction occurs. In the first step, tyrosine and ATP generate enzyme-bound Tyr-AMP and release PP\(_i\). In the second step, the tyrosyl moiety of Tyr-AMP is transferred to the 3’-end of tRNA\(^{\text{Tyr}}\) to generate Tyr-tRNA\(^{\text{Tyr}}\). To address the question of how the distribution of a tRNA synthetase is controlled, we focused on human TyrRS because it is one of the most characterized tRNA synthetases, with crystal structures being solved from 12 different organisms including human (Protein Data Bank (PDB) database). Human TyrRS is also of broad interest because it prominently exemplifies the multifunctionality of tRNA synthetases (10) and is associated with heritable disease (11). Lastly, nuclear localization of TyrRS was previously demonstrated in Saccharomyces cerevisiae (12).

Because the nuclear localization signal (NLS) identified in yeast TyrRS is not found in the human enzyme (12), we started our investigation by detecting nuclear localization of TyrRS in human cells. Through a combination of methods, we identified a distinct NLS sequence for human TyrRS and showed that this sequence was adapted from a key motif used for tRNA anticodon binding. This scenario effectively coordinates nuclear trafficking of the synthetase with its role for protein synthesis in the cytoplasm.

EXPERIMENTAL PROCEDURES

Confocal Immunofluorescence Microscopy—To detect the subcellular localization of TyrRS, HeLa cells were seeded on glass coverslips, grown for 24 h to 80% confluence, and then fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. After permeabilization with 0.5% Triton X-100 in PBS for 2 min and being blocked with 1% bovine serum albumin for 1 h, cells were first stained with rabbit anti-TyrRS antibody (custom-made, 1:10,000 diluted) for 3 h at room temperature. After permeabilization with 0.5% Triton X-100 in PBS for 2 min and being blocked with 1% bovine serum albumin for 1 h, cells were first stained with rabbit anti-TyrRS antibody (custom-made, 1:10,000 diluted) for 3 h at room temperature followed by three washes with PBS. Cells were then incubated for 30 min with goat anti-rabbit IgG conjugated to the fluorescent Alexa Fluor 488 dye (1:3000 diluted) followed by three washes with PBS. Lastly, cells were stained with DAPI (1 μg/ml) for 15 min followed by three washes with PBS. The cells were
viewed with a Bio-Rad (Zeiss) Radiance 2100 Rainbow laser scanning confocal microscope.

**Cell Fractionation**—Human TyrRS or mutants were cloned into pcDNA6 vector and transfected into near-confluent adherent HeLa cells. Cells were harvested after incubating overnight at 37 °C. The pooled cells were pelleted at 500 × g for 5 min and resuspended with PBS after removing the medium. The cytoplasmic and nuclear fractions were separated using the NE-PER® nuclear and cytoplasmic extraction kit (Thermo Scientific). TyrRS was detected by Western blot analysis using the custom-made anti-TyrRS antibody.

**Mutagenesis**—Site-directed mutagenesis of the human TyrRS gene was performed using the method of single mutagenic oligonucleotides and DpnI digestion on the template DNA as described (13).

**Protein Expression and Purification**—Human TyrRS or mutants were cloned into a bacterial expression vector pET-20b(+) with a C-terminal His6 tag and transformed into *Escherichia coli* BL21(DE3) cells. The transformed cells were grown in the presence of ampicillin at room temperature and induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside to express the recombinant protein for 5–6 h. The harvested cells were lysed by ultrasonication, and the supernatants of the cell lysates were applied to a nickel-nitrilotriacetic acid (Novagen) column for purification. The purified proteins were stored in 10 mM HEPES, pH 7.5, 10 mM MgCl2, 100 mM KCl, and 2 mM DTT.

**Aminoacylation and PPi-ATP Exchange Assays**—The assays were performed as described (14). The aminoacylation assay was performed with 100 nM WT or mutant TyrRS proteins in 50 mM HEPES (pH 7.5), 20 mM KCl, 1 mM ATP, 1 mM MgCl2, and 5–6 h. The harvested cells were also subjected to cell fractionation analyses using the Bradford method.

**Filter Binding Assay**—Human tRNATyr transcript was labeled at the 5′-end with [γ-32P]ATP using the KinaseMax™ kit (Ambion) and incubated with a concentration gradient of TyrRS (WT or mutant) from 125 nM to 4 μM in 10 mM HEPES, pH 7.5, 4 mM KCl, 0.02 mg/ml total yeast tRNA. The PPi-ATP exchange assay was performed with 1 μM enzyme in 50 mM HEPES (pH 7.5), 20 mM KCl, 0.5 mM tyrosine, 1 mM total NaPPi, and 1:20 ratio of [32P]NaPPi to cold NaPPi. The concentration of the proteins was determined by the Bradford method.

**Filter Binding Assay**—Human tRNATyr was labeled at the 5′-end with [γ-32P]ATP using the KinaseMax™ kit (Ambion) and incubated with a concentration gradient of TyrRS (WT or mutant) from 125 nM to 4 μM in 10 mM HEPES, pH 7.5, 4 mM KCl, 0.02 mg/ml total yeast tRNA. The PPi-ATP exchange assay was performed with 1 μM enzyme in 50 mM HEPES (pH 7.5), 20 mM KCl, 0.5 mM tyrosine, 1 mM total NaPPi, and 1:20 ratio of [32P]NaPPi to cold NaPPi. The concentration of the proteins was determined by the Bradford method.

**Identification of NLS**—We next attempted to identify the NLS of human TyrRS. The classical NLS is characterized by one or two stretches of basic residues that are surface-exposed and recognized by the acidic binding surfaces of importins (15). Based on an analysis of the primary sequence and of our previously reported high-resolution crystal structures of human TyrRS (16, 17), two potential NLSs, 242KKKLK247 in the anticycle recognition domain and 482NPNN486 in the C-terminal endothelial monocyte-activating protein II-like domain, were identified (Fig. 1C).

To determine which motif is the true NLS, we cloned wild type (WT) TyrRS and created two mutant forms: 242YQFWIN247 TyrRS to replace 242KKKLK247 with the corresponding motif in *E. coli* TyrRS and 482NNPNN486 TyrRS to replace 482KKKLK486. (TyrRS from bacteria, archaea, and lower
eukaryotes does not have endothelial monocyte-activating protein II-like domain.) Each clone was transfected into HeLa cells and analyzed by cell fractionation. Consistent with the previous results with endogenous TyrRS, overexpressed WT TyrRS also distributed to the nucleus (Fig. 1D). In contrast to WT and 482NPNNN486 TyrRS, 242YQFWIN247 TyrRS did not enter the nucleus (Fig. 1D), suggesting that 242KKKLKK247 is the nuclear targeting signal for human TyrRS. To confirm this result, we fused the KKKLKK and, separately, the KPKKK motif to the N terminus of GFP that was chosen as a passenger protein. Cell fractionation analysis showed that KKKLKK, but not KPKKK, facilitated nuclear localization of GFP (Fig. 1E). Thus, the 242KKKLKK247 motif is necessary and sufficient to facilitate nuclear import of human TyrRS.

NLS Is Positioned for tRNA Interaction—The hexapeptide NLS motif 242KKKLKK247 is located on an α-helix in the anticodon recognition domain of human TyrRS (Fig. 2A). According to the crystal structures (18–20), the corresponding motif in *Thermus thermophilus* 234FKKLMR256 is responsible for anticodon recognition (Fig. 2A); Arg-256, the last residue of the motif, makes base-specific and backbone interactions with U35 of the GUA anticodon. In *Methanococcus jannaschii* and *S. cerevisiae*, the corresponding motifs (223RAKIKK228 and 247KKKINS252) maintain strong interactions, through the last two residues of the hexapeptide motif, to the backbone atoms of the GUA anticodon (Fig. 2A); however, base-specific recognition of U35 was taken over by another helix adjacent to the hexapeptide motif. Nevertheless, in all above cases, this hexapeptide motif is involved in tRNA binding.

**Mutagenic Analysis Confirms Dual Role of NLS**—In the absence of a structure for the human TyrRS-tRNA<sup>Tyr</sup> complex, to confirm the role of KKKLKK in tRNA binding, we created a series of mutations of the hexapeptide motif. These mutations progressively replaced charged lysines with neutral residues (asparagine or alanine) to give 242NKKK247, 242NNKLKK247, 242NNKLKK247, 242NNKLNN247, and 242NNALNA247 TyrRSs (the underlining indicates mutated residue(s)). The mutant enzymes were expressed in *E. coli*, purified, and used in three different assays. First, tyrosine-dependent PP<sub>i</sub>-ATP exchange was used to monitor adenylate synthesis in the absence of...
tRNA<sup>Tyr</sup>. Second, the overall aminoacylation activity was determined using tRNA<sup>Tyr</sup> as the acceptor. Lastly, a nitrocceullose filter binding assay was used to determine the binding constant with tRNA<sup>Tyr</sup>. Progressive replacement of Lys-242, Lys-243, and Lys-246 with Asn only modestly affected the aminoacylation activity, whereas the additional K247A mutation (to give NNKLNA) decreased the initial rate of aminoacylation to less than 50% of the WT protein (Fig. 2B). However, the NNKLNA mutant had WT-like activity in the PP<sub>1</sub>-ATP exchange reaction (Fig. 2B), suggesting that the side chain of Lys-247 is important for tRNA binding. Consistently, the filter binding assay indicated that although the <i>K</i><sub>D</i> of NNKLNK TyrRS for tRNA<sup>Tyr</sup> (123 nM) is similar to that of WT TyrRS (119 nM), the <i>K</i><sub>D</sub> of NNKLNA TyrRS has increased to 166 nM (Table 1), suggesting a weakened interaction with the cognate tRNA as a result of K247A mutation. Possibly, the positively charged side chain of Lys-247 binds to a negatively charged phosphate of the anticodon stem loop, in a way similar to that of Lys-228 of <i>M. jannaschii</i> TyrRS (Fig. 2A).

Additional replacement of Lys-244 with Ala (to give NNAI-NA) further decreased the synthetase-tRNA interaction (<i>K</i><sub>D</sub> = 190 nM, Table 1) and affected both aminoacylation and adenylate synthesis activities (Fig. 2B). It is worth noting that Lys-244 forms a conserved salt bridge interaction with Asp-236 and is the most conserved residue of the hexapeptide motif during evolution (Fig. 3). Most likely, Lys-244 plays a structural role to ensure the correct positioning of the hexapeptide, which, in some way, also affects the active site. Consistently, replacement of the entire hexapeptide from human TyrRS with its counterpart from <i>E. coli</i> TyrRS (YQFWIN) dramatically decreased the interaction with tRNA<sup>Tyr</sup> (<i>K</i><sub>D</sub> = 562 nM; Table 1) and completely abolished aminoacylation as well as adenylate synthesis activities (Fig. 2B). Therefore, our mutagenesis and enzymatic analyses suggested a close communication between the active site of the catalytic domain and the site of the NLS motif. However, with regard to tRNA binding, the role of Lys-244 should be indirect.

As for nuclear targeting, each additional replacement of Lys-242, Lys-243, Lys-246, and Lys-247 within the NLS progressively diminished nuclear location of TyrRS (Fig. 2C). This result is consistent with the idea that the charge-charge interactions of TyrRS with importins are dispersed over several contiguous residues within the synthetase.

**Dual Role of NLS Provides tRNA-controlled Nuclear Trafficking**—We surmised from these results that tRNA binding might block full access of the hexapeptide to the nuclear import machinery. If this were the case, then the level of tRNA inside the cell might regulate the nuclear trafficking of TyrRS. To test this possibility, we tried to knock down expression of tRNA<sup>Tyr</sup> by introduction of a specific siRNA directed against tRNA<sup>Tyr</sup>. The knockdown of tRNA<sup>Tyr</sup> in HeLa cells was confirmed by Northern blot analysis. Remarkably, knockdown of tRNA<sup>Tyr</sup> dramatically increased nuclear localization of the endogenous TyrRS in HeLa cells (Fig. 2D). In contrast, use of a nonspecific control siRNA, although causing some diminution in the production of RNA in general, had no effect on nuclear import of TyrRS. Thus, having an NLS and anticodon-binding element overlap makes tRNA<sup>Tyr</sup> a regulator of the nuclear trafficking of its cognate synthetase.

**DISCUSSION**

The dual-function KKKLKK hexapeptide motif first appeared in <i>Drosophila melanogaster</i> and has been stably maintained in evolution ever since (Fig. 3). Thus, from insects to vertebrates and to mammals, the hexapeptide motif is most likely used for tRNA<sup>Tyr</sup> binding as well as for targeting nuclear localization of TyrRS. For most tRNA synthetases, anticodon binding is critical for the essential aminoacylation activity, so there is strong selective pressure for maintenance of this structural element. Interestingly, the hexapeptide motif in TyrRS transitions from predominantly hydrophobic in bacteria to predominantly hydrophilic in archaea and eukaryotes (Fig. 3). The transition in sequence is also accompanied by an outward conformational shift of this hexapeptide to become more surface-exposed and presumably more accessible to the nuclear import machinery. In <i>S. cerevisiae</i>, although this motif contains three lysines, they were not sufficient to direct yeast TyrRS to the nucleus (12). Instead, nuclear localization is driven by a separate lysine-rich peptide that is unique to TyrRS in fungi. Mutations that altered the basic amino acids of that NLS affected the nuclear localization of the synthetase, but did not perturb aminoacylation (12). Thus, in contrast to the mammalian system investigated here, the nuclear targeting motif in yeast is segregated from key elements for aminoacylation.

To our knowledge, this is the first report of nuclear localization of a tRNA synthetase being regulated by its cognate tRNA. In <i>Xenopus laevis</i>, where aminoacylated tRNA<sup>Tyr</sup> was found in the nucleus (5), the nuclear presence of TyrRS was strongly suggested. The aminoacylated tRNA was exported more efficiently than was uncharged tRNA, so that the preference for the charged tRNA serves as a proofreading mechanism to ensure...
that mature and functional tRNAs are exported into the cytoplasm for protein synthesis. In a different vein, from yeast to vertebrates, retrograde trafficking of tRNA from the cytoplasm to the nucleus occurs under stress conditions such as amino acid deprivation (21). This retrograde tRNA trafficking is thought to down-regulate gene expression through general inhibition of translation under conditions that are unfavorable for protein synthesis. This kind of response would presumably deprive tRNA Tyr from the cytoplasm and, according to the results here, would stimulate nuclear localization of TyrRS. In this scenario, the nuclear accumulated TyrRS should not only serve for proofreading tRNA Tyr, but could also affect other to be determined functions in the nucleus. These functions are perhaps related to a physiological response to stress conditions that cut back protein synthesis. However, most importantly, the novel design reported here, which makes tRNA a regulator of that cut back protein synthesis. However, most importantly, the novel design reported here, which makes tRNA a regulator of the nuclear trafficking of its cognate synthetase, still prioritizes the demands of protein synthesis in the cytoplasm.

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