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Human Thg1 displays tRNA-inducible GTPase activity

Titi Rindi Antika[1], Kun Rohmatan Nazilah[1], Yi-Hsueh Lee[1], Ya-Ting Lo[1], Chung-Shu Yeh[2], Fu-Lung Yeh[2], Tien-Hsien Chang[2], Tzu-Ling Wang[3] and Chien-Chia Wang [1,*]

[1]Department of Life Sciences, National Central University, Zhongli District, Taoyuan 320317, Taiwan, [2]Genomics Research Center, Academia Sinica, Nankang District, Taipei 11529, Taiwan and [3]Graduate Institute of Mathematics and Science Education, National Tsing Hua University, Hsinchu City 30014, Taiwan

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ABSTRACT

tRNA^{His} guanylyltransferase (Thg1) catalyzes the 3′-5′ incorporation of guanosine into position -1 (G-1) of tRNA^{His}. G-1 is unique to tRNA^{His} and is crucial for recognition by histidyl-tRNA synthetase (HisRS). Yeast Thg1 requires ATP for G-1 addition to tRNA^{His} opposite A73, whereas archaeal Thg1 requires either ATP or GTP for G-1 addition to tRNA^{His} opposite C73. Paradoxically, human Thg1 (HsThg1) can add G-1 to tRNAs^{His} with A73 (cytoplasmic) and C73 (mitochondrial). As N73 is immediately followed by a CCA end (positions 74–76), how HsThg1 prevents successive 3′-5′ incorporation of G-1/G-2/G-3 into mitochondrial tRNA^{His} (tRNA$_m$^{His}) through a template-dependent mechanism remains a puzzle. We showed herein that mature native human tRNA$_m$^{His} indeed contains only G-1. ATP was absolutely required for G-1 addition to tRNA$_m$^{His} by HsThg1. Although HsThg1 could incorporate more than one GTP into tRNA$_m$^{His} *in vitro*, a single-GTP incorporation prevailed when the relative GTP level was low. Surprisingly, HsThg1 possessed a tRNA-inducible GTPase activity, which could be inhibited by ATP. Similar activity was found in other high-eukaryotic dual-functional Thg1 enzymes, but not in yeast Thg1. This study suggests that HsThg1 may downregulate the level of GTP through its GTPase activity to prevent multiple-GTP incorporation into tRNA$_m$^{His}.

INTRODUCTION

Two distinct pathways for acquisition of G-1 to tRNA^{His}

Nuclear tRNA genes are transcribed by RNA polymerase III as nascent precursor tRNAs (1,2), which then go through a series of modifications to yield mature tRNAs. A unique modification, 3′-5′ addition of a guanosine at position −1 (G-1), has been shown to occur at the 5′ end of tRNA^{His}. Note that position −1 is normally unoccupied in mature tRNAs. In bacteria, G-1 is encoded by the tRNA^{His} genes opposite C73 and retained after removal of the 5′ leader sequence by ribonuclease P (3). An exception to this rule is a small group of α-proteobacteria, whose tRNAs^{His} lack a G-1 residue (4). In eukaryotes, however, G-1 is added posttranscriptionally by tRNA^{His} guanylyltransferase (Thg1) after removal of the 5′ leader sequence (5). The presence of two distinct pathways to ensure the acquisition of G-1 highlights the key role of G-1 for tRNA^{His}. G-1 is a unique distinguishing feature of tRNA^{His} throughout biology (6–9) and is essential for aminoacylation of tRNA^{His} by histidyl-tRNA synthetase (HisRS) (9–11). Most archaea acquire G-1 through a pathway similar to that for bacteria (12), whereas some archaea acquire G-1 through a posttranscriptional pathway (13). Paradoxically, both pathways appear to take place in some organellar tRNAs^{His} (14–16).

tRNA^{His} recognition by Thg1

Unlike HisRS, which identifies its cognate tRNA primarily through recognition of G-1, Thg1 identifies tRNA^{His} through recognition of its anticodon (GUG). Despite the fact that both eukaryal- and archaeal-type Thg1 enzymes recognize the anticodon, the former prefers tRNA^{His} with A73, whereas the latter prefers tRNA^{His} with C73. Eukaryal-type Thg1, such as yeast Thg1, adds G-1 to tRNA^{His} across from A73 via a template-independent mechanism, producing a G:A mismatch (5). In fact, this enzyme can add any nucleotide to tRNA^{His} opposite A73 *in vitro*, albeit with a strong preference for GTP (17). In contrast, archaeal-type Thg1, such as *M. thermoautotrophicus* and *M. kandleri* Thg1 enzymes, adds G-1 to tRNA^{His} across from C73 through a template-dependent mechanism, producing an extra Watson-Crick base pair in the acceptor stem (12,18). Many bacterial, archaeal, and certain eukaryotic microbes, such as *Dictyostelium discoideum*, possess a Thg1-like protein, which is not specific to tRNA^{His} and is involved in tRNA 5′ end repair instead (13,19).

*To whom correspondence should be addressed. Tel: +886 3 422 7151 (Ext 65062); Fax: +886 3 422 8482; Email: dukewang@cc.ncu.edu.tw

Mechanisms of G-1 addition in eukaryotes and archaea

In yeast, Thg1-catalyzed G-1 addition to ribonuclease P-processed $tRNA^{His}$, 5′-monophosphorylated $tRNA^{His}$ (p-$tRNA^{His}$), involves three consecutive steps: **(i)** adenylylation, in which p-$tRNA^{His}$ is activated by ATP, forming an activated intermediate, App-$tRNA^{His}$; (ii) guanylylation, in which GTP replaces the 5′ AMP, producing 5′-triphosphorylated G-1-$tRNA^{His}$ intermediate (ppp-G-1-$tRNA^{His}$) and (iii) pyrophosphate removal, in which the PP_i group is removed from the 5′ end of ppp-G-1-$tRNA^{His}$, yielding a mature $tRNA^{His}$, p-G-1-$tRNA^{His}$ (17) (Figure 1A). *In vitro*, ppp-$tRNA^{His}$ can be used as the substrate for G-1 addition, bypassing the requirement for the activation step (13). In contrast, ATP is not absolutely required for archaeal Thg1, as GTP can also be used to activate p-$tRNA^{His}$ (Figure 1A) (13,20).

Each Thg1 subunit possesses two nucleotide-binding pockets

To date, Thg1 is the only enzyme known to catalyze templated reverse polymerization. Its catalytic domain shares unexpected structural similarity with canonical 5′-3′ DNA polymerases (13,21). Each Thg1 subunit consists of a palm domain and a finger domain. The former is responsible for catalysis, whereas the latter is responsible for binding the anticodon (20,22). Human Thg1 exists as a homotetramer (subunits ABCD), but appears as a dimer of dimers (dimers AB and CD) (21,23). Such an architecture allows the enzyme to contact the opposite ends of the L-shape tRNA simultaneously, with one dimer (AB) capturing the acceptor stem and the other dimer (CD) binding the anticodon (23). Each subunit possesses two nucleotide-binding pockets, one for adenylylation and the other for guanylylation (13,21). The adenylylation site can fit both ATP and GTP, whereas the guanylylation site can fit all four nucleotides. In the adenylylation site, two conserved amino acid residues (D29 and D76) interact with two Mg^{2+} ions, which in turn coordinate the triphosphate of the incoming nucleotide. In the guanylylation site, four conserved amino acid residues (R27, R92, K95 and R130) interact with the triphosphate of the second nucleotide, which in turn holds another Mg^{2+} ion (21). Mutation of any of these residues distinctly reduces G-1 addition to $tRNA^{His}$ (21,24).

A dual-functional human Thg1

A recent study showed that a single HsThg1 gene is responsible for G-1 addition to both cytoplasmic and mitochondrial $tRNA^{His}$ isoacceptors ($tRNA_n^{His}$ and $tRNA_m^{His}$, respectively) (25). Notably, addition of G-1 to $tRNA_n^{His}$ forms a G-1:A73 mismatch, whereas addition of G-1 to $tRNA_m^{His}$ forms a G-1:C73 base pair (Figure 1B). As a CCA end immediately follows the discriminator base (N73), G-2 and G-3 can be incorporated easily via a template-dependent mechanism into $tRNA^{His}$ with C73, but not A73. Unfortunately, $tRNA^{His}$ with an additional G-2:C74 base pair perturbs the universally conserved CCA end of tRNA. This additional tRNA base pair might interfere with a conformational change important for translation (26). This raised the question of how HsThg1 can prevent successive incorporation of GTP into $tRNA_m^{His}$ *in vivo*. We showed herein that a single-GTP incorporation prevails when the GTP level is low (or the ATP/GTP ratio is high), and that HsThg1 possesses a tRNA-inducible GTPase activity. Perhaps HsThg1 can down-regulate the relative GTP level to preclude continuous incorporation of GTP into $tRNA_m^{His}$.

MATERIALS AND METHODS

Gene cloning and protein purification

Cloning of the gene encoding HsThg1 into pET21b (or pADH) followed a standard protocol. In brief, a pair of gene-specific primers was used to amplify the gene via a polymerase chain reaction using human cDNA as the template. The forward primer (containing an SpeI site) was located just upstream of the open reading frame, while the reverse primer (containing an XhoI site) was located just upstream of the stop codon. The amplified DNA fragment was digested with SpeI and XhoI and then cloned into the multiple cloning sites of pET21b (or pADH). Cloning of *Saccharomyces cerevisiae*, *Drosophila melanogaster* and *Bombyx mori* Thg1 genes followed a similar strategy. To construct Mut A (D76A) and Mut G (R27A) of HsThg1, two sets of mutagenesis primer were designed accordingly. Site-directed mutagenesis followed protocols provided by the manufacturer (Agilent, Santa Clara, CA, USA).

For protein purification, the plasmid harboring the Thg1 gene was transformed into an *Escherichia coli* expression strain, BL21-CodonPlus(DE3) and the His_6-tagged protein was purified to homogeneity through Ni-NTA affinity chromatography as previously described (27).

Primer extension analysis

To sequence the 5′ end of mature human $tRNA_m^{His}$, this tRNA was isolated from a 293T cell culture and sequenced by primer extension analysis. Primer used for this assay contains sequences complementary to the anticodon- and D-arm region of $HstRNA_m^{His}$ (5′ TCTGATGTTTTGGTT). Primer was 5′ end labeled with [γ-^{32}P]-ATP (Perkin Elmer, 3000 Ci/mmol) by T4 polynucleotide kinase and purified as described (28). Annealed primer was extended for 5 minute at room temperature, followed by 1-h reverse-transcription at 37°C with 20 μM dNTP and 0.4 U/μl AMV-reverse transcriptase in AMV-RT reaction buffer (Promega). Sequencing reaction additionally contained 10 μM of each corresponding ddNTP. Reactions were terminated by addition of an equal volume of RNA loading buffer (80% formamide and 1 mM EDTA). Products were resolved by 12% PAGE with 4 M urea and visualized by PhosphoImager (Typhoon FLA 9000) (5).

In vitro transcription of $tRNA^{His}$

Preparation of $tRNA^{His}$ transcripts followed a previously described protocol (9). Briefly, a synthetic DNA duplex containing a T7 promoter (class III) and a gene encoding $tRNA^{His}$ (GUG) was cloned into the SmaI site of pUC18 using a pair of complementary primers. The transcription template was enriched by polymerase chain reaction amplification of the insert (containing the T7 promoter and target gene). The *in vitro* transcription reaction of $tRNA^{His}$ with

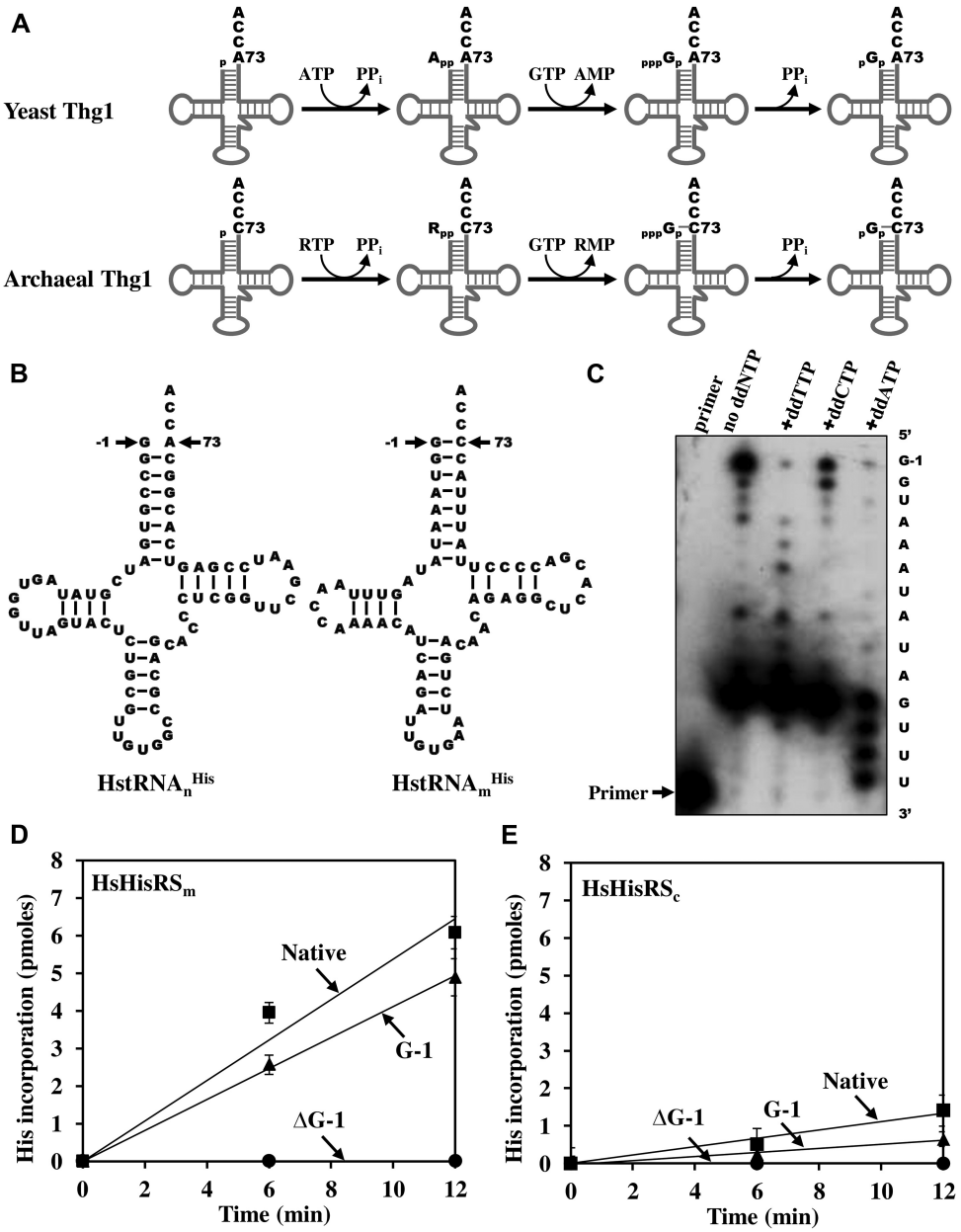

Figure 1. Incorporation of G-1 into $tRNA^{His}$. (**A**) Mechanisms of Thg1-catalyzed G-1 incorporation. Yeast Thg1 adds G-1 to $tRNA^{His}$ opposite A73 through an ATP-activation mechanism, whereas archaeal Thg1 adds G-1 to $tRNA^{His}$ opposite C73 through an ATP(GTP)-activation mechanism. RTP represents ATP or GTP. (**B**) Cloverleaf structures of human cytoplasmic and mitochondrial $tRNA^{His}$ isoacceptors. (**C**) Primer extension analysis of *in vivo*-isolated human mitochondrial $tRNA^{His}$. Aminoacylation of $tRNA_m^{His}$ by human (**D**) mitochondrial and (**E**) cytoplasmic HisRSs. tRNA substrates used included native $tRNA_m^{His}$ and *in vitro*-transcribed $tRNA_m^{His}$ with or without G-1.

a 5′-GMP was performed at 37°C for 3 h with 0.3 μM T7 RNA polymerase in 20 mM Tris–HCl (pH 8.0), 150 mM NaCl, 20 mM $MgCl_2$, 5 mM DTT, 1 mM spermidine, 2 mM of each NTP, and 20 mM GMP. To generate $tRNA^{His}$ with a 5′-GTP, 20 mM GMP was omitted from the above reaction mixture. The $tRNA^{His}$ transcript was purified by a 10% denaturing urea-polyacrylamide gel. After ethanol precipitation and vacuum-drying, the tRNA pellet was dissolved in TE buffer and then refolded by heating to 80°C and gradually cooled to room temperature after addition of 2 mM $MgCl_2$. Approximately 80% of *in vitro*-transcribed $tRNA^{His}$ containing G-1 was active in aminoacylation.

Aminoacylation assay

Aminoacylation was carried out at ambient temperature in a buffer containing 50 mM HEPES (pH 7.5), 50 mM KCl, 15 mM $MgCl_2$, 5 mM dithiothreitol, 10 mM ATP, 0.1 mg/ml bovine serum albumin, and 26.25 μM histidine (6.25 μM ^{14}C-histidine; Perkin Elmer, Waltham, MA, USA), 5 μM $tRNA^{His}$ and 50 nM HisRS (29). At various time points, reactions were quenched by spotting 10-μl aliquots of the reaction mixture onto Whatman filter pads (Maidstone, Kent, UK) that had been presoaked in a solution containing 5% trichloroacetic acid and 2 mM histidine.

The filter pads were washed three times for 15 min each in ice-cold 5% trichloroacetic acid before liquid scintillation counting. Aminoacylation data were obtained from three independent experiments and averaged.

GTP incorporation assay

A GTP incorporation assay was carried out in a buffer containing 25 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 125 mM KCl, 3 mM DTT, 0.5 μM p-$tRNA^{His}$, 1–1000 μM GTP and 0 or 1000 μM ATP. The reaction was initiated by addition of HsThg1 to a final concentration of 0.5 μM. At various times, reactions were quenched by mixing 4-μl aliquots of the reaction mixture with an equal volume of 2× loading buffer (16 M urea, 0.5 M EDTA, 2% bromophenol blue, 2% xylene cyanol and 10% glycerol), and analyzed by 10% urea-polyacrylamide gel electrophoresis. The gel was then stained with SYBR Gold Nucleic Acid Gel Stain (Thermo Fisher Scientific) and visualized using a Typhoon Imager (GE Healthcare).

GTPase assay

The GTPase assay was carried out in a buffer containing 1 μM GTP (0.125 μM α-^{32}P-GTP; PerkinElmer, Boston, USA), 25 mM HEPES (pH 7.4), 125 mM KCl, 10 mM $MgCl_2$, 3 mM DTT, 0 or 5 μM tRNA, 0 or 1000 μM ATP and 0.5 μM Thg1 (unless otherwise indicated). The reaction mixture was incubated at 37°C for 40 min and quenched by addition of 2 μl 0.5 M EDTA. Two-μl aliquots of the quenched reaction mixture were spotted onto a polyethyleneimine cellulose plate (Merck, Germany) and the nucleotides were separated by thin-layer chromatography using 0.4 M KH_2PO_4 (pH 3.65) as the mobile phase for 40 min. The wet TLC plate was air dried in a laminar air flow for 30 min and then covered with plastic wrap before being inserted into a cassette with an X-ray film. The cassette was incubated at either −80°C (for 3–5 h) or room temperature (for overnight) (30,31). To mark the relative positions of GTP, GDP GMP and P_i in the TLC plate, GTP was partially hydrolyzed in a buffer containing 1 μM GTP (0.125 μM α-^{32}P-GTP) and calf intestinal phosphatase (CIP) (10^{-5} unit) at 37°C for 1 min. The relative signal intensities of GTP and GDP were quantified using an ImageJ.

Complementation assay

Construction of a yeast haploid *THG1* knockout (KO) strain (*MATα, thg1::kanMX4, his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) was reported earlier (32). To examine whether a heterologous *THG1* gene can substitute functionally for yeast *THG1*, a test plasmid harboring the target gene was constructed and transformed into the KO strain, and the resultant transformant was tested on a 5-FOA (1 mg/ml) plate for its ability to grow. Because yeast can convert 5-FOA to a toxic compound in the presence of *URA3*, the transformant must get rid of the maintenance plasmid (carrying a yeast *THG1* gene and a *URA3* marker) on 5-FOA to survive. Without the maintenance plasmid, the transformant will die on 5-FOA unless the test plasmid encodes a functional Thg1 enzyme.

RESULTS

Mature human mitochondrial $tRNA^{His}$ contains G-1

Despite G-1 playing a key role in aminoacylation of *in vitro*-transcribed $HstRNA_m^{His}$ by $HisRS_m$ (29), the mitochondrial gene encoding $HstRNA_m^{His}$ lacks a G residue at position -1, and no experimental evidence has been provided so far to verify that the mature $HstRNA_m^{His}$ actually contains G-1. Pursuant to this objective, total RNA was isolated from a human 293T cell culture, followed by purification of $tRNA_m^{His}$ through a streptavidin column using a biotin-conjugated complementary oligonucleotide as the probe. Purified $HstRNA_m^{His}$ was then subjected to sequencing via primer extension. As shown in Figure 1C, the mature $HstRNA_m^{His}$ indeed contained G-1 but no G-2 or G-3. In addition, a prominent band was found across the gel at position G10. G10 is normally modified by human Trm11/Trm12 complex, yielding N^2-methylguanosine (m^2G) (33). This modification is involved in L-shaped structure stabilization by preventing incorrect folding (34), which might hinder primer extension, leading to early termination of polymerization.

An aminoacylation assay was carried out to explore whether *in vivo*-isolated native $HstRNA_m^{His}$ is a suitable substrate for human mitochondrial HisRS ($HsHisRS_m$). As shown in Figure 1D, $HsHisRS_m$ charged the native $HstRNA_m^{His}$ with an efficiency slightly higher than that for *in vitro*-transcribed $HstRNA_m^{His}$ with G-1, but it could hardly charge *in vitro*-transcribed $HstRNA_m^{His}$ without G-1. Therefore, G-1 is an important identity element for $tRNA^{His}$. On the other hand, because human cytoplasmic HisRS ($HsHisRS_c$) strongly prefers $tRNA^{His}$ with A73 (29), it was much less efficient at charging the aforementioned $HstRNA_m^{His}$ (Figure 1E).

ATP/GTP ratios affects GTP incorporation into $tRNA_m^{His}$ by human Thg1

As yeast and archaeal Thg1 enzymes require different nucleotides for $tRNA^{His}$ activation (Figure 1A), we wondered whether ATP is absolutely required for HsThg1-catalyzed G-1 addition to $tRNA_m^{His}$ and whether ATP/GTP ratios affect the number of GTPs incorporated into $HstRNA_m^{His}$. To this end, a G-1 addition assay was carried out using p-$tRNA_m^{His}$ as the substrate under conditions with various concentrations of ATP and GTP, and aliquots of the reaction products were resolved by 8 M urea−10% polyacrylamide gel electrophoresis. Figure 2 shows that G-1 incorporation occurred only when ATP was present (compared lanes 10–12 and 1–9), suggesting that ATP is absolutely required for HsThg1-catalyzed GTP incorporation. In addition, GTP concentrations or ATP/GTP ratios affected the number of GTPs incorporated into $tRNA_m^{His}$. When the reaction was carried out at a relatively low ATP/GTP ratio (e.g. 1000/1000), more than one GTP was incorporated into p-$tRNA_m^{His}$ (see lanes 1–3). In contrast, when the reaction was carried out at a relatively high ATP/GTP ratio (e.g. 1000/10 or 1000/1), no multiple-GTP incorporation was observed (see lanes 4–9). Thus, the ratios of ATP to GTP affect the number of GTPs incorporated into $tRNA^{His}$ with C73. Inspired by this finding, we sought

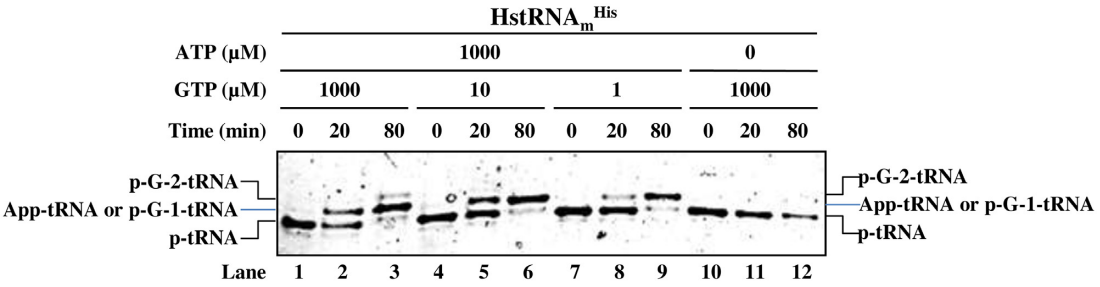

Figure 2. Incorporation of GTP into p-HstRNA$_m^{His}$ by human Thg1. A G-1 addition assay was carried out under conditions with various concentrations of ATP and GTP. The reactions were quenched by addition of 0.5 M EDTA at various times, and the products were resolved by 8 M urea–10% polyacrylamide gel electrophoresis.

to unveil mechanisms that can regulate the ATP/GTP ratio.

Human Thg1 displays tRNA-inducible GTPase activity

We next explored whether HsThg1 displays activities other than adenylylation and guanylylation. As HsThg1 closely interacts with mitofusin 2, a dynamin-like GTPase on the mitochondrial outer membrane (35), we set out to check whether HsThg1 also demonstrates a GTPase activity. Interestingly, HsThg1 could hydrolyze GTP to GDP to a certain extent in the absence of ATP and tRNA^{His}. Further addition of HstRNA$_n^{His}$ or HstRNA$_m^{His}$ to the reaction enhanced its activity (∼5-fold) (Figure 3A). In contrast, a similar activity was not detected in *S. cerevisiae* Thg1 (ScThg1), regardless of the presence or absence of SctRNA$_n^{His}$. To check whether HsThg1 can further hydrolyze GDP to GMP or even guanosine, GTP was treated with various concentrations of HsThg1, ranging from 16 to 0.5 μM. As shown in Figure 3B, no phosphate (P_i) signal was observed at any enzyme concentration, and only a poor GMP signal was observed at the highest enzyme concentration. Thus, HsThg1 predominantly cleaves γ-phosphate from GTP, yielding GDP (Figure 3B).

We next investigated whether this GTPase activity is tRNA^{His}-specific. Surprisingly, tRNA^{Thr} and tRNA^{Ala} were almost as effective as tRNA^{His} in inducing the GTPase activity (∼5-fold increase), but tRNA^{Phe} was less effective than HstRNA$_m^{His}$ (∼2-fold increase) (Figure 3C). Thus, the GTPase activity of HsThg1 is not tRNA^{His}-specific. Additionally, we checked whether an activated tRNA^{His} intermediate (ppp-HstRNA$_m^{His}$) and a mature tRNA$_m^{His}$ (p-G-1-HstRNA$_m^{His}$) can induce GTPase activity at a similar level. The results showed that these two tRNA$_m^{His}$ species could also induce the GTPase activity (3.6- and 2.7-fold, respectively) (Figure 3C). On the contrary, mRNA had little effect on the GTPase activity (Figure 3D). Together, these results suggest that HsThg1 can regulate the cellular GTP level (or ATP/GTP ratio) through its GTPase activity.

ATP inhibits the GTPase activity of human Thg1

As both ATP and GTP are substrates of HsThg1, we wondered whether ATP affects its GTPase activity. Pursuant to this objective, a GTPase assay was carried out with various concentrations of ATP, ranging from 1000 to 10 μM. As shown in Figure 4A, ATP inhibited the GTPase activity of HsThg1 in a dose-dependent manner. The higher the ATP concentration, the stronger the inhibition was. In addition, dATP, an ATP analog, was as effective as ATP in inhibiting the GTPase activity. To map the active site for the GTPase, we made two mutants, D76A (Mut A) in the adenylylation site and R27A (Mut G) in the guanylylation site, and then tested their G-1 addition and GTPase activities. Consistent with an earlier report (21), mutation at D76 impaired both adenylylation and guanylylation, whereas mutation at R27 impaired only guanylylation (Supplementary Figure S1). Our results also showed that mutation at either site abolished its ability to rescue a yeast *THG1* KO strain on 5-FOA (Figure 4B). Western blotting showed that these two mutants could be stably expressed in the yeast, albeit to a level slightly lower than that of its WT counterpart (Figure 4C).

As described earlier, the adenylylation site can accommodate only ATP and GTP, while the guanylylation site can accommodate all four nucleotides (21). We next analyzed the WT, Mut A, and Mut G for their relative GTPase activities under conditions with ATP, CTP or UTP. As shown in Figure 4D, all three nucleotides inhibited the GTPase activity of WT HsThg1 to a certain level, with ATP, UTP, and CTP reducing the activity 6-, 4- and 2-fold, respectively. Mutation at the guanylylation site (yielding Mut G) had little effect on its GTPase activity, and the mutant behaved like its WT counterpart in responding to nucleotide inhibition. In contrast, mutation at the adenylylation site (yielding Mut A) distinctly reduced its GTPase activity (∼2-fold), and the mutant was relatively insensitive to inhibition by ATP, UTP, or CTP. These results suggest that the GTPase activity resides in the adenylylation site of HsThg1.

GTPase activity exists in other high-eukaryotic Thg1 enzymes

As shown earlier, GTPase activity exists in HsThg1 but not in ScThg1 (Figure 3A), this prompted us to ask whether a similar activity exists in other high-eukaryotic Thg1 enzymes, especially those responsible for G-1 addition to both tRNAs^{His} with A73 and C73. To this end, we cloned the

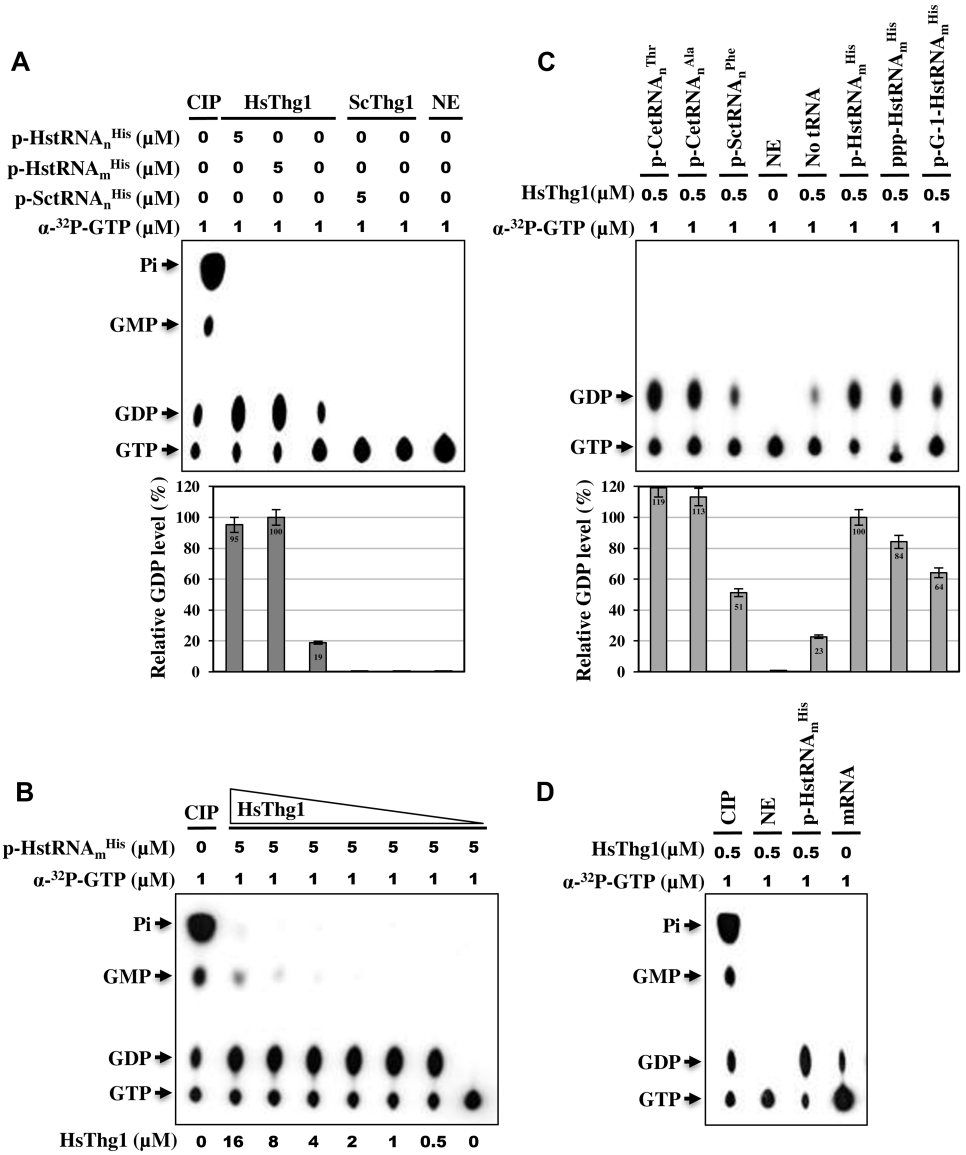

Figure 3. Human Thg1 possesses a tRNA-inducible GTPase activity. A GTPase assay was carried out under conditions (**A**) with or without tRNA^{His}, (**B**) with various concentrations of HsThg1, (**C**) with various tRNA species and (**D**) with mRNA. Relative positions of GTP, GDP, GMP and P_i are marked by partial hydrolysis of α-^{32}P-labeled GTP by CIP. Quantitative data for (A) and (C) were obtained from three independent experiments and averaged.

genes encoding *D. melanogaster* and *B. mori* Thg1 homologues (DmThg1 and BmThg1, respectively) and tested their activities *in vivo* and *in vitro*. As shown in Figure 5A, DmThg1 efficiently rescued the growth defect of the yeast KO strain on 5-FOA. In contrast, BmThg1 only weakly restored the growth phenotype of the KO strain, probably due to its poor expression in the yeast (Figure 5B). We next tested the purified Thg1 homologues for their GTPases activities *in vitro*. Remarkably, both DmThg1 and BmThg1 exhibited a strong GTPase activity, but the major product for DmThg1 was guanosine (removing α-, β- and γ-phosphates) instead of GDP (removing γ-phosphate) (Figure 5C). Thus, the GTPase activity is not unique to HsThg1, but instead is present in many other high-eukaryotic Thg1 homologues.

DISCUSSION

ATP/GTP ratios regulate the number of GTPs incorporated into HstRNA$_m^{His}$

A previous study showed that yeast Thg1 adds only one GTP (as G-1) to tRNA$_n^{His}$ (with A73), but it adds up to three GTPs (as G-1/G-2/G-3) to tRNA once its A73 mutates to C73 (17). Similarly, human Thg1 adds more than one GTP to tRNA$_m^{His}$ (with C73) *in vitro*. This poses a challenge to HisRS, as only p-G-1-tRNA^{His} is a suitable substrate for aminoacylation. GMP can be used as a substrate and incorporated into an activated tRNA$_m^{His}$ intermediate (ppp-tRNA$_m^{His}$), producing mature p-G-1-tRNA$_m^{His}$ *in vitro*. However, GMP is 22-fold less efficient than GTP in incorporation, which makes GMP an inferior substrate

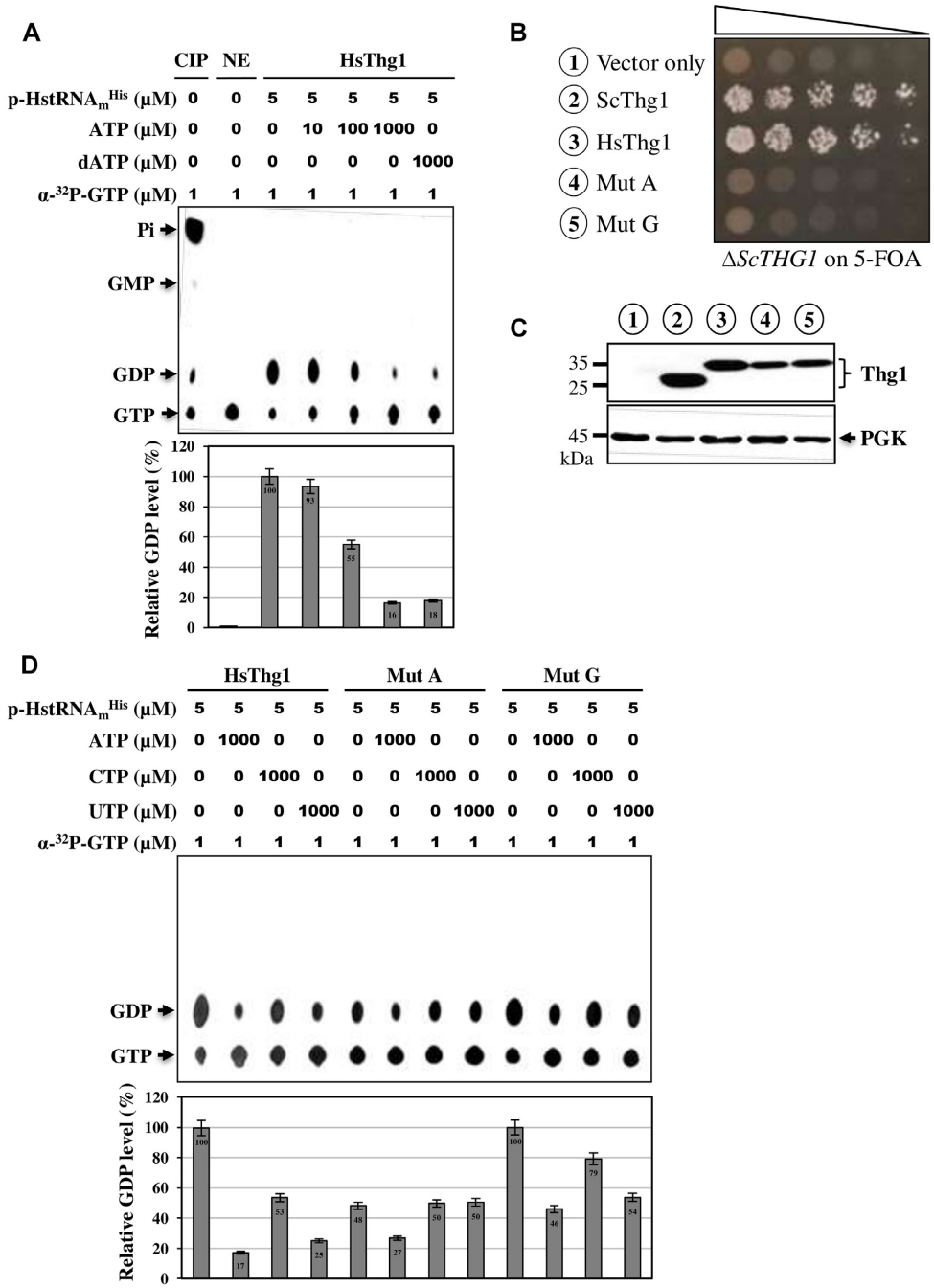

Figure 4. GTPase activity resides in the adenylylation site of human Thg1. (**A**) Inhibition of GTPase activity by ATP. GTPase activity of HsThg1 was tested with various concentrations of ATP. (**B**) Complementation assay. Complementation was determined by transforming the test plasmid harboring a WT or mutant Thg1 gene into a yeast *THG1* KO strain and plating the resultant transformants on 5-FOA. (**C**) Western blotting. The protein expression levels of the constructs used in (**B**) were determined by Western blotting using an anti-His_6-tagged antibody as the probe. Constructs used in (B, C) are numbered for clarity. (**D**) GTPase activities of WT and mutant human Thg1 enzymes. GTPase activities of WT and mutant HsThg1 enzymes were determined in the absence or presence of ATP, CTP, and UTP. Mut A and Mut G refer to D76A and R27A, respectively. Quantitative data for (A) and (D) were obtained from three independent experiments and averaged.

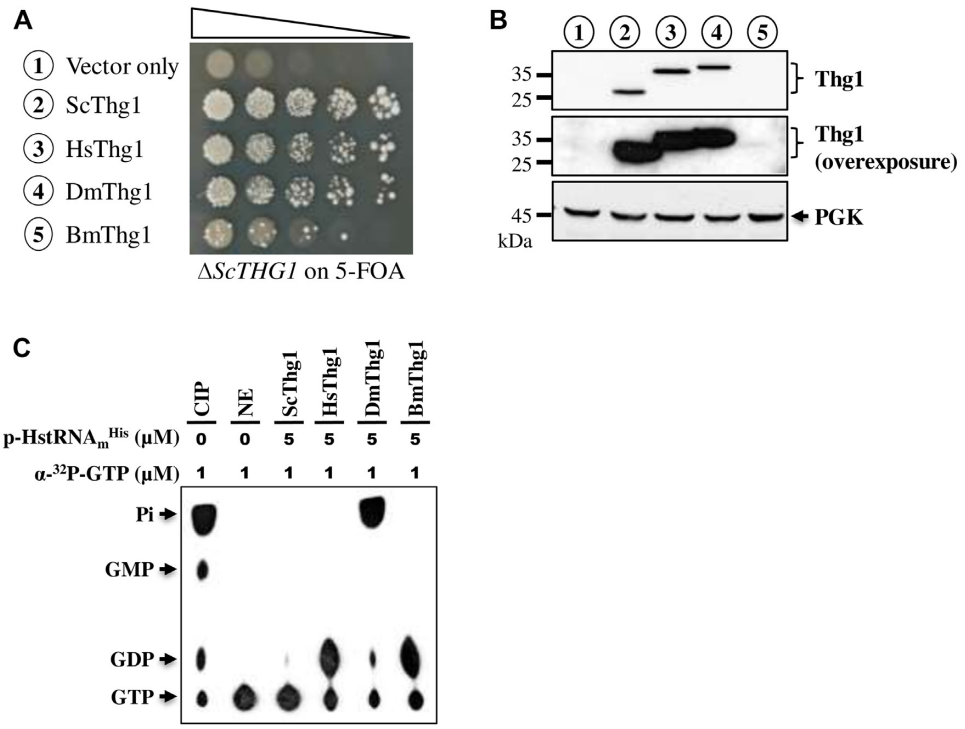

Figure 5. GTPase activity exists in other high-eukaryotic Thg1 homologues. (**A**) Complementation assay. Complementation was determined by transforming the test plasmid harboring a Thg1 homologue into a yeast *THG1* KO strain and plating the resultant transformants on 5-FOA. (**B**) Western blotting. The protein expression levels of the constructs used in (A) were determined by Western blotting using an anti-His_6-tagged antibody as the probe. Constructs used in (A, B) are numbered for clarity. (**C**) GTPase assay for various Thg1 enzymes.

(36,37). In addition, the concentration of GMP is much lower than that of GTP in human cells (38). We demonstrated herein that multiple-GTP incorporation prevailed when the ATP/GTP ratio was low (Figure 2). In contrast, a single-GTP incorporation prevailed when the ATP/GTP ratio was high. As the concentration of ATP is ∼10-fold higher than that of GTP inside the cell (∼1000 μM and ∼100 μM, respectively) (38,39), such a condition might favor a single-GTP incorporation (Figure 2). However, under circumstances when the relative mitochondrial GTP level is increased, reducing the GTP level becomes necessary and pertinent to ensure proper maturation of $tRNA_m^{His}$.

Human Thg1 displays a tRNA-inducible GTPase activity

The GTPase-family enzymes contain a classical G domain that folds structurally into five α helices, six β strands, and five loops interconnecting the helices and strands (40). Interestingly, the palm domain of a human Thg1 monomer holds a similar architechure, with four α helices, six β strands, and five loops interconnecting the helices and strands (21). A structural study revealed that HsThg1 appears as a dimer of dimers (AB and CD), holding two $tRNA^{His}$ molecules facing one another in a parallel fashion. Each $tRNA^{His}$ molecule occupies three subunits, where its acceptor stem is positioned in between subunits A and B, while its anticodon is positioned in subunit D or C (23). Although HsThg1 specifically recognizes the anticodon of $tRNA^{His}$, its GTPase activity can be induced not only by $tRNA^{His}$ of various statuses, including p-$tRNA^{His}$ (an immature $tRNA^{His}$), ppp-$tRNA^{His}$ (an activated $tRNA^{His}$ intermediate), and p-G-1-$tRNA^{His}$ (a mature $tRNA^{His}$), but also by non-$tRNA^{His}$ species, such as $tRNA^{Ala}$, $tRNA^{Thr}$ and $tRNA^{Phe}$, suggesting that this induction activity is not anticodon specific. (Figure 3). As $tRNA^{Phe}$ is not as efficient as other tRNAs tested, other parts of tRNA might be involved in this activity. Sequence and structural comparison showed that $tRNA^{Phe}$ is different from others by having a larger D-loop, a G at position 37, a U at position 52, and an A at position 62. Whether or not these differences play a role in this activity requires further verification. As most GTPase-family enzymes are induced by DNA (41), K^+ (42), or ribosome (43), HsThg1 represents an interesting example of a tRNA-inducible GTPase.

Human Thg1's GTPase activity resides in the adenylylation site

Despite the fact that yeast and human Thg1 enzymes follow a similar pathway for G-1 addition (17,21), only human Thg1 can efficiently hydrolyze GTP to GDP. Our results showed that the GTPase activity of HsThg1 resides in the adenylylation site. Therefore, mutation at the adenylylation site (D76A), but not the guanylylation site (R27A), distinctly reduces its GTPase activity (Figure 4). Moreover, ATP is much more potent than UTP and CTP in inhibiting the GTPase activity (Figure 4). As depicted in Figure 6, the amino acid residues in the adenylylation site make direct contacts with the nitrogen base (A43, D47 and S75), ribose

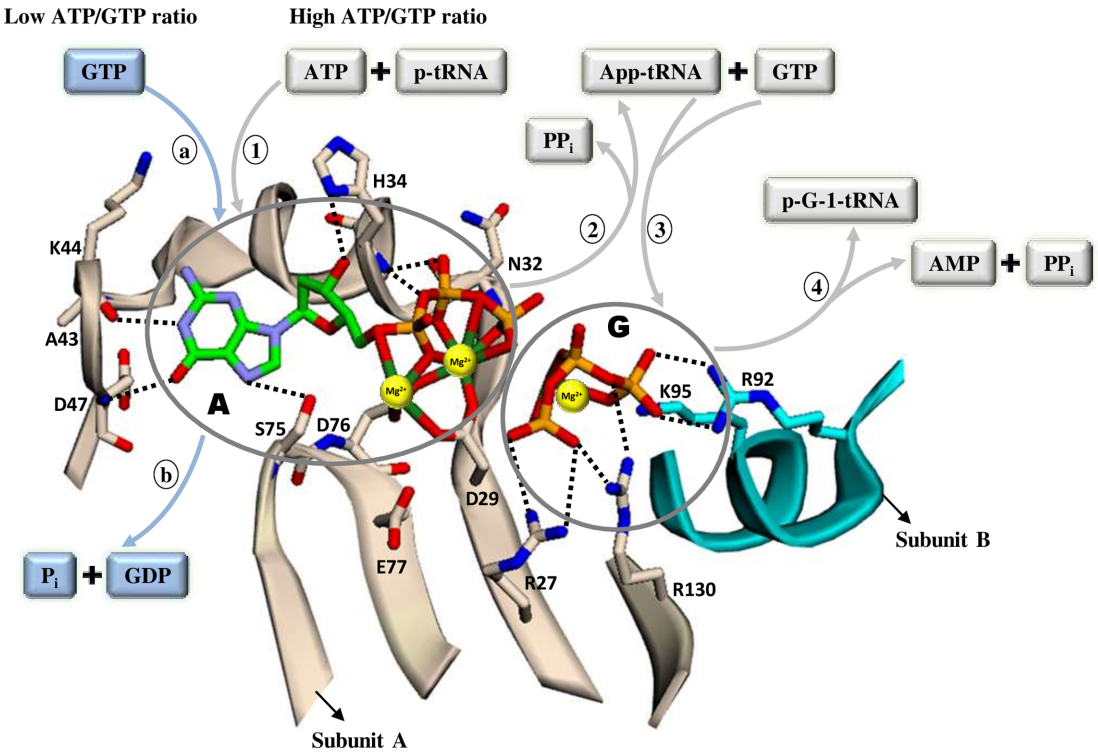

Figure 6. A possible interplay between G-1 addition and GTP hydrolysis by human Thg1. The protein structure was obtained from dimeric human Thg1-dGTP complex (PDB ID: 3OTB). Subunits A and B of human Thg1 are colored in wheat and turquoise, respectively. The adenylylation and guanylylation sites are marked as 'A' and 'G', respectively. Under conditions with a high ATP/GTP ratio, HsThg1 preferentially catalyzes G-1 incorporation into $tRNA_m^{His}$ (reactions 1 to 4), whereas under conditions with a low ATP/GTP ratio, HsThg1 preferentially catalyzes the hydrolysis of GTP to GDP (reactions a to b).

(H34), and triphosphate (N32, H34 and D76) of the purine nucleotide. Therefore, the pyrimidine nucleotides (CTP or UTP) cannot properly fit the pocket. Moreover, mutation at D76 disrupts its interaction with the triphosphate of the purine nucleotide and impairs the GTPase activity. On the contrary, the base and sugar moieties are not seen in the electron density map of the guanylylation site presumably due to lack of specific interactions (21). As a result, the adenylylation site can fit only purine nucleotides (ATP or GTP), while the guanylylation site can accommodate all four nucleotides (ATP, UTP, CTP or GTP) with a preference toward GTP/UTP (37). When the ATP/GTP ratio is high, the enzyme preferentially catalyzes G-1 addition to $tRNA^{His}$, producing a mature $tRNA^{His}$ for protein synthesis. Conversely, when the ATP/GTP ratio is low, the enzyme preferentially hydrolyzes GTP to GDP, precluding multiple-GTP incorporation into $tRNA_m^{His}$. Human Thg1 interacts strongly with both Mfn1 and Mfn2, two dynamine-like GTPase homologues that play a central role in regulating mitochondrial fusion and cell metabolism. Thg1 increases Mfn2′s GTP-binding affinity significantly (44). However, It is unclear whether Mfn2 also increases Thg1′s G-1 addition or GTPase activity.

tRNA-inducible GTPase activity exists in high-eukaryotic Thg1 enzymes

Yeast Thg1 adds G-1 to $tRNA_n^{His}$ across from A73, resulting in a G-1:A73 mismatch, which would terminate continuous 3′-5′ polymerization through a template-dependent mechanism (Figure 1A) (17). However, human Thg1 needs to add G-1 not only to $tRNA_n^{His}$ (with A73) but also to $tRNA_m^{His}$ (with C73) (25). As N73 is immediately followed by a CCA end (Figure 1B), multiple-GTP incorporation (as G-2/G-3) through a template-dependent mechanism can occur with $tRNA_m^{His}$ *in vitro* (Figure 2) (17,36). Because $tRNA^{His}$ with an additional G-2:C74 base pair perturbs the universally conserved CCA end of tRNA and might interfere with a conformational change important for translation (27), an approach to generate mature $tRNA^{His}$ with only G-1 becomes crucial. Our data showed that human Thg1 possesses a tRNA-inducible GTPase activity, which could be involved in down-regulating the GTP concentration inside mitochondria (Figure 6). This activity is particularly pertinent for Thg1 enzymes that must cope with $tRNA^{His}$ carrying C73. Indeed, many high-eukaryotic Thg1 enzymes that act on both $tRNAs^{His}$ with A73 and C73 (or U73) (29) possess a similar activity (Figure 5). However, regardless of the detailed interpretation, our study suggests that HsThg1 exhibits an ATP-inhibitable, tRNA-inducible GTPase activity. Additionally, an earlier report showed that HsThg1 interacts with a mitochondrial dynamin-like GTPase (Mfn1 or Mfn2) to regulate the GTP level. Such an interaction might allow Thg1 to participate in mitochondrial fusion and bioenergetic function (35).

DATA AVAILABILITY

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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REFERENCES

1. Hopper,A.K. and Phizicky,E.M. (2003) tRNA transfers to the limelight. *Genes Dev.*, **17**, 162–180.
2. Kufel,J. and Tollervey,D. (2003) 3′-processing of yeast tRNATrp precedes 5′-processing. *RNA*, **9**, 202–208.
3. Orellana,O., Cooley,L. and Soll,D. (1986) The additional guanylate at the 5′ terminus of *Escherichia coli* $tRNA^{His}$ is the result of unusual processing by RNase P. *Mol. Cell. Biol.*, **6**, 525–529.
4. Wang,C., Sobral,B.W. and Williams,K.P. (2007) Loss of a universal tRNA feature. *J. Bacteriol.*, **189**, 1954–1962.
5. Gu,W., Jackman,J.E., Lohan,A.J., Gray,M.W. and Phizicky,E.M. (2003) tRNAHis maturation: an essential yeast protein catalyzes addition of a guanine nucleotide to the 5′ end of tRNAHis. *Genes Dev.*, **17**, 2889–2901.
6. Rosen,A.E. and Musier-Forsyth,K. (2004) Recognition of G-1:C73 atomic groups by escherichia coli histidyl-tRNA synthetase. *J. Am. Chem. Soc.*, **126**, 64–65.
7. Connolly,S.A., Rosen,A.E., Musier-Forsyth,K. and Francklyn,C.S. (2004) G-1:C73 recognition by an arginine cluster in the active site of escherichia coli histidyl-tRNA synthetase. *Biochemistry*, **43**, 962–969.
8. Rudinger,J., Florentz,C. and Giegé,R. (1994) Histidylation by yeast HisRS of tRNA or tRNA-like structure relies on residues -1 and 73 but is dependent on the RNA context. *Nucleic Acids Res.*, **22**, 5031–5037.
9. Himeno,H., Hasegawa,T., Ueda,T., Watanabe,K., Miura,K. and Shimizu,M. (1989) Role of the extra G-C pair at the end of the acceptor stem of tRNA(His) in aminoacylation. *Nucleic Acids Res.*, **17**, 7855–7863.
10. Preston,M.A. and Phizicky,E.M. (2010) The requirement for the highly conserved G-1 residue of saccharomyces cerevisiae tRNAHis can be circumvented by overexpression of tRNAHis and its synthetase. *RNA*, **16**, 1068–1077.
11. Gu,W., Hurto,R.L., Hopper,A.K., Grayhack,E.J. and Phizicky,E.M. (2005) Depletion of saccharomyces cerevisiae tRNA(His) guanylyltransferase thg1p leads to uncharged tRNAHis with additional m(5)C. *Mol. Cell. Biol.*, **25**, 8191–8201.
12. Heinemann,I.U., O'Donoghue,P., Madinger,C., Benner,J., Randau,L., Noren,C.J. and Söll,D. (2009) The appearance of pyrrolysine in tRNAHis guanylyltransferase by neutral evolution. *Proc. Natl. Acad. Sci. U.S.A.*, **106**, 21103–21108.
13. Heinemann,I.U., Nakamura,A., O'Donoghue,P., Eiler,D. and Söll,D. (2012) tRNAHis-guanylyltransferase establishes tRNAHis identity. *Nucleic Acids Res.*, **40**, 333–344.
14. Unseld,M., Marienfeld,J.R., Brandt,P. and Brennicke,A. (1997) The mitochondrial genome of arabidopsis thaliana contains 57 genes in 366,924 nucleotides. *Nat. Genet.*, **15**, 57–61.
15. Maréchal-Drouard,L., Kumar,R., Remacle,C. and Small,I. (1996) RNA editing of larch mitochondrial tRNA(His) precursors is a prerequisite for processing. *Nucleic Acids Res.*, **24**, 3229–3234.
16. Oda,K., Yamato,K., Ohta,E., Nakamura,Y., Takemura,M., Nozato,N., Akashi,K. and Ohyama,K. (1992) Transfer RNA genes in the mitochondrial genome from a liverwort, marchantia polymorpha: the absence of chloroplast-like tRNAs. *Nucleic Acids Res.*, **20**, 3773–3777.
17. Jackman,J.E. and Phizicky,E.M. (2006) tRNAHis guanylyltransferase catalyzes a 3′-5′ polymerization reaction that is distinct from G-1 addition. *Proc. Natl. Acad. Sci. U.S.A.*, **103**, 8640–8645.
18. Abad,M.G., Rao,B.S. and Jackman,J.E. (2010) Template-dependent 3′-5′ nucleotide addition is a shared feature of tRNAHis guanylyltransferase enzymes from multiple domains of life. *Proc. Natl. Acad. Sci. U.S.A.*, **107**, 674–679.
19. Abad,M.G., Long,Y., Willcox,A., Gott,J.M., Gray,M.W. and Jackman,J.E. (2011) A role for tRNA(His) guanylyltransferase (Thg1)-like proteins from dictyostelium discoideum in mitochondrial 5′-tRNA editing. *RNA*, **17**, 613–623.
20. Heinemann,I.U., Randau,L., Tomko,R.J. Jr and Söll,D. (2010) 3′-5′ tRNAHis guanylyltransferase in bacteria. *FEBS Lett.*, **584**, 3567–3572.
21. Hyde,S.J., Eckenroth,B.E., Smith,B.A., Eberley,W.A., Heintz,N.H., Jackman,J.E. and Doublié,S. (2010) tRNA(His) guanylyltransferase (THG1), a unique 3′-5′ nucleotidyl transferase, shares unexpected structural homology with canonical 5′-3′ DNA polymerases. *Proc. Natl. Acad. Sci. U.S.A.*, **107**, 20305–20310.
22. Rao,B.S., Maris,E.L. and Jackman,J.E. (2011) tRNA 5′-end repair activities of tRNAHis guanylyltransferase (Thg1)-like proteins from bacteria and archaea. *Nucleic Acids Res.*, **39**, 1833–1842.
23. Nakamura,A., Nemoto,T., Heinemann,I.U., Yamashita,K., Sonoda,T., Komoda,K., Tanaka,I., Söll,D. and Yao,M. (2013) Structural basis of reverse nucleotide polymerization. *Proc. Natl. Acad. Sci. U.S.A.*, **110**, 20970–20975.
24. Doublié,S., Tabor,S., Long,A.M., Richardson,C.C. and Ellenberger,T. (1998) Crystal structure of a bacteriophage T7 DNA replication complex at 2.2 a resolution. *Nature*, **391**, 251–258.
25. Nakamura,A., Wang,D. and Komatsu,Y. (2018) Biochemical analysis of human $tRNA^{His}$ guanylyltransferase in mitochondrial $tRNA^{His}$ maturation. *Biochem. Biophys. Res. Commun.*, **503**, 2015–2021.
26. Schmeing,T.M., Huang,K.S., Strobel,S.A. and Steitz,T.A. (2005) An induced-fit mechanism to promote peptide bond formation and exclude hydrolysis of peptidyl-tRNA. *Nature*, **438**, 520–524.
27. Chang,C.P., Lin,G., Chen,S.J., Chiu,W.C., Chen,W.H. and Wang,C.C. (2008) Promoting the formation of an active synthetase/tRNA complex by a nonspecific tRNA-binding domain. *J. Biol. Chem.*, **283**, 30699–30706.
28. Jackman,J.E., Montange,R.K., Malik,H.S. and Phizicky,E.M. (2003) Identification of the yeast gene encoding the tRNA m1G methyltransferase responsible for modification at position 9. *RNA*, **9**, 574–585.
29. Lee,Y.H., Chang,C.P., Cheng,Y.J., Kuo,Y.Y., Lin,Y.S. and Wang,C.C. (2017) Evolutionary gain of highly divergent tRNA specificities by two isoforms of human histidyl-tRNA synthetase. *Cell. Mol. Life Sci.*, **74**, 2663–2677.
30. Choudhury,S.R., Westfall,C.S., Hackenberg,D. and Pandey,S. (2013) Measurement of GTP-binding and GTPase activity of heterotrimeric gα proteins. *Methods Mol. Biol.*, **1043**, 13–20.
31. Hames,B.D. and Rickwood,D. (1981) In: *Gel Electrophoresis of Proteins: A Practical Approach, B.D. Hames, D. Rickwood (eds.)*. IRL Press, London.
32. Lee,Y.-H., Lo,Y.-T., Chang,C.-P., Yeh,C.-S., Chang,T.-H., Chen,Y.-W., Tseng,Y.-K. and Wang,C.-C. (2019) Naturally occurring dual recognition of tRNAHis substrates with and without a universal identity element. *RNA Biol.*, **16**, 1275–1285.
33. Bourgeois,G., Marcoux,J., Saliou,J.-M., Cianférani,S. and Graille,M. (2017) Activation mode of the eukaryotic m2G10 tRNA methyltransferase trm11 by its partner protein trm112. *Nucleic Acids Res.*, **45**, 1971–1982.
34. Urbonavicius,J., Armengaud,J. and Grosjean,H. (2006) Identity elements required for enzymatic formation of N2,N2-dimethylguanosine from N2-monomethylated derivative and its possible role in avoiding alternative conformations in archaeal tRNA. *J. Mol. Biol.*, **357**, 387–399.
35. Hickey,F.B., Corcoran,J.B., Griffin,B., Bhreathnach,U., Mortiboys,H., Reid,H.M., Andrews,D., Byrne,S., Furlong,F., Martin,F. *et al.* (2014) IHG-1 increases mitochondrial fusion and bioenergetic function. *Diabetes*, **63**, 4314–4325.
36. Nakamura,A., Wang,D. and Komatsu,Y. (2018) Biochemical analysis of human tRNA(His) guanylyltransferase in mitochondrial tRNA(His) maturation. *Biochem. Biophys. Res. Commun.*, **503**, 2015–2021.
37. Nakamura,A., Wang,D. and Komatsu,Y. (2021) Analysis of GTP addition in the reverse (3′-5′) direction by human tRNA(His) guanylyltransferase. RNA, **27**, 665–675.
38. Traut,T.W. (1994) Physiological concentrations of purines and pyrimidines. *Mol. Cell. Biochem.*, **140**, 1–22.

39. Zala,D., Schlattner,U., Desvignes,T., Bobe,J., Roux,A., Chavrier,P. and Boissan,M. (2017) The advantage of channeling nucleotides for very processive functions. *F1000Res*, **6**, 724.
40. Wittinghofer,A. and Vetter,I.R. (2011) Structure-function relationships of the g domain, a canonical switch motif. *Annu. Rev. Biochem.*, **80**, 943–971.
41. Liu,C.C. and Alberts,B.M. (1981) Characterization of the DNA-dependent GTPase activity of T4 gene 41 protein, an essential component of the T4 bacteriophage DNA replication apparatus. *J. Biol. Chem.*, **256**, 2813–2820.
42. Manikas,R.G., Thomson,E., Thoms,M. and Hurt,E. (2016) The K^+-dependent GTPase nug1 is implicated in the association of the helicase dbp10 to the immature peptidyl transferase centre during ribosome maturation. *Nucleic. Acids. Res.*, **44**, 1800–1812.
43. Kimura,T., Takagi,K., Hirata,Y., Hase,Y., Muto,A. and Himeno,H. (2008) Ribosome-small-subunit-dependent GTPase interacts with tRNA-binding sites on the ribosome. *J. Mol. Biol.*, **381**, 467–477.
44. Li,Y.J., Cao,Y.L., Feng,J.X., Qi,Y., Meng,S., Yang,J.F., Zhong,Y.T., Kang,S., Chen,X., Lan,L. *et al.* (2019) Structural insights of human mitofusin-2 into mitochondrial fusion and CMT2A onset. *Nat. Commun.*, **10**, 4914.