Biogenesis of glutaminyl-mt tRNA^{Gln} in human mitochondria

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Mammalian mitochondrial (mt) tRNAs, which are required for mitochondrial protein synthesis, are all encoded in the mitochondrial genome, while mt aminoacyl-tRNA synthetases (aaRSs) are encoded in the nuclear genome. However, no mitochondrial homolog of glutaminyl-tRNA synthetase (GlnRS) has been identified in mammalian genomes, implying that Gln-tRNA^{Gln} is synthesized via an indirect pathway in the mammalian mitochondria. We demonstrate here that human mt glutamyl-tRNA synthetase (mtGluRS) efficiently misaminoacylates mt tRNA^{Gln} to form Glu-tRNA^{Gln}. In addition, we have identified a human homolog of the Glu-tRNA^{Gln} amidotransferase, the hGatCAB heterotrimer. When any of the hGatCAB subunits were inactivated by siRNA-mediated knock down in human cells, the Glu-charged form of tRNA^{Gln} accumulated and defects in respiration could be observed. We successfully reconstituted in vitro Gln-tRNA^{Gln} formation catalyzed by the recombinant mtGluRS and hGatCAB. The misaminoacylated form of tRNA^{Gln} has a weak binding affinity to the mt elongation factor Tu (mtEF-Tu), indicating that the misaminoacylated form of tRNA^{Gln} is rejected from the translational apparatus to maintain the accuracy of mitochondrial protein synthesis.

EF-Tu | Glu-tRNAGln amidotransferase | glutamyl-tRNA synthetase | mitochondrial tRNA

Translational accuracy is required to properly decipher the genetic code to form proteins. The fidelity of protein synthesis largely depends on the formation of correct aminoacyl-tRNAs (aa-tRNAs) by the corresponding aminoacyl-tRNA synthetases (aaRSs). In the classical model, each species of aaRS strictly discriminates one amino acid among the 20 canonical amino acids, as well as its cognate tRNA isoacceptor, from non-cognate tRNA species. However, recent genomic analyses have revealed that the full complement of 20 aaRSs is used only in eukaryotic cytoplasm and a minority of bacteria, whereas a majority of the bacterial and all of the known archaeal genomes do not encode a glutaminyl-tRNA synthetase (GlnRS) (1). In addition, an asparaginyl-tRNA synthetase (AsnRS) is not encoded in most bacterial and archaeal genomes (1). In these organisms, the amide-amino acid (Gln or Asn) on its cognate tRNA is synthesized via an indirect pathway (2). In most bacteria and archaea, in which GlnRS is absent, tRNA^{Gln} is first misaminoacylated with Glu catalyzed by a non-discriminating GluRS (ND-GluRS) that can aminoacylate both tRNA^{Glu} and tRNA^{Gln}. The Glu moiety on the Glu-tRNA^{Gln} is then transamidated by a glutamyl-tRNA^{Gln} amidotransferase (Glu-AdT) in the presence of ATP and Gln as an amide donor to synthesize Gln-tRNA^{Gln}. Similarly, in the case of Asn-tRNA^{Asn} formation in organisms lacking AsnRS, Asn-tRNA^{Asn} is synthesized by a non-discriminating aspartyl-tRNA synthetase and an aspartyl-tRNA^{Asn} amidotransferase (Asp-AdT).

Two types of tRNA-dependent amidotransferase exist, the heterotrimeric GatCAB (3) and heterodimeric GatDE (1). Bacterial GatCAB functions as a Glu-AdT or an Asp-AdT in a species-specific manner. In some bacteria lacking both AsnRS and GlnRS, GatCAB has a dual activity as both a Glu-AdT and an Asp-AdT (2). As each subunit of GatCAB is encoded only in archaeal genomes lacking a gene for AsnRS, archaeal GatCAB

seems to function as an Asp-AdT. GatDE is only found in archaea, and serves as a Glu-AdT (2).

GatB and GatE are paralogous subunits having a tRNA-dependent kinase activity, while GatA and GatD are the glutaminase subunits of the AdT. For transamidation of Glu on the tRNAs, Glu-AdT catalyzes three sequential reactions. First, the GatB (or GatE) subunit uses ATP to phosphorylate the Glu moiety on the Glu-tRNA^{Gln}, forming a γ-phosphoryl-Glu-tRNA^{Gln} as an activated intermediate. Second, the GatA (or GatD) subunit catalyzes the liberation of ammonia from Gln as an amide donor. Finally, Glu-AdT amidates the activated intermediate using the liberated ammonia to form Gln-tRNA^{Gln}.

Mitochondria have own translational apparatus that synthesizes proteins encoded in mitochondrial (mt) genome. While with the exception of some protozoans the required rRNA and a full set of tRNAs are encoded in the mtDNA (4), all other factors in the mt translational apparatus are encoded in nuclear genome, translated in the cytoplasm, and then imported into the mitochondria. However, according to eukaryotic genomic analyses, to date, it is known that mitochondrial ortholog of GlnRS is not encoded in nuclear genome (4). This fact is consistent with the observation that no GlnRS activity could be detected in mitochondria purified from some eukaryotic species (5), implying that mitochondrial Gln-tRNA^{Gln} formation proceeds via the indirect pathway. In *Saccharomyces cerevisiae*, it is known that *gatA* and *gatB* orthologs encoded in nuclear genome are involved in mitochondrial function (6–8), although *gatC* ortholog was not identified. These observations strongly indicated that Gln-tRNA^{Gln} is formed via the indirect pathway in yeast mitochondria. However, it was reported that yeast mtGluRS is not an ND-GluRS that does not generate Glu-tRNA^{Gln}, obligatory intermediate of the indirect pathway (9, 10). These contradictory facts complicated the issue of Gln-tRNA^{Gln} formation in yeast mitochondria. Recent study has reported that cytoplasmic GluRS is imported into yeast mitochondria, and acts as an ND-GluRS that glutamylates mt tRNA^{Gln} to synthesize Glu-tRNA^{Gln} (9). In addition, they have found a new type heterotrimeric amidotransferase GatFAB that converts Glu-tRNA^{Gln} into Gln-tRNA^{Gln} (9). GatF is a fungi-specific subunit of Glu-AdT. In higher plants, dual targeted GluRS and GatCAB are responsible for both mitochondrial and chloroplastic Gln-tRNA^{Gln} formation in the respective organelles (11). Thus, mitochondrial Gln-tRNA^{Gln} formation is likely species-specific, which might make it difficult to predict what pathway and factors generate mitochon-

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drial Gln-tRNA^{Gln} for mitochondrial translation in a given eukaryote.

Mammalian mitochondria have a translational apparatus that synthesizes 13 proteins encoded in the mitochondrial (mt) genome. While two rRNAs and a full set of tRNAs are encoded in the mtDNA, all other translational factors including aaRSs are encoded in the nuclear genome. To our knowledge, 11 species of mammalian mt aaRSs have been characterized (12). Among them, only two aaRSs, mtLysRS and mtGlyRS, share the same genes as the cytoplasmic LysRS and GlyRS, respectively. The other aaRSs which are mitochondria-specific have been bioinformatically predicted as bacterial homologs in mammalian genomes (12). However, no mitochondrial homolog of GlnRS has been predicted in a mammalian genome, implying that Gln-tRNA^{Gln} is synthesized via the indirect pathway in mammalian mitochondria. This speculation is supported by the observation that mitochondrial extracts from mouse liver do not exhibit GlnRS activity (5). It was recently reported that cytoplasmic tRNA^{Gln} is imported into human mitochondria (13), indicating an involvement of this tRNA in the biogenesis of Gln-tRNA^{Gln} in human mitochondria. However, fate of the imported tRNA and the enzymatic pathway for generating mitochondrial Gln-tRNA^{Gln} were unknown. In this study, we aimed to clarify the pathway and mechanism of Gln-tRNA^{Gln} formation in human mitochondria by characterizing the mtGluRS and a human homolog of GatCAB in vitro as well as in vivo.

Results

Post-Transcriptional Modifications of Mammalian Mitochondrial tRNA^{Glu} and tRNA^{Gln} Isolated from Bovine Liver. In *E. coli*, the wobble modification of tRNA^{Glu} is essential for glutamylation by GluRS (14). Therefore, to study Gln-tRNA^{Gln} formation in mammalian mitochondria, we needed to examine an aminoacylation of native mt tRNAs which are fully modified, instead of using in vitro transcribed tRNAs with no modification. We isolated mt tRNAs for Glu and Gln from bovine liver using chaplet column chromatography (15). The primary structures, including the posttranscriptional modifications, of the isolated tRNAs were determined by mass spectrometric analysis (16, 17) (Fig. S1 and Tables S1 and S2). As shown in Fig. 1A, both tRNAs contain modified bases at several positions. At the wobble positions, the tRNAs commonly contain 5-taurinomethyl-2-thiouridine (τm^{5}s^{2}U), which is a mammalian mitochondria-specific uridine derivative we previously identified at the wobble position in mt tRNA^{Lys} (18). In addition, we also isolated mt tRNAs for Glu and Gln from human placenta (19), and confirmed the presence of τm^{5}s^{2}U at the wobble positions.

Human Mitochondrial GluRS Is a Non-Discriminating GluRS. Since most mitochondrial proteins are homologous to their bacterial counterparts, we searched for a gene having sequence similarity to bacterial GluRS in the non-redundant NCBI database, and retrieved a candidate gene for the human ortholog of the bacterial GluRS (protein ID = NP_001077083). This gene had been previously predicted to be a GluRS by other group (12). Sequence alignment of the putative human mtGluRS with other putative mitochondrial and bacterial GluRS proteins (Fig. S2) revealed that the mtGluRS has an extended N-terminal sequence, which is predicted to be a mitochondria-targeting signal with a high probability value (0.994) by MitoProt (http://ihg.gsf.de/ihg/mitoprot.html) (20), strongly suggesting that the putative mtGluRS is indeed localized in mitochondria. To examine the subcellular localization of the mtGluRS, full length mtGluRS with EGFP fused to its C terminus was transiently expressed in HeLa cells. As observed by confocal microscopy (Fig. 1B, *Top*), mtGluRS-EGFP was clearly localized to mitochondria.

As we were unable to identify a candidate gene for a human ortholog of the bacterial GlnRS, consistent with previous reports
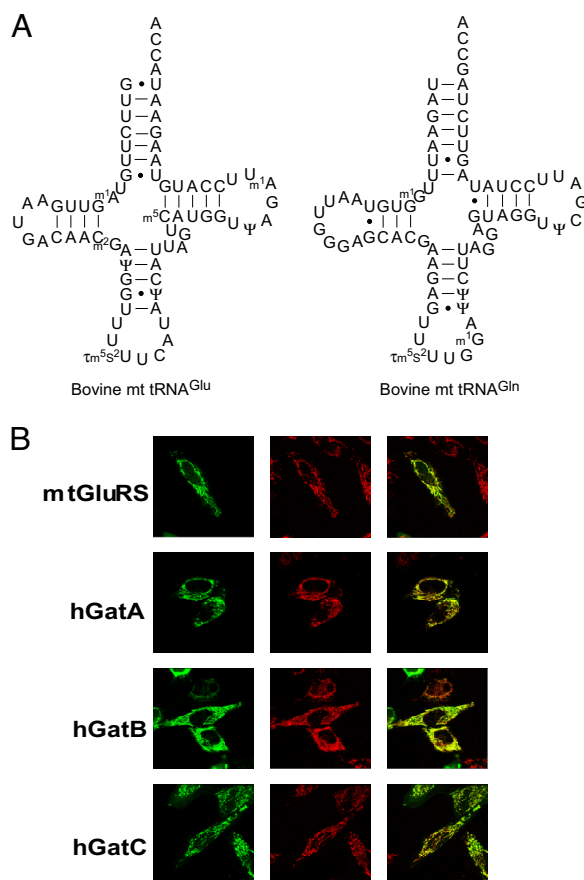

Fig. 1. Primary structures of mitochondrial tRNA^{Glu} and tRNA^{Gln}, and subcellular localization of mtGluRS and hGatCAB. (*A*) Nucleotide sequences of bovine mt tRNA^{Glu} (*Left*) and mt tRNA^{Gln} (*Right*). Modified nucleosides: τm^{5}s^{2}U, 5-taurinomethyl-2-thiouridine; Ψ, pseudouridine; m^{1}A, 1-methyladenosine; m^{2}G, N^2-methylguanosine; m^{1}G, 1-methylguanine; m^{5}C, 5-methylcytidine. (*B*) Subcellular localization of mtGluRS and three subunits of hGatCAB. Confocal fluorescence microscopic image of HeLa cells transfected with pEGFP-mtGluRS (top row), pEGFP-hGatA (second row), pEGFP-hGatB (third row) and pEGFP-hGatC (bottom row). Left panels show EGFP fluorescence. Middle images show mitochondria stained by MitoTracker Red. The right column shows the merged images.

(12), we hypothesized that Gln-tRNA^{Gln} is synthesized via the indirect pathway in human mitochondria. Thus, mtGluRS may be a non-discriminating GluRS (ND-GluRS), which can aminoacylate both mt tRNA^{Glu} and mt tRNA^{Gln} as natural substrates.

We therefore examined the aminoacylation ability of mt-GluRS. The cDNA of mtGluRS lacking the N-terminal mitochondria-targeting sequence was cloned into an expression vector. The N-terminal His-tagged mtGluRS was recombinantly expressed in *E. coli*, and purified to homogeneity. To investigate whether the recombinant mtGluRS is an ND-GluRS, we carried out glutamylation of mt tRNAs for Glu and Gln isolated from bovine liver, as well as an in vitro transcribed mt tRNA^{Glu} as a control. The mtGluRS efficiently glutamylated both mt tRNA^{Gln} and mt tRNA^{Glu} (Table S3), demonstrating that mtGluRS is an ND-GluRS. Since the in vitro transcribed mt tRNA^{Glu} was not glutamylated, it was found that posttranscriptional modifications in mt tRNA^{Glu} are necessary for glutamylation. As reported for bacterial GluRS, mtGluRS presumably recognizes the wobble modifications of the mt tRNAs for Glu and Gln. We also confirmed that human mt tRNAs for Glu and Gln isolated from placenta were efficiently glutamylated by mtGluRS, and our

observations are, therefore, not due to the heterologous combination of human mtGluRS and bovine mt tRNAs.

The kinetic parameters of glutamylation of the bovine mt tRNAs for Glu and Gln by the recombinant mtGluRS were determined (Table S3). Although the K_m value (1.7 μM) for the mt tRNA^{Gln} was $\approx$6 fold higher than that (0.27 μM) for mt tRNA^{Glu}, the k_{cat}/K_m of mt tRNA^{Gln} was only 2-fold lower than that of mt tRNA^{Glu}. The ND-GluRS from *Thermosynechococcus elongatus* exhibits a K_m value for glutamylation of tRNA^{Gln} that is approximately 5-fold higher than that of tRNA^{Glu} (21). Therefore, the higher K_m value for glutamylation of tRNA^{Gln} may be a common feature of ND-GluRS.

Identification and Characterization of Human Glu-tRNA^{Gln} Amidotransferase. We next searched for human orthologs of bacterial *gatA*, *gatB*, and *gatC* from the human non-redundant NCBI database, and retrieved candidate genes for each: NM_018292 (NP_060762) as a *gatA*-like gene, NM_004564 (NP_004555) as a *gatB*-like gene, and NM_176818 (NP_789788) as a *gatC*-like gene (named hGatA, hGatB and hGatC, respectively). The sequence alignment of each of the human homologs with bacterial and mitochondrial counterparts revealed that hGatB and hGatC possess N-terminal mitochondria-targeting sequences, while hGatA does not (Fig. S3). The MitoProt values for mitochondria-targeting of hGatA (0.18), hGatB (0.82), and hGatC (0.16) are not indicative as to whether all of the homologs are actually localized in mitochondria and form a heterotrimeric amidotransferase. To determine the subcellular localization of each subunit, the full-length cDNA of each protein fused to EGFP at its C terminus was transiently expressed in HeLa cells. As shown in Fig. 1B (*Lower*), all three proteins are clearly localized in mitochondria.

We then performed siRNA-mediated knockdowns of each subunit of the hGatCAB in HeLa cells and analyzed the growth properties of the siRNA-treated cells in glucose- or galactose-containing medium. Cells with defective mitochondrial function cannot proliferate in a non-fermentable growth medium, the carbon source of which is only consisted of galactose (22). The knockdown efficiency for each subunit was estimated by qRT-PCR and demonstrated that each mRNA in the siRNA-treated cells was decreased to less than 15% of the level in control cells. As shown in Fig. 2A, the growth of HeLa cells treated with siRNAs targeting each of the three subunits and cultured in galactose medium was significantly decreased as compared to siRNA-treated cells cultured in glucose medium. As a negative control, cells treated with a siRNA targeting luciferase were able to grow in galactose medium. These results clearly demonstrate that all of the subunits are required for mitochondrial function.

To obtain direct evidence that hGatCAB is a bona-fide Glu-AdT that can generate Gln-tRNA^{Gln} in mitochondria, we devised a method to directly analyze amino acids attached to individual tRNAs isolated from cells (23). The method is outlined in Fig. 2B. If the indirect pathway is involved in Gln-tRNA^{Gln} formation in human mitochondria, once the activity of hGatCAB is down-regulated by siRNAs targeting one of the subunits, Glu-tRNA^{Gln} (the misaminoacylated form) should accumulate in the cell. HeLa cells were harvested 72 h after transfection with three siRNAs targeting each of the hGatCAB subunits, and total RNA, including aa-tRNAs, was immediately extracted under acidic conditions at low temperature. To stabilize the aminoacyl-bond of each aa-tRNA, the α-amino group of the aminoacyl moiety was acetylated by acetic anhydride as described previously (23). The mt tRNA^{Gln} was isolated using a solid-phase DNA probe method (15, 23), and then completely digested by RNase I, yielding acetyl-aminoacyl-adenosines (Ac-aa-Ado) from the 3′-terminus of the tRNA. The Ac-aa-Ado species were determined by LC/MS analysis. Known control samples of Ac-Glu-Ado (*m/z* 439) and Ac-Gln-Ado (*m/z* 438)
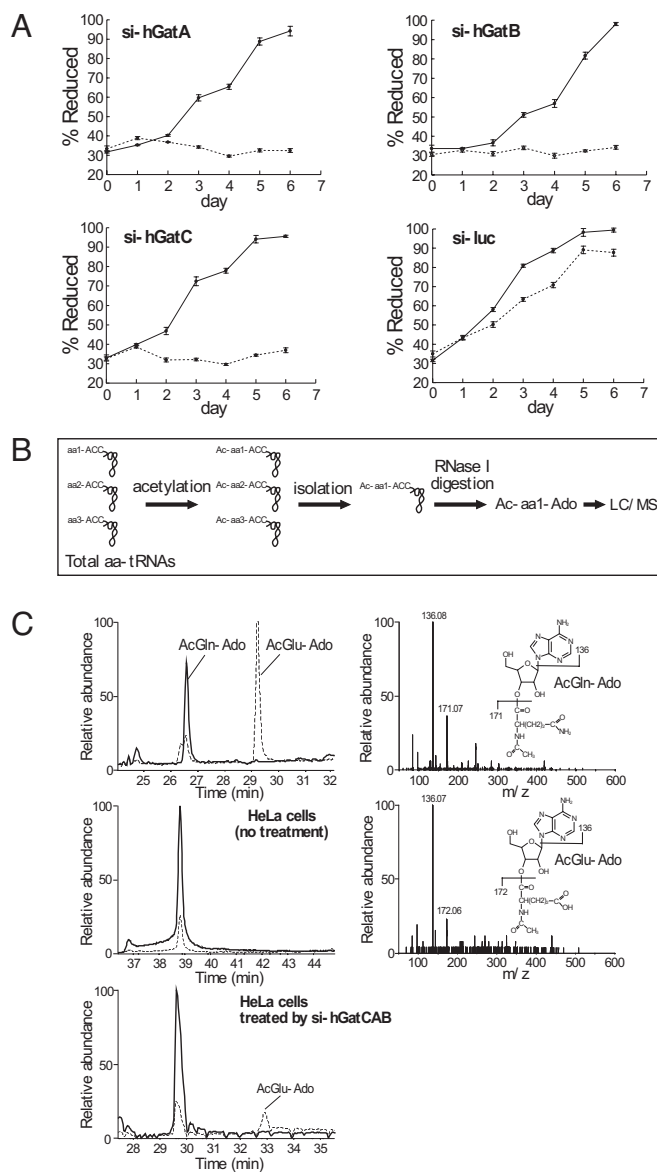

Fig. 2. Growth of HeLa cells treated with siRNAs targeting hGatCAB and direct identification Glu-tRNA^{Gln} in the cells. (*A*) Growth curves for HeLa cells treated with siRNAs for hGatA (*Upper Left*), hGatB (*Upper Right*), hGatC (*Lower Left*), or luciferase (control, *Lower Right*). The siRNA-treated cells were cultured in glucose (solid line) or galactose (dotted line) containing medium. Each plot is the average of three independent cultures (bars, ± SD). (*B*) A schematic of the procedure for direct analysis of amino acids attached to an aa-tRNA is shown. Total aa-tRNAs obtained from the cells are acetylated and individual acetyl-aa-tRNAs are affinity-purified using the solid-phase DNA probe method, and are then digested by RNase I. The acetyl-aminoacyl-adenosine (Ac-aa-Ado) fragments originating from the 3′-terminus were analyzed by LC/MS. (*C*) Mass chromatograms detecting Ac-Gln-Ado (*m/z* 438, solid line) and Ac-Glu-Ado (*m/z* 439, dotted line); authentic Ac-Gln-Ado and Ac-Glu-Ado (*Top Left*) separately prepared from mouse liver, HeLa cells (no treatment, *Middle Left*) and HeLa cells treated by siRNAs targeting for all subunits of hGatCAB (*Bottom Left*). The small peak at *m/z* 439 (dotted line) that overlaps with the Ac-Gln-Ado (*m/z* 438) peak was assigned to the isotopic ion of Ac-Gln-Ado. Collision-induced dissociation (CID) spectra of Ac-Gln-Ado (*Upper Right*) and Ac-Glu-Ado (*Lower Right*) are shown. Product ions for the adenine base (BH$_2^+$, *m/z* 136), AcGln moiety (*m/z* 171), and AcGlu moiety (*m/z* 172) were clearly detected.

were clearly detected as singly-charged positive ions in separated peaks of a mass chromatogram (Fig. 2C). In mt tRNA^{Gln} isolated from untreated HeLa cells, only Ac-Gln-Ado was detected. In
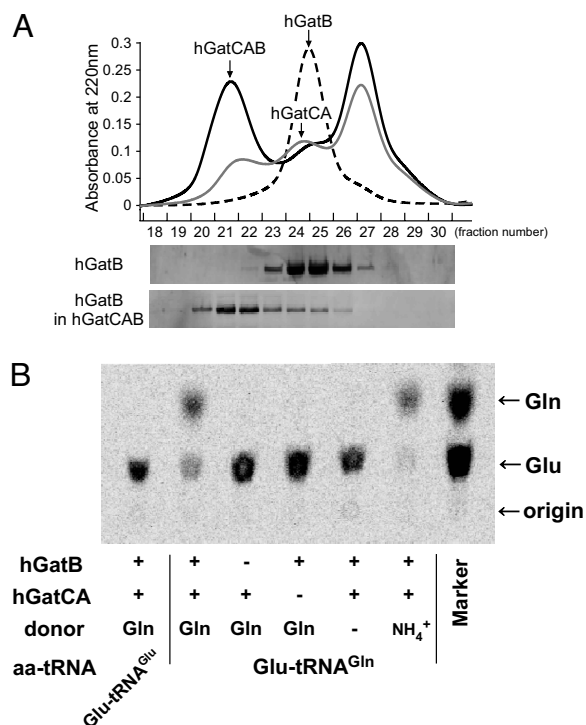
Fig. 3. In vitro reconstitution of Gln-tRNA^{Gln} formation by hGatCAB. (*A*) Gel filtration chromatography (Superdex 200) of hGatCA (gray line), hGatB (dotted line), and a mixture of hGatCA and hGatB (black line) detected by UV absorption at 220 nm. Elution profiles of hGatB alone (*Upper*) and hGatB in the hGatCAB complex (*Lower*) were analyzed by Western blotting using an anti-His-tag antibody. (*B*) In vitro reconstitution of Gln-tRNA^{Gln} formation by the recombinant hGatCAB. Phosphor-image of the TLC analysis of [^{14}C]-labeled Gln and Glu deacylated from aa-tRNAs in transamidation experiments. The assays were performed in the presence (+) or absence (−) of hGatB or hGatCA. Gln or NH$_4^+$ was used as an amide donor. [^{14}C]-labeled Glu-tRNA^{Glu} or Glu-tRNA^{Gln} was used as a substrate, as indicated below the panel. Positions of Gln and Glu on TLC were determined using [^{14}C]Gln and [^{14}C]Glu as markers (right lane).

Fig. 4. Status of Gln-tRNA^{Gln} in cells treated with siRNAs targeting hGatCAB and measuring the binding affinity of mtEF-Tu with Glu-tRNA^{Gln}. (*A*) Total aa-tRNAs were separated by acid-urea PAGE, and mt tRNA^{Gln} (*Left*) and mt tRNA^{Glu} (*Right*) from HeLa cells treated with the siRNAs targeting luciferase (lane 2), hGatA (lane 3) or all three subunits of hGatCAB (lane 4) were detected by northern blotting. The lane 1 signal in each panel represents the deacylated tRNAs prepared by alkaline-treatment of aa-tRNAs. Positions of the aa-tRNA and deacyl-tRNA are shown by the closed and open arrowheads, respectively. (*B*) (*Left*) Time course experiments of the hydrolysis protection assay for Glu-tRNA^{Glu} (square), Glu-tRNA^{Gln} (circle), and Gln-tRNA^{Gln} (triangle) in the presence (closed symbols) or absence (open symbols) of bovine mtEF-Tu. The remaining aa-tRNAs were measured and plotted. (*Right*) The rates of hydrolysis of aa-tRNAs measured in the presence of different concentrations of mtEF-Tu.

contrast, when hGatCAB was knocked down, Ac-Glu-Ado was clearly detected in the isolated mt tRNA^{Gln} samples (Fig. 2C). The chemical structure of Ac-Glu-Ado was confirmed by assignment of product ions produced by collision-induced dissociation (Fig. 2C, *Right*). These results provide the direct evidence that Gln-tRNA^{Gln} is generated from Glu-tRNA^{Gln} mediated by hGatCAB in human mitochondria.

In Vitro Reconstitution of Amidotransfer Reaction by hGatCAB. To reconstitute Gln-tRNA^{Gln} formation in vitro by hGatCAB, each subunit was recombinantly expressed in *E. coli*. According to the crystal structure of bacterial GatCAB (24), GatC interacts extensively with the hydrophobic core of GatA. Therefore, hGatC was coexpressed with hGatA to obtain an hGatCA complex in soluble form. To test the molecular interaction between recombinant hGatCA and hGatB, mixtures were analyzed by gel filtration chromatography (Fig. 3A). Each fraction was analyzed by SDS/PAGE and the His-tag of hGatB was detected by Western blotting (Fig. 3A). When hGatB alone was subjected to gel filtration chromatography, hGatB eluted at peak fractions 24 and 25. When hGatB was mixed with hGatCA, the hGatB signal was detected in higher molecular weight fractions (fractions 21 and 22). According to the retention times of molecular size markers, a heterotrimeric hGatCAB of approximately 132 kDa was formed.

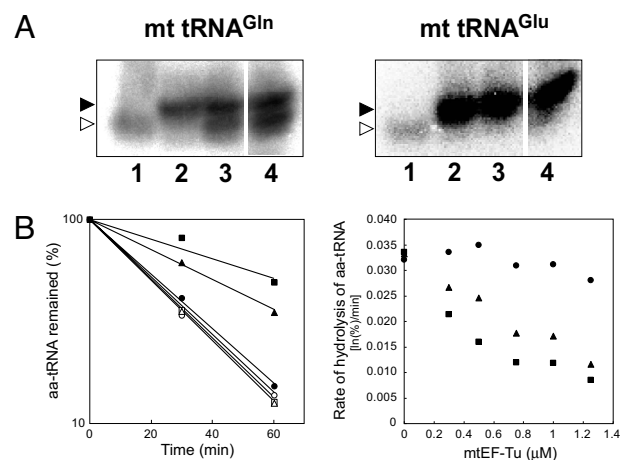We next examined the in vitro amidotransferase activity of the reconstituted hGatCAB (Fig. 3B). When Glu-tRNA^{Gln} was used as a substrate, Gln-tRNA^{Gln} was clearly formed in the presence of hGatCAB and an amide donor, Gln or NH$_4^+$. This reaction did not occur when Glu-tRNA^{Glu} was used as a substrate, in the absence of hGatB or hGatCA, or in the absence of an amide donor (Gln or NH$_4^+$). We were able to successfully reconstitute Gln-tRNA^{Gln} formation in vitro using the recombinant mt-GluRS and hGatCAB.

Exclusion of the Mischarged Intermediate of tRNA^{Gln} by Mitochondrial EF-Tu Surveillance. The indirect pathway involves a mischarged intermediate of tRNA^{Gln}. If Glu-tRNA^{Gln} were to participate in translation, incorporation of the incorrect amino acid would reduce the fidelity of mitochondrial protein synthesis. A previous report has demonstrated that chloroplast EF-Tu does not recognize Glu-tRNA^{Gln}, generated as a mischarged intermediate in the biogenesis of Gln-tRNA^{Gln}, in the organelle (25). Thus, we hypothesized that a similar mechanism may maintain the fidelity of translation in human mitochondria. If mtEF-Tu only weakly binds Glu-tRNA^{Gln}, Glu-tRNA^{Gln} should be rapidly deacylated in mitochondria. To examine this possibility, we separated tRNAs by acid-urea polyacrylamide gel electrophoresis and examined the state of the aa-tRNAs in human mitochondria by northern blotting (26). In the control cells treated by siRNA targeting luciferase, both mt tRNAs for Gln and Glu were fully aminoacylated (Fig. 4A, lane 2). When hGatA was knocked down by siRNA, the deacylated form of mt tRNA^{Gln} appeared (Fig. 4A, lane 3), while no change in the status of aminoacylation of mt tRNA^{Glu} was detected. Moreover, when the three subunits of hGatCAB were inactivated simultaneously, the deacylated form of mt tRNA^{Gln} increased, while mt tRNA^{Glu} was still fully aminoacylated (Fig. 4A, lane 4). These results suggest that Glu-tRNA^{Gln} generated by siRNA-mediated knock down of hGatCAB tends to be rapidly deacylated in mitochondria, probably due to the low affinity of Glu-tRNA^{Gln} for mtEF-Tu.

To investigate whether mtEF-Tu plays a role in the exclusion of Glu-tRNA^{Gln} from mitochondrial protein synthesis, we conducted hydrolysis protection assays to estimate the binding affinity of mtEF-Tu for Glu-tRNA^{Gln}. The aminoacyl moiety of an aa-tRNA is spontaneously hydrolyzed with a characteristic half-life. In the presence of EF-Tu/GTP, the tRNA is protected from deacylation by the specific binding of EF-Tu/GTP to the aminoacyl moiety of the aa-tRNA. We compared the rate of deacylation for Glu-tRNA^{Gln}, Gln-tRNA^{Gln}, and Glu-tRNA^{Glu} in the absence or presence of mtEF-Tu/GTP (Fig. 4B). Without mtEF-Tu/GTP, the three aa-tRNAs were rapidly deacylated at similar rates with a half-life of ≈20 min. In the presence of mtEF-Tu/GTP, the deacylation rate of both Gln-tRNA^{Gln} and Glu-tRNA^{Glu} clearly decreased, indicating that mtEF-Tu/GTP efficiently binds to aa-tRNAs charged with their cognate amino acid and protects them from deacylation. In contrast, the rate of deacylation of the misaminoacylated Glu-tRNA^{Gln} was unchanged in the presence of mtEF-Tu/GTP (Fig. 4B, Left). To confirm these results, we plotted the rate of hydrolysis by titrating mtEF-Tu. The rate of deacylation for both cognate aa-tRNAs decreased with increasing concentrations of mtEF-Tu (Fig. 4B, Right). In contrast, the rapid deacylation of Glu-tRNA^{Gln} was maintained even in the presence of high concentrations of mtEF-Tu (Fig. 4B, Right). These results demonstrate that Glu-tRNA^{Gln} has a low binding affinity for mtEF-Tu, suggesting a mechanism by which mischarged aa-tRNAs are rejected by the mitochondrial translation system. After Glu-tRNA^{Gln} is converted to Gln-tRNA^{Gln} by hGatCAB, it recovers a strong binding affinity to mtEF-Tu and is available for mitochondrial protein synthesis.

Cytoplasmic tRNA^{Gln} Is a Potential Substrate for mtGluRS and hGatCAB. It was reported that cytoplasmic (cyto) tRNA^{Gln} is imported into human mitochondria (13), indicating cyto tRNA^{Gln} might be charged with Gln, and participate in mitochondrial translation, although we have revealed in this study that mt tRNA^{Gln} is a major substrate in the indirect pathway in human mitochondria. To examine cyto tRNA^{Gln} to be a substrate for mtGluRS and hGatCAB, we isolated cyto tRNA^{Gln} from human placenta using the chaplet column chromatography (15), and carried out glutaminylation of cyto tRNA^{Gln} by recombinant mtGluRS and hGatCAB. In fact, cyto tRNA^{Gln} was slightly glutamylated by mtGluRS (Fig. S4A) and converted to Gln-cyto tRNA^{Gln} (Fig. S4B), but with low activity compared with mt tRNAs for Glu and Gln, implying that the imported cyto tRNA^{Gln} can be slightly glutaminylated in human mitochondria.

Discussion

Approximately two decades ago, Söll's group reported that no GlnRS activity was detected in the S100 fraction of mouse liver mitochondria (5), strongly suggesting that the indirect pathway consisted of ND-GluRS and Glu-AdT is involved in Gln-tRNA^{Gln} formation in mammalian mitochondria. We have shown here that the recombinant mtGluRS was indeed an ND-GluRS that efficiently glutamylated mt tRNAs for both Glu and Gln. In the crystal structure of the GluRS-tRNA^{Glu} complex (27), Arg-358 in discriminating type GluRS (D-GluRS) from T. thermophilus specifically recognizes C36 of tRNA^{Glu}. The D-GluRS Arg-358 is supposed to sterically interfere with the G36 position in the tRNA^{Gln}, resulting in a lack of recognition of tRNA^{Gln}. Arg-358 is considered to be a signature residue of D-GluRS. In the ND-GluRS of T. elongatus (21), this position is replaced by Gly so that it accommodates the G36 of tRNA^{Gln}, resulting in recognition of the tRNAs for both Glu and Gln. The residue corresponding to Arg-358 in mammalian mtGluRS is His (Fig. S2), which is expected to recognize both C36 and G36 of mt tRNAs$^{Glu/Gln}$ as an ND-GluRS.

Homologs of bacterial Glu-AdT subunits are found in the nuclear genomes of eukaryotes. In Saccharomyces cerevisiae, pet112 encodes a mitochondrial protein that is homologous to gatB (8). Pet112 is essential for mitochondrial translation, and loss of Pet112 can be complemented by the Bacillus gatB gene (7). YMR293c/HER2 is a gatA homolog of S. cerevisiae (28), and its gene product is localized in mitochondria (29). A null mutant of YMR293c exhibited abnormal mitochondrial morphology (6) and respiratory defects (28). Although an apparent homolog of the bacterial gatC is not encoded in the S. cerevisiae genome, a fungi-specific subunit gatF was recently discovered, and a new heterotrimeric GatFAB has been found as Glu-AdT in yeast mitochondria (9). However, yeast mtGluRS is a D-GluRS and unable to aminoacylate mt tRNA^{Gln} in vitro (9, 10). This discrepancy was solved by the finding that cyto GluRS is imported in mitochondria and glutamylates mt tRNA^{Gln}, which is then subjected to Gln-tRNA^{Gln} formation by GatFAB (9). In plants, mitochondrial Gln-tRNA^{Gln} formation is achieved by the indirect pathway mediated by ND-GluRS and a GatCAB-type Glu-AdT, and both of these enzymes are also imported into chloroplasts (11). Therefore, the dual-targeted ND-GluRS and Glu-AdT ensure both mitochondrial and chloroplastic Gln-tRNA^{Gln} formation in plants. In Drosophila, the mutant benedict (bene) was shown to disrupt the Drosophila homolog of the gatA gene (30). Null mutants of the gatA homolog grew slowly and never achieved wild-type size, and gatA null larvae died before pupariation (31). Thus, gatA is required for mitotic and endoreplicating tissues in the larval stages of Drosophila. Here, we have identified the human homologs of the bacterial gatA, gatB, and gatC. These homologs were localized in the mitochondria and are required for respiratory function. In addition, we directly observed Glu-tRNA^{Gln} as a misaminoacylated form of Gln-tRNA^{Gln} in cells in which hGatCAB was inactivated by RNAi. These results demonstrate that Gln-tRNA^{Gln} formation in human mitochondria is mediated by ND-GluRS and hGatCAB, which acts as a Glu-AdT. We successfully reconstituted a heterotrimeric complex of hGatCAB and demonstrated its transamidation activity in vitro.

It was reported that mammalian cyto tRNA^{Gln} is imported into mitochondria (13). To investigate the fate of the imported cyto tRNA^{Gln}, we examined cyto tRNA^{Gln} to be converted to Gln-tRNA^{Gln} in vitro using the recombinant mtGluRS and hGatCAB. It was shown that cyto tRNA^{Gln} could be charged with Glu and converted to Gln-tRNA^{Gln}, although its activity is not as efficiently as that of mt tRNA^{Gln}. Thus, the imported cyto tRNA^{Gln} potentially might have a supportive role to be used in mitochondrial translation. Use of the cyto tRNA in mitochondria may be required for adaptation of the cell to some environmental stresses (32). Further studies are needed to clarify this issue.

According to biochemical and structural studies (24), the bacterial GatCAB strictly recognizes the first U1–72A base pair and a specific number of bases in the D-loop of tRNA^{Gln} as identity elements. The first U1–72A base pair is conserved in mt tRNA^{Gln} (Fig. 1A), while the corresponding base pair of mt tRNA^{Glu} is G1–72U, indicating that the first base pair also acts as a positive determinant recognized by hGatCAB. According to the crystal structure of the archaeal GatDE complexed with tRNA^{Gln} (33), the tail domain of GatE closely contacts the D-loop/T-loop assembly region, in particular the G18-C56 base pair, which indicates that the interaction between the D- and T-loop is critical for the correct and efficient recognition of tRNA^{Gln} by Glu-AdT. In most tRNAs from the three domains of life, G18G19 in the D-loop and T54Ψ55C56 in the T-loop are well conserved (34, 35). In contrast, most mammalian mt tRNAs have variations in the numbers of bases with no consensus sequences in either the D- or T-loop (36). In fact, mammalian mt tRNAs frequently lack the conserved D- and T-loop interactions

(37). However, unlike other mt tRNAs, mammalian mt tRNA^{Gln} species commonly have the canonical D- and T-loops, containing a G18G19 D-loop and U54U55C56 T-loop sequence (Fig. S5) (38). Although the complex structure of GatCAB-tRNA^{Gln} is not available, considering that GatCAB strictly recognizes the number of bases in the D-loop of tRNA^{Gln} (24), we suggest that hGatCAB recognizes the canonical D-loop/T-loop interaction of mt tRNA^{Gln}. Although there has been a high mutation pressure in the D- and T-loops of mt tRNAs during the evolution of the mitochondrial translation system, the canonical D-loop/T-loop sequence of the mt tRNA^{Gln} may be preserved since it functions as an identity element recognized by hGatCAB.

In 1994, Sprinzl and coworkers reported that chloroplast EF-Tu did not efficiently bind Glu-tRNA^{Gln} (25). This was the first reported instance of translational fidelity monitored by EF-Tu. Similarly, we demonstrated here that Glu-tRNA^{Gln} was not recognized by mtEF-Tu and, after conversion to Gln-tRNA^{Gln}, mtEF-Tu binding affinity was recovered. These observations suggest that the rejection of misaminoacylated tRNAs appears to be a common feature of EF-Tu. Molecular recognition of the amino acid moiety of an aa-tRNA by EF-Tu has been studied by Uhlenbeck and coworkers (39, 40). tRNA^{Glu} binds more tightly to EF-Tu than tRNA^{Gln} does. In addition, EF-Tu exhibits the strongest binding affinity to the Gln moiety among 20 species of aminoacyl moieties. Our data, here, suggest that mtEF-Tu may have a similar preference for the amino acid moieties attached to their cognate tRNAs.

Materials and Methods

Identification of the Amino Acid Attached to mt tRNA^{Gln} in the Cell. The isolation of individual species of aa-tRNA and analysis of the attached amino acids were conducted according to the method of Suzuki et al. (23). Briefly, HeLa cells (7.5×10^5 cells) were cultured and transfected with a mixture of three siRNAs targeting all subunits of hGatCAB (final concentration, 3.3 nM for each). At 72 h post-transfection, total RNA was extracted from the cells, as described in *SI Text*. The amino groups of the amino acid moieties of the aa-tRNAs in the total RNA fraction were acetylated with acetic anhydride to stabilize the aminoacyl bond (23). The acetylaminoacyl-tRNAs were then isolated from the total RNA by a solid-phase DNA probe method using a 3′-biotinylated oligonucleotide with a sequence identical to that used for northern blotting, which was performed by immobilizing the oligonucleotide on streptavidin-agarose (GE Healthcare). For further purification, the isolated aa-tRNAs were electrophoresed on a 12% polyacrylamide gel containing 7 M urea. The purified acetylaminoacyl-tRNA was digested with RNase I in 50 mM NaOAc at 37 °C for 1 h and subjected to an LC/MS analysis as described (16, 17). Other methods are decribed in *SI Text*.

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