Human Mitochondrial Leucyl-tRNA Synthetase Corrects Mitochondrial Dysfunctions Due to the tRNA\textsubscript{Leu(UUR)} A3243G Mutation, Associated with Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-Like Symptoms and Diabetes

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Respiration defects (5, 21, 24, 51).

ing the reduced rate of mitochondrial protein synthesis and mutation in the tRNALeu(UUR) gene causes mitochondrial en-

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Mutations in mitochondrial tRNA genes are associated with a wide spectrum of human diseases. In particular, the tRNA leucyl-tRNA synthetase (LARS2) in the cytoplasmic hybrid (cybrid) cells carrying the A3243G mutation corrects the mitochondrial dysfunctions. Human LARS2 localizes exclusively to mitochondria, and LARS2 is expressed ubiquitously but most abundantly in tissues with high metabolic rates. We showed that the alteration of aminoacylation tRNALeu(UUR) caused by the A3243G mutation led to mitochondrial translational defects and thereby reduced the aminoacylated efficiencies of tRNA Leuc(UUR) as well as tRNAAla and tRNAMet. We demonstrated that the transfer of human mitochondrial leucyl-tRNA synthetase into the cybrid cells carrying the A3243G mutation improved the efficiency of aminoacylation and stability of mitochondrial tRNAs and then increased the rates of mitochondrial translation and respiration, consequently correcting the mitochondrial dysfunction. These findings provide new insights into the molecular mechanism of maternally inherited diseases and a step toward therapeutic interventions for these disorders.

Mutations in mitochondrial DNA (mtDNA) have been associated with a wide spectrum of clinical abnormalities, including neuromuscular disorders, heart failure, diabetes, and hearing and visual loss (2, 20, 28, 47). More than 50% of these mtDNA mutations are located in the 22 mitochondrial tRNA genes, including tRNALeu(UUR) (2). In particular, the A3243G mutation in the tRNALeu(UUR) gene causes mitochondrial encephalomyopathy, lactic acidosis, and stroke-like symptoms (MELAS) (14). This mtDNA mutation is also one of the most important causes of maternally inherited diseases and deafness (35, 37, 45). The primary defect in this mutation was an inefficient aminoacylation of the tRNALeu(UUR) (1, 5, 9, 24, 38, 50). This mutation also affected the processing of the longer mitochondrial RNA precursors (22, 24, 41) and the base posttranscriptional modification of the tRNALeu(UUR) (19, 26, 50). In cytoplasmic hybrids (cybrids) harboring the nearly homoplasmic A3243G mutation, the level of aminoacylated tRNALeu(UUR) was reduced approximately 70% to 75% (5, 9, 12, 50). The deficient aminoacylation of tRNALeu(UUR) mainly contributed to a shortage of tRNALeu(UUR) (20), thereby causing the reduced rate of mitochondrial protein synthesis and respiration defects (5, 21, 24, 51).

The formation of aminoacylated mitochondrial tRNALeu(UUR) was catalyzed by the human mitochondrial leucyl-tRNA synthetase (LARS2) belonging to class I of aminocysl-tRNA synthetases (3, 27, 43). This evolutionarily conservative tRNA synthetase, encoded by the nuclear gene LARS2 at chromosome 3p21.3, was composed of 903 amino acids with a mitochondrial signal sequence (3, 18, 27). In the yeast Saccharomyces cerevisiae, mitochondrial defects due to the yeast counterpart of the human tRNA leucyl-tRNA synthetase (LARS2) (A3243G mutation were rescued by overexpression of the translation elongation factor EF-Tu (11) and mitochondrial leucyl-tRNA synthetase (6). Thus, it is anticipated that the overexpression of human LARS2 in the cybrid cells carrying the A3243G mutation would improve the efficiency of aminoacylation of tRNA Leuc(UUR), enhance the stability of tRNA, and then increase the rates of mitochondrial translation and respiration, consequently correcting the mitochondrial dysfunction. To test this hypothesis, stable transfectants were constructed by transferring a human LARS2 cDNA into a cybrid cell line carrying the nearly homoplasmic A3243G mutation and an isogenic control cybrid cell line harboring the homoplasmic wild-type version of tRNALeu(UUR). These stable transfectants were analyzed for the aminoacylation capacity of tRNAs, the stability of the tRNALeu(UUR), and the rates of mitochondrial translation and respiration as well as RNA processing. Furthermore, human LARS2 was further characterized by examining the gene expression in different tissues and subcellular locations.

MATERIALS AND METHODS

Cell lines and culture conditions. 143B.TK is a human osteosarcoma-derived cell line (ATCC CRL 8303). The 43B cybrid cell line carrying the nearly homoplasmic A3243G mutation in the tRNALeu(UUR) gene and the isogenic HSI cybrid cell line carrying the homoplasmic wild-type version of the tRNALeu(UUR)
gene were isolated by transfer mitochondria from myoblasts of the same MELAS subject carrying the heteroplasmic A3243G mutation into human mtDNA-less p"206 cells (51). The 143B.TK- cells and cybrid cells were grown in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS). The stable transfectants were grown in DMEM supplemented with 10% FBS and 15 ng/ml of phleomycin (Zeocin).

Isolation of human LARS2 cDNA. To construct the plasmid pLARS2 containing the entire coding region of LARS2 cDNA, reverse transcription-PCR (RT-PCR) was performed using Taq DNA polymerase (Promega) and total RNA isolated from 143B cells as template, with the primers 5′-ATGGCTTCGTCTTTGGGAGCAG (nucleotides [nt] 186 to 205) and 5′-TGTTTCTTCTCTCCCTTGG (nt 2980 to 2998) (GenBank accession no. NM_015340) (3). The predominant PCR product was purified by agarose gel electrophoresis and subsequently cloned into a PCR 2.1-Topo vector (Invitrogen). Nucleotide sequencing was done using a Dye Terminator cycle sequencing kit (Perkin-Elmer) and an ABI Prism 3700 genetic analyzer.

Northern blot analysis of LARS2 expression. A 12-lane human multiple tissue RNA blot (Clontech) containing 2 μg poly(A)" RNA/lane was used for this study. An 1.124-bp LARS2 cDNA fragment corresponding to the nucleotide positions 1309 to 2432 (3) was random prime labeled with [32P]dATP and hybridized with the RNA blots according to the manufacturer’s instructions. Membranes were then washed to a final stringency of 0.1 × SSC (0.1 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS) at 65°C for 40 min. As an internal control, the human RNA blots were stripped and rehybridized with [32P]dATP-labeled human actin β subunit cDNA as a control.

Subcellular localization of human LARS2. The coding region of LARS2 cDNA lacking its natural stop codon was obtained by PCR using pLARS2 cDNA as the template. Primers 5′-CTCGAGATGGCTTCTGGAGCAG (nt 186 to 205) and 5′-GAGA CCT TTG CAC CAG GAA GTT GAT GA (nt 2870 to 2891) were used for the PCR amplification. PCR products were digested with EcoRI and cloned into pBluescript KS (+) (Promega). After sequence determination, the inserts were subcloned into pEGFP-N1 (Clontech) to generate the pEGFP-N1-LARS2. Resultant constructs were transfected into 143B cells using the SuperFect transfection reagent (Qiagen, Inc.) according to the manufacturer’s protocol. Immunofluorescence analysis was performed as detailed elsewhere (29, 30, 49).

Construction of stable transfectants. The insert of pLARS2 was subcloned into pcDNA3.1/Zeo (+) (Invitrogen). The resultant constructs or vector only were transfected into 41B and HSI cell lines using the SuperFect transfection reagent (Qiagen, Inc.) according to the manufacturer’s protocol. The stable transfectants were isolated by culturing cells in DMEM supplemented with 15 ng/ml of phleomycin and 10% FBS for 4 weeks. The resultant clones were derived from the same heteroplasmic patient cells (5, 51).

Mitochondrial tRNA analysis. Equal amounts (5 μg) of total mitochondrial RNA were fractionated by electrophoresis through a 1.8% agarose-formaldehyde gel, transferred onto a positively charged membrane (Roche), and hybridized with the RNA probes specific for ND1, ND4, ND6, 12S rRNA, and 16 rRNA, respectively (31).

Analysis of mitochondrial protein synthesis. Pulse-labeling of the cell lines for 30 min with [35S]methionine-[35S]cysteine in methionine-free DMEM in the presence of emetine, electrophoretic analysis of the translation products, and quantification of radioactivity in the whole electrophoretic patterns or in individual well-resolved bands were carried out as detailed previously (4, 15, 16).

RESULTS

Expression analysis and subcellular localization of human LARS2. To investigate the tissue-specific expression, a LARS2 cDNA probe was hybridized with RNA blots of multiple human tissues. Figure 1A illustrates that LARS2 was ubiquitously expressed, but that there were significant variations in the steady-state LARS2 mRNA levels among tissues. Compared to a high-level expression of LARS2 in the tissues with a high metabolic rate such as skeletal muscle, heart, and kidney, the expression levels of LARS2 in small intestine, spleen, thymus, and leukocytes were very low, while LARS2 was expressed moderately in the brain, colon, placenta, liver, spleen, and lung.

To determine the cellular localization of human LARS2, pEGFP-N1-LARS2 expressing the LARS2-green fluorescent protein (GFP) fusion protein was transfected into the 143B cell line. Figure 1B shows that the immunofluorescence pattern of transfected 143B cells was double labeled with a monoclonal antibody specific for the GFP and mouse monoclonal antibody to COXI, a subunit of cytochrome c oxidase (COX) complex in the mitochondrial inner membrane. A typical mitochondrial staining pattern was observed with both antibodies, and superimposition of two panels showed complete overlap of the two patterns, demonstrating that human LARS2 localizes exclusively to mitochondria. This suggested that this protein, like other nucleus-encoding mitochondrial proteins such as MTO1, GTPBP3, and TRMU (29, 30, 49), was synthesized on cytoplasmic ribosomes and was imported into and then functioned in the mitochondrion.

Construction of stable transfectants expressing the human LARS2. A 2.8-kb human LARS2 cDNA expressed in a pcDNA3.1 vector or the vector only was transfected into the 43B cybrid cell line carrying the A3243G mutation and the isogenic HSI cell line with the wild-type version of tRNA\textsubscript{Leu(UUR)} (5). The HSI cell line carrying the homoplasmic wild-type version of the tRNA\textsubscript{Leu(UUR)} gene was isogenic with its respective mutant 43B cell line, as the wild-type and mutated mtDNA were derived from the same heteroplasmic patient cells (5, 51). These stable transfectants were isolated by culturing cells in DMEM supplemented with 15 ng/ml of phleomycin and 10% FBS for 4 weeks. The expression level of the LARS2 cDNA in
resultant stable transfectants was examined by a Northern blot analysis, as shown in Fig. 2A. Here, the levels of exogenous LARS2 mRNA (2.8 kb) harboring only the coding region were more than 5-fold higher than those of endogenous LARS2 mRNA (4.2 kb) representing full-length mRNA in transfec-
tants 43B-LARS2 and HSI-LARS2. To test if the transfer of LARS2 may change the proportion of mutated mtDNA mol-
ecules and mtDNA copy number in transfectant lines carrying the A3243G mutation, the presence and degree of the A3243G mutation in the transfectants 43B-LARS2, expressing the LARS2 cDNA, and 43B-V, carrying the vector only, were comparable with those of the parental cybrid cell line 43B carrying the A3243G mutation. Furthermore, an analysis of the mtDNA copy numbers of these 43B- and HSI-
derived transfectants expressing the LARS2 cDNA and HSI-V carrying the vector as well as parental cybrids failed to reveal any significant difference in the mtDNA/nuclear ribosomal DNA (rDNA) ratios between these transfectants and parental cybrids. These four transfectants (43B-LARS2, 43B-V, HSI-
LARS2, and HSI-V) were then used for further characteriza-
tion.

Aminoacylation capacity of mitochondrial tRNAs. We first investigated the effects of the transfer of human LARS2 on the aminoacylation deficiency of tRNA\(^{\text{Leu(UUR)}}\) in transfectants 43B-LARS2 and 43B-V and their parental mutant cybrid cell line 43B as well as transfectants HSI-LARS2 and HSI-V and their parental wild-type cybrid cell line HSI \textit{in vivo}. Total mitochondrial RNA was isolated from cell lines under acidic con-
ditions to preserve the aminoacyl-tRNA linkage (46). The aminoacylated tRNAs were separated from nonaminoacylated tRNA species on acidic denaturing polyacrylamide-urea gels and then electroblotted and hybridized with specific probes for tRNA\(^{\text{Leu(UUR)}}\), as well as tRNA\(^{\text{Leu(UUN)}}\), tRNA\(^{\text{Ala}}\), tRNA\(^{\text{Ser(UCN)}}\), and tRNA\(^{\text{Met}}\), respectively. To further distin-
guish nonaminoacylated tRNA from aminoacylated tRNA, samples of mitochondrial tRNAs were deacylated by being heated for 10 min at 60°C at pH 8.3 and then run in parallel
Notably, the efficiencies of aminoacylated tRNA Met and tRNA Ala, in addition to those of tRNA Leu(UUR), as shown in Fig. 3, were markedly reduced in mutant 43B cells, relative to wild-type HSI cells. In particular, the proportions of aminoacylated tRNAs in the 43B cells were 24.9%, 61.8%, 66.3%, 38.7%, 71.2%, and 56.1% in the tRNA Leu(UUR), tRNA Leu(CUN), tRNA Lys, tRNA Met, tRNA Ser(UCN), and tRNA Ala, respectively, while 64.1% of tRNA Leu(UUR), 66% of tRNA Leu(CUN), 67.4% of tRNA Lys, 73.1% of tRNA Met, 66.1% of tRNA Ser(UCN), and 73% of tRNA Ala were aminoacylated in the isogenic HSI cells. In contrast, the aminoacylated levels of these six tRNAs in the 43B-V cells and HSI-V cells carrying only the vector as well as HSI-LARS2 cells expressing the LARS2 cDNA only were comparable with those of 43B cells and HSI cells, respectively. Strikingly, the expression of LARS2 in the 43B cybrids led to an ~100% increase in the efficiency of aminoacylation of tRNA Leu(UUR) (from 24.9% to 50.5%), but the level was still below that of controls (64.1%). Furthermore, the efficiencies of aminoacylation of tRNA Leu(CUN), tRNA Lys, tRNA Met, tRNA Ser(UCN), and tRNA Ala in the 43B-LARS2 cell line expressing LARS2 were 106.6%, 111.6%, 133.33%, 98.5%, and 118% of those in parental 43B cell line, respectively. These suggested that the deficiencies of aminoacylation of tRNAs were partially restored by the overexpression of human LARS2 in the 43B cells.

The steady-state level of mitochondrial tRNA. To evaluate if the overexpression of LARS2 increased the stability of tRNA Leu(UUR) in transfected cells carrying the A3243G mutation, we determined the steady-state level of the tRNA Leu(UUR) by isolating total mitochondrial RNAs from cell lines, separating them on a 10% polyacrylamide-7 M urea gel, and electro-blotting and hybridizing with a nonradioactive DIG-labeled oligodeoxynucleotide probe specific for the mitochondrial tRNA Leu(UUR). The blots were then stripped and rehybridized with DIG-labeled oligodeoxynucleotide probes specific for tRNA Leu(UUR), tRNA Leu(CUN), tRNA Lys, tRNA Met, tRNA Ser(UCN), and tRNA Ala, respectively. Samples were deacylated (DA) by being heated for 10 min at 60°C in a buffer containing 1 M KCl, 10 mM Tris (pH 7.5), 10 mM MgCl₂, 100 mM DTT, 1% SDS, and 10 μg/ml pronase K, and then neutralized with an equal volume of water. The blots were then treated with 0.2% SDS and 0.05% Pronase K for 30 min at 37°C to remove the tRNA and then washed with 0.1× SSC at 65°C. The blots were then stripped and rehybridized with DIG-labeled RNA oligonucleotide probes specific for tRNA Leu(UUR), tRNA Leu(CUN), tRNA Lys, tRNA Met, tRNA Ser(UCN), and tRNA Ala, respectively. The blots were then stripped and rehybridized with DIG-labeled RNA oligonucleotide probes specific for tRNA Leu(UUR), tRNA Leu(CUN), tRNA Lys, tRNA Met, tRNA Ser(UCN), and tRNA Ala, respectively. The blots were then stripped and rehybridized with DIG-labeled RNA oligonucleotide probes specific for tRNA Leu(UUR), tRNA Leu(CUN), tRNA Lys, tRNA Met, tRNA Ser(UCN), and tRNA Ala, respectively.

Mitochondrial RNA processing. We then tested if the transfer of human LARS2 restored the defects in mitochondrial RNA processing in the 43B cell line carrying the A3243G mutation. For this purpose, RNA transfer hybridization experiments were performed with total mitochondrial RNA from transfected and parental cybrids using the nonradioactive DIG-labeled ND1 RNA probe (16, 30). After the blots were stripped, a set of DIG-labeled RNA probes (ND4, ND6, 12S rRNA, and 16S rRNA) were rehybridized with the same blots for normalization purposes. As shown in Fig. 5, RNA19 comprising the 12S rRNA plus tRNA Val plus 16S rRNA plus tRNA Leu(UUR) plus ND1 was accumulated in the 43B cell line, confirming the defects in mitochondrial RNA processing caused by the A3243G mutation (22, 24, 41). Interestingly, the reduction in the levels of RNA19, ND1, ND4, and ND6 mRNA was observed in the 43B-LARS2 transfected, while the...
levels of 16S rRNA plus tRNA^Lue(UUR) plus ND1 as well as 12S rRNA appeared to be unchanged in this transfectant compared with those of the parental 43B cell line.

Mitochondrial protein synthesis. To assess if the overexpression of LARS2 enhanced the rate of overall mitochondrial protein synthesis in the transfectant carrying the A3243G mutation, samples of cultures of transfectants as well as their parental cybrid cell lines were labeled with short [35S]methionine pulses in the presence of emetine to inhibit the cytosolic protein synthesis (4). As shown in Fig. 6A, the patterns of the mtDNA-encoded polypeptides of parental cybrid cell lines and their transfectants were qualitatively identical, in terms of electrophoretic mobility of the various polypeptides, to those of the isogenic control cybrid cell line HSI. However, the 43B cells exhibited a decrease in the total rate of labeling of mitochondrial translation products, relative to the control cybrid. In contrast, the overexpression of LARS2 apparently increased the total rate of labeling of mitochondrial translation products in the 43B-LARS2 transfectant line. Figure 6B illustrates a quantification of the results of three labeling experiments and three electrophoretic runs, which was carried out by ImageQuant program analysis of appropriate exposures of the fluorograms and normalization to the data obtained for the HSI sample included in each gel. The overall rates of labeling of the mitochondrial translation products in the 43B cell line and 43B-V and 43B-LARS2 transfectants were 32.3%, 31.1%, and 48.3%, respectively, of the mean value measured in the control HSI cell line. This translated to a 51% increase of the rate of mitochondrial translation in 43B-LARS2 cells, compared to the parental cybrid cell line 43B. In contrast, the rates of mitochondrial translation of HSI-LARS2 and HSI-V transfec-
tants were comparable with that of the parental HSI cell line.

Analysis of respiration. To examine if the overexpression of LARS2 rescued the respiratory deficiencies caused by the A3243G mutation, the endogenous respiration rates of transfectants and their parental cybrid cell lines were measured by determining the O2 consumption rate in intact cells, as described previously (23). As can be seen in Fig. 7, the rate of total O2 consumption in 43B cell lines was 24%, relative to the mean value measured in the control HSI cell line. However, the rate of total O2 consumption in the 43B-LARS2 transfectant cell line expressing LARS2 was 142% of that of the parental 43B cell line, while the rate of total oxygen consumption...
of HSI-LARS2 was 92% of that of the parental HSI cell line. However, rates for 43B-V and HSI-V transfectants were comparable with those of parental 43B and HSI cell lines. These data demonstrated that the increase of the rate of mitochondrial translation products by overexpression of LARS2 indeed enhanced the rate of respiration in the transfectant 43B-LARS2 carrying the A3243G mutation.

**DISCUSSION**

In the present study, we have further investigated the molecular pathogenesis of the MELAS-associated tRNALeu(UUR) A3243G mutation and whether the overexpression of human LARS2 in the cybrid cells carrying the A3243G mutation corrects the mitochondrial dysfunctions. The A3243G mutation changes the A to G at conventional position 14 (A14) of the mitochondrial tRNALeu(UUR) (13) and thus alters the structure and function of tRNALeu(UUR). In particular, the tRNALeu(UUR) carrying the A3243G mutation was less charged to a lesser extent by the leucyl-tRNA synthetase, thereby altering aminoacylation. Alteration in aminoacylation of tRNALeu(UUR) may result in stalling of ribosomes at leucine codons, mistranslation of UUR codons, or translational shifting (1, 21, 42). This was evidenced by the disproportionate decrease of translation in some polypeptides (42). The idea that an inefficient aminoacylation of tRNALeu(UUR) is the primary defect in MELAS was further supported by the recent isolation of a suppressor mutation in the anticodon of the mitochondrial tRNALeu(UUR) gene in an A3243G lung carcinoma cell line (8, 9). Furthermore, the A3243G mutation disturbed the processing of the tRNALeu(UUR) precursor (22, 24, 41) as well as the posttranscriptional modification of uridine at the first position of the anticodon in the tRNALeu(UUR) (19, 26, 50). The mutant tRNALeu(UUR) may be metabolically less stable and subject to turnover, thereby lowering the steady-state level of tRNALeu(UUR). As a result, a failure in tRNA metabolism is responsible for defective mitochondrial protein synthesis and an impaired oxidative phosphorylation. In this investigation, it is interesting that the lowered efficiencies of aminoacylated mitochondrial tRNAMet and tRNA Ala were also observed in the cybrid cell line carrying the A3243G mutation. It is likely that the reduced ratio of charged to uncharged tRNALeu(UUR) may mediate mitochondrial tRNA metabolism, thereby reducing the aminoacylated efficiencies of other mitochondrial tRNAs. Indeed, the mutation in TRMU, encoding a highly conserved 5-methylaminomethyl-2-thiouridylate-methyltransferase responsible for the biosynthesis of 5-taurinomethyl-2-thiouridine (tm5s2U) of mitochondrial tRNA^Gln^, tRNA^Glu^, and tRNA^Aaa^ in the wobble position, not only lowered the steady-state level of tRNA^Gln^, tRNA^Glu^, and tRNA^Aaa^ but also reduced the steady-state level of other...
tRNAs such as tRNA^{Leu(UUR)}, tRNA^{Ser(UCN)}, tRNA^{Met}, and tRNA^{His} (17).

In the present investigation, we have shown that the overexpression of human LARS2 in the cybrid cells carrying the A3243G mutation corrected the mitochondrial dysfunctions. In particular, the overexpression of LARS2 in the 43B cybrid cell line carrying the A3243G mutation raised 50% of the mitophagy even though it still was far short of the mitochondrial translation was responsible for the increasing oxidative phosphorylation. However, this result was not in good agreement with the observation that the full recovery of respiration chain function occurred by means of overexpression of LARS2 in a mutant cybrid cell line carrying the A3243G mutation (39). This discrepancy may be attributed to different nuclear backgrounds, as in the case of cell lines carrying other mtDNA mutations (7, 15). In conclusion, we demonstrated that the overexpression of human mitochondrial leucyl-tRNA synthetase corrected the mitochondrial dysfunction caused by the MELAS-associated tRNA^{Leu(UUR)} A3243G mutation. These findings will provide new insights into the pathophysiology of maternally inherited diseases and a step toward therapeutic interventions for these disorders.

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