Aminoacylation and translational quality control strategy employed by leucyl-tRNA synthetase from a human pathogen with genetic code ambiguity

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ABSTRACT

Aminoacyl-tRNA synthetases should ensure high accuracy in tRNA aminoacylation. However, the absence of significant structural differences between amino acids always poses a direct challenge for some aminoacyl-tRNA synthetases, such as leucyl-tRNA synthetase (LeuRS), which require editing function to remove mis-activated amino acids. In the cytoplasm of the human pathogen Candida albicans, the CUG codon is translated as both Ser and Leu by a uniquely evolved CattRNASer(CAG). Its cytoplasmic LeuRS (CaLeuRS) is a crucial component for CUG codon ambiguity and harbors only one CUG codon at position 919. Comparison of the activity of CaLeuRS-Ser919 and CaLeuRS-Leu919 revealed yeast LeuRSs have a relaxed tRNA recognition capacity. We also studied the mis-activation and editing of non-cognate amino acids by CaLeuRS. Interestingly, we found that CaLeuRS is naturally deficient in tRNA-dependent pre-transfer editing for non-cognate norvaline while displaying a weak tRNA-dependent pre-transfer editing capacity for non-cognate α-amino butyric acid. We also demonstrated that post-transfer editing of CaLeuRS is not tRNALeu species-specific. In addition, other eukaryotic but not archaeal or bacterial LeuRSs were found to recognize CattRNASer(CAG). Overall, we systematically studied the aminoacylation and editing properties of CaLeuRS and established a characteristic LeuRS model with naturally deficient tRNA-dependent pre-transfer editing, which increases LeuRS types with unique editing patterns.

INTRODUCTION

Aminoacyl-tRNA synthetases (aaRSs) are essential components required to establish the genetic code during protein biosynthesis by coupling specific amino acids with their cognate tRNAs in a two-step aminoacylation reaction (1,2). This process requires amino acid activation by condensation with ATP to form the aminoacyl-adenylate (aa-AMP) and pyrophosphate; the activated amino acid is then transferred to the cognate tRNA to yield the aminoacyl-tRNA (aa-tRNA), which is then transferred to the protein biosynthesis machinery as a building block (1). Aminoacylation of tRNA requires adequate efficiency and accuracy, which requires tightly regulated control of the speed of the aa-tRNA production for the ribosome and the risk of generation of aberrant aa-tRNA pairs (3–5). Transfer RNA always harbors various identity determinants and/or anti-determinants, facilitating selection of the correct tRNA from a large pool of tRNA species (6). However, the specificity of aaRS is greatly challenged by the presence of various types of amino acids and their analogues and the fact that amino acids differ only in the side-chain. AaRSs that do not show an overall selectivity above 1 in 3000 are predicted to require some form of proofreading (editing) mechanism to maintain sufficient accuracy during aa-tRNA synthesis (5,7,8). Editing activity has evolved in half of the currently identified aaRSs to remove any aberrantly produced aa-AMP (pre-transfer editing) and/or aa-tRNA (post-transfer editing). This is an essential checkpoint that ensures translational fidelity (5). Pre-transfer editing can be further divided into tRNA-independent and tRNA-dependent pre-transfer editing. In tRNA-independent pre-transfer editing, the non-cognate aa-AMP is hydrolyzed into the amino acid and AMP molecules without the presence of cognate tRNA, whereas in tRNA-dependent pre-transfer editing, aa-AMP hydrolysis is triggered by the addition of the cognate tRNA (9–11).

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Mis-translation due to the impairment or loss of editing activity can lead to ambiguity of the proteome, having a seriously negative effect on the cellular function of most organisms and causing neuron-degeneration in a mouse model (12).

Leucyl-tRNA synthetase (LeuRS) is a large multi-domain class Ia aaRS with both aminoacylation activity to generate Leu-tRNA\textsubscript{Leu} and editing activity to clear non-cognate aa-AMP and aa-tRNA (13). It can be divided into bacterial and archaeal/eukaryotic types based on primary sequence and domain location (14). Both types of LeuRSs usually consist of a Rossmann-fold domain (for amino acid activation and aminoacylation), an \(\alpha\)-helix bundle, a C-terminal domain (for tRNA binding) and a CP1 domain (for editing) (15–17). Extensive studies of various LeuRS species all found that non-cognate norvaline (Nva) is the most significantly mis-activated amino acid among all the non-cognate amino acids tested, including Ile, Val, Met and \(\alpha\)-amino butyric acid (ABA). For instance, compared with cognate Leu, Nva is mis-activated by Aquifex aeolicus LeuRS (AaLeuRS) (9), Saccharomyces cerevisiae LeuRS (ScLeuRS) (18), human cytoplasmic LeuRS (hcLeuRS) (19), Mycoplasma mobile LeuRS (MmLeuRS) (20), human mitochondrial LeuRS (hmtLeuRS) (unpublished data) 72-, 105-, 100-, 122- and 180-fold less efficiently, respectively. Nva is a non-proteinogenic amino acid differing from Leu only by the absence of a side-chain methyl group. Nva is naturally present in vivo and is a by-product of the Leu biosynthesis pathway (21). Its synthesis is predominantly related to an imbalance in the synthesis of the branched-chain amino acids under pyruvate-high conditions. In addition, Nva significantly accumulates immediately after a shift from aerobic culture conditions to oxygen limitation at high glucose concentrations (22). Therefore, the amount of Nva is dynamic and varies according to the environment. The incorporation of Nva in proteins at Leu codons has been clearly demonstrated. It has been reported to be a natural component of an antifungal peptide of Bacillus subtilis (23) and can be intentionally inserted into heterologous proteins by culturing Escherichia coli in the presence of Nva (US patent, Nov 7, 1989, 4879223). Accompanied by conditions of an elevated ratio of available Nva to Leu in the medium, increasing mis-incorporation of Nva at Leu codons has been observed in recombinant human hemoglobin produced in E. coli as a result of mis-aminoacylation of tRNA\textsubscript{Leu} by E. coli LeuRS (EcLeuRS) (24). It is proposed that Nva replacement may disrupt the correct folding and assembly of hemoglobin and other proteins (24). All this evidence suggests that Nva mis-activation by LeuRS is a non-artificial event that occurs in vivo, and that mis-charged Nva-tRNA\textsubscript{Leu} can be accommodated and used by the ribosome. Therefore, editing of Nva by LeuRS seems to be essential for the correct functioning of organisms.

Based on significant mis-activation of Nva, editing catalyzed by LeuRS (with a functional CP1 domain) has been shown to be one of the most interesting editing mechanisms. This process is predominantly mediated by three diverse pathways (tRNA-independent, tRNA-dependent pre-transfer and post-transfer editing) (10). Both types of LeuRS critically depend on the editing active site embedded in the CP1 domain to perform post-transfer editing (15–17,25). However, MmLeuRS harbors only tRNA-independent pre-transfer editing activity owing to its natural lack of the CP1 domain (20). Another example of a unique LeuRS is hmtLeuRS, which possesses a degenerate editing active site in the CP1 domain as well as defunct post-transfer editing (26) and tRNA-dependent pre-transfer editing activities (unpublished data). Combining site-directed mutagenesis and AMP formation methodology, the contribution of different pathways to the overall editing process can be quantified (9,10,19). Strikingly, there are quantitative and species-specific differences in the contribution of a specific pathway to the total editing activity of a LeuRS (9,10,19). To evaluate the significance of each mechanism, we have attempted to generate LeuRSs lacking one or more editing mechanisms; to date, two types have been successfully established. One type contains LeuRSs with abolished post-transfer editing activity, obtained by introducing mutations at key residues (e.g. EcLeuRS-T252R, AaLeuRS-T273R, AcLeuRS-D373A, ScLeuRS-D419A, hcLeuRS-D399A, Gi LeuRS-D444A) (9,10,18,19,27) or by the inclusion of a small molecule inhibitor (AN2690) of the CP1 editing domain (10). The second type includes LeuRSs for which both the post-transfer editing and tRNA-dependent pre-transfer editing activities (EcLeuRS-Y330D, AaLeuRS-Y358D) have been abolished (10). Our aim was to determine whether a LeuRS with defective tRNA-dependent pre-transfer editing activity but intact post-transfer editing would produce mis-charged tRNAs. However, extensive efforts to establish such a LeuRS model failed.

The protein biosynthesis machinery of Candida albicans is of great interest, not only because it is a human pathogen but also in its cytoplasm, the universal Leu codon CUG is translated as both Ser (97%) and Leu (3%) (28,29). This genetic code alteration is mediated by a newly evolved tRNA, which bears a CAG anti-codon [C. albicans tRNA\textsubscript{Ser}(CAG), CatRNA\textsubscript{Ser}] and can be aminoacylated either with Ser by C. albicans seryl-tRNA synthetase (CaSerRS) and with Leu by leucyl-tRNA synthetase (CaLeuRS) (29). Therefore, the proteome of C. albicans is ambiguous with some proteins exhibiting differences in primary sequences. For example, a key player in CUG reassignment, CaSerRS, has two isoforms (SerRS-Leu\textsuperscript{197} and SerRS-Ser\textsuperscript{197}). The residue at position 197 is located at the SerRS dimer interface, and replacement of Ser by Leu at this site induces a local structural rearrangement, leading to a slightly higher (27%) activity of SerRS-Leu\textsuperscript{197} compared with SerRS-Ser\textsuperscript{197} (30). These data indicate that distribution of the CUG codon and its ambiguity is not random and has potential significance. CaLeuRS is another critical molecule in the CUG reassignment in C. albicans, which charges CatRNA\textsubscript{Ser} with Leu to produce Leu-CatRNA\textsubscript{Ser}. CaLeuRS comprises 1098 residues and has a molecular mass of 126 kDa. A single CUG codon is present at position of 919 of CaLeuRS, which is located at the C-terminal domain. Thus, CaLeuRS should also have
two isoforms, C. albicans-Leu19 and C. albicans-Leu19. Based on the decoding rule of C. albicans (28,29), C. albicans exists mainly as C. albicans-Ser19 (~97%), and this was used here as the wild-type form.

In this study, we compared the activity of two LeuRS isoforms and analyzed the cross-species tRNA^{Leu} recognition and editing capacity of C. albicans. Interestingly, we showed that C. albicans-Leu19 is naturally deficient in tRNA-dependent pre-transfer editing activity but with obvious tRNA-independent pre-transfer editing and efficient post-transfer editing of Nve. However, it harbored a measurable level of tRNA-dependent pre-transfer editing of ABA when specific tRNA was present, although editing of ABA seemed not to be a necessity, as the rejection of ABA was efficient at the aminocacylation active site. Furthermore, post-transfer editing of C. albicans-Leu19 was not tRNA^{Leu} species-specific but was functional for mischarged CatrNA^{Ser}{(CAG)}, being recognized by other eukaryotic LeuRSs.

MATERIALS AND METHODS

Materials

- L-leucine (Leu), L-norvaline (Nva), L-isoleucine (Ile), L-valine (Val), L-methionine (Met), L-serine (Ser), ABA, dithiothreitol (DTT), ATP, CTP, GTP, UTP, 5'GMP, tetrasodium pyrophosphate, inorganic pyrophosphate, ATP, Tris–HCl, MgCl₂, NaCl, yeast total tRNA and activated charcoal were purchased from Sigma (St. Louis, MO, USA).
- [³H]Leu, [²⁵P]tetrasodium pyrophosphate and [γ³²P]ATP were obtained from PerkinElmer Life Sciences (Boston, MA, USA).
- Pfu DNA polymerase, a DNA fragment rapid purification kit and a plasmid extraction kit were purchased from YPH Company (China).
- The KOD-plus mutagenesis kit was obtained from TOYOBO (Japan).
- N4 ligase, nuclease S1 and restriction endonucleases were obtained from MBI Fermentas (Pittsburgh, PA, USA).
- Oligonucleotide primers were synthesized by Biosune (China). Escherichia coli BL21 (DE3) was purchased from Stratagene (USA).

Gene cloning, mutagenesis and protein expression

The C. albicans genome was kindly provided by Prof. Jiang-Ye Chen of our institute and was used as the template for amplifying genes encoding C. albicans LeuRS, C. albicans SerRS (CaSerRS) and C. albicans mitochondrial LeuRS (CmtLeuRS). Gene sequences of C. albicans LeuRS, C. albicans SerRS and C. albicans mitochondrial LeuRS were cloned into pET28a at the NdeI and XhoI sites with N-terminal His₆-tag (the mitochondrial targeting sequence of CmtLeuRS had been removed). Plasmids containing E. coli LeuRS (10), ScLeuRS (18) and Pyrococcus horikoshii LeuRS (9) were constructed previously. The E. coli tRNA{m(1)G37} methyltransferase (TrmD) gene was amplified from the E. coli genome and inserted between the EcoRI and XhoI of sites of pET28a. The plasmid expressing E. coli tRNA nucleotidytransferase (CCase) was provided by Dr. Gilbert Eriani (Strasbourg, CNRS, France). Mutations at Asp422 of the C. albicans LeuRS gene was performed with the KOD-plus mutagenesis kit according to the manufacturer’s instructions. Asc422 corresponds to Asp373, Asp419, Asp444 and Asp499 of AaLeuRS, ScLeuRS, GlLeuRS and hclLeuRS, respectively, which are crucial for post-transfer editing of these LeuRSs (9,10,18,19,27). The CTG and TCG codons at position 919 in the C. albicans LeuRS gene were used to over-express the gene encoding C. albicans-Leu19 and C. albicans-Ser19, respectively. All constructs were confirmed by DNA sequencing. E. coli BL21 (DE3) was transformed with various constructs. A single colony of each of the transformants was chosen and cultured in 500 ml of 2 × YT medium at 37°C. When the cells reached mid-log phase (A₆₀₀ = 0.6), expression of the recombinant proteins was induced by the addition of 0.2 mM isopropyl-1-thio-β-D-galactopyranoside for 8 h at 22°C. Protein purification was performed according to a previously described method (32).

tRNA gene cloning, transcription and methylation

CatrNA^{Leu}(UAU) and CatrNA^{Ser}(CAG) genes were cloned between the PsI and EcoRI sites of pTrc99b with an N-terminal T7 promoter. Detailed T7 in vitro run-off transcription of CatrNA^{Leu} and CatrNA^{Ser} has been described previously (33). The amino acid accepting activities of CatrNA^{Leu}(UAU) or CatrNA^{Ser}(CAG) are 1390 and 1208 pmol/A₂₆₀, respectively. The methyl group of m¹G37 of CatrNA^{Ser} is a critical element for recognition by LeuRS (29). The purified CatrNA^{Ser} transcript was methylated at position G37 with E. coli TrmD (34) in a mixture containing 0.1 M Tris–HCl (pH 8.0), 1 mM DTT, 0.1 mM EDTA, 6 mM MgCl₂, 24 mM NH₄Cl, 7.5 µg of bovine serum albumin, 5 µM CatrNA^{Ser} transcript, 100 µM S-adenosylmethionine, 1 µM RNase inhibitor and 10 µM TrmD at 37°C for 1.5 h. Approximately 45% of transcripts were methylated in this reaction as estimated in a control experiment with ³H-labeled S-adenosylmethionine. m¹G37-CatrNA^{Ser} was ethanol-precipitated at −20°C after phenol/chloroform extraction (twice) and dissolved in 5 mM MgCl₂. All CatrNA^{Ser} used in this study refers to m¹G37-CatrNA^{Ser}. Transcribed or over-expressed E. coli tRNA^{Leu}(GAG) (EctRNA^{Leu}) and human cytoplasmic tRNA^{Leu}(CAG) (hctRNA^{Leu}) were obtained according to methods described elsewhere, and their amino acid accepting activity was ~1500 pmol/A₂₆₀ (19,35).

³²P-labeling of CatrNA^{Leu} or CatrNA^{Ser}

³²P-labeling of CatrNA^{Leu} or CatrNA^{Ser} was performed at 37°C in a mixture containing 60 mM Tris–HCl (pH 8.0), 12 mM MgCl₂, 15 µM CatrNA^{Leu} or CatrNA^{Ser}, 0.5 mM DTT, 15 µM ATP, 50 µM tetrasodium pyrophosphate,
In vitro activity assays

ATP-PPi exchange measurement was carried out at 30°C in a reaction mixture containing 60 mM Tris–HCl (pH 7.5), 10 mM MgCl₂, 2 mM DTT, 4 mM ATP, 20 mM [³²P]tetrasodium pyrophosphate, 1 mM Leu or 50 mM non-cognate ABA, Nva, Val, Ile, Met, Ser and 20 nM CaLeuRS. The kinetics of amino acid activation were measured in the presence of Leu (3–1000 μM) or Nva (0.3–50 mM) or ABA (3–940 mM). Samples of the reaction mixture were removed at specific time-points, added to 200 μl of quenching solution containing 2% activated charcoal, 3.5% HClO₄ and 50 mM tetradsodium pyrophosphate and mixed by vortexing for 20 s. The solution was filtered through a Whatman GF/C filter, followed by washing with 20 μl of 10 mM tetradsodium pyrophosphate solution and 10 μl of 100% ethanol. The filters were dried, and [³²P]ATP was counted using a scintillation counter (Beckman Coulter).

Aminoacylation of CatRNA⁰⁰⁰ with Leu was performed in a reaction mixture containing 60 mM Tris–HCl (pH 7.5), 10 mM MgCl₂, 2 mM DTT, 4 mM ATP, 10 μM CatRNA⁰⁰⁰, 20 μM [³²P]Leu and 20 nM CaLeuRS at 30°C. The kinetics of CaLeuRS aminoacylation were measured in the presence of CatRNA⁰⁰⁰ (0.6–15.8 μM) or transcribed or over-expressed EctRNA⁰⁰⁰ (0.6–10 μM) or yeast total tRNA (0.2–6 μM) or over-expressed hctRNA⁰⁰⁰ (0.2–6 μM) or transcribed hctRNA⁰⁰⁰ (0.6–10 μM).

Mis-aminoacylation of [³²P]CatRNA⁰⁰⁰ with Nva or ABA was carried out at 30°C in a reaction mixture containing 60 mM Tris–HCl (pH 7.5), 10 mM MgCl₂, 2 mM DTT, 4 mM ATP, 5 μM ‘cold’ CatRNA⁰⁰⁰, 1 μM [³²P]CatRNA⁰⁰⁰, 20 mM Nva or 376 mM ABA and 1 μM CaLeuRS or CaLeuRS-D422A. Samples at specific time-points were taken for ethanol precipitation with 0.8 U/ml PPIase, 10 μl of each sample) were spotted on polyethyleneimine cellulose plates pre-washed with water. Separation of Nva/ABA-[³²P]AMP, [³²P]AMP and [³²P]ATP was performed in 0.1 M NH₄Ac and 5% acetic acid. Quantification of [³²P]AMP was achieved by densitometry in comparison with [³²P]ATP samples of known concentrations.

RESULTS

CaLeuRS-Leu⁹¹⁹ is more active than CaLeuRS-Ser⁹¹⁹

Determination of the crystal structure of the PhLeuRS-tRNA⁰⁰⁰ complex (Protein Data Bank, PDB 1WZ2) shows that the amino acid at position 919 of archaeal/eukaryotic LeuRSs is located in the 29 helix of the C-terminal domain (Figure 1A and B). The primary sequence of the 919-containing 29 helix is not conserved; thus, it is difficult to identify its homologous site in the crystal structure of PhLeuRS. The CUG codon in E. coli is uniformly translated as Leu. Therefore, we introduced CTG and TCG codons at this position in the CaLeuRS gene to facilitate expression of CaLeuRS-Leu⁹¹⁹ and CaLeuRS-Ser⁹¹⁹, respectively, in E. coli.

No differences were observed in amino acid activation by CaLeuRS-Ser⁹¹⁹ and CaLeuRS-Leu⁹¹⁹ (Figure 1C), indicating that Leu or Ser insertion at this position has no direct effect on the structure or function of the aminoacylation active site located in the Rossmann-fold domain. This is consistent with the fact that residue 919 is spatially distant from the aminoacylation active site (>50 Å in the PhLeuRS-tRNA⁰⁰⁰ structure) (Figure 1B).
Subsequent comparisons of the aminoacylation kinetics of CaLeuRS-Ser919 and CaLeuRS-Leu919 revealed that CaLeuRS-Leu919 displayed a higher $K_m$ (2.91 ± 0.37 μM) and a higher $k_{cat}$ (0.62 ± 0.08 s⁻¹/C₀) compared with the values determined for CaLeuRS-Ser919 ($K_m$: 1.87 ± 0.23 μM, $k_{cat}$: 0.31 ± 0.05 s⁻¹/C₀). These data indicated that CaLeuRS-Ser919 has a stronger binding affinity for transcribed Ca$tRNALeu(UAA)$ during aminoacylation (Table 1). Based on the structure, we suggested that the presence of Ser in this helix may facilitate binding with the variable stem-loop element of Ca$tRNALeu(UAA)$. The catalytic efficiency of CaLeuRS-Leu919 (213.06 s⁻¹/C₀ 1 mM⁻¹) is ~30% higher than that of CaLeuRS-Ser919 (165.78 s⁻¹/C₀ 1). This phenomenon is similar to that observed in the case of CaSerRS, for which no differences were observed in the amino acid activation of CaSerRS-Leu197 and CaSerRS-Ser197, whereas CaSerRS-Leu197 showed a slightly (27%) higher activity than CaSerRS-Ser197 (30).

According to the decoding rule of C. albicans, CaLeuRS is present in the cytoplasm mainly in the form of CaLeuRS-Ser919. Thus, in the following study, we used CaLeuRS-Ser919 as the wild-type CaLeuRS.

Yeast LeuRSs efficiently recognized bacterial, yeast and human tRNA$^{Leu}$. Species-specific charging of tRNA is common for some aaRS systems. The aaRSs from higher organisms often have the capacity to charge tRNA from lower species, whereas aaRSs from lower organisms fail to aminoacylate tRNA from higher ones. It is unclear whether yeast LeuRS is able to recognize various tRNA$^{Leu}$s from other species. In this study, we investigated the tRNA$^{Leu}$ recognition capacity in detail using CaLeuRS as a model system.

The CaRNA$^{Leu}$ gene could not be over-expressed in E. coli and was obtained by T7 in vitro transcription. We also obtained transcribed and over-expressed EctRNA$^{Leu}$ and hetRNA$^{Leu}$ to reveal any potential role of base modification in recognition. Moreover, as transcribed S. cerevisiae tRNA$^{Leu}$ without modification showed no Leu accepting activity (data not shown), commercial S. cerevisiae yeast total tRNA was used. CaLeuRS recognized all the available tRNAs. It aminoacylated transcribed or over-expressed EctRNA$^{Leu}$ with similar $k_{cat}$ values (0.474 ± 0.026 and

![Figure 1. Location of residue 919 and its effect on amino acid activation in CaLeuRS. (A) Primary sequence alignment of LeuRSs from three domains of life with position of 919 indicated. Amino acid sequences homologous to those from α27 to α29 helix of PhLeuRS are aligned. (B) Crystal structure of PhLeuRS-tRNA$^{Leu}$ structure showing the position of 919-containing α29 helix. (C) Amino acid activation measurement of CaLeuRS-Leu$^{919}$ (black circle) and CaLeuRS-Ser$^{919}$ (black square). Ph, Pyrococcus horikoshii; Ca, C. albicans; Sc, S. cerevisiae; Gl, Giardia lamblia; Hs, Homo sapiens; Mj, Methanococcus jannaschii; Ce, Caenorhabditis elegans; Ec, E. coli; Tt, Thermus thermophiles.](image-url)
0.555 ± 0.061 s\(^{-1}\) respectively), although the \(K_m\) for transcribed \(E.\) coli tRNA\(^{\text{Leu}}\) (2.68 ± 0.39 \(\mu\)M) was nearly 4-fold greater than that for over-expressed \(E.\) coli tRNA\(^{\text{Leu}}\) (0.74 ± 0.08 \(\mu\)M). Interestingly, \(C.\) elegans LeuRS efficiently charged both transcribed and over-expressed hctRNA\(^{\text{Leu}}\), which was from a higher organism. A similar recognition pattern as seen with the two tRNA\(^{\text{Leu}}\)\(^\text{S}^{\text{S}}\) was also observed, with comparable \(k_{\text{cat}}\) values but a smaller \(K_m\) for over-expressed hctRNA\(^{\text{Leu}}\), indicating base modification was important for tRNA recognition. Additionally, \(C.\) elegans LeuRS obviously charged yeast total tRNA with \(K_m\) and \(k_{\text{cat}}\) values of 0.39 ± 0.05 \(\mu\)M and 0.174 ± 0.019 s\(^{-1}\), respectively, and with the greatest catalytic efficiency (1486.05 s\(^{-1}\)mM\(^{-1}\)) for over-expressed hctRNA\(^{\text{Leu}}\) among all the tested tRNAs (Table 2).

Owing to recognition ability of \(C.\) elegans LeuRS for hctRNA\(^{\text{Leu}}\), we further explored the capacity of \(S.\) cerevisiae LeuRS to aminoacylate bacterial and human tRNA\(^{\text{Leu}}\)\(^\text{S}^{\text{S}}\) as well as yeast total tRNA. \(S.\) cerevisiae aminoacylated yeast total tRNA with \(K_m\) and \(k_{\text{cat}}\) values of 0.332 ± 0.037 \(\mu\)M and 0.188 ± 0.025 s\(^{-1}\), respectively. However, its \(k_{\text{cat}}\) values for over-expressed \(E.\) coli tRNA\(^{\text{Leu}}\) or hctRNA\(^{\text{Leu}}\) increased >10-fold (2.09 ± 0.16 and 2.19 ± 0.22 s\(^{-1}\), respectively), although the \(K_m\) values differed from each other remarkably (2.19 ± 0.47 \(\mu\)M for over-expressed \(E.\) coli tRNA\(^{\text{Leu}}\) and 0.111 ± 0.027 \(\mu\)M for over-expressed hctRNA\(^{\text{Leu}}\)). These data demonstrated that over-expressed hctRNA\(^{\text{Leu}}\) was the best aminoacylation substrate for \(S.\) cerevisiae LeuRS (catalytic efficiency 19729.73 s\(^{-1}\)mM\(^{-1}\)) and furthermore suggested that base modification was important during recognition or catalysis. \(S.\) cerevisiae LeuRS recognized transcribed \(E.\) coli tRNA\(^{\text{Leu}}\) with a similar \(K_m\) (1.71 ± 0.22 \(\mu\)M) but a sharply decreased \(k_{\text{cat}}\) (0.134 ± 0.011 s\(^{-1}\)) compared with the values of over-expressed \(E.\) coli tRNA\(^{\text{Leu}}\). It also recognized transcribed hctRNA\(^{\text{Leu}}\) with an increased \(K_m\) (0.926 ± 0.170 \(\mu\)M) and a decreased \(k_{\text{cat}}\) (0.887 ± 0.114 s\(^{-1}\)) compared with the values for over-expressed hctRNA\(^{\text{Leu}}\) (Table 2).

Overall, both \(C.\) elegans and \(S.\) cerevisiae LeuRS recognized bacterial, yeast and human tRNA\(^{\text{Leu}}\). Interestingly, recognition of \(C.\) elegans tRNA\(^{\text{Leu}}\) by \(S.\) cerevisiae LeuRS was negligible (Supplementary Figure S1A). Furthermore, \(E.\) coli LeuRS failed to acylate \(C.\) elegans tRNA\(^{\text{Leu}}\) (Supplementary Figure S1B). These results were unexpected because it is widely accepted that aaRSs from higher organisms are able to aminoacylate tRNAs from lower organisms.

### Amino acid activation capacity of \(C.\) elegans LeuRS

Various aaRSs have been shown to mis-activate a series of non-cognate amino acids. To investigate mis-activation of non-cognate amino acids by \(C.\) elegans LeuRS, we included ABA, Nva, Val, Ile, Met, Ser in the ATP-PPi exchange reaction. The data clearly showed that \(C.\) elegans LeuRS significantly mis-activated Nva; furthermore, ABA was also mis-activated to an obvious level compared with the control reaction conducted in the absence of amino acids. In contrast, mis-activation of Val, Ile, Met and Ser was comparable with that of the control reaction conducted in the absence of amino acids (Figure 2). To further define the quantitative discrimination capacity of the aminoacylation active site of \(C.\) elegans LeuRS, we measured the activation kinetics for cognate Leu and non-cognate Nva and ABA of \(C.\) elegans LeuRS. \(C.\) elegans LeuRS gave much higher \(K_m\) values for Nva (5487 ± 645 \(\mu\)M) and ABA (120387 ± 1698 \(\mu\)M) than for Leu (1054 ± 123 \(\mu\)M) (Table 3). These results indicated that Nva is a real challenge for \(C.\) elegans LeuRS and that removal of Nva-AMP and/or Nva-tRNA\(^{\text{Leu}}\) is required to maintain the translational quality control. However, the discrimination against ABA was below the proposed threshold of 1/3000, indicating that editing of ABA may not be necessary.

### \(C.\) elegans LeuRS exhibited little tRNA-dependent pre-transfer editing for Nva

The hydrolysis of Nva-AMP or Nva-tRNA\(^{\text{Leu}}\) may be separately or simultaneously catalyzed by \(C.\) elegans LeuRS. Editing leads to the net consumption of ATP (yielding AMP) due to repetitive cycles of synthesis-hydrolysis of the non-cognate products. This is the basis of the TLC-based AMP formation methodology, in which the editing capacity is measured by monitoring the quantity

**Table 2. Aminoacylation kinetic parameters of \(C.\) elegans LeuRS and \(S.\) cerevisiae LeuRS for various tRNAs**

| Enzyme   | tRNA            | \(K_m\) (\(\mu\)M) | \(k_{\text{cat}}\) (s\(^{-1}\)) | \(k_{\text{cat}}/K_m\) (s\(^{-1}\)mM\(^{-1}\)) |
|----------|-----------------|---------------------|-------------------------------|-----------------------------------------------|
| \(C.\) elegans LeuRS | OE\(^{\text{E}}\)-E. coli tRNA\(^{\text{Leu}}\) | 0.74 ± 0.08          | 0.474 ± 0.026       | 640.54                                        |
|          | TS-E. coli tRNA\(^{\text{Leu}}\)     | 2.68 ± 0.39          | 0.555 ± 0.061       | 207.09                                        |
|          | yeast total tRNA | 0.39 ± 0.05          | 0.174 ± 0.019       | 446.15                                        |
|          | OE-hctRNA\(^{\text{Leu}}\)           | 0.43 ± 0.05          | 0.639 ± 0.055       | 1486.05                                       |
|          | TS-hctRNA\(^{\text{Leu}}\)           | 1.30 ± 0.23          | 0.719 ± 0.094       | 553.08                                        |
| \(S.\) cerevisiae LeuRS | OE-E. coli tRNA\(^{\text{Leu}}\) | 2.19 ± 0.47          | 2.09 ± 0.16         | 954.34                                        |
|          | TS-E. coli tRNA\(^{\text{Leu}}\)     | 1.71 ± 0.22          | 0.134 ± 0.011       | 78.36                                         |
|          | yeast total tRNA | 0.332 ± 0.037        | 0.188 ± 0.025       | 566.27                                        |
|          | OE-hctRNA\(^{\text{Leu}}\)           | 0.111 ± 0.027        | 2.19 ± 0.22         | 19729.73                                       |
|          | TS-hctRNA\(^{\text{Leu}}\)           | 0.926 ± 0.170        | 0.887 ± 0.114       | 957.88                                        |

*The results are the average of three independent repeats with standard deviations indicated.\(^{\text{OE}}\) OE, over-expressed.\(^{\text{TS}}\) TS, transcribed.
of AMP produced (9–11,36,37). In the presence of tRNA and non-cognate amino acid, the TLC assay measures the global editing activity, including the tRNA-independent and tRNA-dependent pre-transfer editing in addition to the post-transfer editing. In the absence of tRNA, but with non-cognate amino acid, AMP is produced only with non-cognate amino acid, AMP is produced only with non-cognate amino acid, the TLC assay measures the relative to Leu.

Table 3. Amino acid activation kinetics of CaLeuRS for various amino acids

| Amino acid | K_m (μM) | k_cat (s⁻¹) | k_cat/K_m (s⁻¹ M⁻¹) | Discrimination factor |
|------------|---------|------------|-----------------|---------------------|
| Leu        | 40.11 ± 3.84 | 93.05 ± 10.25 | 2319.87 | 1 |
| Nva        | 5487 ± 645  | 57.77 ± 6.35  | 10.53   | 220  |
| ABA        | 120387 ± 1698 | 80.28 ± 10.75 | 0.67 | 3462 |

The results are the average of three independent repeats with standard deviations indicated.

The lack of availability of over-expressed CatRNA_{Leu} impaired exploration of the potential role of modified bases in editing. Therefore, we performed AMP formation assays with Nva in the presence of transcribed or over-produced EctRNA_{Leu} in E. coli, which could be leucylated by CaLeuRS. In accordance with our findings, the k_{obs} values with unmodified or modified EctRNA_{Leu} were 0.40 ± 0.06 or 0.51 ± 0.04 s⁻¹, respectively. Similarity, transcribed or over-produced hctRNA_{Leu} in E. coli, both of which were effectively aminoacylated by CaLeuRS, stimulated Nva-editing of CaLeuRS with k_{obs} values of 0.33 ± 0.02 or 0.38 ± 0.05 s⁻¹, respectively (Table 4). These data showed that the modified bases of tRNA_{Leu} had little effect on the tRNA-dependent editing of CaLeuRS. Based on data from various transcripts or the tRNA_{Leu} with modified bases, we concluded that CaLeuRS has little tRNA-dependent editing activity for Nva. Whether it was deficient in post-transfer editing would be explored later in the text. By comparing the k_{obs} values with or without tRNAs, we also observed that post-transfer editing, if it occurred, contributed little to the total editing, and that the observed k_{obs} with tRNAs was almost a reflection of the tRNA-independent pre-transfer editing.

Table 4. k_{obs} values of CaLeuRSs for editing Nva with various tRNAs

| Enzyme            | tRNA                  | k_{obs} (s⁻¹) |
|-------------------|-----------------------|---------------|
|                   | No tRNA               | 0.25 ± 0.03   |
| CatRNA_{Leu}      | 0.28 ± 0.04           |
| TS_{Ec}-EctRNA_{Leu} | 0.40 ± 0.06          |
| OE_{Ec}-EctRNA_{Leu} | 0.51 ± 0.04          |
| TS-hctRNA_{Leu}   | 0.33 ± 0.02           |
| OE-hctRNA_{Leu}   | 0.38 ± 0.05           |
|                   | No tRNA               | 0.22 ± 0.06   |
| CaLeuRS-D422A     | 0.25 ± 0.04           |
|                   | No tRNA               | 0.22 ± 0.02   |
| CaLeuRS-Leu_{919} | 0.25 ± 0.05           |

The results are the average of three independent repeats with standard deviations indicated.

TS, transcribed.

OE, over-expressed.

CaLeuRS exhibited obvious and efficient post-transfer editing to prevent synthesis of Nva-tRNA_{Leu}

The absence of significant stimulation of editing of Nva by CaLeuRS with various tRNA_{Leu}’s prompted us to investigate its post-transfer editing capability. Usually, the post-transfer editing ability of various LeuRSs is monitored by hydrolysis of Ile- or Met-tRNA_{Leu}, which are easily obtained by mis-charging tRNA_{Leu} with commercially available radioactive Ile or Met using a LeuRS mutant without post-transfer editing capability. Because we focused on the Nva-editing properties of CaLeuRS and Nva labeled with radioactive isotope was not commercially available, the 3’ end of CatRNA_{Leu} was first labeled with [z-32P]ATP by E. coli CCase, and then Nva-[32P]CatRNA_{Leu} was prepared by editing-deficient...
ScLeuRS-D419A (13,18). Hydrolytic analysis clearly showed that CaLeuRS edited Nva-[^32]P]tRNA^Leu when compared with the control experiment conducted in the absence of the enzyme (Figure 3A and B). To confirm the post-transfer editing reaction catalyzed by CaLeuRS, we mutated the conserved and post-transfer editing-essential Asp422 to generate CaLeuRS-D422A. Asp422 corresponds to Asp373, Asp419, Asp444 and Asp399 of AaLeuRS, ScLeuRS, GlLeuRS and hctRNA^Leu, respectively, which are crucial to post-transfer editing by these LeuRSs (9,10,18,19,27). Indeed, CaLeuRS-D422A did not hydrolyze Nva-[^32]P]tRNA^Leu and was deficient in post-transfer editing, indicating that this mutation inactivated the CP1 domain of CaLeuRS (Figure 3A and B). Further mis-aminoacylation of[^32]P]tRNA^Leu with non-cognate Nva (left) or ABA (right). Free[^32]P]tRNA^Leu and mis-charged[^32]P]tRNA^Leu are represented by[^32]P]AMP and Nva[^32]P]AMP or ABA[^32]P]AMP, respectively. Known amounts of [α-[^32]P]ATP were serially diluted and loaded onto the TLC plate after separation for quantification. (D) Quantitative analysis of Nva-[^32]P]tRNA^Leu or ABA[^32]P]tRNA^Leu generated by CaLeuRS (black square) and CaLeuRS-D422A (black up-pointing triangle) or ABA[^32]P]tRNA^Leu by CaLeuRS (white up-pointing triangle) and CaLeuRS-D422A (white down-pointing triangle) in (C).

CaLeuRS inhibited synthesis of Nva-tRNA^Ser by non tRNA species-specific post-transfer editing

The ability of CaLeuRS to efficiently mis-activate non-cognate Nva and recognize non-cognate CatRNA^Ser raises the interesting question of how to prevent the formation of Nva-CatRNA^Ser. To test for the presence of post-transfer editing activity that hydrolyzes potentially synthesized Nva-CatRNA^Ser, mis-aminoacylation of[^32]P]CatRNA^Ser with Nva by CaLeuRS was compared with that of the post-transfer editing-deficient CaLeuRS-D422A. The data clearly showed that, mutation of Asp422 resulted in significant synthesis of Nva[^32]P]CatRNA^Ser by the mutant, in contrast to wild-type enzyme, which generated negligible amounts of Nva[^32]P]CatRNA^Ser, indicating that CaLeuRS used post-transfer editing to prevent Nva-CatRNA^Ser synthesis (Figure 4A and B).
We then prepared Nva-Ca\textsubscript{t}tRNA\textsubscript{Ser} for use in hydrolysis assays to more directly monitor the post-transfer editing activity. Obvious hydrolysis of Nva-[\textsuperscript{32}P]Ca\textsubscript{t}tRNA\textsubscript{Ser} was mediated by Ca\textsubscript{Leu}RS but not Ca\textsubscript{Leu}RS-D422A (Figure 4C and D). Above all, these data showed that the post-transfer editing by Ca\textsubscript{Leu}RS was not only Ca\textsubscript{t}tRNA\textsubscript{Leu} specific but also efficient for Ca\textsubscript{t}tRNA\textsubscript{Ser} to inhibit synthesis of both Nva-Ca\textsubscript{t}tRNA\textsubscript{Leu} and Nva-Ca\textsubscript{t}tRNA\textsubscript{Ser}.

**CaLeuRS possessed weak tRNA-dependent pre-transfer editing capacity for ABA**

ABA was selected to test whether CaLeuRS possessed any tRNA-dependent pre-transfer editing of other non-cognate amino acids because it was obviously activated by CaLeuRS. Ca\textsubscript{t}tRNA\textsubscript{Leu} transcript, transcribed or over-produced Ect\textsubscript{RNA}\textsubscript{Leu} and hct\textsubscript{RNA}\textsubscript{Leu} were used to trigger editing of ABA by CaLeuRS (Table 5). The data showed that over-produced hct\textsubscript{RNA}\textsubscript{Leu} obviously stimulated editing by increasing the $k_{\text{obs}}$ 5-fold \([23.19 \pm 3.62] \times 10^{-3}\text{ s}^{-1}\) compared with that in the absence of tRNA \([4.69 \pm 0.51] \times 10^{-3}\text{ s}^{-1}\). Over-expressed Ect\textsubscript{RNA}\textsubscript{Leu} led only to an \(~3\)-fold increase in $k_{\text{obs}}$ \([14.58 \pm 2.14] \times 10^{-3}\text{ s}^{-1}\). However, Ca\textsubscript{t}tRNA\textsubscript{Leu}, Ect\textsubscript{RNA}\textsubscript{Leu} and hct\textsubscript{RNA}\textsubscript{Leu} transcripts had little effect on the rate of ABA-editing (Table 5). These data implied that editing of ABA was tRNA modification-dependent.

![Figure 4. Mis-charging of [\textsuperscript{32}P]Ca\textsubscript{t}tRNA\textsubscript{Ser} with Nva and post-transfer editing of Nva-[\textsuperscript{32}P]Ca\textsubscript{t}tRNA\textsubscript{Ser} by CaLeuRS and CaLeuRS-D422A. (A) A representative graph showing mis-charging of [\textsuperscript{32}P]Ca\textsubscript{t}tRNA\textsubscript{Ser} with non-cognate Nva. Free [\textsuperscript{32}P]Ca\textsubscript{t}tRNA\textsubscript{Ser} and mis-charged [\textsuperscript{32}P]Ca\textsubscript{t}tRNA\textsubscript{Ser} are represented by [\textsuperscript{32}P]AMP and Nva-[\textsuperscript{32}P]AMP after digestion of nuclease S1. Known amounts of [\textsuperscript{\alpha}-\textsuperscript{32}P]ATP were serially diluted and loaded onto the TLC plate after separation for quantification. (B) Quantitative analysis of Nva-[\textsuperscript{32}P]Ca\textsubscript{t}tRNA\textsubscript{Ser} generated by CaLeuRS (black square) and CaLeuRS-D422A (black up-pointing triangle) in (A). (C) A representative graph showing hydrolysis of Nva-[\textsuperscript{32}P]Ca\textsubscript{t}tRNA\textsubscript{Ser} by CaLeuRS and CaLeuRS-D422A. A control reaction represented the spontaneous hydrolysis of Nva-[\textsuperscript{32}P]Ca\textsubscript{t}tRNA\textsubscript{Ser} without the addition of enzyme. (D) Analysis of post-transfer editing of Nva-[\textsuperscript{32}P]Ca\textsubscript{t}tRNA\textsubscript{Ser} by CaLeuRS (black square) and CaLeuRS-D422A (black up-pointing triangle) in (C).](image)

| Enzyme          | tRNA                  | $k_{\text{obs}}$ (\(10^3\text{ s}^{-1}\))$^a$ |
|-----------------|-----------------------|------------------------------------------|
| CaLeuRS         | No tRNA               | 4.69 ± 0.51                              |
|                 | C\textsubscript{t}tRNA\textsubscript{Leu} | 4.90 ± 0.37                              |
|                 | TS\textsuperscript{b}-Ect\textsubscript{RNA}\textsubscript{Leu} | 4.78 ± 0.26                              |
|                 | OE\textsuperscript{c}-Ect\textsubscript{RNA}\textsubscript{Leu} | 14.58 ± 2.14                             |
|                 | TS-hct\textsubscript{RNA}\textsubscript{Leu} | 10.93 ± 0.85                             |
|                 | OE-hct\textsubscript{RNA}\textsubscript{Leu} | 23.19 ± 3.62                             |
| CaLeuRS-D422A   | OE-hct\textsubscript{RNA}\textsubscript{Leu} | 12.16 ± 1.98                             |

$^a$The results are the average of three independent repeats with standard deviations indicated.

$^b$TS, transcribed.

$^c$OE, over-expressed.

As over-produced hct\textsubscript{RNA}\textsubscript{Leu} was the most efficient stimulator of ABA-editing, we measured AMP formation by the editing-deficient CaLeuRS-D422A mutant in the presence of ABA with over-produced hct\textsubscript{RNA}\textsubscript{Leu}. Mutation of Asp\textsuperscript{422}, which abolished post-transfer editing, apparently decreased the rate of AMP formation with a $k_{\text{obs}}$ of \([12.16 \pm 1.98] \times 10^{-3}\text{ s}^{-1}\). Therefore, with over-produced hct\textsubscript{RNA}\textsubscript{Leu}, post-transfer editing of ABA by CaLeuRS accounted for 47.6% of the total editing \([23.19 - 12.16]/23.19\), whereas tRNA-independent and
tRNA-dependent pre-transfer editing of ABA only accounted for 20.2% (4.69/23.19) and 32.2% [12.16 – 4.69]/23.19, respectively, of the total editing of ABA by CaLeuRS.

We further performed aminoacylation of $^{32}$P-CatRNA$_{Leu}$ by CaLeuRS and CaLeuRS-D422A with saturating concentrations of ABA. The data showed that defective post-transfer editing resulted in the generation of significantly more ABA-$^{32}$P-CatRNA$_{Leu}$, however, surprisingly, even CaLeuRS yielded a significant amount of ABA-$^{32}$P-CatRNA$_{Leu}$ (Figure 3C and D). These data implied that editing of ABA by CaLeuRS was not sufficient to prevent the synthesis of ABA-$^{32}$P-CatRNA$_{Leu}$ in the presence of saturating ABA concentrations. This paradox between ABA-misaminoacylation and charging accuracy may be solved by fine discrimination against ABA at the aminoacylation active site (Table 3).

These results revealed that CaLeuRS exhibits a weak level of tRNA-dependent pre-transfer editing activity for ABA. In addition, the total ABA-editing capacity is not sufficient to avoid the formation of mis-charged tRNA$_{Leu}$, which is different from Nva-editing capacity.

ScLeuRS, like CaLeuRS, also exhibited little tRNA-dependent pre-transfer editing capacity for Nva

The natural deficiency in tRNA-dependent pre-transfer editing of Nva by CaLeuRS prompted us to investigate whether it is a common characteristic of other yeast LeuRS. Therefore, we assayed the Nva-included AMP formation catalyzed by ScLeuRS in the absence or presence various tRNA$_{Leu}$s. The data showed that over-produced hctRNA$_{Leu}$ obviously stimulated editing ($k_{obs}$ of 0.64 ± 0.04 s$^{-1}$) compared with that observed in the absence of tRNA (0.10 ± 0.02 s$^{-1}$). However, transcribed CatRNA$_{Leu}$, EctRNA$_{Leu}$, hctRNA$_{Leu}$ and over-expressed EctRNA$_{Leu}$ failed to trigger further editing by ScLeuRS (Table 6). The formation of AMP stimulated by tRNA$_{Leu}$ should be derived from its combined tRNA$_{Leu}$ and tRNA$_{Ser}$. To test whether other eukaryotic, archaeal, bacterial or mitochondrial LeuRSs could potentially recognize CatRNA$_{Ser}$, we performed aminoacylation of $^{32}$P-CatRNA$_{Ser}$ with Leu by CamtLeuRS, EtcLeuRS, ScLeuRS, hctLeuRS and PhLeuRS. The data showed that only eukaryotic LeuRSs (including CaLeuRS, ScLeuRS and hctLeuRS) could aminoacylate CatRNA$_{Ser}$ with Leu; however, other LeuRSs, including bacterial EtcLeuRS, mitochondrial CamtLeuRS and archaeal PhLeuRS, failed to charge it (Figure 5). Strikingly, under the same conditions, ScLeuRS and hctLeuRS mediated more efficient aminoacylation of CatRNA$_{Ser}$.

**DISCUSSION**

Insertion of Ser or Leu at CUG codons might not be incidental

In *C. albicans* and most other CUG clade species, a mutant tRNA$_{Ser}$(CAG) has evolved to decode the Leu CUG codon both as Ser and Leu (28,29). This peculiarity is derived from its combined tRNA$_{Leu}$ and tRNA$_{Ser}$ identity elements (38). This tRNA is mainly aminoacylated by SerRS and charged by LeuRS to a small extent (29). Both biochemical and structural data have revealed that ambiguity at the single CUG codon of SerRS induces local structural rearrangement, leading to a slightly increased activity (27%) of CaSerRS-Leu$^{197}$ compared with the wild-type CaSerRS-Ser$^{197}$ (30). Furthermore, genetic studies showed that increased Leu incorporation across all the CUG codons of *C. albicans* had no visible effect on the growth phenotype but had an impressive impact on cell morphology (39). Therefore, it was proposed that CUG decoding ambiguity has a potential regulatory role in protein structure and/or function (30). CaLeuRS is another crucial player in this genetic code alteration and also contains only one CUG codon at position 919. This site is located at the C-terminal domain of LeuRS, which has been shown to be responsible for binding the variable loop of tRNA$_{Leu}$ and involved in the aminoacylation activity; however, this

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**Table 6.** $k_{obs}$ values of ScLeuRSs for editing Nva with various tRNAs

| Enzyme     | tRNA      | $k_{obs}$ (s$^{-1}$)$^a$ |
|------------|-----------|--------------------------|
| no tRNA    | 0.10 ± 0.02 |
| TS$^c$-EtRNA$_{Leu}$ | 0.10 ± 0.02 |
| OE$^c$-EtRNA$_{Leu}$ | 0.12 ± 0.03 |
| CatRNA$_{Leu}$ | 0.10 ± 0.03 |
| TS-hcRNA$_{Leu}$ | 0.10 ± 0.01 |
| OE-hcRNA$_{Leu}$ | 0.64 ± 0.04 |
| ScLeuRS    | no tRNA    | 0.094 ± 0.001 |
| D419A      | OE-hcRNA$_{Leu}$ | 0.100 ± 0.010 |

$a$The results are the average of three independent repeats with standard deviations indicated.

$^c$TS, transcribed.

$^e$OE, over-expressed.
domain is not strictly conserved among archaeal/eukaryotic LeuRSs (Figure 1A and B). Here, we revealed that both CaLeuRS-Leu919 and CaLeuRS-Ser919 catalyzed Leu activation and aminoacylation, but the former was more active (30%) than the latter, indicating that the conformation of the 919-containing α29 helix might be finely controlled by the introduction of either Ser or Leu. This phenomenon was also observed in another crucial player in the CUG decoding alteration pathway, CaSerRS (30). We suggested that insertion of either Ser or Leu at the CUG codon was not random and incidental. The relative amounts of CaLeuRS-Ser919/CaLeuRS-Leu919 should be strictly regulated by an unidentified but precise molecular mechanism in vivo. Whether the fine balance of CaLeuRS-Ser919/CaLeuRS-Leu919 is critical for decoding other Leu codons and correlates with the ratio of CaSerRS-Ser197/CaSerRS-Leu197 requires further investigation.

Yeast LeuRS exhibited a relaxed tRNA recognition capacity

In tRNA aminoacylation, species-specific charging, where a tRNA from one taxonomic domain is not aminoacylated by an aaRS from another, is widespread. This may be as a result of the co-evolution of synthetase/tRNA pairs by the addition of species-specific elements. For instance, human tyrosyl-tRNA synthetase does not recognize bacterial tRNA\textsubscript{Tyr}, and \textit{E. coli} tyrosyl-tRNA synthetase is unable to charge eukaryotic tRNA\textsubscript{Tyr} (40), and there is no cross-recognition of \textit{E. coli} and human tRNA\textsubscript{Gly} by the respective glycyl-tRNA synthetases (41). Similarly, \textit{E. coli} isoleucyl-tRNA synthetase is unable to charge eukaryotic tRNA\textsubscript{Ile} (42). Yeast ArgRS charges \textit{E. coli} tRNA\textsubscript{Arg}, however, \textit{E. coli} ArgRS acylates only its cognate \textit{E. coli} tRNA (43). Human cysteinyl-tRNA synthetase charges bacterial tRNA\textsubscript{Cys}, but \textit{E. coli} cysteinyl-tRNA synthetase is non-functional in aminoacylating human tRNA\textsubscript{Cys} (44). Here, we showed that both hcLeuRS and EcLeuRS failed to aminoacylate Ca\textsubscript{tRNASer}, however, both CaLeuRS and ScLeuRS readily aminoacylated bacterial, yeast and even human tRNA\textsubscript{Leu}. These results showed that yeast LeuRSs exhibit a more relaxed recognition specificity compared with other LeuRSs. Indeed, Ca\textsubscript{tRNASer} itself harbors only tRNA\textsubscript{Leu} recognition elements in the anticodon loop with other parts being crucial for SerRS recognition. In addition, G33 is also unfavorable for LeuRS; even in this adverse state, CaLeuRS aminoacylates it in vivo (29). Comparison between transcribed and over-expressed tRNA\textsubscript{Leu} showed that base modification of tRNA\textsubscript{Leu} plays an important role in both binding and catalysis.
CaLeuRS was deficient in tRNA-dependent pre-transfer editing but exhibited efficient post-transfer editing for Nva

Nva is inherently mis-activated by various LeuRSs to a significant level that requires editing for translational accuracy (9,18–20). With an elevated ratio of Nva to Leu, Nva can escape the safeguarding of EcLeuRS and replace Leu in proteins rich in Leu codons, indicating that Nva-tRNA^{Leu}_E can escape further checking by the ribosome and pose a direct threat to the accuracy of newly synthesized proteins (24). From the viewpoint of editing, some LeuRSs with degenerated (e.g. hmtLeuRS) or deleted CP1 (e.g. MmLeuRS) domains are exceptional examples, which use alternative pathways (efficient discrimination at the active site) for translational quality control (hmtLeuRS) (26) or do not edit mis-aminoacylation product to produce proteome ambiguity (MmLeuRS) (20). However, all LeuRSs with functional CP1 domains studied so far display tRNA-independent, tRNA-dependent pre-transfer editing and post-transfer editing for Nva. Through inactivation of CP1 or utilization of LeuRS inhibitors, post-transfer editing has been successfully isolated (9,10,18,19,27). Similarly, by mutating a crucial Tyr residue to Asp in EcLeuRS and AaLeuRS, both tRNA-dependent pre-transfer and post-transfer editing are inactivated (10,33). Interestingly, this study identified that CaLeuRS itself is naturally defective in tRNA-dependent pre-transfer editing for Nva. With CatRNA^{Leu}_E, no tRNA-dependent pre-transfer editing was identified. In contrast, weak tRNA-dependent pre-transfer editing for ABA in the presence of specific tRNA^{Leu}_E was observed, despite the indication that ABA-editing might not be necessary in vivo based on fine discrimination at the active site. Similarly, ScLeuRS did not mediate tRNA-dependent pre-transfer editing. These results indicate that the capacity for tRNA-dependent pre-transfer editing for Nva has been lost by CaLeuRS (also ScLeuRS), and that ABA is also rarely induced. The reason for this deficiency in tRNA-dependent pre-transfer editing and the pathway by which this deficiency was introduced remains to be elucidated.

Post-transfer editing contributed little or negligibly to the total Nva-editing since addition of any tRNAs in the AMP formation assays did not significantly induce additional AMP. Thus, the produced AMP was mainly derived from tRNA-independent pre-transfer editing. However, the energy-saving post-transfer editing pathway critically controls the accuracy of aminoacylation. Mutation at the conserved Asp^{222} of CaLeuRS led to a LeuRS with abolished post-transfer editing capacity; consequently, Nva-tRNA^{Leu}_E was synthesized. Similarly, ScLeuRS did not synthesize Ile-tRNA^{Leu}_E; however, ScLeuRS-D419A readily generated significant amounts of Ile-tRNA^{Leu}_E (13,18). Using these unique CaLeuRS and ScLeuRS models devoid of tRNA-dependent pre-transfer editing capacity, we concluded that the post-transfer editing pathway is the most economic but efficient editing mechanism for LeuRS. Consistent with other LeuRS models and even other aaRS systems, once post-transfer editing is impaired, the mis-charged tRNA is unavoidably accumulated (9–11,18,19,27,45,46).

Our results also revealed that post-transfer editing by CaLeuRS is not tRNA-species specific, as Nva-tRNA^{Ser}_Ser was also a substrate. Indeed, based on the poor discrimination against Nva in the active site, Nva-tRNA^{Ser}_Ser is possibly synthesized but should be removed. Otherwise, the CUG codon might be decoded as Ser, Leu and Nva in vivo. It has been proposed that the acceptor end of the tRNA switches from a hairpin conformation to a helical conformation for editing by class I aaRSs, whereas the reverse change in conformation occurs at the acceptor end of the tRNA for editing by class II aaRSs (47). Notably, CatRNA^{Ser}_Ser corresponds to a class II SerRS; however, results here showed that CatRNA^{Ser}_Ser could switch from a hairpin to a helical conformation for editing by a class I LeuRS.

Eukaryotic LeuRSs recognized CatRNA^{Ser}_Ser

In addition, we revealed that other eukaryotic LeuRSs could efficiently aminoacylate CatRNA^{Ser}_Ser, implying that the introduction or evolution of this type of tRNA in other eukaryotic systems would reprogram or discombobulate the genetic code, leading to proteome chaos. In other words, a specific eukaryotic genetic code could be artificially reprogrammed by expression of this tRNA^{Ser}_Ser. Indeed, CatRNA^{Ser}_Ser has been shown to be efficiently produced, processed and aminoacylated in S. cerevisiae, with its expression triggering a stress response, blocking mating and re-defining the gene expression model of S. cerevisiae (48). Notably, archaeal LeuRS is in the same group with eukaryotic LeuRS according to primary or higher structure (14) and only differs at the C-terminal tRNA binding domain, indicating that this domain in eukaryotic LeuRSs is a key element for recognition of CatRNA^{Ser}_Ser. This observation is consistent with the structural and biochemical results showing that the C-terminal domain of archaeal LeuRS specifically contacts the variable loop but not the anti-codon loop of archaeal tRNA^{Leu}_A (16,49). However, the anti-codon loop, which is a key recognition element in both CatRNA^{Ser}_Ser (28,29,38) and yeast tRNA^{Leu}_A (49), is likely to be bound by the C-terminal domain of eukaryotic LeuRS. This proposal requires confirmation from eukaryotic LeuRS-tRNA^{Leu}_E/tRNA^{Ser}_Ser structures.

Concluding remarks

Translational machinery of human pathogen C. albicans is of particular interest because its CUG codon in the genome is decoded as both Ser and Leu by a unique CatRNA^{Ser}_Ser leading to proteome ambiguity (28,29). One of the most crucial components in this decoding process is CaLeuRS, which catalyzes two successive steps, aminoacylation and editing reactions, which together are essential for ensuring high specificity of tRNA charging. In aminoacylation, we showed that Leu isofrom was more active than Ser isofrom of CaLeuRS in charging CatRNA^{Leu}_E, implying the existence of an in vivo mechanism regulated by balance of CaLeuRS-Leu^{919} and CaLeuRS-Ser^{919}. In addition, as a yeast LeuRS model,
Furthermore, the capacity of eukaryotic LeuRSs at and revealed interesting properties of both mechanism and significance of genetic code ambiguity in C. albicans and revealed interesting properties of both aminoacylation and editing reactions by CaLeuRS. Furthermore, the capacity of eukaryotic LeuRSs at aminoacylating CatRNA Ser suggests the possibility of reconstructing proteome of other eukaryotes by simply introducing this unique tRNA Ser.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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