C-terminal Domain of Leucyl-tRNA Synthetase from Pathogenic Candida albicans Recognizes both tRNA<sup>Ser</sup> and tRNA<sup>Leu</sup>∗

Leucyl-tRNA synthetase (LeuRS) is a multidomain enzyme that catalyzes Leu-tRNA<sup>Leu</sup> formation and is classified into bacterial and archaeal/eukaryotic types with significant diversity in the C-terminal domain (CTD). CTDs of both bacterial and archaeal LeuRSs have been reported to recognize tRNA<sup>Leu</sup> through different modes of interaction. In the human pathogen Candida albicans, the cytoplasmic LeuRS (CaLeuRS) is distinguished by its capacity to recognize a uniquely evolved chimeric tRNA<sup>Ser</sup> (CatRNA<sup>Ser</sup>(CAG)) in addition to its cognate CatRNA<sup>Leu</sup>, leading to CUG codon reassignment. Our previous study showed that eukaryotic but not archaeal LeuRSs recognize this peculiar tRNA<sup>Ser</sup>, suggesting the significance of their highly divergent CTDs in tRNA<sup>Ser</sup> recognition. The results of this study provided the first evidence of the indispensable function of the CTD of eukaryotic LeuRS in recognizing non-cognate CatRNA<sup>Ser</sup> and cognate CatRNA<sup>Leu</sup>. Three lysine residues were identified as involved in mediating enzyme-tRNA interaction in the leucylation process: mutation of all three sites totally ablated the leucylation activity. The importance of the three lysine residues was further verified by gel mobility shift assays and complementation of a yeast leu5<sup>+</sup> gene knock-out strain.

Aminoacyl-tRNA synthetases (aaRSs) are a family of enzymes that catalyze aminoacyl-tRNA formation, playing a pivotal role in protein translation (1). In general, this catalytic process occurs in two steps. First, the amino acid is activated by ATP to form an aminoacyl-adenylate (aa-AMP) intermediate; second, the activated amino acid is transferred to the CCA terminus of the cognate tRNA to form aminoacyl-tRNA (1, 2). Based on the conserved sequences and properties of structural motifs, the 20 aaRSs that account for this catalytic process are divided into two classes (3, 4). Class I aaRSs utilize a Rossmann fold (characterized by “HIGH” and “KMSKS” motifs) to perform their tRNA-charging activities (5). Class II aaRSs dimerize to bind ATP and amino acids through an antiparallel β-fold catalytic site with signature motifs (3, 4). Leucyl-tRNA synthetase (LeuRS), which is a class I aaRS, is further classified into bacterial and archaeal/eukaryotic types on the basis of CP1 domain insertion site and orientation (6–8). Both types of LeuRS possess a catalytic domain (for amino acid activation and tRNA charging), a CP1 domain (for editing), an α-helix bundle domain, and a C-terminal domain (CTD; for tRNA binding) (9). However, the two types of LeuRS show divergence in the primary sequence and tertiary folding of the CTD. The CTD of bacterial LeuRS is compacted into an αβ domain surrounding by a four-stranded β-sheet (7), and that of archaeal LeuRS comprises a three-stranded β-sheet surrounded by α-helices (10). Seryl-tRNA synthetase (SerRS) belongs to class II aaRSs and is a homodimer, which is distinct from LeuRS in structure and the tRNA recognition mechanism (11, 12).

Candida albicans, a human fungal pathogen, has an ambiguous codon deciphering mechanism in which the universal leucine codon CUG is decoded as both Ser (97%) and Leu (3%) during ribosome translation, leading to proteome ambiguity (11). This ambiguity may create novel protein functionalities to speed up evolution or block sexual reproduction (13). Interestingly, it is a uniquely evolved C. albicans tRNA<sup>Ser</sup>(CAG) (CatRNA<sup>Ser</sup>(CAG); CatRNA<sup>Ser</sup>) that mediates this codon reassignment with no tRNA<sup>Leu</sup>(CAG) isoacceptor existing (14). CatRNA<sup>Ser</sup>(CAG) is a chimeric tRNA containing the main body (including acceptor stem, D-stem/loop, T/C stem/loop, and long variable stem/loop) of tRNA<sup>Ser</sup> and anticodon stem/loop of tRNA<sup>Leu</sup> (Fig. 1A) (14–16). Both yeast tRNA<sup>Ser</sup> and tRNA<sup>Leu</sup> are class II tRNAs, which have a long variable arm and share similar L-shaped tertiary structures (17–19). Therefore, CatRNA<sup>Ser</sup>(CAG) could be recognized by CaLeuRS through the anticodon triplet and methylated G37 (m<sup>1</sup>G37), which are characteristics of yeast tRNA<sup>Leu</sup> (Fig. 1A), in addition to being recognized by CaSerRS through the (GC)<sub>2</sub> helix of the long variable arm and the discriminator G73 of tRNA<sup>Ser</sup> (16). However, G33, which distorts the anticodon stem, leads to decreased leucylation efficiency of CatRNA<sup>Ser</sup>(CAG) (Fig. 1A) (20, 21). Overall, the elements of CatRNA<sup>Ser</sup>(CAG) to be rec-
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FIGURE 1. Structure of CatRNA<sup>Ser</sup>(CAG) and PhLeuRS. A, cloverleaf structure of CatRNA<sup>Ser</sup>(CAG). CaSerRS recognizes the (GC)<sub>3</sub> helix and G73 (shown in cyan). m<sup>1</sup>G37 and A35 are crucial for CaLeuRS recognition (shown in pink). G33 distorts the anticodon arm (shown in pink). B, crystal structure of PhLeuRS-tRNA<sup>Leu</sup> (Protein Data Bank code 1WZ2).

ognized by both CaSerRS and CaLeuRS have been clearly established (16, 20, 21).

CaSerRS, a key aaRS in CUG reassignment, contains only one CUG codon-encoded residue at position 197 in the dimerization interface of the enzyme (22). Replacement of Ser<sup>197</sup> by Leu<sup>197</sup> in CaSerRS causes a local structure rearrangement and induces slightly higher catalytic activity without affecting Ser activation. The N-terminal catalytic domain of CaSerRS interacts with the long variable arm of tRNA<sup>Ser</sup> with its CTD stabilizing the intramonomer interaction (22, 23). Thus, the element in CaSerRS that recognizes tRNA<sup>Ser</sup> has been revealed. Another critical aaRS involved in CUG decoding ambiguity, CaLeuRS, with 1,097 residues, also contains a single CUG codon-encoded residue at position 919, located in its CTD (from Gly<sup>894</sup> to Gln<sup>1097</sup>) (24). The two isoforms of CaLeuRS (CaLeuRS-Ser<sup>919</sup> and -Leu<sup>919</sup>) do not differ in amino acid activation activity; however, CaLeuRS-Leu<sup>919</sup> has higher efficiency in leucylating CatRNA<sup>Leu</sup> (24). CaLeuRS-Ser<sup>919</sup> predominates in vivo; therefore, this isoform is designated as the wild-type (WT) enzyme. For the interaction between CaLeuRS and CatRNA<sup>Ser</sup>, the elements in CaLeuRS that recognize CatRNA<sup>Ser</sup>(CAG) have not been identified.

On the basis of crystal structures of bacterial and archaeal LeuRSs, the CTD folds into a separated domain and is disordered in the absence of tRNA (7, 25). Indeed, deletion analysis showed that the CTDs of Escherichia coli LeuRS (EcLeuRS), Thermus thermophilus LeuRS (TtLeuRS), Pyrococcus horikoshii LeuRS (PhLeuRS), and Natrithba magadii LeuRS (NmLeuRS) are all indispensable for leucylating tRNA<sup>Leu</sup> (6, 7, 10, 26–28). However, deletion of the CTD enhanced the leucylating activity of yeast mitochondrial LeuRS, emphasizing its adaptation in RNA splicing (28). Several studies have explored the concrete recognition elements in the CTD of LeuRSs for cognate tRNA<sup>Leu</sup>. In EcLeuRS, Ala or Asp mutations of several conserved residues had only minimal effects on the aminoacylation activity as did the corresponding double and triple site mutants (28). In another bacterial LeuRS, Mycobacterium tuberculosis LeuRS (MtLeuRS), several residues were shown to maintain the hydrophobic environment to stabilize the conformation of its CTD and to orient the tRNA (27). In addition, the main chains of the C-terminal Pro<sup>962</sup> and Glu<sup>967</sup> of PhLeuRS are crucial for recognizing the A47c and G47d of PhlRNA<sup>Leu</sup> (10). Our previous study showed that, in addition to CaLeuRS, other eukaryotic LeuRSs, including Saccharomyces cerevisiae LeuRS (ScLeuRS) and Homo sapiens LeuRS (HsLeuRS), can also leucylate CatRNA<sup>Ser</sup>; however, both bacterial and archaeal LeuRSs, including EcLeuRS, C. albicans mitochondrial LeuRS (CamtLeuRS), and PhLeuRS, could not (24). In particular, eukaryotic LeuRS and archaeal PhLeuRS only exhibit obvious divergence in their CTD, indicating the potential significance of the CTD of eukaryotic LeuRS in tRNA<sup>Ser</sup>(CAG) recognition.

Above all, the only unexplored question concerning interaction between CaLeuRS and CatRNA<sup>Ser</sup>(CAG) seems to be identification of the elements in CaLeuRS that recognize CatRNA<sup>Ser</sup>. Besides, although the function of the CTD of bacterial and archaeal LeuRSs in recognition of tRNA<sup>Leu</sup> has been studied extensively by structural and biochemical methods, the potential role of the CTD of eukaryotic LeuRSs in the recognition of tRNA<sup>Leu</sup> has never been reported. Here, our results first showed that CTD of CaLeuRS (CaCTD) is indispensable for leucylating both CatRNA<sup>Ser</sup>(CAG) and CatRNA<sup>Leu</sup> (CatRNAs). Additionally, three highly conserved lysine residues within CaCTD were identified as important for leucylating both tRNAs in an additive manner both in vitro and in vivo. In combination, our data identified the specific mechanism concerning recognition of CatRNA<sup>Ser</sup>(CAG) by CaLeuRS and improved our understanding of the recognition of tRNA<sup>Leu</sup> by eukaryotic LeuRS.

Experimental Procedures

Materials—t-Leu, t-norvaline, dithiothreitol (DTT), ATP, CTP, GTP, UTp, 5′-GMP, tetrasodium pyrophosphate, inorganic pyrophosphate, Tris-HCl, MgCl<sub>2</sub>, NaCl, and activated charcoal were purchased from Sigma-Aldrich. [3H]Leu, tetrasodium [32P]pyrophosphate, and [α-32P]ATP were obtained from PerkinElmer Life Sciences. Pfu DNA polymerase, the DNA fragment rapid purification kits, and the plasmid extraction kits were purchased from Tiangen (China). The KOD-plus mutagenesis kits were obtained from Toyobo (Japan). T4 ligase, nuclease S1, and restriction endonucleases were obtained from Thermo Scientific (Pittsburgh, PA). Ni<sup>2+</sup>-nitrilotriacetic acid Superflow was purchased from Qiagen Inc. (Germany). Poly-
ethylenimine cellulose plates were purchased from Merck. Pyrophosphatase was obtained from Roche Applied Science. The dNTP mixture was obtained from Sangon (China). Oligonucleotide primers were synthesized by Biosune Bioscience (Shanghai, China). Competent *E. coli* Top10 and Rosetta™ 2 (DE3) cells were prepared in our laboratory.

**Gene Cloning, Mutagenesis and Expression, and Protein Purification**—The plasmids containing genes encoding CaluRS, ScLeuRS, HsLeuRS, and EcTrmD, pET28a-CaluS (24), pET28a-ScleuS (29), pET22b(+)-HsleuS (30), and pET28a-EcTrmD (24), respectively, were constructed in our laboratory. The plasmid expressing *E. coli* tRNA nucleotidyltransferase was provided by Dr. Gilbert Eriani (CNRS, Strasbourg, France). Five deletion mutants of the C terminus of *E. coli* provided by Dr. Gilbert Eriani (CNRS, Strasbourg, France). Five deletion mutants of the C terminus of *C. albicans* and no tRNALeu(CAG) exists. Therefore, in this study, we used *C. albicans* and no tRNALeu(CAG) was performed as described previously (30).

**In Vitro Activity Assays**—For the first step of the aminoacylation reaction, amino acid activation was measured by an ATP-PP, exchange assay at 30 °C in a reaction mixture containing 60 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 2 mM DTT, 4 mM ATP, 2 mM [³²P]tetrasodium pyrophosphate (22 cpm/mol), 1 mM Leu, and 20 mM CaLeuRS or its variants. Samples of the reaction mixture (9 μl) were removed at 5-min intervals and immediately added to 200 μl of quenching solution (2% activated charcoal, 3.5% HClO₄, and 50 mM tetrasodium pyrophosphate) and mixed by vortexing for 20 s. The solution was filtered through a Whatman GF/C filter followed by washing with 20 ml of Milli-Q water and 10 ml of 95% ethanol. The filter was dried, and [³²P]ATP was measured using a scintillation counter (Beckman Coulter).

Leucylation of CatRNA⁴⁴⁵uma was performed 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 μM [¹⁴C]Leu, 10 μM CatRNA⁴⁴⁵uma, and 20 mM CaLeuRS or its variants. Samples of the reaction mixture (9 μl) were removed onto a Whatman filter at 2-min intervals. After washing with 5% trichloroacetic acid (three times) and 95% ethanol (twice), the filters containing precipitated [¹⁴C]leucyl-tRNALeu were dried, and radioactivity was quantified using a scintillation counter. Leucylation of CatRNA⁴⁴⁵uma by ScLeuRS and its variant was performed under the same conditions, and that of HsRNA⁴⁴⁵uma (CAG) by HsLeuRS and its variant was performed as described previously (33). The kinetics of CatRNA⁴⁴⁵uma aminoacylation by *CaLeuRS* and its variants were determined in the presence of varying concentrations of RNA⁴⁴⁵uma (0.5–32 μM).

Misleucylation of [³²P]CatRNA⁴⁴⁵uma 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, 40 mM Leu, 5 μM “cold” CatRNA⁴⁴⁵uma, 1 μM [³²P]CatRNA⁴⁴⁵uma, and 1 mM CaLeuRS or its variants. Aliquots (9 μl) were removed at specific time points for ethanol precipitation with sodium acetate (pH 5.2) at −20 °C overnight. The precipitated samples were centrifuged at 12,000 × g at 4 °C for 30 min, dried at room temperature for 30 min, and digested with 6 μl of nuclease S1 (25 units) for 1 h at 37 °C. After treatment with nuclease S1, leucyl-[³²P]tRNA should produce leucyl-[³²P]AMP, and free [³²P]tRNA should produce [³²P]AMP. Quenched aliquots (2 μl) of the digestion mixture were spotted on a thin polyethylenimine cellulose plate and separated by the thin layer chromatography (TLC) in 0.1 M ammonium acetate and 5% acetic acid. Known amounts of [α-³²P]ATP were diluted stepwise and spotted onto the plate for quantification. After visualization by phosphorimaging, the data were analyzed using Multi-Gauge Version 3.0 software (Fujifilm). Misaminoacylation of [³²P]CatRNA⁴⁴⁵uma by ScLeuRS, HsLeuRS, and their variants was performed under the same
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**FIGURE 2. Construction of ScΔleuS and the yeast expression plasmid.** A, schematic representation of the construction of ScΔleuS. The rescue plasmid p416GPD-ScleuS was introduced into the diploid yeast BY4743-ScleuS<sup>−/−</sup> strain. After sporulation and selection, ScΔleuS strain was obtained. B, schematic representation of the reconstruction of the yeast expression plasmid. The TEF promoter was replaced by the yeast gene promoter to acquire p425ScPr vector, the gene encoding CaLeuRS was inserted, and mutations were introduced. The constructs were then transformed into ScΔleuS. Chr<sub>H</sub>, chromosome.

conditions except that misaminoacylation by HsLeuRS and its variant was performed at 37 °C.

**Gel Mobility Shift Assay—**100 nm m<sup>1</sup>G37 CatRNA<sup>Ser</sup>(CAG) or CatRNA<sup>Leu</sup>(UAA) was incubated with a range of CaLeuRS (0–12 μM) or its mutants in 20 μl of buffer (20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 15% glycerol) on ice for 20 min. After incubation, 1 μl of loading buffer (0.25% bromphenol blue and 30% glycerol) was added to the sample, and then the sample was loaded into a 6% polyacrylamide native gel. The electrophoresis was carried out at 4 °C at a constant voltage of 100 V for 80 min using 50 mM Tris-glycine buffer. The gel was then stained with ethidium bromide. The RNA bands were quantified by using a Fujifilm imaging analyzer.

**Yeast Complementation Assay—**The yeast leu<sup>−</sup> gene was amplified by PCR using pET28a-ScleuS as a template, digested with BamHI and EcoRI, and then ligated into the p416GPD plasmid (predigested with BamHI and EcoRI) to generate p416GPD-ScleuS (leu<sup>−</sup>, ura<sup>−</sup>) (Fig. 2). The diploid yeast strain BY4743-ScleuS<sup>−/−</sup> (Dharmacon), which contains only one chromosomal copy of leu<sup>−</sup> with the other replaced by a G418<sup>−</sup> resistance gene, was transformed with the rescue plasmid p416GPD-ScleuS. The transformants were cultured on synthetic dropout medium without uracil (SD/Ura<sup>−</sup>) plate and failed to grow on SD/Ura<sup>−</sup>/5-fluoroorotic acid plate to induce the loss of the rescue plasmid (p416GPD-ScleuS). Complementation was observed by comparing the growth rates of ScΔleuS expressing CaLeuRS or its different variants.

**Preparation of Yeast Lysates and Western Blotting Assay—**Yeast transformants were grown in 20 ml of liquid SD/Leu<sup>−</sup> medium. The yeast were then harvested; resuspended in ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM PMSF, and 1 mM DTT; mixed with a few glass beads; vortexed rigorously for 10 s (three times at 1-min intervals); and then centrifuged at 10,000 g for 10 min. The supernatant was separated by SDS–PAGE (10% gel) and transferred to a PVDF membrane. The membrane was blocked with PBST (phosphate-buffered saline containing 0.05% Tween 20) with 5% nonfat dried milk for 3 h at room temperature. Membranes were hybridized overnight with anti-His<sub>6</sub> antibody (Abmart; M20001) or anti-α-tubulin antibody (Cell Signaling Technology, 3873S) separately at 4 °C. After incubating with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, immunoreactivity was detected using LAS4000 (Fujifilm) and the SuperSignal West Pico Trial kit (Thermo Fisher Scientific). The results were quantified using

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The CTD is crucial for recognizing both CatRNA<sub>Ser</sub>(CAG) and CatRNA<sub>Leu</sub>—Our previous study showed that only eukaryotic LeuRSs (e.g. CaLeuRS, ScLeuRS, and HsLeuRS) could charge CatRNA<sub>Ser</sub>(CAG); however, archaeal LeuRS (e.g. PhLeuRS) and bacterial/mitochondrial LeuRS (e.g. EcLeuRS and CmLeuRS) could not (24). The archaeal and eukaryotic LeuRSs appear to be derived from the same ancestor with the most divergence in the CTD, implying that this region may be critical for recognizing CatRNA<sub>Ser</sub>. To address this question, we truncated the CTD of CaLeuRS (from Gly<sup>994</sup> to Glu<sup>1097</sup>), based on the crystal structure of PhLeuRS (Protein Data Bank code 1WKB), obtaining a CTD-truncated mutant (CaLeuRS-ΔC) (Fig. 1B) (6). CaLeuRS-ΔC could be purified to high homogeneity and retained intact leucyl-adenylate synthesis activity (data not shown), suggesting that deletion of CTD had no direct effect on the secondary structure of CaLeuRS and the catalytic active site for amino acid activation. However, it totally lacked the leucylation activity for CatRNA<sub>Ser</sub>(CAG), indicating the indispensable role of the CTD in Leu-CatRNA<sub>Ser</sub> formation (Fig. 3, A and B). Similar results were obtained in assays of the leucylation of CatRNA<sub>Leu</sub> by CaLeuRS-ΔC (Fig. 3C), supporting the hypothesis that the CTD was critical for aminoacylation of tRNA. To further determine the importance of CTD in leucylating CatRNA<sub>Ser</sub> and CatRNA<sub>Leu</sub>, we substituted CaCTD for PhCTD in PhLeuRS to obtain a mosaic enzyme, PhLeuRS-CaCTD. Compared with PhLeuRS, the mosaic enzyme gained the ability to leucylate CatRNA<sub>Ser</sub> and CatRNA<sub>Leu</sub>, further indicating the importance of CaCTD in capturing CatRNAs for aminoacylation (Fig. 3, D, E, and F).

PhLeuRS shows high homology with CaLeuRS (Fig. 4A), and simultaneous deletion of its C-terminal Ile<sup>966</sup> and Glu<sup>967</sup> completely abolishes the leucylation activity (10). Primary sequence alignment showed that Ile<sup>1093</sup> of CaLeuRS is highly conserved among yeast and archaeal LeuRSs and corresponds to Ile<sup>966</sup> of PhLeuRS (Fig. 4A). To explore whether CaLeuRS interacts with tRNAs in a similar manner to PhLeuRS, we performed progressive deletions from the C terminus of CaLeuRS, obtaining five mutants, CaLeuRS-ΔC1 to -ΔC5, all of which retained intact amino acid activation activity (data not shown). Further data showed that CaLeuRS-ΔC1 and -ΔC2 showed similar leucylation activity to the WT in the presence of CatRNA<sub>Ser</sub>; CaLeuRS-ΔC3 and -ΔC4 gradually decreased their leucylation activity, and CaLeuRS-ΔC5 abolished the leucylation activity (Figs. 4B). Similar results were obtained in assays of the leucylation of CatRNA<sub>Leu</sub> by the five deletion mutants (Fig. 4C).

However, the substitution of Ala for the five terminal residues (Glu<sup>997</sup>, Val<sup>1096</sup>, Asn<sup>1095</sup>, Lys<sup>1094</sup>, and Ile<sup>1093</sup>) of CaLeuRS showed similar leucylation activities to the native enzyme (data not shown), suggesting that either the interaction mode between CatRNA and residues at the C terminus of CaLeuRS is similar to that of the PhLeuRS-tRNA<sub>Leu</sub> system (10) or the C terminus is essential for maintaining proper conformation (the latter issue was subsequently addressed using the ScΔH<sub>leu</sub>S strain). Overall, the results show that CaCTD is critical for leucylating both CatRNA<sub>Ser</sub> and CatRNA<sub>Leu</sub>; however, the specific elements within CaCTD for charging tRNAs remain to be explored.

Identification of Important Residues within CaCTD—The above data showed that the C-terminal five residues, especially Ile<sup>1093</sup>, are important for the leucylation activity of CaLeuRS for both CatRNA<sub>Ser</sub>(CAG) and CatRNA<sub>Leu</sub>. Despite PhLeuRS having Ile<sup>966</sup> corresponding to Ile<sup>1093</sup>, it still failed to recognize both CatRNAs, indicating that the terminal five residues of CaLeuRS may act to affect the leucylation activity in a manner different from that of PhLeuRS. Therefore, CaCTD must harbor other specific elements for charging CatRNAs. Phylogenetic analysis of 13 LeuRSs from various yeast species revealed a high degree of conservation in their CTDs. To identify key residues within CaCTD that may interact with tRNA directly, 10 conserved basic residues (Arg<sup>923</sup>, Lys<sup>997</sup>, Lys<sup>1007</sup>, Arg<sup>1009</sup>, Arg<sup>1021</sup>, and 938KKKKGGK<sup>943</sup>) were mutated to Ala, and the leucylation activities of these mutants were assayed. Additionally, we substituted the conserved Pro residues (Pro<sup>963</sup>, Pro<sup>1086</sup>, and Pro<sup>1089</sup>), Tyr<sup>921</sup>, and Gln<sup>966</sup>, which were either polar or potentially structure-crucial, with Ala (Fig. 5). Among the 15 Ala mutants, only CaLeuRS-R923A, -K938A, -K941A, -K1007A, and -P1086A showed marked decreases in leucylation activity.
FIGURE 4. The leucylation activity of the C terminus-deleted mutants. A, sequence alignment of the C terminus of different LeuRSs. Mj, Methanocaldococcus jannaschii; Si1, Sulfolobus islandicus LeuRS1 (22); Ma, Methanosarcina acetivorans; Nm1, Natrrialba magadii LeuRS1 (22); Kt, Kluyveromycyes thermofermentans; Ec, Eremothecium cymbalariae; Dh, Debaryomyces Hansenii; Ct, Candida tropicalis; L, Lodderomyces elongisporus; Ps, Pichia stipitis. B, leucylation of 1 mM CatRNA\textsuperscript{Ser} by 10 nM CaLeuRS (●), -1 mM CatRNA\textsuperscript{Ser} (●), -1 mM CatRNA\textsuperscript{Ser} (●), and -1 mM CatRNA\textsuperscript{Ser} (●). C, leucylation of 10 μM CatRNA\textsuperscript{Ser} by 20 nM CaLeuRS (●), -1 mM CatRNA\textsuperscript{Ser} (●), -1 mM CatRNA\textsuperscript{Ser} (●), and control (▲). Error bars represent S.D.

compared with the WT (Table 1). To further investigate their mode of interaction with tRNA, we replaced the five residues with Asp, obtaining CaLeuRS-R923D, -K938D, -K941D, -K1007D, and -P1086D. The residue Arg\textsuperscript{923} may contribute to maintaining stability/conformation of the enzyme because CaLeuRS-R923D mainly produced inclusion bodies during expression of its gene in E. coli. All the other four mutants retained intact Leu activation activity comparable with the WT (data not shown), indicating that these mutations had no effect on the catalytic active site. In the subsequent aminoacylation reaction, we assayed and compared the kinetic constants of the WT and the mutants (Table 1). CaLeuRS-K938D, -K941D, -K1007D, and -P1086D showed a decreased K\textsubscript{m} values (5.43, 10.95, 12.36, and 12.08 μM, respectively) compared with the WT (2.01 μM), showing that the affinity between the mutants and tRNA was disrupted. In addition, the k_{cat} values of the four mutants decreased to different extents, accounting for 33, 63, 18, and 25% of that of the WT, respectively. Because of the increased K\textsubscript{m} and decreased k_{cat} values, their catalytic efficiencies (k_{cat}/K\textsubscript{m}) were approximately 12, 12, 3, and 4% of that of the WT, respectively. Substituting of these three lysine residues with acidic Asp affects the tRNA\textsuperscript{Leu} leucylating activity, indicating that the positive charge of the three lysines may be important during the leucylation process. This hypothesis was confirmed by the data that the Gln mutants (CaLeuRS-K938Q, -K941Q, and -K1007Q) also showed decreased catalytic efficiency (about 53, 29, and 38% of that of the WT, respectively) although not as obvious as that of their corresponding Asp mutants (Table 1). And the K\textsubscript{m} values of all three Gln mutants increased (3.33, 5.54, and 4.01 μM, respectively), indicating a reduction of the affinity between the mutants and tRNA (Table 1).

To explore whether the residues important for Leu-tRNA\textsuperscript{Leu} synthesis also affect Leu-tRNA\textsuperscript{Ser} formation, we investigated leucylation of CatRNA\textsuperscript{Ser} by CaLeuRS-K938D, -K941D, -K1007D, and -P1086D. All the mutants showed obvious decreased k_{obs} values (0.95 × 10^{-4}, 0.14 × 10^{-3}, 0.25 × 10^{-4}, and 0.28 × 10^{-4} s\textsuperscript{-1}) in CatRNA\textsuperscript{Ser} leucylation activity, representing about 50, 70, 10, and 10% of that of the WT (0.20 × 10^{-3} s\textsuperscript{-1}), respectively (Fig. 6A). However, their corresponding Ala mutants, CaLeuRS-K938A, -K941A, and -P1086A, showed a subtle decrease in the CatRNA\textsuperscript{Ser}-charging activity (Fig. 6B), indicating that introduction of negatively charged residues at Lys\textsuperscript{938}, Lys\textsuperscript{941}, and Pro\textsuperscript{1086} hindered the formation of Leu-tRNA\textsuperscript{Ser}. However, CaLeuRS-K1007A still lost almost half of the CatRNA\textsuperscript{Ser}-charging activity (k_{obs} = 0.14 × 10^{-3} s\textsuperscript{-1}) compared with the WT (Fig. 6B). These results indicated that the basic residues (Lys\textsuperscript{938}, Lys\textsuperscript{941}, and Lys\textsuperscript{1007}) contribute significantly to the leucylation of both CatRNA\textsuperscript{Leu} and CatRNA\textsuperscript{Ser}, affecting the affinity of enzyme for the tRNA and/or the efficiency of transition in the aminoacylation reaction, which further explains their high conservation within the CTD of yeast LeuRSs.

Lys\textsuperscript{938}, Lys\textsuperscript{941}, and Lys\textsuperscript{1007} Made an Additive Contribution to Leucylation—We identified three basic residues, Lys\textsuperscript{938}, Lys\textsuperscript{941}, and Lys\textsuperscript{1007}, that were implicated as tRNA-interacting elements within the CTD based on the positive charge of their side chains. However, none of their Asp mutants completely lost the
leucylation activity for CatRNA\textsuperscript{Ser} and CatRNA\textsuperscript{Leu}. To check whether these residues interact with tRNA in an additive or cooperative manner, we combined the K938D, K941D, and K1007D in the form of either double site mutants (CaLeuRS\textsuperscript{-K938D/K941D}, -K938D/K1007D, and -K941D/K1007D) or

![Diagram](https://example.com/diagram.png)

**FIGURE 5.** Sequence alignment of CTDs of LeuRSs from different yeast species. The residues studied by alanine-scanning mutagenesis are indicated by a black line, and the position is annotated. The residues of CaLeuRS included in further studies are indicated by a blue line, and the position is annotated.

**FIGURE 6.** Recognition of CatRNASer by CaLeuRS and its mutants. A, leucylation of $\frac{1}{2}$H9262\textsuperscript{M} [\textsuperscript{32}P]CatRNASer by 1 mM CaLeuRS (●), -K938D (○), -K941D (△), -K1007D (□), and -P1086D (▼). B, leucylation of 1 mM [\textsuperscript{32}P]CatRNASer by 1 mM CaLeuRS (●), K938D (○), K941D (△), and K1007D (□). Error bars represent S.D.

**TABLE 1**

| Enzyme | $K_m$ (\textmu M) | $k_{cat}$ (s\textsuperscript{-1}) | $k_{cat}/K_m$ (s\textsuperscript{-1} \textmu M\textsuperscript{-1}) | Relative $k_{cat}/K_m$ |
|--------|-----------------|-----------------|----------------|------------------|
| CaLeuRS | 2.01 ± 0.03 | 0.51 ± 0.03 | 253.7 | 100 |
| Lys\textsuperscript{938} | 5.43 ± 0.95 | 0.17 ± 0.03 | 31.3 | 12.3 |
| Ala | 2.70 ± 0.12 | 0.29 ± 0.06 | 106.7 | 42.0 |
| Gln | 3.33 ± 0.02 | 0.45 ± 0.05 | 155.1 | 53.3 |
| Lys\textsuperscript{941} | 10.95 ± 0.21 | 0.32 ± 0.04 | 29.2 | 11.5 |
| Ala | 2.89 ± 0.04 | 0.24 ± 0.01 | 83.2 | 32.8 |
| Gln | 5.54 ± 0.33 | 0.41 ± 0.22 | 74.0 | 29.2 |
| Lys\textsuperscript{1007} | 12.36 ± 0.09 | 0.09 ± 0.01 | 7.3 | 2.9 |
| Ala | 1.18 ± 0.01 | 0.17 ± 0.02 | 144.3 | 56.9 |
| Gln | 4.01 ± 0.77 | 0.39 ± 0.01 | 97.3 | 38.3 |
| Pro\textsuperscript{1086} | 12.08 ± 0.83 | 0.13 ± 0.01 | 10.8 | 4.4 |
| Ala | 1.42 ± 0.03 | 0.34 ± 0.04 | 239.4 | 94.3 |

\(a\) Data represent the average of at least two independent repeats with standard deviations indicated.
Recognition of CatRNA\textsuperscript{Ser} and CatRNA\textsuperscript{Leu} by CaLeuRS

The Counterparts of Lys\textsuperscript{1007} in Other Eukaryotic LeuRSs Are Also Important for Leucylating tRNA—The K1007D mutation showed the most severe impact on the leucylation activity. Primary sequence alignment revealed that Lys\textsuperscript{1007} is highly conserved in eukaryotic LeuRSs, ranging from yeast species such as \textit{S. cerevisiae} and \textit{Candida tropicalis} to higher organisms, such as \textit{H. sapiens}, \textit{Mus musculus}, and \textit{Xenopus laevis}. The counterparts of CaLeuRS-Lys\textsuperscript{1007} are identified as Lys\textsuperscript{1002} in ScLeuRS and Lys\textsuperscript{997} in \textit{HsLeuRS}, respectively (Fig. 9A). To assess any functional conservation of this site, we replaced them with Asp in ScLeuRS and \textit{HsLeuRS} to obtain ScLeuRS-K1002D and \textit{HsLeuRS}-K997D, respectively. In the presence of CatRNA\textsuperscript{Ser}, the $k_{\text{obs}}$ value of ScLeuRS-K1002D (0.35 \times 10^{-3} \text{ s}^{-1}) was about 40% of that of ScLeuRS (0.92 \times 10^{-3} \text{ s}^{-1}), and that of \textit{HsLeuRS}-K997D (0.11 \times 10^{-2} \text{ s}^{-1}) was about 70% of that of \textit{HsLeuRS} (0.16 \times 10^{-2} \text{ s}^{-1}) (Fig. 9B). Further assay of their tRNA\textsuperscript{Leu}-charging activity showed similar results. The $k_{\text{obs}}$ value of ScLeuRS-K1002D (0.03 \text{ s}^{-1}) was approximately 10% of that of ScLeuRS (0.26 \text{ s}^{-1}) (Fig. 9C), and that of \textit{HsLeuRS}-K997D (0.28 \text{ s}^{-1}) was about 40% of that of \textit{HsLeuRS} (0.67 \text{ s}^{-1}) (Fig. 9D).

These data showed that the counterparts of CaLeuRS-Lys\textsuperscript{1007} in ScLeuRS and \textit{HsLeuRS} are also important for the recognition of both CatRNA\textsuperscript{Ser}(CAG) and their cognate tRNA\textsuperscript{Leu}s by CTD, emphasizing the importance of this highly conserved basic residue for the recognition function of CTD.

Confirmation of tRNA Binding Capacity Using Sc\textit{ScleuS}—Previous studies showed that CaLeuRS was able to rescue an \textit{ScleuS} strain because CaLeuRS showed high sequence homology with ScLeuRS (64.9%) and could aminoacylate SctRNA\textsuperscript{Ser} efficiently (34, 35). Hence, the \textit{ScleuS} strain is a good tool to test the \textit{in vivo} effect of residues identified in \textit{in vitro} screens.

CaLeuRS-Leu\textsuperscript{919} and CaLeuRS-Ser\textsuperscript{919} are two isoforms differing at position 919, and CaLeuRS-Leu\textsuperscript{919} has a higher catalytic efficiency for CatRNA\textsuperscript{Leu} in the aminoacylation assay (24). Here, both isoforms supported \textit{ScleuS} strain growth to a similar level (Fig. 10A). However, by comparing steady-state protein levels of CaLeuRS-Leu\textsuperscript{919} and -Ser\textsuperscript{919} from yeast transformants containing their genes, we found that the amount of CaLeuRS-Leu\textsuperscript{919} was only 32% of that of CaLeuRS-Ser\textsuperscript{919}, indicating that the insertion of Ser or Leu at position 919 may affect gene expression or protein stability (Fig. 10, C and D).

The growth of \textit{ScleuS} containing CaLeuRS-\textit{ΔC1}, -\textit{ΔC2}, -\textit{ΔC3}, and -\textit{ΔC4} was similar to that containing CaLeuRS-Ser\textsuperscript{919} (Fig. 10A). However, the growth of \textit{ScleuS} containing CaLeuRS-\textit{ΔC5} was severely disrupted (Fig. 10A). Western blotting showed that the genes encoding all the truncated proteins were expressed to a similar level in yeast (Fig. 10, C and D). Thus, the inability of CaLeuRS-\textit{ΔC5} to support \textit{ScleuS} viability was probably caused by the total loss of aminoacylation capacity, not by non- or decreased expression. Compared with CaLeuRS-Ser\textsuperscript{919}, the five deletion mutants were expressed at lower levels, suggesting that the terminal five residues may act to stabilize the conformation of CaCTD and affect protein expression.

\textit{ScleuS} strains transformed of CaLeuRS-K938D, -K941D, -K1007D, -P1086D, and -K938D/K941D had growth rates similar to that transformed with CaLeuRS-Ser\textsuperscript{919} (Fig. 10B), suggesting that the mutants exogenously expressed by a high copy plasmid were sufficient in supporting yeast growth despite their low aminoacylation activity (see “Discussion”). However, the growth of \textit{ScleuS} transformed with CaLeuRS-K938D/K1007D and -K941D/K1007D showed an apparent delay to different degrees compared with that of CaLeuRS-Ser\textsuperscript{919}, espe-
**FIGURE 8.** Gel mobility shift assay of CaLeuRS and its mutants with tRNAs. A, 100 nm CatRNA\textsuperscript{Ser} was incubated with increasing concentrations (0–12 \( \mu \text{M} \)) of CaLeuRS, -K938D/K941D, -K938D/K1007D, -K941D/K1007D, and -K938D/K941D/K1007D. B, apparent \( K_d \) of CaLeuRS was estimated using a Scatchard plot after quantification of the free and retarded tRNA\textsuperscript{Ser} and calculation of the amount of free and bound enzyme. C, 100 nm CatRNA\textsuperscript{Leu} was incubated with increasing concentrations (0–12 \( \mu \text{M} \)) of CaLeuRS, -K938D/K941D, -K938D/K1007D, -K941D/K1007D, and -K938D/K941D/K1007D. D, Scatchard analysis of the binding of tRNA\textsuperscript{Leu} by CaLeuRS.

**FIGURE 9.** Role of counterparts of CaLeuRS-Lys1007D in recognition of CatRNA\textsuperscript{Ser} (CAG) and CatRNA\textsuperscript{Leu}. A, primary sequence alignment of CTDs from CaLeuRS, ScLeuRS, C. tropicalis (Ct) LeuRS, HsLeuRS, M. musculus (Mm) LeuRS, and X. laevis (Xl) LeuRS. Counterpart residues of CaLeuRS-Lys1007 are highlighted in black (Lys1002 and Lys997, respectively, in ScLeuRS and HsLeuRS). B, leucylation of 1 \( \mu \text{M} \) CatRNA\textsuperscript{Ser} by 1 nm ScLeuRS (●), ScLeuRS-K1002D (○), HsLeuRS (■), and HsLeuRS-K997D (□). C, leucylation of 10 \( \mu \text{M} \) CatRNA\textsuperscript{Leu} by 20 nm ScLeuRS (●), ScLeuRS-K1002D (○), and control (▲). D, leucylation of 10 \( \mu \text{M} \) HstRNA\textsuperscript{Leu} by 20 nm HsLeuRS (●), HsLeuRS-K997D (□), and control (▲). Error bars represent S.D.
cially CaLeuRS-K938D/K1007D (Fig. 10B). The ScΔleuS containing CaLeuRS-K938D/K941D/K1007D could not grow on SD/Leu^-/-5-fluoroorotic acid plates, suggesting that the triple site mutant had totally lost tRNA binding ability even when present in sufficient amount. All the results indicated the significance of Lys^938, Lys^941, and Lys^1007 in binding tRNA in vivo, which is consistent with the results obtained in vitro.

**Discussion**

CTDs from Three Kingdoms Are Essential for Recognizing tRNA^{Leu}_--aaRSs aminoacylate their cognate tRNAs to produce materials for protein biosynthesis. Structural studies have shown that they evolved N-/C-domains appended to the catalytic center to capture tRNAs for efficient and accurate aminoacylation. For example, the N-terminal domain, containing...
lysin-rich motifs, significantly enhances tRNA binding in yeast asparyl-tRNA synthetase and mammalian lysyl-tRNA synthetase (36, 37). The CTDs of Staphylococcus aureus isoleucyl-tRNA synthetase, Thermus thermophilus valyl-tRNA synthetase, and E. coli histidyl-tRNA synthetase are also responsible for recognizing tRNA (38–40).

Generally, LeuRS are classified into two types (6–8). In fact, based on their divergent CTDs, they are further classified into three types (bacterial, archaean, and eukaryotic LeuRSs) (6, 7). CTDs of both bacterial and archaean LeuRSs are responsible for recognizing tRNA_{Leu} as negligible leucyl-tRNA_{Leu} synthesis was observed in the CTD-deleted mutants of EcLeuRS, TfLeuRS, PhLeuRS, and NmLeuRS (7, 10, 26, 28). However, their concrete recognition mechanisms are divergent. A yeast three-hybrid system and band shift assay showed that the CTD of the β-subunit of Aquifex aeolicus LeuRS was responsible for binding tRNA both in vivo and in vitro; however, the recognition elements remained unclear (41). Although several conserved, positively charged residues were identified within the CTD of EcLeuRS, none of them provided site-specific interaction with tRNA_{Leu} (28). Asp scanning of the MtLeuRS CTD identified several residues (Val914, Leu939, Gln913, and Leu964) that contributed to maintain the hydrophobic environment to orient tRNA_{Leu} in the correct aminoacylation conformation (27). By contrast, based on the crystal structure of PhLeuRS-tRNA_{Leu} complex (Protein Data Bank code 1WZ2), some residues (Asp854, Ile849, Pro962, Ile964, Ile966, and Glu967) within the CTD of PhLeuRS formed base-specific interactions with tRNA_{Leu} mainly through van der Waals interactions or hydrogen bonds (10).

Our present study, for the first time, used CaLeuRS as a eukaryotic LeuRS model, revealing that the CTD from a eukaryotic LeuRS is also indispensable for recognizing tRNA_{Leu}. Notably, three basic residues (Lys938, Lys941, and Lys1007) within CaCTD were important during the leucylation process; their positively charged side chains may interact with tRNA, consistent with the Arg921 of bacterial MtLeuRS and Lys961 of archaean PhLeuRS (10, 27). In addition to the three key lysine residues, the C-terminal five residues of CaLeuRS may maintain the proper conformation of the CTD during leucylation but not interact with tRNA_{Leu} directly, which contrasts with archaean PhLeuRS (10). In conclusion, although CTDs from the three kingdoms are all essential for recognizing tRNA_{Leu}, the recognition mechanisms have evolved to be distinct, allowing more specific and efficient discrimination of their different cognate tRNAs.

CaLeuRS Recognizes CatRNA{Ser}(CAG) and CatRNA{Leu} Using the Same Elements—CaLeuRS is unique because it recognizes two types of tRNAs (cognate CatRNA{Leu} and non-cognate CatRNA{Ser}(CAG)) (24). In S. cerevisiae, substituting the anticodon arm of SctRNA{Ser} with that of SctRNA{Leu} endowed it with leucine charging capacity, emphasizing that the anticodon arm/loop of SctRNA{Leu} plays a dominant role in recognition by ScLeuRS (19). CatRNA{Ser}(CAG) is a naturally chimeric tRNA with the main body of tRNA{Ser} and the anticodon of tRNA{Leu} (13, 14). Mutation analysis of tRNA determinants showed that the anticodon loops/arms of both CatRNA{Ser}(CAG) and CatRNA{Leu} are crucial for recognition by CaLeuRS (13, 19).

In our study, Lys938, Lys941, and Lys1007 within CaCTD contribute to recognizing both CatRNA{Leu} and CatRNA{Ser}(CAG), suggesting a similar interaction mode between the CaLeuRS and CatRNA{Leu}/CatRNA{Ser} in the aminoacylation state. In addition, the affinities between the CatLeuRS and CatRNA{Leu}/CatRNA{Ser} were similar in vitro. All of these observations were consistent with the mutation analysis of the tRNA determinants that indicated the crucial importance of the anticodon loop/arm of CatRNA{Ser} and CatRNA{Leu} in the recognition by LeuRS (13, 19). In addition, both CatRNA{Ser} and CatRNA{Leu} belong to class II tRNAs with a long variable arm, sharing similar tertiary structures. Therefore, the hypothesis that CaLeuRS recognizes the two tRNAs using the same elements is reasonable. Although the recognition elements within CaCTD for CatRNA{Ser}(CAG) and CatRNA{Leu} are similar, CatRNA{Ser}(CAG) may still possess some antideterminants for CaLeuRS, leading to tRNA{Ser} being a poorer charging tRNA compared with tRNA_{Leu}, contributing to the low abundance of Leu in CUG sites.

Lys938, Lys941, and Lys1007 Affect the Binding Capacity of CaLeuRS for Both CatRNA{Ser}(CAG) and CatRNA{Leu} with Additive Effect—Although the key recognition elements of bacterial or archaean CTD have been elucidated, neither single nor double/triple site mutants lost the aminoacylation activity completely (28). In this study, we identified three lysine residues (Lys938, Lys941, and Lys1007) in CaLeuRS and first verified that their double/triple site mutants had more severe disruption of even destruction of the leucylation activity compared with the single site mutants, differing from that of EcLeuRS (28).

Furthermore, the gel mobility shift assays showed that CaLeuRS-K938D/K941D -K938D/K1007D, -K941D/K1007D, and -K938D/K941D/K1007D could not form obvious enzyme-tRNA_{Leu} shifted bands with incalculable $K_d$ values, indicating the importance of the three lysine residues in binding tRNA. However, in vivo complementation assays showed that the three double site mutants still supported yeast growth to some extent. This was probably caused by the double site mutants also binding yeast tRNA_{Leu} in vivo because the $K_d$ value may not be accurately captured/reflected by the gel mobility shift assay, which was measured under limited enzyme concentration. Besides, it is possible that expression of double site mutants by the high copy p425ScPr plasmid would produce excess protein to complement the impaired tRNA binding capacity of the mutants. Despite the fact that the double site mutants showed negligible aminoacylation activity and $K_d$ in vitro, they could still complement yeast growth to varying degrees. However, both the in vitro and in vivo data supported the view that the combination of the triple site mutant (CaLeuRS-K938D/K941D/K1007D) had completely lost the aminoacylation activity, suggesting an additive effect of these lysine residues on leucylating CatRNA{Leu}.

The Leucylating Capacity of the Mutants Was Further Confirmed Using ScΔLeuS—Our research focused on exploring the interaction between CaLeuRS and CatRNA{Ser}/CatRNA{Leu}. The in vitro data showed that Lys938, Lys941, and Lys1007 are important for leucylating both tRNAs. As CaLeuRS can com-
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plement ScLeuRS in vivo (34). We used an ScLeuS strain in which the gene encoding LeuRS was deleted to investigate the catalytic function of the various mutants mentioned in vitro.

In C. albicans, CaLeuRS has two isoforms, CaLeuRS-Ser^{919} and -Leu^{919}, because the CUG codon at position 919 could be decoded by CattRNA^{Ser}(CAG) as Ser or Leu. CaLeuRS-Ser^{919} is the main form; therefore, we used it as the wild type in the in vitro aminoacylation assay. However, in the complementation assay, both isoforms were introduced into the ScLeuS to compare their expression levels and ability to rescue the yeast. In our study, although there was a 68% decrease in the relative expression level of CaLeuRS-Leu^{919} compared with that of CaLeuRS-Ser^{919}, ScLeuS transformed with the gene encoding CaLeuRS-Leu^{919} grew slightly better than that transformed with CaLeuRS-Ser^{919}, which reflects the higher catalytic efficiency of CaLeuRS-Leu^{919} compared with CaLeuRS-Ser^{919} (24). This strict regulation of the relative amounts of CaLeuRS-Ser^{919}/CaLeuRS-Leu^{919} might balance the CUG decoding with CaSerRS-Ser^{919}/CaSerRS-Leu^{919} as well as relieve the potential effect on phenotypic diversity caused by excessive Leu misincorporation (16). Moreover, mutants of CaLeuRS with low aminoacylation activity (such as CaLeuRS-K938D, -K941D, -K1007D, -P1086D, and -K938D/K941D) complemented the loss of ScLeuS as well as the native enzyme, suggesting that mutants exogenously expressed from high copy plasmids were sufficient to support yeast growth although with minimal activity. A similar phenotype was also observed in complementation with ScLeuS transformed with the gene encoding ScLeuS^{919} grown in vitro (42).

ScLeuS containing CaLeuRS-K938D/K1007D and -K941D/K1007D showed a growth-retarded phenotype, and ScLeuS containing CaLeuRS-K938D/K941D/K1007D showed a lethal phenotype, which further emphasized the significance and additive effect of Lys^{938}, Lys^{941}, and Lys^{1007} in leucylating tRNA in vivo. In addition, CaLeuRS-K938D/K941D/K1007D and -ΔC5 could not rescue ScLeuS, which suggests that both the three lysine residues and the terminus five residues are indispensable for leucylating tRNA through either direct interaction or maintenance of the proper conformation. Therefore, we believe that the CTD acts to recognize tRNA through complex interactions.

Concluding Remarks—C. albicans, an opportunistic pathogen, has evolved a chimeric CattRNA^{Ser}(CAG) that leads to protein translation ambiguity. The resultant ambiguity at the CTG codon is crucial in the morphological switch and virulence of this pathogen. The elements in CaLeuRS that mediate this crucial genetic code ambiguity were undetermined. In our study, we have provided the first evidence and clarified the interaction mechanism of the indispensable function of the CTD of CaLeuRS in recognizing both CattRNA^{Ser} and CattRNA^{Leu}. Our results deepen our understanding of the mechanism that mediates CTG codon ambiguity in C. albicans and improve our knowledge concerning tRNA^{Leu} recognition by eukaryotic LeuRSs.

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