A Unique Insertion in the CP1 Domain of *Giardia lamblia* Leucyl-tRNA Synthetase[†]

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ABSTRACT: Leucyl-tRNA synthetase (LeuRS) catalyzes the esterification of the $tRNA^{Leu}$ isoacceptor with leucine. It contains a large insertion domain, connective peptide 1 (CP1), for amino acid editing. Here, we cloned the gene encoding LeuRS from *Giardia lamblia* (*Gl*LeuRS), one of the most ancient eukaryotes. *Gl*LeuRS was purified from an *Escherichia coli* overproduction strain, and its properties were investigated. The isolated CP1 domain of *Gl*LeuRS (*Gl*LeuRS-CP1) was an active protein for editing mischarged *G. lamblia* $tRNA^{Leu}$(AAG) (*Gl*$tRNA^{Leu}$). Insertion of 49 amino acid residues within the CP1 domain (the so-called 49-amino acid motif) was important for the optimal aminoacylation activity of *Gl*LeuRS and was crucial for the editing capacity of *Gl*LeuRS-CP1. Additionally, the motif can confer editing activity on the editing-defective isolated CP1 domain from *E. coli* LeuRS (*Ec*LeuRS-CP1). We also found that *Gl*LeuRS could not rescue a *Saccharomyces cerevisiae leuS* null strain, suggesting different recognition modes for these two LeuRSs with respect to $tRNA^{Leu}$.

Aminoacyl-tRNA synthetases (aaRSs)[1] catalyze aminoacylation of their cognate tRNAs with their cognate amino acids to form correct aminoacyl-tRNAs, which are the substrates of protein biosynthesis in the ribosome (*1*). Aminoacylation of tRNA is performed by a two-step reaction: activation of the amino acid with ATP and subsequent transfer of the activated amino acid to the 3′ end of tRNA (*1*). On the basis of common structures and signature sequences of the active domains, aaRSs can be divided into two classes, each of which can be further divided into three subclasses (*2*).

Class I aaRSs are characterized by a catalytic center based on a Rossmann fold of alternating β-strands and α-helices (*2*). The class Ia aaRSs, leucyl-tRNA synthetase (LeuRS), isoleucyl-tRNA synthetase, and valyl-tRNA synthetase, have a long insertion peptide [connective peptide 1 (CP1)] inserted into the Rossmann fold (*2*). Leucyl-tRNA synthetase catalyzes the precise leucylation of $tRNA^{Leu}$ isoacceptors (*3*). In the amino acid activation reaction, amino acids that are similar in size and shape (e.g., Leu and Ile, Ile and Val, Val and Thr, etc.) substantially challenge the specificity of class Ia aaRSs (*4*). It has been shown that the CP1 domain of LeuRS is the editing domain for removing mischarged $tRNA^{Leu}$ (*5, 6*). The X-ray crystal structure has shown that the CP1 domain of *Thermus thermophilus* LeuRS is located ~30 Å from the catalytic site for aminoacylation (*3*).

The isolated CP1 domain of *Escherichia coli* LeuRS (*Ec*LeuRS-CP1), extending from residue 126 to 389 (*7*), is unable to edit mischarged $tRNA^{Leu}$ (*5*); however, the domain ranging from residue 216 to 430 and fused with enough flanking sequences behaves as a functional protein for editing Ile-$tRNA^{Leu}$ (*8*). The isolated CP1 domain of *Aquifex aeolicus* LeuRS (*Aa*LeuRS-CP1), which is the only known heterodimeric LeuRS, can edit mischarged $tRNA^{Leu}$ (*9*). A unique 20-amino acid motif within the CP1 domain of *Aa*LeuRS is crucial to the editing function of *Aa*LeuRS-CP1 and can confer this editing function on inactive *Ec*LeuRS-CP1 but is unimportant to *Aa*LeuRS (*9*) and seems to be a relic of LeuRS evolution (*9*).

Giardia lamblia is a unicellular, flagellated, protozoan parasite that colonizes and reproduces in the small intestine, causing giardiasis. Its genome has been determined (*10*), and sequence alignment of LeuRSs from different species has revealed that *G. lamblia* LeuRS consists of 1173 amino acid residues (*11*). Here, we cloned and overexpressed the gene encoding LeuRS from *G. lamblia* (*GlleuS*) in *E. coli*. We then investigated the properties of purified *Gl*LeuRS, the editing function of its isolated CP1 domain (*Gl*LeuRS-CP1, 309 amino acids from G253 to V561), and the effect of a 49-amino acid insertion within this domain on the editing of *Gl*LeuRS, *Gl*LeuRS-CP1, and *Ec*LeuRS-CP1.

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[1] Abbreviations: aaRS, aminoacyl-tRNA synthetase; CD, circular dichroism; CP1, connective peptide 1; IPTG, isopropyl 1-thio-β-D-galactopyranoside; LeuRS, leucyl-tRNA synthetase; 5-FOA, 5-fluoroorotic acid.

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MATERIALS AND METHODS

Materials. L-Leucine, dithiothreitol, NTP, 5′-GMP, tetrasodium pyrophosphate, inorganic pyrophosphate, ATP, Tris-HCl, magnesium chloride, sodium chloride, and activated charcoal were purchased from Sigma. [^{3}H]-L-Leucine and [^{3}H]-L-isoleucine were obtained from Amersham Biosciences. The *Pfu* DNA polymerase, DNA fragment rapid purification kit, and plasmid extraction kit were purchased from Biotech Company. The T4 DNA ligase and restriction endonucleases were obtained from MBI Fermentas. Ni^{2+}-NTA Superflow was purchased from Qiagen Inc. Pyrobest DNA polymerase and the dNTP mixture were obtained from Takara. Oligonucleotide primers were synthesized by Invitrogen. The pET28a(+) and pUC19 vectors were acquired from Novagen. T7 RNA polymerase was purified from an overproduction strain in our laboratory (*9*). The genome DNA of *G. lamblia* (WB clone C6, ATCC50803) was isolated in the laboratory of L.-H.Q.

Cloning of the Gene Encoding GlLeuRS and Purification of the Enzyme. Using PCR, *GlleuS* was amplified using the *G. lamblia* genome as a template and forward (5′ CATGCCATGGGCCATCATCATCATCATCATGGCGGCGGCGGCATGAAGTGCCTTTCTCGACTTCAGG 3′) and reverse (5′ CGCGGATCCTTACTCAAAGAATAC TATTGGCTTTC 3′) primers, in which the cleavage sites with *Nco*I and *Bam*HI, respectively, are underlined. The PCR product was digested with *Nco*I and *Bam*HI and then inserted into the gap between the *Nco*I and *Bam*HI sites of pET28a(+) to produce recombinant plasmid pET28a(+)-*GlleuS*. The sequence of *GlleuS* was confirmed by DNA sequencing.

E. coli BL21-Codon Plus (DE3)-RIL cells were transformed with pET28a(+)-*GlleuS* and induced with isopropyl 1-thio-β-D-galactopyranoside (IPTG) to overproduce *Gl*LeuRS with a His_6 tag at its N-terminus. The DNA fragment encoding *Ec*LeuRS-CP1-∇49 was amplified and constructed in plasmid $pMFT_7H_6$-*Ec*CP1 (*5*) according to the protocol provided by the In-Fusion 2.0 dry-down PCR cloning kit (Clontech). The genes of the deletion and single-point mutants of *Gl*LeuRS were constructed by PCR using pET28a(+)-*GlleuS* as a template, according to the protocol provided with the KOD-Plus mutagenesis kit (TOYOBO), and were expressed by the same method as *Gl*LeuRS. His_6-tagged *Gl*LeuRS and its mutants were purified according to the method described previously (*11*). All purification steps were carried out at 4 °C. Protein concentrations were determined by the Bradford method (*12*).

Preparation of tRNA. In the *G. lamblia* genome, five isoacceptors of $tRNA^{Leu}$ were found, and their sequences are listed in Table 1 of the Supporting Information. By sequence alignment, the five $tRNA^{Leu}$ species shared conserved D-stem and loop, conserved TΨC-stem and loop, and A73 (data not shown). In this study, *G. lamblia* $tRNA^{Leu}$(AAG) (*Gl*$tRNA^{Leu}$) was used and obtained by in vitro T7 RNA polymerase transcription (its secondary structure is shown in Figure 1 of the Supporting Information), as described previously by our laboratory (*9*). The accepting activity of transcripts of *Gl*$tRNA^{Leu}$ reached 660 pmol/A_{260}. *E. coli* $tRNA^{Leu}$(GAG) (*Ec*$tRNA^{Leu}$) and *A. aeolicus* $tRNA^{Leu}$(GAG) (*Aa*$tRNA^{Leu}$) were obtained in vivo from overproduction strains in our laboratory, and their accepting activity was >1300 pmol/A_{260} (*13*). [H^3]Ile-$tRNA^{Leu}$ was obtained by isoleucylation of *Gl*$tRNA^{Leu}$ or *Ec*$tRNA^{Leu}$ by editing defective *Gl*LeuRSD444A or *Ec*LeuRST252E with [^{3}H]-L-isoleucine, as described previously by our laboratory (*9*).

Assays of Aminoacylation, ATP−PP_i Exchange, and Kinetic Parameters. Optimal temperatures, pHs, and concentrations of ATP and Mg^{2+} were determined by varying each individual factor while holding the others constant. The aminoacylation reaction of *Gl*LeuRS was performed at 45 °C. The aminoacylation or misaminoacylation activities were determined in a reaction mixture containing 60 mM Tris-HCl (pH 8.2), 10 mM $MgCl_2$, 2 mM dithiothreitol, 4 mM ATP, 10 μM $tRNA^{Leu}$, 20 μM [^{14}H]Leu or [^{3}H]Ile, and the indicated amount of *Gl*LeuRS or its mutants, respectively. ATP−PP_i exchange was performed in a reaction buffer containing 60 mM Tris-HCl (pH 8.2), 10 mM $MgCl_2$, 2 mM dithiothreitol, 4 mM ATP, 1 mM Leu, 2 mM tetrasodium [^{32}P]pyrophosphate, and 20 nM *Gl*LeuRS. The kinetic constants of enzymes were determined using various concentrations of the relevant substrates, as described previously (*14*).

Assay of ATP Consumption. When LeuRS edits misactivated amino acids and mischarged $tRNA^{Leu}$, ATP is consumed, allowing editing to be measured via ATP breakdown (to AMP and PP_i) in the presence of noncognate amino acids. The ATP consumption assay was performed in reaction buffer containing 60 mM Tris-HCl (pH 8.2), 10 mM $MgCl_2$, 5 mM dithiothreitol, 5 units/mL PPase, 3 mM [γ-^{32}P]ATP, 10 μM $tRNA^{Leu}$, 20 mM norvaline, and 1 μM *Gl*LeuRS or its mutants. Aliquots (10 μL) of the editing reaction mixture were removed at appropriate time intervals and reactions quenched in 350 μL of a mixture containing 10 mM tetrasodium pyrophosphate, 7% $HClO_4$, and 6% activated charcoal. After centrifugation, the amount of inorganic phosphate (^{32}P) in 50 μL of supernatant was quantified by scintillation counting.

Ile-$tRNA^{Leu}$ Hydrolysis Assay. Hydrolytic activities toward [^{3}H]Ile-$tRNA^{Leu}$ were determined in a reaction mixture containing 60 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 2 mM dithiothreitol, and 1 μM [^{3}H]Ile-$tRNA^{Leu}$ at 37 °C. The reaction was initiated by adding 20 nM *Gl*LeuRS or its deletion mutants, 5 μM *Gl*LeuRS-CP1, *Sc*LeuRS-CP1, *Ec*LeuRS-CP1, *Gl*LeuRS-CP1-Δ49, *Sc*LeuRS-CP1-∇49, or *Ec*LeuRS-CP1-∇49 (see the text below), separately. Aliquots were removed at various time intervals, and the amount of the label on the tRNA was determined by precipitation in 5% trichloroacetic acid. Spontaneous hydrolysis of the mischarged tRNA was performed under the same conditions in the absence of enzymes (*9*).

Circular Dichroism (CD) Analysis. We measured the amount of *Gl*LeuRS or its mutants (0.20 mg/mL) in 20 mM potassium phosphate buffer (pH 7.5) on a Jasco J-715 spectropolarimeter at room temperature. A path length of 0.1 cm was used, and spectra were accumulated over five scans. Background signals from the cuvette and the buffer were subtracted from each spectrum.

In Vivo Complementation Assay by a ScleuS Null Strain. We constructed a knockout *Saccharomyces cerevisiae* strain for *leuS*, Sc*ΔleuS* (*15*). The knockout strain could not survive without the rescuing plasmid pAL5, showing that *leuS* is an essential gene. In Sc*ΔleuS*, *URA3* in the rescuing plasmid (pALR5: Ura^+, Ade^+, $leuS^+$) confers 5-FOA sensitivity on the transformed cells. Therefore, introducing a functionally

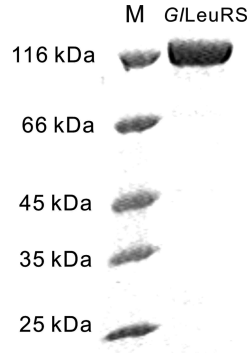

FIGURE 1: SDS−PAGE of *Gl*LeuRS. Fourteen micrograms of *Gl*LeuRS was loaded onto a 10% SDS−PAGE gel. Standard protein markers are indicated are in lane M, and the molecular mass is given at the left.

active LeuRS into Sc*ΔleuS* and then spreading it on proper solid medium with 5-FOA can eliminate pALR5 (Ura^+, Ade^+, $leuS^+$) and enable us to assess the in vivo activity of the introduced LeuRS and its mutants according to the growth rate of the transformants (*15*).

The carrier plasmid p414GPD with a *gpd* promoter (TRP selection marker gene) has a slightly stronger promoter than plasmid p414TEF with a *tef* promoter (TRP selection marker gene), all of which allow a moderate level of constitutive gene expression (*16*). The plasmids carrying the genes encoding LeuRSs from *S. cerevisiae* and *G. lamblia*, and the vector p414GPD, were transformed separately into the Sc*ΔleuS* strain. Transformants were grown at 30 °C on a plate with synthetic defined (SD) medium without tryptophan ($SD\text{-}Trp^-$). Within 2−3 days, colonies formed and were further analyzed for the compensation phenotype. By being cultured in liquid $SD\text{-}Trp^-$ medium, cells were grown to an initial A_{600} of 0.1, and 5 μL of the yeast culture was dropped onto 5-fluoroorotic acid (5-FOA) plates without tryptophan and with uracil to allow loss of the pAL5 maintenance plasmid. The growth of the yeast cells was then observed (*15*).

RESULTS

Cloning and Expression of GlleuS and the Gene Encoding GltRNA^{Leu}. The *G. lamblia* genome database (www.mbl.edu/Giardia) was screened and analyzed for the alignment of *leuS* genes from various species to obtain the complete sequence of *GlleuS*. Since there are rarely introns in the *G. lamblia* gene (*17*), the genome was utilized directly to amplify *GlleuS*. *GlleuS* was obtained and confirmed by DNA sequencing. *GlleuS* can be overexpressed in *E. coli* BL21-Codon Plus (DE3)-RIL transformants. The gene encoding *Gl*tRNA^{Leu} was cloned in *E. coli* MT102 by the same methods in our laboratory (*13*); however, it could not be overexpressed (data not shown).

Preparation of GlLeuRS, Optimization of Reaction Conditions, and Kinetics. *Gl*LeuRS was purified from *E. coli* BL21-Codon Plus (DE3)-RIL transformants by Ni-NTA affinity chromatography (Figure 1). The molecular mass was consistent with the theoretical molecular mass deduced from the DNA sequence. The reaction conditions of *Gl*LeuRS were optimized for further biochemical studies. The optimal pH and temperature were 8.2 (actual pH was 7.7 at 45 °C) and 45 °C, respectively, and *Gl*LeuRS was thermally stable up to 55 °C (data not shown). The optimal ratio of magnesium to ATP for activation and charging reactions was ∼2.5; in this work, 10 mM magnesium chloride was used.

Because *Gl*tRNA^{Leu} could not be efficiently overproduced in *E. coli* (data not shown), transcribed *Gl*tRNA^{Leu} was used as the substrate. The kinetic parameters of *Gl*LeuRS were assayed at 45 °C and compared with those of *Aa*LeuRS (*18*) and *Ec*LeuRS (*19*) in Table 1. In the amino acid activation reaction, as compared with *Ec*LeuRS, for the Leu the K_m of *Gl*LeuRS was half and the k_{cat} was one-fifth of that of *Ec*LeuRS; for ATP, the K_m was the same while k_{cat} was one-fourth of that of *Ec*LeuRS. In the aminoacylation reaction, *Gl*LeuRS could leucylate transcribed *Gl*tRNA^{Leu} without modified bases and *E. coli* tRNA^{Leu} with similar k_{cat} values. The K_m values of *Gl*LeuRS for both tRNA^{Leu}s were comparable and larger than those of *Ec*LeuRS.

Compared with *Aa*LeuRS, in the amino acid activation reaction for Leu, the K_m and k_{cat} of *Gl*LeuRS were ∼9 and ∼2 times those of *Aa*LeuRS, respectively; for ATP, the values were ∼2 times of those of *Aa*LeuRS. *Gl*LeuRS could leucylate *Aa*tRNA^{Leu} purified in vivo, and the K_m of *Gl*LeuRS for *Aa*tRNA^{Leu} was comparable to that of *Gl*LeuRS for the *Gl*tRNA^{Leu} transcript without modified bases. The k_{cat} of *Gl*LeuRS for *Aa*tRNA^{Leu} was one-third of that of *Gl*LeuRS for *Gl*tRNA^{Leu}.

GlLeuRS Has Slight but Negligible Activity toward S. cerevisiae tRNA^{Leu} in Vivo or in Vitro. We further took advantage of a *ScleuS* knockout strain, Sc*ΔleuS*, constructed in our laboratory to test whether *Gl*LeuRS could substitute for *Sc*LeuRS in vivo (*15*). Two recombinant plasmids, p414GPD-*GlleuS* and p414TEF-*ScleuS* (as a positive control), were constructed by insertion of *GlleuS* (encoding *Gl*LeuRS with six histidines at the N-terminus) and *ScleuS* (encoding *Sc*LeuRS) into p414GPD (Trp^+) and p414TEF (Trp^+). P414GPD-*GlleuS* and p414TEF-*Scleus* were then introduced into Sc*ΔleuS*, and *Gl*LeuRS expression was confirmed by Western blot using an antibody specific to the six histidines (data not shown). Surprisingly, the strain containing p414GPD-*GlleuS* could not eliminate pALR5 (Ura^+, Ade^+, $leuS^+$) to grow on the $SD\text{-}Trp^-$ medium with 5-FOA, in contrast with the strain containing p414TEF-*ScleuS*, despite the fact that p414GPD has a stronger promoter than p414TEF (*16*). This indicates that *Gl*LeuRS cannot leucylate *S. cerevisiae* tRNA^{Leu} in vivo with sufficient efficiency to support normal growth of yeast (Figure 2A). *Gl*LeuRS in vitro aminoacylated *S. cerevisiae* tRNA^{Leu} (yeast total tRNA) slightly as compared with *Sc*LeuRS, showing that *Gl*LeuRS had little activity toward *S. cerevisiae* tRNA^{Leu} (Figure 2B).

The CP1 Domain of GlLeuRS Is Active in Editing Mischarged tRNA^{Leu}. Besides the aminoacylation reaction, editing is of great significance for LeuRS to clear mistakes during amino acid selection (*4*). The sequence of *Gl*LeuRS is 27 and 15% identical with those of *Pyrococcus horikoshii* LeuRS (*Ph*LeuRS) and *Aa*LeuRS, respectively. The crystal structure of *Ph*LeuRS has been determined (*20*), and sequence alignment of *Gl*LeuRS and *Ph*LeuRS reveals that the CP1 domain of *Gl*LeuRS spans from G253 to V561 (*20*). To determine whether the isolated CP1 domain of *Gl*LeuRS (*Gl*LeuRS-CP1) has a hydrolytic editing function toward Ile-tRNA^{Leu} similar to that of *Aa*LeuRS-CP1 (*9*), *Gl*LeuRS-CP1 was purified through gene cloning and overexpression in *E.*

Table 1: Kinetic Constants of *Gl*LeuRS at 45 °C

substrate	constant	ATP−PP_i exchange			aminoacylation		
		*Gl*LeuRS[a] at 45 °C	*Aa*LeuRS[b] at 60 °C	*Ec*LeuRS[b] at 37 °C	*Gl*LeuRS[a] at 45 °C	*Aa*LeuRS[b] at 60 °C	*Ec*LeuRS[b] at 37 °C
leucine	K_m (μM)	14.2 ± 1.5	1.6	30	9.0 ± 0.5	6.0	15
	k_{cat} (s^{-1})	30.5 ± 3.5	15.5	155	2.8 ± 0.2	1.4	3.0
	k_{cat}/K_m (s^{-1} mM^{-1})	2147.8	9688	5167	311.1	233	200
ATP	K_m (μM)	750.4 ± 61.2	380	750	687.6 ± 54.2	112.3	280
	k_{cat} (s^{-1})	34.5 ± 2.3	14.5	140	3.1 ± 0.2	1.8	3.6
	k_{cat}/K_m (s^{-1} mM^{-1})	46.0	38.2	187	4.5	16.0	12.9
*Gl*tRNA^{Leu} transcribed in vitro	K_m (μM)				6.1 ± 0.4	ND[c]	ND[c]
	k_{cat} (s^{-1})				2.7 ± 0.2	ND[c]	ND[c]
	k_{cat}/K_m (s^{-1} mM^{-1})				442.6	ND[c]	ND[c]
*Ec*tRNA^{Leu} purified in vivo	K_m (μM)				7.2 ± 0.4	1.5	1.5
	k_{cat} (s^{-1})				2.6 ± 0.3	0.4	2.9
	k_{cat}/K_m (s^{-1} mM^{-1})				361.1	267	1933
*Aa*tRNA^{Leu} purified in vivo	K_m (μM)				7.5 ± 0.7	0.3	1.1
	k_{cat} (s^{-1})				0.8 ± 0.1	1.5	1.6
	k_{cat}/K_m (s^{-1} mM^{-1})			106.7	5000	1455	

[a] The values of *Gl*LeuRS represent the means ± the standard deviation of three independent experiments. [b] Data from ref *18*. [c] Not determined.

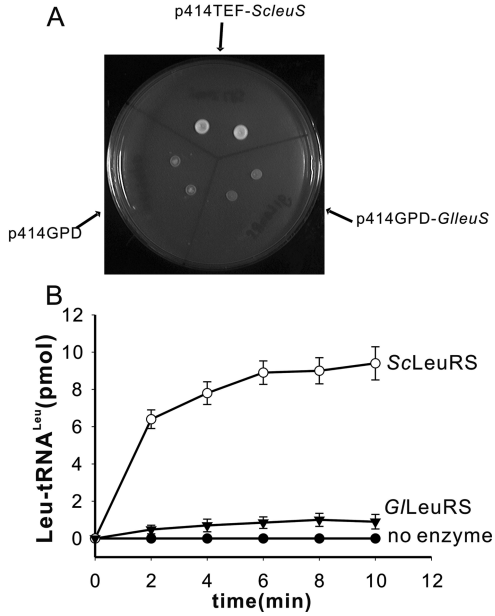

FIGURE 2: *Gl*LeuRS has slight but negligible activity toward *Sc*tRNA^{Leu} in vivo or in vitro. (A) Plasmid p414GPD (negative control), p414GPD-*GlleuS*, or p414TEF-*ScleuS* was introduced into a haploid strain whose chromosomal *ScleuS* gene was depleted. *Sc*LeuRS can rescue the growth by eliminating the rescuing plasmid in contrast to *Gl*LeuRS. (B) *Sc*LeuRS (50 nM) (○), but not *Gl*LeuRS (50 nM) (▼), could aminoacylate yeast total tRNA with leucine. A control was performed in the absence of any enzyme (●).

coli to a >95% homogeneity by Ni-NTA chromatography (data not shown). We found that *Gl*LeuRS-CP1 could hydrolyze mischarged Ile-*Gl*tRNA^{Leu} (Figure 3), like *Aa*LeuRS-CP1.

Effect of the 49-Amino Acid Motif on Aminoacylation and Editing Functions of GlLeuRS. By sequence alignment of LeuRSs from various species, an insertion of 49 amino acid residues (designated as the 49-amino acid motif) within CP1 is identified from Q281 to K329. This insertion is absent from all 50 LeuRSs we aligned, including LeuRSs from lower eukaryotes such as *Trypanosoma brucei*, *Leishmania major*, *Entamoeba histolytica*, and *Theileria annulata*. The location is just before one of the two conserved "TLRPDT" regions (*6*) (Figure 4A) that corresponds to a 20-amino acid motif in *Aa*LeuRS-CP1 that is crucial for its editing function

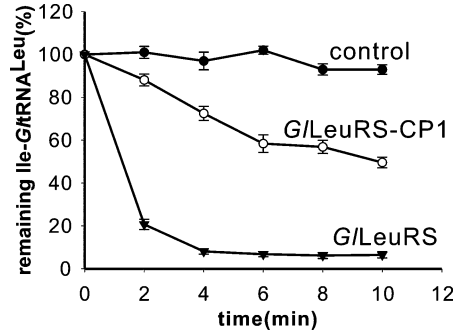

FIGURE 3: Hydrolytic editing activity assay of *Gl*LeuRS-CP1. Hydrolysis of Ile-*Gl*tRNA^{Leu} by 20 nM *Gl*LeuRS (▼) or 5 μM *Gl*LeuRS-CP1 (○) and a control (spontaneous hydrolysis) (●).

(*9*). To study the role of the 49-amino acid motif in aminoacylation and editing of *Gl*LeuRS, we constructed deletion mutants within the 49-amino acid motif: *Gl*LeuRS-A (deleted Q281−D294), *Gl*LeuRS-B (deleted S295−L304), *Gl*LeuRS-C (deleted R306−F317), *Gl*LeuRS-D (deleted D318−A328), and *Gl*LeuRS-Δ49 (deleted the whole 49-amino acid motif) (Figure 4B). During gene expression in *E. coli*, *Gl*LeuRS-A and *Gl*LeuRS-B, similar to wild-type *Gl*LeuRS, could be produced as soluble proteins and purified by Ni-NTA affinity chromatography (data not shown). However, *Gl*LeuRS-C, *Gl*LeuRS-D, and *Gl*LeuRS-Δ49 formed inclusion bodies, despite our extensive efforts (data not shown).

The aminoacylation kinetics of *Gl*LeuRS, *Gl*LeuRS-A, and *Gl*LeuRS-B were measured under optimal conditions. The K_m values of the two mutants for leucine and ATP decreased slightly and for tRNA increased slightly. The k_{cat} values for the three substrates decreased, and the catalytic efficiency (k_{cat}/K_m) for three substrates decreased to some extent compared with that of *Gl*LeuRS (Table 2). Because the aminoacylation efficiency of *Gl*LeuRS-B was decreased more than that of *Gl*LeuRS-A, we substituted alanine for five polar amino acid residues, S295, E298, K299, N301, and K303, between S295 and L304 which was absent in *Gl*LeuRS-B and allowed us to obtain five mutants, *Gl*LeuRS-S295A, *Gl*LeuRS-E298A, *Gl*LeuRS-K299A, *Gl*LeuRS-N301A, and *Gl*LeuRS-K303A, respectively. The aminoacylation activities of these mutants showed that all the single-point mutants had the same catalytic activity as wild-type *Gl*LeuRS (data

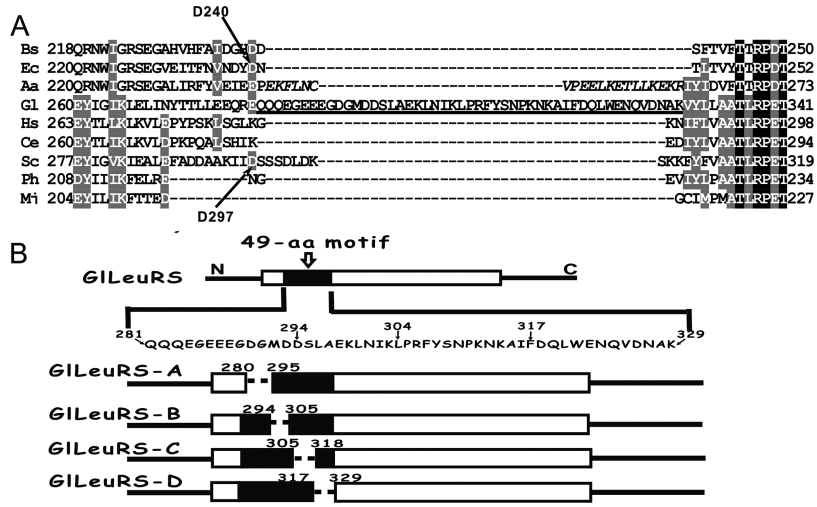

FIGURE 4: Primary sequence alignment of the sequence just before the conserved "TLRPET" region (*6*) in the CP1 domain of LeuRS from different species and construction of the mutants. (A) The 49-amino acid motif is underlined, and the 20-amino acid motif crucial for the editing of *Aa*LeuRS-CP1 is in italics (*9*). D297 of *Sc*LeuRS and D240 of *Ec*LeuRS are marked by arrows. (B) Schematic representation of the 49-amino acid motif. Mutants lacking the 49-amino acid motif in whole or in part were constructed according to their sequences. These mutants were *Gl*LeuRS-A (lacking Q281−D294), *Gl*LeuRS-B (lacking S295−L304), *Gl*LeuRS-C (lacking R306−F317), *Gl*LeuRS-D (lacking D318−A328), and *Gl*LeuRS-Δ49 (lacking Q281−K329). Abbreviations: Bs, *Bacillus subtilis*; Ec, *E. coli*; Aa, *A. aeolicus*; Gl, *G. lamblia*; Hs, *Homo sapiens*; Ce, *Caenorhabditis elegans*; Sc, *S. cerevisiae*; Ph, *P. horikoshii*; Mj, *Methanococcus jannishii*.

Table 2: Kinetics of *Gl*LeuRS, *Gl*LeuRS-A, and *Gl*LeuRS-B[a]

substrate	constant	*Gl*LeuRS	*Gl*LeuRS-A	*Gl*LeuRS-B
leucine	K_m (μM)	9.0 ± 0.5	5.9 ± 0.4	7.7 ± 0.5
	k_{cat} (s^{-1})	2.8 ± 0.2	1.1 ± 0.1	0.6 ± 0.1
	k_{cat}/K_m (s^{-1} mM^{-1})	311.1	186.4	77.9
ATP	K_m (μM)	687.6 ± 54.2	330.0 ± 32.1	415.3 ± 38.9
	k_{cat} (s^{-1})	3.1 ± 0.2	1.2 ± 0.2	0.9 ± 0.2
	k_{cat}/K_m (s^{-1} mM^{-1})	4.5	3.6	2.2
*Gl*tRNA^{Leu}	K_m (μM)	6.1 ± 0.4	10.1 ± 0.8	8.2 ± 0.5
	k_{cat} (s^{-1})	2.7 ± 0.2	1.3 ± 0.2	0.6 ± 0.1
	k_{cat}/K_m (s^{-1} mM^{-1})	442.6	128.7	73.2

[a] The values represent the means ± the standard deviation of three independent experiments.

not shown), indicating that the structural change of *Gl*LeuRS-B by deletion of S295−L304 may decrease the aminoacylation activity. Although the CD spectrometry data showed that the secondary structures of *Gl*LeuRS-A and *Gl*LeuRS-B were not significantly changed compared to that of wild-type *Gl*LeuRS, subtle conformational changes may have occurred, resulting in decreased activity (data not shown).

Misaminoacylation of *Gl*tRNA^{Leu} by *Gl*LeuRS-A or *Gl*LeuRS-B was the same as that by *Gl*LeuRS, and no detectable amount of Ile-*Gl*tRNA^{Leu} was formed (Figure 5A). Sequence alignment of LeuRSs of various species previously showed that D444 of *Gl*LeuRS in the CP1 domain is homologous with D419 of *Sc*LeuRS and is responsible for post-transfer editing (*6, 15*). *Gl*LeuRS-D444A could obviously isoleucylate *Gl*tRNA^{Leu} (Figure 5A). An ATP hydrolysis assay was also carried out in the presence of *Gl*tRNA^{Leu} and revealed that *Gl*LeuRS, *Gl*LeuRS-A, and *Gl*LeuRS-B exhibited no differences in total editing, including pre- and post-transfer editing (*21*) (Figure 5B). *Gl*LeuRS and its two mutants hydrolyzed Ile-*Gl*tRNA^{Leu} at the same rate, indicating that they have the same post-transfer editing activity (Figure 5C). These data show that the deletion in *Gl*LeuRS-A or *Gl*LeuRS-B had no effect on the editing activity of *Gl*LeuRS.

Deletion of the 49-Amino Acid Motif Abolishes the Editing Activity of GlLeuRS-CP1. Although *Gl*LeuRS-Δ49 formed inclusion bodies during its gene expression, the mutant with deletion of the 49-amino acid motif from the isolated CP1 domain, *Gl*LeuRS-CP1-Δ49, could be purified as a soluble protein by expression of its gene in *E. coli*. In contrast, *Gl*LeuRS-CP1-A (lacking Q281−D294), *Gl*LeuRS-CP1-B (lacking S295−L304), *Gl*LeuRS-CP1-C (lacking R306−F317), and *Gl*LeuRS-CP1-D (lacking D318−A328) formed inclusion bodies during expression of their gene fragments (data not shown).

*Gl*LeuRS-CP1-Δ49 could not hydrolyze Ile-*Gl*tRNA^{Leu} at all, indicating that the 49-amino acid motif is indispensable to the editing activity of *Gl*LeuRS-CP1 (Figure 6A). The β-subunit of *Aa*LeuRS is a tRNA binding subunit, and fusion of the β-subunit to inactive *Ec*LeuRS-CP1 activates its editing function (*9*). When the β-subunit was added to the editing reaction mixture of *Gl*LeuRS-CP1-Δ49, Ile-*Gl*tRNA^{Leu} could be hydrolyzed slightly; however, the β-subunit or *Gl*LeuRS-CP1-Δ49 alone could not (Figure 6B).

The 49-Amino Acid Motif Can Confer Editing Activity on Inactive EcLeuRS-CP1. Our previous studies have shown that *Ec*LeuRS-CP1, extending from residue 126 to 389 (*7*), is inactive in hydrolytically edit mischarged tRNA^{Leu} (*5, 9*), in contrast to *Aa*LeuRS-CP1 (*9*). However, the *Ec*LeuRS-

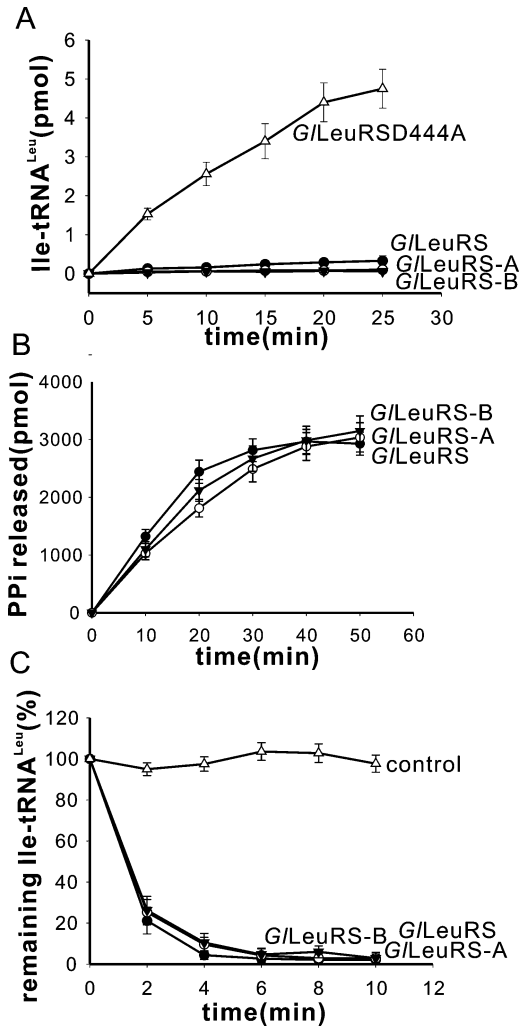

FIGURE 5: Characteristics of *Gl*LeuRS, *Gl*LeuRS-A, and *Gl*LeuRS-B. (A) Misaminoacylation, (B) ATP hydrolysis with norvaline, and (C) post-transfer editing assay of *Gl*LeuRS (●), *Gl*LeuRS-A (○), and *Gl*LeuRS-B (▼). Controls (△) were performed with editing-defective *Gl*LeuRSD444A and by adding no enzyme in the misaminoacylation and post-transfer editing assays.

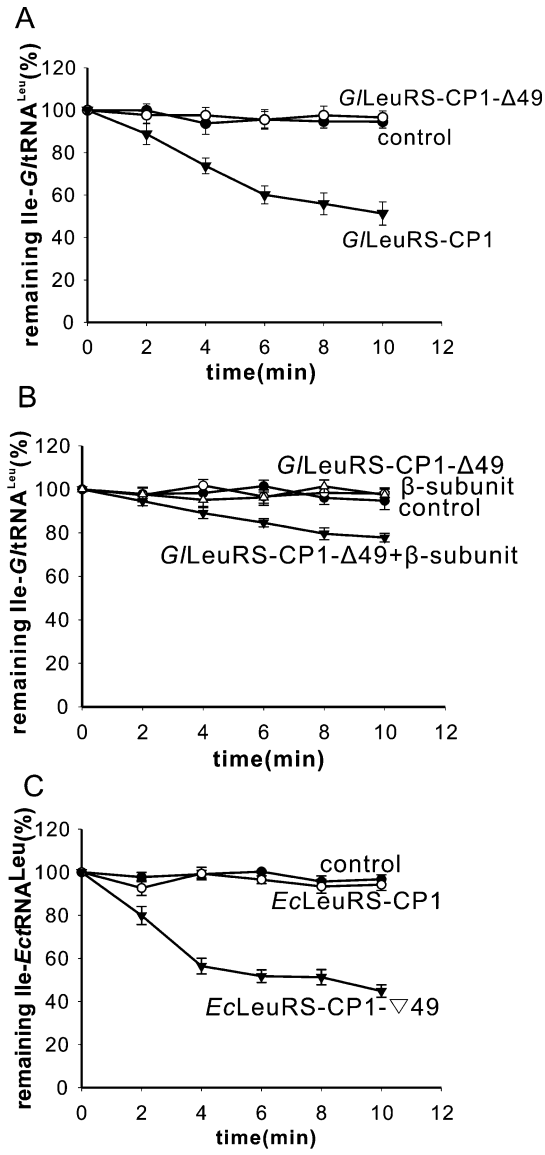

FIGURE 6: Role of the 49-amino acid motif in the editing functions of *Gl*LeuRS-CP1 and *Ec*LeuRS-CP1. A control (spontaneous hydrolysis) (●) was performed in the absence of any enzyme. (A) Post-transfer editing of Ile-tRNA^{Leu} from *Gl*tRNA^{Leu} by 5 μM *Gl*LeuRS-CP1 (▼) or 5 μM *Gl*LeuRS-CP1-Δ49 (○) or a control. (B) Post-transfer editing of Ile-tRNA^{Leu} from *Gl*tRNA^{Leu} by 5 μM *Gl*LeuRS-CP1-Δ49 (○), *Gl*LeuRS-CP1-Δ49 with the 5 μM β-subunit of *Aa*LeuRS (▼), or 5 μM β-subunit of *Aa*LeuRS (△) or a control. (C) Post-transfer editing of Ile-tRNA^{Leu} from *Ec*tRNA^{Leu} by 5 μM *Ec*LeuRS-CP1 (○) or 5 μM *Ec*LeuRS-CP1-∇49 (▼) or a control.

CP1 mutant with an insertion of the 20-amino acid motif within *Aa*LeuRS-CP1 (*Ec*LeuRS-CP1-∇20) gained the editing function. The 20-amino acid motif within *Aa*LeuRS-CP1 is crucial for hydrolyzing mischarged tRNA^{Leu} of the isolated CP1 domain (*9*). According to alignment of sequences, the location of the 49-amino acid motif within *Gl*LeuRS-CP1 corresponds to that of the 20-amino acid motif of *Aa*LeuRS-CP1, both preceding the conserved TLRPDT region. To clarify whether the 49-amino acid motif can confer editing activity on inactive *Ec*LeuRS-CP1 like the 20-amino acid motif, *Ec*LeuRS-CP1-∇49 was constructed by transplanting the 49-amino acid motif between D240 and N241 of *Ec*LeuRS (Figure 4A) (*9*). The data from the post-transfer editing assay showed that *Ec*LeuRS-CP1-∇49 could hydrolyze mischarged Ile-*Ec*tRNA^{Leu}, like *Ec*LeuRS-CP1-∇20 (*9*), indicating that the 49-amino acid motif could confer editing activity on previously inactive *Ec*LeuRS-CP1 (Figure 6C).

We also explored the effect of this motif on the editing function of the isolated CP1 domain of LeuRS from *S. cerevisiae* (*Sc*LeuRS-CP1). The CP1 domain of *Sc*LeuRS spans residues G270−V530 according to the crystal structure of *Ph*LeuRS and its sequence alignment with *Ph*LeuRS (*20*). *Sc*LeuRS-CP1 was isolated by the same method as *Gl*LeuRS-CP1. *Sc*LeuRS-CP1 is inactive in hydrolyzing Ile-*Ec*tRNA^{Leu} (Figure 2 of the Supporting Information), in contrast with *Gl*LeuRS-CP1 (Figure 3), *Aa*LeuRS-CP1 (*9*), and *Ec*LeuRS-CP1 (*8*). We constructed an insertion mutant *Sc*LeuRS-CP1-∇49, which had the 49-amino acid motif of *Gl*LeuRS inserted into the same position of *Sc*LeuRS-CP1 (between D297 and S298) (Figure 4A), and its hydrolytic activity with respect to Ile-*Ec*tRNA^{Leu} was assayed. *Sc*LeuRS-CP1-∇49 was inactive in editing Ile-*Ec*tRNA^{Leu}, similar to *Sc*LeuRS-CP1 (Figure 2 of the Supporting Information), indicating that the 49-amino acid motif could not confer editing activity on *Sc*LeuRS-CP1.

DISCUSSION

Some aaRSs, such as ProRS and AlaRS, from *G. lamblia,* which is generally believed to be among the most basal of the extant eukaryotes, are archaeal-type. Investigation of these aaRSs has led to an improved understanding of eukaryotic translation systems and has enabled support of some evolutionary hypotheses (*22, 23*). The determined genome of *G. lamblia* has revealed that sequences of some aaRSs, such as tyrosyl- and tryptophanyl-tRNA synthetases, and LeuRS, commonly show an insertion of an average of 20 amino acid residues compared to their counterparts in other organisms; however, the functions of these unusual insertions remain to be determined (*10*).

Here, we cloned the gene encoding LeuRS from *G. lamblia* and purified *Gl*LeuRS from *E. coli* for the first time. Under optimal conditions, *Gl*LeuRS bound leucine and ATP looser than *Aa*LeuRS and tighter than *Ec*LeuRS. However, *Gl*LeuRS bound tRNAs from either *Ec*tRNA^{Leu} or *Aa*tRNA^{Leu} with less affinity than *Ec*LeuRS and *Aa*LeuRS. It was previously shown that *Aa*LeuRS can only leucylate *Ec*tRNA^{Leu} at one-sixth the rate of *Ec*LeuRS, although *Ec*LeuRS can leucylate *Aa*tRNA^{Leu} at the same rate as *Aa*LeuRS (*18*). In the work presented here, we found that *Gl*LeuRS could leucylate *Ec*tRNA^{Leu} at the same rate as *Ec*LeuRS and could leucylate *Aa*tRNA^{Leu} at half the rate of *Aa*LeuRS, indicating that *Gl*LeuRS can recognize the identity elements of *Ec*tRNA^{Leu} very well. Because we could not obtain an overproduction strain of *Gl*tRNA^{Leu} by gene expression, *Ec*tRNA^{Leu} can be used as a substrate of *Gl*LeuRS in assaying aminoacylation activity instead. In contrast, *Gl*LeuRS cannot substitute for *Sc*LeuRS in vivo, indicating a possibly different tRNA recognition mode. Crystal structures of LeuRS and biochemical data showed that C-terminal extension serves as the tRNA binding domain in bacterial and eukaryal/archaeal LeuRSs (*24*−*26*). C-Terminal extension is shown biochemically to be crucial for *Sc*LeuRS in that deletion of the five extreme C-terminal amino acid residues in *Sc*LeuRS completely abolishes the aminoacylation activity of the enzyme, and three conserved residues in the C-terminal extension (P1080, P1083, and N1089) are essential for tRNA aminoacylation (ref *15* and data not shown). However, C-terminal extensions of *Gl*LeuRS and *Sc*LeuRS exhibit the lowest levels of similarity and identity among the entire group enzymes (data not shown). This is the possible reason why *Gl*LeuRS cannot in vivo instead of *Sc*LeuRS (Figure 2A) and in vitro effectively recognize *Sc*tRNA^{Leu} (Figure 2B).

To find the effect of the 49-amino acid motif on aminoacylation and editing functions of *Gl*LeuRS, we constructed five complete or partial deletion mutants within the 49-amino acid motif of *Gl*LeuRS. Only two mutants could be obtained as soluble proteins. Compared with the native *Gl*LeuRS, although their secondary structures had no obvious changes as seen in CD spectra, both of these mutants (*Gl*LeuRS-A and *Gl*LeuRS-B) bound ATP and leucine more tightly, and tRNA^{Leu} more loosely, decreasing their aminoacylation activities. Surprisingly, *Gl*LeuRS-A and *Gl*LeuRS-B had no effect on amino acid editing function, showing that the exact structure of the editing active site in the context of the full enzyme was not impaired. Therefore, we suggest that slight changes in the CP1 domain (which does not influence fine editing active site structure in this work) may alter the exact structure of the aminoacylation active site. Similar phenomena have previously been reported in other LeuRS systems. For example, the split in *Ec*LeuRS around T252/F253, E292/A293, L327/M328, and P368/D369, which are all located in the CP1 domain, impairs aminoacylation activity to various extents (*19*). Mutations of R322 in the *Aa*LeuRS editing domain interrupt the aminoacylation ability of the enzyme, but the editing activity is not affected at all (B. Zhu et al., unpublished results).

The isolated CP1 domain of *Gl*LeuRS functions as an active protein to edit mischarged *Gl*tRNA^{Leu}. It is the third example of a functional, isolated CP1 domain of LeuRS (*8, 9*). We were interested in the unique 49-amino acid motif insertion within the hydrolytic editing domain of *Gl*LeuRS. Previously, we showed that the insertion of an idiosyncratic 20-amino acid motif from the CP1 domain of *Aa*LeuRS stimulated the hydrolytic editing activity of *Ec*LeuRS-CP1. Fusion of the β-subunit of the split *Aa*LeuRS, which is a tRNA binding domain, to *Ec*LeuRS-CP1 conferred post-transfer editing activity on the mischarged tRNA^{Leu} (*9*). We therefore postulated that the 20-amino acid motif and the β-subunit help to enhance the interaction between RNA and protein (*9*). In this work, the editing function was lost when the 49-amino acid motif was removed from *Gl*LeuRS-CP1. Similarly, adding the nonspecific nucleic acid binding domain, the β-subunit of *Aa*LeuRS (*26*), to the editing reaction of *Gl*LeuRS-CP1-Δ49 partially restored this lost editing activity. Additionally, the editing function of *Ec*LeuRS-CP1 was stimulated by the insertion of the 49-amino acid motif. Therefore, we suggest that the unique 49-amino acid motif insertion may also stabilize the interaction between the mischarged tRNA and the CP1 domain, like the role of the 20-amino acid motif in *Aa*LeuRS, and that this stabilization is important to the editing function of isolated CP1. This is possibly the reason why *Sc*LeuRS-CP1, which is homologous to *Gl*LeuRS-CP1 and lacks such an insertion at its corresponding site, lacks post-transfer editing ability. Nevertheless, the stabilization derived from the 49-amino acid motif might be insufficient for conferring hydrolytic editing activity on an editing-inactive *Sc*LeuRS-CP1.

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SUPPORTING INFORMATION AVAILABLE

Five tRNA^{Leu} isoacceptors of *G. lamblia* (Table 1), cloverleaf structure of the *G. lamblia* tRNA^{Leu}(AAG) isoacceptor (Figure 1), and role of the 49-amino acid motif in the editing functions of *Sc*LeuRS-CP1 (Figure 2). This material is available free of charge via the Internet at http://pubs.acs.org.

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