Unique Residues Crucial for Optimal Editing in Yeast Cytoplasmic Leucyl-tRNA Synthetase Are Revealed by Using a Novel Knockout Yeast Strain*

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Leucyl-tRNA synthetase (LeuRS) contains an editing domain that discriminates leucine from noncognate amino acids to ensure translational fidelity. In this study, a knock-out strain for Saccharomyces cerevisiae LeuRS was constructed to analyze in vivo the tRNA aminoacylation properties of S. cerevisiae and human cytoplasmic LeuRSs. The activities of several editing-defective mutants of ycLeuRS were determined in vivo and compared with those obtained in vitro in a complementation assay performed in the knock-out strain. The editing activities of these mutants were analyzed in the presence of either norvaline, a leucine analogue, or AN2690, a specific inhibitor that targets the editing active site. In general, the in vivo data are consistent with those obtained in vitro. Our results show that ycLeuRS post-transfer editing plays a crucial role in the establishment of the aminoacylation fidelity. When impaired, the viability of cells bearing editing-defective mutants is drastically decreased in the presence of noncognate amino acid. This study also emphasizes the crucial function of some semi-conserved residues around the editing site in modulating the editing efficiency. The assay system can be used to test the effect of compounds that potentially target the aminoacylation or editing active site of fungal LeuRSs.

It is presumed that the genetic code underwent an initial period during which codons did not specify an unalterable amino acid. The earliest proteins were a mixture of statistical products due to unclear code assignment from various similar amino acids (1). The modern unalterable genetic code arose with the evolution of aminoacyl-tRNA synthetases (aaRSs), which catalyze the esterification reaction that links an amino acid with the corresponding anticodon triplet on its cognate tRNA (2, 3). As components of the translation apparatus, aaRSs are responsible for the fidelity of protein synthesis, and aaRS errors can lead to genetic code ambiguity and disruption of cellular functions (4). Thus, many aaRSs have developed an editing mechanism to remove their errors and maintain translational fidelity (5).

Leucyl-tRNA synthetase (LeuRS) belongs to class Ia aaRSs and catalyzes the attachment of leucine to tRNA^Leu. In this reaction, aliphatic amino acids with similar size or structure, such as isoleucine, methionine, valine, norvaline, and norleucine, are easy to be mischarged with tRNA^Leu by LeuRS. LeuRS contains a classic editing domain, connecting polypeptide 1 (CP1), which can hydrolyze the aminoacyl bond of mischarged aa-tRNA^Leu (6–8). The molecular mechanisms of the editing function of prokaryotic LeuRS have been investigated in vitro (6–11), and the crystallographic structure of prokaryotic or archaeal LeuRS complexed with the cognate tRNA has been reported (12, 13). However, most previous studies on LeuRS editing have been performed in in vitro systems, and very few studies have shed light on the effect of editing deficiency in vivo (14–18). Recently, an Escherichia coli-based assay was established to identify amino acids that compromise the fidelity of E. coli and yeast mitochondrial LeuRS (EcLeuRS and ymLeuRS) during translation (19, 20).

Little is known regarding the structural basis and functional implementation of the editing reaction carried out by eukaryotic LeuRS. We previously found that the human cytoplasmic LeuRS (hcLeuRS) is quite active as a free enzyme, and its kinetic constants for ATP, leucine, and tRNA^Leu in the ATP-PP exchange and tRNA leucylation reactions were determined (21); however, the editing function of this enzyme has not yet been addressed. The Saccharomyces cerevisiae cytoplasmic LeuRS (ycLeuRS) consists of 1090 amino acid residues and has a molecular mass of 120 kDa. It is encoded by the gene yceleuS. ycLeuRS has been reported to recognize its cognate tRNA^Leu with the anticodon loop acting as a major identity determinant (22) and aminoacylate E. coli tRNA^Leu efficiently (23). In addition, ycLeuRS performs the editing function through both pre-transfer hydrolysis as a major pathway in vitro (24) and post-transfer editing in dependence on an essential conserved aspartic acid residue (9).
Yeast Strain Reveals Residues for LeuRS Editing

In the present study, we created several LeuRS mutants for highly conserved residues and assayed their in vitro enzymatic activities. Then, we constructed a leuS knock-out yeast strain, SacleuS, and determined the growth rates of compensated SacleuS strains expressing these mutants to assess their effect on editing. We found that ycLeuRS activates norvaline (Nva) with a much higher efficiency than isoleucine, and this poses a strong challenge to the editing step of ycLeuRS in vivo. We demonstrate that the post-transfer editing pathway is the major proofreading pathway in vivo and is critical for yeast cell survival in the presence of noncognate amino acid. Our results also suggest that several unique residues in ycLeuRS play important roles in optimal editing reaction to maintain translational fidelity in vivo. The in vivo data obtained by this method match up to those obtained in vitro, and the former seemed to be more sensitive to slight changes in the editing activity of ycLeuRS than the latter.

EXPERIMENTAL PROCEDURES

Materials, Strains, and Vectors—Amino acids, ATP, dithiothreitol, tetrasodium pyrophosphate, and 10 mg/ml yeast total tRNA were purchased from Sigma. [L-14C]Leucine (300 Ci/mol), [1-3H]isoleucine (1 mCi/ml), [γ-32P]ATP, and tetrasodium [32P]pyrophosphate were obtained from Amersham Biosciences. GF/C filters were from Whatman Co. (Mainstone, England). T4 DNA ligase and restriction endonucleases were obtained from Sangon Co. (Canada, Shanghai Branch); nickel-nitrilotriacetic acid Superflow was purchased from Qiagen Inc. (Germany). All oligonucleotides for amplifying DNA fragments were synthesized by Invitrogen. Aquifex aeolicus tRNA\textsubscript{GAG} (Aa\textsuperscript{tRNALeu\textsuperscript{L}}) with an accepting activity of 1400 pmol/A\textsubscript{260} was prepared from an overexpressing strain carrying this gene. The strain was constructed in our laboratory (26).

E. coli—DH5α and BL21 (DE3) were used for cloning and producing recombinant proteins. The pET28a plasmid was used to construct the recombinant plasmid with ycleuS. Compound AN2690 and the recombinant pET16-hcLeuRS plasmid harboring the gene that encodes human cytoplasmic LeuRS (hcLeuRS) were gifts from Dr. Alley, Anacor Co., in 2006. The haploid (hcLeuRS) were used to construct the recombinant plasmid with ycleuS gene was amplified from the aforementioned DNA by PCR using the following primers: forward (5'-GCAGATCCATGTCTTTGAGTTGGTCTTAGAA-3') and reverse (5'-CCGGAATCTTAAATTTGGGAAGACACCACTGGG-3'). Then the PCR product was digested with BamHI-EcoRI and inserted into the BamHI-EcoRI sites in p414TEF and pET28a to obtain p414TEF-ycleuS and pET28a-ycleuS, respectively. The single-point mutation in ycLeuRS was performed by PCR from p414TEF-ycleuS or pET28a-ycleuS by a previously described method (26), and mutants were confirmed by DNA sequencing (Invitrogen). p414TEF and pET28a recombinant plasmids containing leuS or various leuS mutants were used to perform the complementation assay in S. cerevisiae and to produce recombinant proteins in E. coli, respectively. The gene encoding human cytoplasmic LeuRS (hcLeuRS) and its mutant D399K were amplified from pET16-hcLeuRS by PCR and inserted into p414TEF by the aforementioned method to obtain recombinant plasmids for the complementation assay in S. cerevisiae.

Production and Purification of His\textsubscript{6}-ycLeuRS and Its Mutants—BL21 (DE3) was transformed with the pET28a recombinant plasmids containing ycleuS and its mutants, and these cells were used to produce ycLeuRS protein along with various mutant proteins. The enzymes were purified by nickel-nitrilotriacetic acid affinity chromatography, as described previously (27). Protein concentration was determined by using the Bradford method (28).

Determination of the Aminoacylation, ATP-PPi Exchange, and Hydrolytic Editing Activity—The aminoacylation activities of ycLeuRS or its various mutants were determined at 30 °C in a reaction mixture containing 60 mM Tris-HCl (pH 7.5), 10 mM MgCl\textsubscript{2}, 2 mM dithiothreitol, 4 mM ATP, 20 μM [L-14C]leucine, 1 mg/ml yeast total tRNA, and 5 mM enzyme.

The kinetics of ycLeuRS in an ATP-PPi exchange reaction were determined at 30 °C in the presence of 0.02–0.2 mM Leu and 0.01–1 mM Nva and 1–125 mM Ile, as described previously (7). The reaction mixture contained 60 mM Tris-HCl (pH 7.5), 10 mM MgCl\textsubscript{2}, 2 mM dithiothreitol, 4 mM ATP, and 2 mM [32P]pyrophosphate (10 cpm/pmole). The reaction was initiated with 50 nM enzyme. Misaminoacylation assays were carried out using the same procedure as the aminoacylation assay except that 20 μM [1-3H]Ile (30 Ci/mmol) and 1 μM enzyme were used (7).

The charging capacity of the yctRNA\textsuperscript{L} transcript by ycLeuRS cannot be detected. However, ycLeuRS could charge AatRNA\textsuperscript{L} and hydrolyze misacylated Ile-AatRNA\textsuperscript{Leu} efficiently. Thus, we used Ile-AatRNA\textsuperscript{L} as the substrate in the hydrolytic editing assay. Ile-AatRNA\textsuperscript{L} was obtained by charging AatRNA\textsuperscript{L} with Ile by catalyzed by AaLeuRS D373A (a post-editing defective mutant enzyme), which was purified as described previously (8). The hydrolytic editing activity of ycLeuRS was measured at 30 °C in 60 mM Tris-HCl (pH 7.5), 10 mM MgCl\textsubscript{2}, 2 mM dithiothreitol, and 0.5 μM [3H]Ile-AatRNA\textsuperscript{L}. The reaction was initiated with 2.5 nM of each enzyme. At appropriate time intervals, aliquots were quenched on Whatman 3MM filter pads and precipitated with 5% trichloroacetic acid. The radioactive counts were measured in a scintillation counter, as described.
previously (8). All values represent the average from three experiments.

**Generation of ycleuS Null Strain**—The 5′ 556-bp and the 3′ 548-bp DNA fragments of ycleuS were amplified by PCR and digested by EcoRI-BamHI and BamHI-XbaI, respectively. The 1.8-kb HIS3 DNA fragment was amplified by PCR from the YAC4 plasmid and digested by BamHI. These three fragments were ligated and inserted into pUC19 using the EcoRI-XbaI sites to produce pUC19-ΔycleuS-HIS3. In pUC19-ΔycleuS-HIS3, a 2.166-kb fragment from ycleuS that encodes the middle 722 amino acid residues (Ile-186 through Val-908) of ycleuS was replaced by HIS3 and ycleuS was disrupted by the HIS3 marker gene. The linear 2.8-kb ΔycleuS-HIS3 sequence cleaved by EcoRI and XbaI from pUC19-ΔycleuS-HIS3 was transformed into the YAMB4 yeast strain. His+ colonies were selected and induced on sporulation plates, tetrads were dissected, and then haploids were plated on YPD (1% yeast extract, 2% peptone, 2% glucose) plates. Two spores were dissected, and then haploids were selected and induced on sporulation plates, tetrads were dissected, and then haploids were plated on YPD (1% yeast extract, 2% peptone, 2% glucose) plates. Two spores were unable to grow, whereas the other two growing spores were His+. When a rescue plasmid encoding leuS (pAL5: Ura+, Ade+, and leuS+) was transformed, the disrupted diploid produced four viable spores after segregation. Dissection showed four viable haploids per tetrad, a result of the complementation by the plasmid-encoded ycLeuRS. Two of these were His+ and could grow on 5-fluoroorotic acid (5-FOA) medium, whereas the other two were His+ and 5-FOA-sensitive (5-FOA+). We selected the His+ and 5-FOA+ haploids and confirmed the integration event by PCR analysis of the genomic DNA. The resulting YAMB4 haploid strain that was disrupted for leuS was designated ScΔleuS.

**In Vivo Complementation Assay**—The carrier plasmid p414TEF with a tef promoter (TRP selection marker gene) allows a moderate level of constitutive gene expression (29). The plasmids carrying the genes encoding ycLeuRS, hclLeuRS, and EcLeuRS were transformed separately into the ScΔleuS strain. Transformants were grown at 30 °C on a plate with synthetic defined (SD) medium minus tryptophan (SD-TRp−). In 2–3 days, colonies formed and were further analyzed for the compensation phenotype. By culturing in liquid SD-TRp− media, cells were grown to an A600 = 0.1. And then 5 μl of yeast culture was dropped to 5-FOA plates without tryptophan and with uracil to allow the loss of the pAL5 maintenance plasmid. The growth of the yeast cells was then observed.

**Measurement of the Growth Rate**—Knock-out yeast cells containing the different rescuing plasmids p414TEF-leuS were grown at 30 °C in liquid SD-TRp− media. Each culture was diluted in liquid SD-TRp− media to an initial A600 = 0.1. The diluted culture was shakered at 30 °C. A parallel assay in which the medium was supplemented with various concentrations of Nva, 1 mM Ile, or 1.25 μg/ml AN2690 was performed separately. The A600 value was measured at various intervals, and the growth rate was calculated according to the logistic growth equation (14).

**RESULTS**

**Construction of Recombinant Plasmids Harboring the ycleuS Mutants**—Alignment of the editing domain sequence from prokaryotic, archael, and eukaryotic LeuRSs shows that only the “T-rich,” “GTG,” and “editing catalytic” regions are well conserved (Fig. 1A). We define three types of residues based on conservation: 1) absolutely conserved residues through all species, e.g., Thr-319 and Asp-419; 2) conserved residues in the eukaryotic cytoplasmic LeuRSs from yeast to human, e.g., Thr-347 and Thr-410; and 3) conserved residues of each kingdom, e.g., Lys-404, Ser-416, and Asp-418. Although the equivalent residues of Thr-319 and Thr-410 in prokaryotic LeuRS (10) and Asp-419 in ycLeuRS (9) have been studied before, the in vivo effects of these mutations remain unclear. Thr-347 is located in the large peptidic region between the T-rich and GTG regions. This region is poorly conserved in prokaryotic LeuRS, and little information is available on its role. Residues Lys-404 and Asp-418 have not yet been investigated in LeuRS, whereas the homologue of Asp-416 in prokaryotic LeuRS has been shown to have no effect on editing (9).

As shown in Fig. 1A, the aforementioned seven residues were selected for mutagenesis to study their effects on the editing function of ycLeuRS. A diagram of the homologous structure of the editing domain of *Thermus thermophilus* LeuRS (TrLeuRS), in which the mutated residues are highlighted, is provided (Fig. 1B). Recombinant plasmids containing ycleuS single-point mutations at these seven positions were constructed to assay the aminocytlation and editing properties (see “Experimental Procedures”).

**Construction of the S. cerevisiae leuS Null Allele Strain ScΔleuS and Compensation of ScΔleuS with LeuRSs from Various Species**—To test the enzymatic activity of ycLeuRS mutants in vivo, a knock-out *S. cerevisiae* strain for leuS, ScΔleuS, was constructed. ScΔleuS could not survive without the rescuing plasmid pAL5, showing that leuS is an essential gene. Human cytoplasmic LeuRS could rescue ScΔleuS lethality but not *E. coli* LeuRS (Fig. 2A). This suggests that human enzyme hclLeuRS can charge the yeast cytoplasmic tRNALeu in vivo, whereas prokaryotic EcLeuRS cannot cross the species barrier of recognition.

**Yeast Strains Harboring yclLeuRS and the Enzymes Mutated in the Editing Domain Grow at the Same Rate in the Absence of Excess Leu Analogue**—Native ycLeuRS and its seven mutated derivatives were overproduced in *E. coli* and purified to 90% homogeneity, as assessed by SDS-PAGE (data not shown). The aminocytlation of yctRNALeu by the eight proteins was assayed and was not found to be obviously different (Fig. 3A).

The ScΔleuS yeast strain was transformed with plasmids expressing each of the wild-type or mutant ycLeuRS enzymes. All the mutants were able to rescue the knock-out strain as shown by the ability of these plasmids to substitute the rescuing plasmid pAL5 on 5-FOA plates (data not shown). The growth rates of the 8 ScΔleuS strains were monitored in liquid SD-TRp−. All were comparable to the native one (Fig. 3B), suggesting that the mutation of these residues did not impair significantly the aminocytlation properties of LeuRS, at least under normal growth condition without excess Leu analogues (Ile, Met, Val, and Nva, etc.).

**Mischarging Capacity with Ile and Deacylation Activity of Mutant Enzymes in Vitro**—To investigate the editing activity of ycLeuRS mutants, we examined the accumulation of mischarged Ile-tRNAβLeu in vitro. Among the seven mutants, only
ycLeuRS-D419A and ycLeuRS-T319E yielded mischarged Ile-tRNALeu (Fig. 4A); the other five mutants did not noticeably mischarge tRNALeu with Ile (data not shown). Decylation of Aale-tRNALeu by ycLeuRS and its seven mutants was also studied (Fig. 4B). Aale-tRNALeu was rapidly hydrolyzed by ycLeuRS; however, the post-transfer editing activity of ycLeuRS-D419A or ycLeuRS-T319E was completely abolished, while the deacylation ability was impaired to various degrees with the five other mutants.

ycLeuRS Misactivates Nva Efficiently—The kinetics of ycLeuRS for Leu, Nva, and Ile in the ATP-PP_i exchange reaction were assayed. Nva was efficiently misactivated with a catalytic efficiency decreased only 105-fold (Table 1). Ile was also misactivated by ycLeuRS, but the catalytic efficiency for Ile was 69-fold lower than that for Nva (Table 1). Because Nva was misactivated more efficiently than Ile, it was used in the following in vivo experiments.

Effect of Exogenous Nva on the Growth Rate of ScΔleuS Carrying the Editing-defective LeuRS Mutant—Under physiological conditions, yeast cells are not exposed to excess of noncognate amino acids. Thus weak mischarging of tRNA may mask the toxicity of possible editing defects to cells. To circumvent this problem, we measured the growth rates of the eight strains in liquid SD-Trp^- media containing a low concentration of Leu (0.51 mM) and a large excess of noncognate amino acids Nva (10 mM). Under these growth conditions we were expecting to increase the amount of noncognate amino acid Nva in the cytoplasm.

The growth rate of ScΔleuS containing the gene encoding wild-type ycLeuRS cultured with 10 mM Nva was decreased by 35% in comparison to that without Nva (Figs. 3B and 4C). This indicates that ycLeuRS could not completely prevent the mischarging of Nva in vivo due to the highly efficient misacti-
vation with Nva. The growth rate of the strains containing LeuRS-T319E or LeuRS-D419A mutant enzymes was severely inhibited (13% or 34% of the growth rate of the Sc/hr004/leuS/ycLeuRS strain) compared with wild-type ycLeuRS. These data show that the catalytic editing pocket composed of the T-rich region and hydrolytic Asp residue is crucial for cell viability in the presence of Nva. Substitution of the semi-conserved residues Lys-404, Ser-416, and Asp-418 with their equivalent residues from prokaryotic LeuRS reduced the growth rate to 45%, 62%, and 66% of the wild-type growth rate, respectively (Fig. 4C). The last two mutants T347A and T410A exhibited a more severely decreased growth rate of 51% and 40% of the wild-type strain, respectively (Fig. 4C). In summary, the data show that a toxic effect is clearly observable in the presence of an excess of noncognate amino acid. This contrasts with the absence of effect on the growth rate in a standard culture medium and suggests that editing is really crucial in extreme growth conditions exhibiting elevated concentrations of noncognate amino acid analogues.

We also tested the growth of all the editing-deficient strains at the low concentration of 1 mM Nva. For the crucial editing mutants such as T319E and D419A, both yeast strains displayed severe growth inhibition (data not shown). The other editing mutant yeast strains showed less conspicuous but still significant growth inhibition (data not shown). Further, we studied

FIGURE 1. Editing mutations lie in the editing sites of ycLeuRS. A, the alignments of the sequences for the editing sites of LeuRSs, including the T-rich region, GTG region, and catalytic region, from various organisms are shown. Abbreviations: 1) prokaryotic organisms: Aa, Aquifex aeolicus; Ec, E. coli; Tt, Thermus thermophilus; 2) archaeal organisms: Ph, P. horikoshii; Pa, Pyrococcus abyssi; and 3) eukaryotic organisms: Ce, Caenorhabditis elegans; Dm, Drosophila melanogaster; Sc, S. cerevisiae; Hs, Homo sapiens. All the mutants studied here are highlighted. B, diagram of a tube model of the editing domain of TtLeuRS with the AMP-AN2690 adduct showing selected ycLeuRS homologue residues mutated in this study (25). The editing domain of LeuRS is colored in green. The mutated residues are colored in yellow. The boron atom is colored in orange, the fluorine atom is in aqua, the oxygens are in red, the nitrogens are in blue, and the carbons are in gray.

FIGURE 2. hcLeuRS can rescue ScΔleuS and possesses editing activity in vivo. A, hcLeuRS can rescue S. cerevisiae leuS knock-out strain ScΔleuS. B, effects of Nva or AN2690 on the growth of the compensated strain ScΔleuS bearing editing-defective mutant hcLeuRS D399K. Each culture was diluted to initial A000 = 0.1 in SD-Trp medium with 10 mM Nva or 1.25 μg/ml AN2690. Absorbance was measured at 600 nm after growth for 12 h.

FIGURE 3. Aminoacylation of tRNALeu by ycLeuRS mutant enzymes and the growth rates of ycLeuRS mutant strains under normal conditions. A, ycLeuRS mutant enzymes (5 μl) efficiently charge yctRNALeu from yeast total tRNA with leucine at rates comparable to that of wild-type ycLeuRS. All values represent the average from three independent experiments. B, growth rates of ycLeuRS mutant strains in SD-Trp media. Error bars indicate the standard deviation of the growth rates from three replicate experiments.
the growth of the strain carrying the severe D419A mutation in the presence of a modest excess of noncognate amino acid Ile (1 mM excess compared with no addition of Ile in the standard SD-Trp⁻ media). Although Ile is involved in protein biosynthesis as a natural amino acid, we could observe a significant inhibitory effect on the growth rate of the editing-defect strain bearing D419A mutation (data not shown).

The Yeast Knock-out Strain Can Be Used to Study the Binding of AN2690—A recently developed antibiotic, AN2690 (see Fig. 5A), specifically targets the editing active site of LeuRS (25). AN2690 with tRNA⁷Leu forms a stable adduct in the editing pocket of ycLeuRS and thus prevents the catalytic turnover and the synthesis of Leu-tRNA⁷Leu (25). The LeuRS mutants T319E, D419A, K404Y, S416D, and D418R were significantly resistant to AN2690 as shown by the normal growth rates of the yeast strains compared with wild-type ycLeuRS (Fig. 5B). This shows that the substitutions of these residues may hinder the binding of AN2690 to the editing site (Fig. 5). On the opposite, the yeast strains carrying the T347A and T410A mutant enzymes were sensitive to AN2690 as the wild-type LeuRS, suggesting that they still bind the compound in their editing site (Fig. 5B).

In one additional assay, the human cytoplasmic enzyme hcLeuRS was tested for its ability to rescue the yeast strain in the presence of AN2690. hcLeuRS was able to rescue the yeast knock-out strain in the standard growth medium. However, in the presence of AN2690 the strain growth was severely impaired (Fig. 2B). This shows that the yeast assay system can also be used to study the binding properties of AN2690 by hcLeuRS. Therefore, we mutated the invariant Asp residue from the editing site of hcLeuRS, namely hcD399K, and showed that this mutant is resistant to AN2690, and additionally more sensitive to Nva inhibition (Fig. 2B). This confirms that the invariant Asp-399

**FIGURE 4.** Mutant ycLeuRS enzymes mediated mischarging of tRNA⁷Leu, declined deacylation activity, and weakened cell viability. A, mischarging of yctRNA⁷Leu from yeast total tRNA with isoleucine by 1 μM enzyme. Wild-type ycLeuRS does not accumulate mischarged tRNA⁷Leu. Editing-defective ycLeuRS-D419A and ycLeuRS-T319E enzymes can mischarge tRNA⁷Leu with isoleucine. B, deacylation of 0.5 μM preformed [³H]Ile-tRNA⁷Leu by 2.5 nM enzyme. The wild-type ycLeuRS enzyme and various mutant enzymes were assayed. All values represent the average from three experiments. C, growth rates of ycLeuRS mutant strains in SD-Trp⁻ media supplemented with 10 mM Nva. Error bars indicate the standard deviation of the growth rates from three replicate experiments.
residue from hcLeuRS also plays a pivotal function in the editing reaction catalyzed by the human enzyme and shows that the yeast knock-out strain can be used in drug design studies.

Editing Activity Is Dispensable for Yeast Cell Viability Except When Subjected to Non-physiological Stresses—To further elucidate the importance of CP1 editing domain and the editing activity to the aminoacylation activity and cell viability, we constructed two mutants, \( \Delta \text{CP1} \) (deletion of the entire CP1 domain Gly-270 through Val-530 according to the structure of homologous \( \text{Pyrococcus horikoshii} \) LeuRS (12)) and \( \Delta \text{T-rich} \) (deletion of the T-rich region Thr-314 through Thr-319), and tested their growth in normal conditions and under the stress induced by an excess of noncognate amino acid. We found that the \( \Delta \text{CP1} \) mutant could not rescue the LeuRS knock-out strain (Fig. 6A), suggesting that the deletion may prevent the correct folding of the enzyme or its synthetic activity. On the other hand, the \( \Delta \text{T-rich} \) mutant could rescue the knock-out strain with a growth rate nearly similar to the wild-type under usual growth conditions in SD-Trp medium (Fig. 6A and B). Theoretically, this mutant should lose its editing capacity completely. We here showed that the growth rate of the strain harboring this mutation is reduced in the presence of a moderate excess of Nva or Ile (Fig. 6B and data not shown). We conclude that the editing activity of ycLeuRS is dispensable for the yeast viability under normal growth conditions but is necessary in the presence of excess of related noncognate amino acids.

DISCUSSION

A number of \textit{in vitro} studies have been performed on the editing properties of ycLeuRS (9, 24). However, few studies have demonstrated the \textit{in vivo} importance of the editing reaction. The first \textit{in vivo} study on the editing reactions catalyzed by eukaryotic aaRS was performed on yeast cytoplasmic methionyl-tRNA synthetase (MetRS) (30). This enzyme edits homocysteine by converting it into the homocysteine thiolactone (30). Although several aaRSs (MetRS, IleRS, ValRS, and LeuRS) catalyze the same reaction \textit{in vitro}, only MetRS is involved in thiolactone synthesis in living cells (31). These observations

\begin{table}[h]
\centering
\caption{Kinetic constants of activation and misactivation by ycLeuRS at 30 °C}
\begin{tabularx}{\textwidth}{|c|c|c|c|c|}
\hline
 & \( K_m \) & \( k_{cat} \) & \( k_{cat}/K_m \) & \( k_{cat}/K_m \) (relative) \\
\hline
\text{Leu} & 4.41 ± 0.83 & 37.5 ± 0.05 & 8.5 & 1 \\
\text{Nva} & 167 ± 4.9 & 13.5 ± 2.7 & 0.081 & 9.53e-3 \\
\text{Ile} & 8713 ± 1242 & 10.2 ± 1.6 & 1.17e-3 & 1.38e-4 \\
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\end{table}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure5}
\caption{Effect of AN2690 on the growth of ycLeuRS mutant strains. A, the chemical structure of AN2690 (25). B, the growth rates of ycLeuRS mutant strains in SD-Trp medium supplemented with 1.25 \( \mu \)g/ml AN2690. Error bars indicate the standard deviation of the growth rates from three replicate experiments.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure6}
\caption{Nva dose-dependent viability decrease of \( \text{ScLeuS} \) with complementation assay. A, rescue of null \( \text{ycLeuS} \) phenotype of yeast strain \( \text{ScLeuS} \). \( \text{ScLeuS} \) was transformed with either an empty plasmid p414TEF, p414TEF-ycLeuRS, p414TEF-ycLeuRS-\( \Delta \text{CP1} \), or p414TEF-ycLeuRS-\( \Delta \text{T-rich} \). Transformants were streaked on SD-Trp medium plate and grown at 30 °C for 3 days. B, Nva dose-dependent viability decrease of \( \text{ScLeuS} \) that is dependent on wild-type or editing-defective \( \Delta \text{T-rich} \) mutant LeuRS. Each culture of \( \text{ScLeuS} \) harboring wild-type (black bars) or \( \Delta \text{T-rich} \) LeuRS (gray bars) was diluted to an initial \( A_{600} = 0.1 \) in SD-Trp media with increasing concentrations of Nva. Absorbance readings were measured at 600 nm after growth for 30 h.}
\end{figure}
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indicate that some specific editing reactions detected in vitro may not in fact occur or be needed in vivo.

In this study, we constructed a yeast knock-out strain to study the editing reactions of cytoplasmic LeuRS. Various knock-out systems have been designed and constructed to study the structure-function relationships of aaRSs in E. coli and yeast (14–20, 32–38); however, our system is the first to investigate the editing function of a specific yeast cytoplasmic aaRS. Other knock-out systems used to study editing have focused on E. coli (14–19) and the yeast mitochondrion (20). Mutation analysis of the yeast mitochondrial LeuRS showed that the enzyme maintains an editing active site that is competent in post-transfer editing. However, compensation assays in which the editing-deficient ymLeuRS was used indicated that neither mitochondrial function nor cell viability was affected by the presence of high levels of noncognate amino acids (20).

Compared with previous approaches, our novel assay system based on the ScΔleuS strain has several advantages. First, with this in vivo system, the editing efficiency of ycLeuRS and hcLeuRS can be directly determined by measuring yeast cell growth. Another advantage afforded by the in vivo nature of this system is that it obviates the need for enzyme purification or tRNA synthesis. Second, compared with mitochondrial in vivo assays, this ycLeuRS system is sensitive to the stress induced by elevated concentrations of leucine competitors in the growth medium, such as Nva, Ile, or the AN2690 antibiotic. Thus, this assay can be used to test the effect of compounds that potentially target aminoaclylation or editing active sites in LeuRS in yeast cells. By using this system, three residues, Lys-404, Asp-418, and Asp-419, that induce resistance against AN2690 were identified for the first time (25).

The Editing Activity of ycLeuRS Is Essential for Maintaining Cell Viability When the Concentration of Leucine Competitors Is Increased—Because branched-chain amino acids are imported through an active transport pathway and accumulate in the cytoplasm, it is possible to measure the impact of mutations on cell viability. It was recently shown that both standard and nonstandard noncognate amino acids are cytotoxic to E. coli strain KL231, which harbors the editing-defective EcLeuRS-TT/NV (19). The growth of this editing-defective strain was inhibited when cultured in liquid minimal media supplemented with 4 mM Ile and was inhibited to a considerably greater extent when cultured in liquid minimal media containing 4 mM Nva (19).

In this study, we show that in vitro, ycLeuRS misactivates Ile at a rate that is lower than the general error level of translation (1:3000) (39). This suggests that the editing of Ile may not be essential for cell viability. However, in vivo, the growth of the yeast knock-out strain rescued by the editing-defective mutant ycLeuRS-ΔT-rich was inhibited when 1 mM Ile was added to the SD-Trp+ growth medium (data not shown). This result suggests that a moderate increase in Ile can induce cytotoxicity in yeast cells despite the very low rate of misactivation rate of Ile. To validate these data obtained with the deletion mutant ΔT-rich, we repeated the growth experiments with the editing-defective mutant D419A. The growth of D419A in the presence of 1 mM Ile was comparable to that of the deletion mutant ΔT-rich (data not shown). Taken together, these data suggest that the editing of Ile is more crucial than previously thought. Low misactivation rates can lead to the synthesis of significant amounts of aberrant proteins, which can be deleterious for cell viability.

Nva Is an Efficient Competitor of Leucine Incorporation—Nva differs from Leu by a single -CH3 group. The structure of Nva is thus very similar to that of Leu. Nva can fit into the synthetic site of ycLeuRS. Our data showed that the rate of Nva misactivation by ycLeuRS was only 105-fold lower than that of Leu (Table 1). In addition, we showed that editing defects are highly toxic to cells in vivo in the presence of Nva. The growth of the yeast leuS knock-out strain bearing seven editing-defective mutations was severely inhibited by 10 mM Nva (Fig. 4C). The growth of mutants T319E and D419A, which were completely defective in post-transfer editing in vitro (Fig. 4B), was more severely inhibited. The growth of other mutants was significantly inhibited despite modest defects in in vitro editing activity (Fig. 4B). The cytotoxic effect of editing defects was also clearly observed when the growth medium contained only 1 mM Nva (data not shown). The cytotoxic effect on the deletion mutant ΔT-rich was also observed when the medium contained as little as 0.25 mM Nva (Fig. 6B). These results suggest that the addition of a small amount of Nva to the growth medium is deleterious for cells expressing LeuRS defective in editing activity.

According to previous reports, the physiological concentrations of Leu and Ile in the yeast cytoplasm vary from 0.06 to 0.48 mM and from 0.025 to 0.5 mM, respectively (40). The physiological concentration of Nva in yeast cells has not yet been measured, probably because this product is usually not found in the cells and appears as a byproduct of the synthetic pathways of the branched-chain amino acids isoleucine and valine (31). In addition, it has been reported that an E. coli strain can release and accumulate up to 2.4 mM of Nva in the growth medium when overexpressing the recombinant bovine somatotropin, a naturally leucine-rich protein (41). This shows that, under certain growth conditions, cells can produce non-negligible concentrations of Nva or other compounds that can compete with cognate amino acids for tRNA charging and subsequent incorporation into proteins.

The Crucial Residues of Editing Sites in Bacterial, Yeast, and Human LeuRSs Are Functionally Equivalent—Our study shows that the Thr residue from the T-rich region and the invariant Asp residue from the catalytic region (Fig. 1) of LeuRS are essential for both in vivo and in vitro editing activity. The yeast LeuRS residues ycT319 and ycD419 are equivalent to ecT252 and ecD345 found in E. coli LeuRS (9–11, 20). It has been shown that the substitution ecT252E makes the E. coli strain KL231 sensitive to α-aminobutyrate when a halo assay was performed (10). The residue ecT252 controls the size of the binding pocket of the editing site, allowing the entry of a wide array of smaller amino acids while preventing the binding of the cognate leucine (11). Similarly, E. coli strains with a mutated ecD345 LeuRS residue exhibited inhibited growth in the presence of 10 mM Ile (20). ecD345 forms a hydrogen bond with the amino moiety of the bound amino acid (9). In this study, we show that in ycLeuRS, the residues ycT319 and ycD419 are equally critical for editing in vitro. Yeast cells with mutations in
these residues show growth phenotypes very similar to those exhibited by E. coli LeuRS mutants. This suggests that the two residues are functionally equivalent in both organisms.

The ycleuS knock-out strain can be used as a tool to assay the in vivo effect of AN2690, an inhibitor of LeuRS that targets the editing site and inhibits the aminoacylation reaction. Using this yeast strain, we showed that ycLeuRS and hcLeuRS were inhibited by the AN2690 compound, suggesting that both enzymes were able to bind AN2690 at their editing sites. The strain enabled human mutated LeuRS to be assayed in vivo and also enabled comparison of the effects of the equivalent mutations in human and yeast cytoplasmic LeuRS. For instance, when the invariant Asp residue from the catalytic region was mutated in yeast or in human LeuRS (ycD419 and hcD399, respectively), both enzymes became resistant to AN2690. Simultaneously, both mutated enzymes induced cytoxic effects in the presence of Nva, suggesting a concomitant loss of editing capacity. The similar responses of bacterial, yeast, and human LeuRSs to noncognate amino acids and AN2690 suggest that the topology of the editing catalytic site, including the critical residues from the T-rich and catalytic regions, has been conserved during evolution.

Semi-conserved Residues Involved in Editing Capacity of ycLeuRS Are Modulated during Evolution—Our in vivo data indicate that semi-conserved residues that are unique to ycLeuRS contribute to its editing function. ycT347 is located within a unique peptide at a distance from the editing active site. It exhibits little similarity with its corresponding residue in prokaryotic and archaeal LeuRSs. The ycT347A mutation does not confer resistance to AN2690, because the residue is distant from the editing active site bound by AN2690 (Fig. 1B). However, it decreases both in vitro and in vivo editing capacity, suggesting that this residue might exert subtle long distance effects on editing.

Another residue, ycT410, is at a site homologous to Met-338 of TtLeuRS. Our data showed that the alteration of the side chain by T410A mutation reduced the editing activity. It seems that ycT410 plays a role in binding and positioning the editing substrates for hydrolysis similar to ttM338 (9). Interestingly, ScΔleuS strains bearing genes encoding ycT410A do not exhibit resistance to AN2690, suggesting that the side-chain alteration does not disrupt the binding of AN2690 to ycLeuRS.

The Lys-404, Ser-416, and Asp-418 residues are close to the GTG and catalytic region. The homologous site of ycLys-404 has never been investigated in the LeuRS system. We found that this residue could modulate the editing efficiency. Previous work demonstrated that Asp-344 of TtLeuRS, which is a homologue of ycS416, has little effect on the editing reaction in vitro (9). However, the substitution of ycS416 was obviously sensitive to Nva, suggesting that it is still important in editing in vivo. Neither ycAsp-418 nor its homologue has been investigated before. We show that it contributes to optimal editing capacity in vivo despite its substitution by an Arg or Phe in prokaryotic or archaeal LeuRS, respectively. The homologous sites of the above three residues do not seem to directly perform the catalytic reaction in view of the structure of TtLeuRS complexed with the analogue of the post-transfer editing substrate (9). Because the structure of neither apo-ycLeuRS nor its complex with tRNA_Leu has been reported, we can only suppose that these residues may facilitate the formation of suble configurations for optimal editing activities of ycLeuRS.

Concluding Remarks—aaRSs play a crucial role in the selection of amino acids for protein synthesis. However, the active sites of several of these enzymes lack the ability to discriminate between closely similar amino acids at a level sufficient to explain the high accuracy of the genetic code. To correct the errors resulting from the misactivation of near-cognate amino acids, aaRSs have evolved editing mechanisms that take place in a separate active site. Although discovered 40 years ago, and extensively studied in vitro, the cellular importance of editing in restricting the genetic code to 20 amino acids has only been demonstrated recently in E. coli (15). It is now accepted that editing may have played a central role in the establishment and maintenance of the genetic code, and editing defects are rarely detected in living organisms. An editing defect has recently been reported in mammalian cells. The mild editing-defective mutation A734E found in mouse cytoplasmic AlaRS causes cerebellar Purkinje cell loss and ataxia through the accumulation of misfolded proteins in neurons (42). This disease highlights the importance of optimal editing activity in living organisms. It also offers insights into new targets for drug design directed against the editing site of aaRSs.

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Yeast Strain Reveals Residues for LeuRS Editing