Identification of Lethal Mutations in Yeast Threonyl-tRNA Synthetase Revealing Critical Residues in Its Human Homolog*

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Background: Eukaryotic ThrRSs exhibit four structural domains similar to bacterial ThrRSs and evolve a eukaryote-specific N-terminal extension domain.

Results: Essential roles of 12 crucial residues in aminoacylation and editing reactions were revealed.

Conclusion: The identified critical residues affected activities of ThrRS in various manners.

Significance: We elucidated the synthetic and editing functions of selected residues and confirmed the functional consistency between yeast and human ThrRSs.

Aminoacyl-tRNA synthetases (aaRSs) are a group of ancient enzymes catalyzing aminoacylation and editing reactions for protein biosynthesis. Increasing evidence suggests that these critical enzymes are often associated with mammalian disorders. Therefore, complete determination of the enzymes functions is essential for informed diagnosis and treatment. Here, we show that a yeast knock-out strain for the threonyl-tRNA synthetase (ThrRS) gene is an excellent platform for such an investigation. Saccharomyces cerevisiae ThrRS has a unique modular structure containing four structural domains and a eukaryote-specific N-terminal extension. Using randomly mutated libraries of the ThrRS gene (thrS) and a genetic screen, a set of loss-of-function mutants were identified. The mutations affected the synthetic and editing activities and influenced the diimer interface. The results also highlighted the role of the N-terminal extension for enzymatic activity and protein stability. To gain insights into the pathological mechanisms induced by mutated aaRSs, we systematically introduced the loss-of-function mutations into the human cytoplasmic ThrRS gene. All mutations induced similar detrimental effects, showing that the yeast model could be used to study pathology-associated point mutations in mammalian aaRSs.

During protein biosynthesis, aminoacyl-tRNA synthetases (aaRSs) must attach the correct amino acid to the corresponding tRNA molecule (1). This process supplies the ribosome with the aminoacyl-tRNAs that are essential for protein synthesis in all cells. The aaRSs are critical in ribosomal protein translation because each canonical aaRS accurately pairs its correct amino acid to the cognate tRNA isoaceptor(s). The attachment of amino acids to tRNAs by aaRSs is performed in two steps. The amino acid is first activated with ATP to form an activated aminoacyl adenylate and then transferred to the 3'-end of the tRNA (1). Generally, there are 20 different aaRSs in living organisms, and these enzymes can be divided into two classes (I and II) of 10 members each. The active site of class I aaRSs is based on a Rossmann fold containing two consensus sequences, HIGH and KMSKS (2), whereas that of class II aaRSs contains an antiparallel β-fold catalytic center with three motifs: motifs 1, 2, and 3 (3, 4).

Accurate aminoacylation of tRNA with its cognate amino acids by aaRSs is essential for quality control of protein synthesis, because a mischarged non-cognate amino acid may be incorporated into the nascent peptide chain after aminoacylation (1). Challenged by a large pool of proteinaceous amino acids and amino acid analogs, some aaRSs cannot discriminate accurately between cognate and non-cognate amino acids. For example, Ile is structurally similar to Leu, differing only by a branched methyl group, and can be activated by leucyl-tRNA synthetase (LeuRS, one specific aaRS is abbreviated as its three-letter amino acid code and “RS”) (5). To maintain translational fidelity, several aaRSs evolved proofreading (editing) mechanisms to hydrolyze either misactivated amino acids or misacylated tRNAs, which are termed pretransfer editing or post-transfer editing, respectively (6, 7). Editing activities catalyzed by aaRSs are essential for organisms to sustain normal metabolism. Editing dysfunction causes growth defects in bacteria (8) and mitochondrial abnormality in yeast (9). In the last decade, more and more disease-causing mutations have been found in the aaRS-coding genes. For example, a collection of mutations...
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Located at the aminoacylation domain of both GlyRS and TyrRS leads to Charcot-Marie-Tooth disease (10–12). A mutation located in the editing domain of AlaRS causes cerebellar ataxia (13).

Together with SerRS, AlaRS, GlyRS, ProRS, and HisRS, ThrRS belongs to the class Ia aaRSs and is a dimer (14, 15). _Escherichia coli_ ThrRS (EcThrRS) forms the dimeric core comprising the synthetic catalytic and C-terminal tRNA anticodon binding domain. Two extra domains on the N-terminal side of each monomer protrude outside the core, forming the editing domains of the dimer, which also provide a binding interface to the minor groove of the tRNA acceptor stem (14). In contrast to other synthetases, ThrRS only activates a small number of non-cognate amino acids, such as β-hydroxyornithine and Ser (16). A zinc ion found in the synthetic active site is implicated in Thr recognition and discrimination against non-cognate amino acids. The zinc atom imposes a strict structural geometry and controls the interacting distance with the amino acid substrate. With Thr, the side chain hydroxyl is at the ideal distance of 2.38 Å from the zinc atom. In the case of the isosteric amino acid Val, an obvious clash with one of its methyl groups would create steric hindrance alone and prevent its activation. In this way, ThrRS efficiently discriminates against amino acids possessing methyl groups but discriminates less well against amino acids with a hydroxyl group, such as Ser (17). Hydrolysis of the erroneously aminoacylated Ser occurs in the distant N2 editing domain by a mechanism of post-transfer editing ensuring faithful aminoacylation. In the editing site, two His residues (His<sup>73</sup> and His<sup>77</sup>) are responsible for hydrolyzing the misacylated Ser-tRNA<sub>Thr</sub> (17). In contrast, most archaean ThrRSs possess a unique N-terminal editing domain unrelated to the bacterial N2 domain (18, 19) but homologous to the D-aminoacyl-tRNA deacylase that removes D-amino acids from mischarged tRNAs. Moreover, several partenarchaeal species contain two genes to encode either the catalytic (ThrRS-cat) or editing (ThrRS-ed) enzymes. ThrRS-cat performs aminoacylation, and ThrRS-ed contains an editing domain to hydrolyze mischarged Ser-tRNA<sub>Thr</sub> in _trans_ (19, 20).

Theoretically, the molecular mass of a subunit in the dimeric ThrRS from _Saccharomyces cerevisiae_ (ScThrRS) is 84.5 kDa, comprising 734 amino acid residues. In terms of primary sequence, eukaryotic ThrRSs, such as _ScThrRS_ and _Homo sapiens_ cytoplasmic ThrRS (_HsThrRS_), resemble their bacterial counterparts. However, detailed analysis revealed several different characteristics between them. First, despite retaining crucial editing amino acid residues (such as His<sup>151</sup> and His<sup>155</sup> in ScThrRS, counterparts of His<sup>73</sup> and His<sup>77</sup> of EcThrRS) in eukaryotic ThrRSs, the periphery elements in both the N1 and N2 domains of eukaryotic ThrRSs display significant differences from their bacterial counterparts. Second, the most striking difference is the fusion of a unique eukaryotic ThrRSs-specific N-terminal extension (N-extension) whose relevance in aminoacylation, editing, or protein structure is not completely understood (Fig. 1, A and B). A previous study found that a partial deletion mutant at the N-extension of _ScThrRS_ (_ScThrRS_Δ40) affected catalytic parameters; however, the possibility of a major structural effect could not be ruled out (21). Furthermore, some conserved residues exist in the N-extension-containing ThrRSs, highlighting their potential role in protein function and/or structure. For example, an arginine residue (Arg<sup>52</sup> in _ScThrRS_) is absolutely conserved in the N-extension of eukaryotic ThrRSs (Fig. 1B), suggesting its potential critical role for eukaryotic N-extension/ThrRSs. Therefore, further detailed investigation of the effect of conserved Arg<sup>52</sup> on the enzymatic properties using various _in vivo_ and _in vitro_ methods is required.

Although the active site of bacterial ThrRS has been studied extensively by structural and functional approaches (14, 22, 23), little research has been done on eukaryotic ThrRSs. Here, we aimed to identify the functional features of _ScThrRS_ using a genetic tool that is useful for capturing important residues in the dynamic process of aminoacylation and editing (24). Instead of a rational targeting of residues on the protein, we constructed two randomly mutated ScThrRS libraries, a yeast _thrS_ random mutagenesis library (_thrS_ library) and an Arg<sup>52</sup>-specific mutagenesis library (Arg<sup>52</sup> library), to identify important amino acid residues for enzymatic activities and to characterize the function of Arg<sup>52</sup> in the N-extension domain, respectively. The two libraries were transformed into a yeast _thrS_ knock-out strain. By plasmid shuffling, we isolated and identified 12 lethal or growth-deficient mutants of _ScThrRS_. The selection procedure could map the critical residues and enzyme functions efficiently, because the 12 mutants were found in the four modules of _ScThrRS_, including the N-extension, N2, aminoacylation, and anticodon binding domains. We present a picture of the functions of several residues in the aminoacylation and editing reactions. The results also show the importance of the dimer interface for enzyme activity. Several mutants whose functional importance cannot be assessed from the structural data are selected, demonstrating the importance of this type of approach in the context of a structure-function relationship study. The essential role of the conserved Arg<sup>52</sup> of the N-extension in maintaining protein structure/function is described. Finally, we show that introducing the same mutation into _HsThrRS_ also leads to inactive proteins and exhibits a protein amount similar to that of the corresponding _ScThrRS_ mutant, indicating functional consistency of eukaryotic ThrRSs. This opens perspectives on further studies on the human enzyme and its potential derived mutants that cause various types of human diseases.

**EXPERIMENTAL PROCEDURES**

**Materials—**_L_ Thr, _L_ Ser, 5′-GMP, GTP, UTP, CTP, ATP, MgCl₂, Tris-HCl (pH 7.5), dithiorthio, isopropyl β-D-1-thio-galactopyranoside, bovine serum albumin (BSA), tetrasodium pyrophosphate, inorganic pyrophosphate, and activated charcoal were obtained from Sigma-Aldrich. [H]<sub>3</sub>Thr was from Biotrend Chemicals (Destin, FL). [35S]Ser and [α-<sup>32</sup>P]ATP were obtained from PerkinElmer Life Sciences. The gel extraction kit, plasmid extraction kit, and yeast plasmid extraction kit were purchased from TIANGEN Biotech (Beijing, China). The DNP mixture, KOD-Plus-Neo high fidelity DNA polymerase, and KOD-plus mutagenesis kit were purchased from TOYOBO (Osaka, Japan). 5-fluorooctacid (5-FOA), pyrophosphatase, T4 ligase, and restriction endonucleases were purchased from Thermo Fisher Scientific. Ni<sup>2+</sup>-nitritrotric acid Superflow was from Qiagen (Hilden, Germany). BioSune (Shanghai,
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China) synthesized the oligonucleotides. The yeast transformation kit was from Clontech. The protease inhibitor mixture was from Roche Applied Science. The mouse anti-His\(_6\) tag (M20001), anti-\(\beta\)-actin, anti-FLAG (M20028), and anti-c-Myc (M20002) antibodies were purchased from Abmart (Shanghai, China).

Construction of Libraries and Selection of ScThrRS Mutants—The construction of the yeast recipient strain with a disrupted \(thrS\) gene (Sc\(\Delta\)thrS) (BY4743, \(thr^+\), pRS426-Sc\(\Delta\)ThrRS) has been described in our laboratory (21). In this knock-out strain, the G418 resistance gene replaced the chromosomal \(thrS\) gene. The wild-type \(thrS\) gene was cloned into pRS426 (containing \(URA3\)) and p425TEF (containing \(LEI12\)) (25) to generate the rescuing plasmid pRS426-Sc\(\Delta\)ThrRS (\(thr^+, Ura^+\)) and the selection plasmid p425TEF-Sc\(\Delta\)ThrRS (\(thr^+, Ura^+\)), respectively. Two libraries based on the selection plasmid were constructed.

The first yeast \(thrS\) library was obtained by random mutagenesis of p425TEF-Sc\(\Delta\)ThrRS. Random mutagenesis can be achieved by several methods, such as error-prone PCR amplification, the use of \(E.\ coli\) mutator strain, or mutagenic agents (26, 27). We used hydroxyamine hydrochloride treatment of p425TEF- Sc\(\Delta\)ThrRS. Hydroxylamine causes C to T and G to A transitions by deamination, with a frequency of less than 0.3% (27). Briefly, p425TEF-Sc\(\Delta\)ThrRS (50 \(\mu\)g) was incubated in 2 ml of 0.8 M hydroxylamine hydrochloride, 50 mM sodium phosphate, 1 mM EDTA, pH 7.0, for 48 h at 37 °C. The DNA was then precipitated by ethanol at −20 °C overnight and recovered by centrifugation at 12,000 \(\times\) g for 30 min. DNA sequencing of 20 \(thrS\) genes from this library was employed to confirm the mutagenic efficiency and the quality of this library. Twelve genes were found to have a single mutation in their ORFs; thus, the mutation efficiency of this library was about 60%.

The second Arg\(^{\mathrm{52}}\) library was produced by PCR using oligonucleotide-directed site-specific mutagenesis to fully randomize residue Arg\(^{\mathrm{52}}\) of Sc\(\Delta\)ThrRS. The oligonucleotide for saturating mutagenesis was 5′-NNSATTGAAATGTTTGACAGATT-3′, where N represents an equimolar mixture of the four deoxyribonucleotides, and S represents an equimolar mixture of G and C (the reading frame of NNS decodes Arg\(^{\mathrm{52}}\) in the native enzyme). A conventional reverse primer (5′-TTTCTCTTGCATGAAAGTTGGTT-3′) was used. PCR amplification was performed using p425TEF-Sc\(\Delta\)ThrRS as the template, following the protocol of the KOD-plus mutagenesis kit. Theoretically, such an Arg\(^{\mathrm{52}}\) library should contain 32 variants (4 \(\times\) 4 \(\times\) 2) generating the 19 other amino acids, and the mutation efficiency should be nearly 100% because the p425TEF-Sc\(\Delta\)ThrRS template was digested by DpnI according to the KOD-plus mutagenesis kit. Indeed, DNA sequencing of 10 \(thrS\) genes from the Arg\(^{\mathrm{52}}\) library confirmed the mutagenesis efficiency; nine genes were found to contain a single mutation at position 52, suggesting that mutagenic efficiency was around 90%.

The \(thrS\) and Arg\(^{\mathrm{52}}\) libraries were introduced separately into the yeast strain Sc\(\Delta\)thrS, according to the Clontech yeast transformation manual, and transformants were selected on SD/Leu\(^-\)/Ura\(^-\) plates. Drop tests were subsequently performed on SD/Leu\(^-\)/Ura\(^-\) and SD/Leu\(^-\)/5-FOA plates. After incubation for 48–96 h, clones that grew very slowly or could not grow on the SD/Leu\(^-\)/5-FOA plate (5-FOA\(^+\)) and thus could not lose the rescuing \(thrS^+\) plasmid were chosen as candidates harboring inactive ThrRS genes. Mutated p425TEF- Sc\(\Delta\)ThrRS plasmids were recovered from the colonies that appeared on original SD/Leu\(^-\)/Ura\(^-\) plates, and the \(thrS\) genes were sequenced.

Subsequently, control wild-type \(thrS\) and mutated \(thrS\) genes were cloned into a modified p425TEF(His) vector, which allows expression of the mutated \(thrS\) genes with a C-terminal His tag, facilitating subsequent quantification of the protein amount. The resulting constructs were introduced into Sc\(\Delta\)thrS to confirm the phenotypes, eliminating any side effects of mutations occurring at other sites in the selection plasmid.

Preparation of Yeast Cell Lysates and Western Blot Assay—Clones sensitive to 5-FOA were grown on SD/Leu\(^-\)/Ura\(^-\) liquid medium. The yeast expressed the genes from both the rescue-plasmid (pRS426-Sc\(\Delta\)ThrRS (without His\(_6\) tag)) and the selection plasmid (p425TEF(His)-Sc\(\Delta\)ThrRS mutant (with His\(_6\) tag)). Western blot was performed with an antibody directed against the His\(_6\) tag to detect the amount of Sc\(\Delta\)ThrRS and its variants in vivo. Yeast cells were harvested by centrifugation at 3000 \(\times\) g for 5 min at 4 °C. Cells were resuspended in ice-cold lysis buffer containing 50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10% glycerol and lysed in a glass bead grinder at 4 °C for 20 min (28).

Proteins were separated by 10% SDS-PAGE gel electrophoresis and transferred to PVDF membranes. The membranes were blocked in PBS plus 0.05% tween 20 buffer containing 5% nonfat dry milk. Membranes were separately immunoblotted with either anti-His\(_6\) antibody or anti-GAPDH antibody. After incubation with the horseradish peroxidase-conjugated secondary antibody, the light emission was monitored using LAS4000 (FUJIFILM) and the SuperSignal West Pico Trial Kit (Thermo Fisher Scientific). Results were quantified using Multi Gauge version 3.0 software (FUJIFILM), and the amounts of Sc\(\Delta\)ThrRS or variants were normalized to the GAPDH amount. The amount of wild-type Sc\(\Delta\)ThrRS from the selection plasmid was designed as 100%.

Cloning and Expression of ScThrRS Gene and tRNA\(^{\text{Thr}}\) Transcription—pET22b-ScThrRS was constructed to obtain C-terminal His\(_6\)-tagged ThrRS in \(E.\ coli\) in our laboratory (21). Here, the mutated \(thrS\) genes (including point and deletion mutations) were constructed using the KOD-plus mutagenesis kit, and mutants were identified by DNA sequencing. Gene expression and protein purification of ScThrRS and its mutants were performed as previously reported (21). Transcripts of Sc\(\text{rRNA}^{\text{Thr}}\) (AGU, CGU, and UGU) were obtained by \textit{in vitro} T7 RNA polymerase run-off transcription, as described previously (21).

Measurements of Kinetic Parameters, Decacylation, and AMP Formation—\(k_m\) and \(k_{cat}\) values of Sc\(\Delta\)ThrRS for amino acid in the Thr activation reaction and for tRNA\(^{\text{Thr}}\) isoacceptors (including AGU, CGU, and UGU) in the aminocacylation reaction were determined as described previously (21).

Misaminoacylated Ser-tRNA\(^{\text{Thr}}\) (AGU) (Ser-tRNA\(^{\text{Thr}}\)) was obtained via mischarging with Ser by Sc\(\Delta\)ThrRS-H151A/H155A (21). The decacylation activities of Sc\(\Delta\)ThrRS variants for mis-
charged [14C]Ser-tRNAThr were measured as reported previously (21). Editing activities were also determined by measuring the AMP formation in the presence of the non-cognate Ser. AMP formation was monitored by thin-layer chromatography (TLC) plates according to previous studies (29, 30). 

Measurement of Equilibrium Dissociation Constants (Kd) for tRNA by Filter Binding Assays—The nitrocellulose filter binding method was used to monitor ScThrRS-[32P]tRNAThr complex formation (31). Nitrocellulose membranes (0.22 µm) were presoaked in washing buffer (50 mM potassium phosphate (pH 7.5), 100 mM NaCl, 0.2 mM EDTA, 10% glycerol, 1 mM KF, 0.02 mg/ml protease inhibitor mixture). Cell lysates were centrifuged at 12,000 × g for 15 min at 4 °C. To immunoprecipitate ScThrRS and its variants, the cell lysates were incubated overnight at 4 °C with anti-FLAG antibodies and then conjugated to agarose beads according to the Protein A/agarose manual (Abmart). Samples of whole cell lysates and immune complexes were separated by 10% SDS-PAGE and detected by Western blot using anti-FLAG or anti-c-Myc antibody.

Complementation Assays by HsThrRS Mutants—Wild-type HsThrRS was cloned into p425TEF(His). Mutations corresponding to selected mutants in ScThrRS were introduced into the HsThrRS gene by site-directed mutagenesis. The genes of mutated HsThrRSs were introduced into ScΔthrS and selected on SD/Leu−/Ura− plates. A single colony of each transformant was grown in liquid SD/Leu−/Ura− medium. To perform comparative drop tests, yeast cultures were diluted to 1 A600 and the same volume was dropped on SD/Leu−/5-FOA plates. The level of growth and complementation was roughly quantified on the basis of growth and complementation was roughly quantified as reported previously (32). The same method was used to introduce two nonsense mutations (Q704stop and -Q719stop, were found in the HsThrRS-G145D, -S308F, -G458D, -R461H, -G604E, -P627L, -G685D, -Q629stop, and -Q719stop, were found in the HsThrRS library harboring single or few mutations. The library was screened in the yeast thrS knock-out strain ScΔthrS for the ability of the mutated genes to replace the rescuing plasmid harboring a URA3 gene. 5000 colonies from the library were screened, and 31 clones were shown to be 5-FOA-sensitive and unable to lose the rescuing thrS+, Ura+ plasmid. The full-length ScThrRS gene of the 31 mutants was sequenced. All 31 mutations were found to be G→A or C→T transitions, which is consistent with the deamination properties of hydroxylamine (26). Among them, 20 of the selected genes carried nonsense mutations at variable positions. In addition, two lethal clones simultaneously harbored two mutations in their ORFs (M70K/Q629stop and W150stop/S201F). To determine which mutation was responsible for the lethal phenotype, we isolated the two missense mutations and two nonsense mutations by mutagenesis and tested them in ScΔthrS strain. The assay confirmed that mutations W150stop and Q629stop were lethal, whereas M70K and S201F were viable (data not shown). Thus, two nonsense mutations (W150stop and Q629stop) were responsible for the lethality of the two lethal clones. However, too many residues were truncated in these two nonsense mutations, with 585 and 106 residues, respectively, in W150stop and Q629stop. Therefore, no further investigation was performed on ScThrRS-W150stop or -Q629stop mutant. Interestingly, two lethal nonsense mutations (Q704stop and Q719stop) introduced stop codons that were only 31 and 16 residues from the C-terminus, suggesting an important function of the C-terminal domain of the enzyme. The remaining 11 mutations were missense mutations. Two of them (G145D and P627L) were identified twice, and the other seven (S308F, G356N, S457F, G458D, R461H, G604E, and G685D) were found only once. Therefore, 11 different mutants with growth-deficient or lethal phenotypes, including ScThrRS-G145D, -S308F, -G356N, -S457F, -G458D, -R461H, -G604E, -P627L, -G685D, -Q704stop, and -Q719stop, were found in the thrS library (Fig. 1A and Table 1). The 11 mutated thrS genes were cloned into vector p425TEF(His), and the constructs were re-introduced into ScΔthrS strain to confirm the lethal or growth-impairing phenotypes on SD/Leu−/5-FOA medium. The yeast ScΔthrS containing ScThrRS-G145D and -S457F was growth-deficient; the other mutations were lethal (Fig. 2A). Western blot showed that the genes of all mutants could express in yeast (Fig. 2B). Therefore, the inability of those mutants to support yeast growth was probably due to their reduced amount and/or their decreased enzymatic activities and not to an incapacity for mutated gene expression. The amounts of the mutant proteins were different, from 5% (G145D) to about 80% (S457F, G458D, and G685D) of the amount of the wild-type ScThrRS (Fig. 2C), which was probably caused by protein instability and/or degradation.
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A

B

C

FIGURE 1. Domain and sequence analysis of the selected residues of ScThrRS. A, schematic representation of the domain composition of ScThrRS, based on the structural modules of EcThrRS. 12 selected ScThrRS mutations were found in four domains of ScThrRS. Eight mutated residues were conserved in ScThrRS and HsThrRS, which are shown with dotted lines. B, sequence alignment of N-terminal peptidic extensions of various eukaryotic ThrRSs, showing the strict conservation of an arginine residue (highlighted by a box) at position 52 in ScThrRS. Multiple sequence analysis of several peptides containing the selected residues, which are highlighted by arrows; other parts of various ThrRSs are omitted for clarity. Ca, Candida albicans; Ce, Caenorhabditis elegans; Cf, Canis familiaris; Dr, Danio rerio; Gi, Giardia lamblia; Hs, Homo sapiens; Lm, Leishmania braziliensis; Lm, Leishmania major; Mm, Mus musculus; Sc, S. cerevisiae; Tb, Trypanosoma brucei; T. co, Trypanosoma congolense; Tv, Trypanosoma vivax.

TABLE 1
Selected ScThrRS mutations, their growth phenotypes, corresponding residues in EcThrRS, and location in the protein domains

| ScThrRS mutations | Growth phenotype | Corresponding EcThrRS residues | Protein domain |
|-------------------|------------------|--------------------------------|----------------|
| R52F              | Lethal           | No equivalent<sup>a</sup>      | N-extension    |
| M70K              | Normal           | Not analyzed<sup>a</sup>       |                |
| G145D             | Deficient        | Gly<sup>b</sup>                |                |
| S201F             | Normal           | Not analyzed<sup>a</sup>       |                |
| S308F             | Lethal           | Ala<sup>c</sup>                |                |
| G356N             | Lethal           | Gly<sup>d</sup>                | Aminoacylation |
| S457F             | Deficient        | His<sup>d</sup>                |                |
| G458D             | Lethal           | Gly<sup>d</sup>                |                |
| R641H             | Lethal           | Arg<sup>d</sup>                |                |
| G604E             | Lethal           | Gly<sup>e</sup>                |                |
| P627L             | Lethal           | Pro<sup>f</sup>                |                |
| G685D             | Lethal           | Gly<sup>g</sup>                |                |
| Q704Stop          | Lethal           | Asp<sup>h</sup>                |                |
| Q719Stop          | Lethal           | Glu<sup>i</sup>                |                |

<sup>a</sup> Arg<sup>a</sup> is located in the eukaryote-specific N-extension domain and thus has no equivalent in EcThrRS.

<sup>b</sup> M70K and S201F were not studied (see "Results").

According to the crystallographic structure of the EcThrRS-tRNA<sub>Thr</sub> complex (14), the mutations in the enzyme were localized. The mutations were clustered in the three main functional domains of ScThrRS. Two mutations, G145D and S308F, were located in the editing domain; six mutations (G356N, S457F, G458D, R461H, G604E, and P627L) were clustered in the aminoacylation active site; and three mutations (G685D, Q704Stop, and Q719Stop) were co-localized in the tRNA anticodon binding domain (Figs. 1A and 1D).

Mutations in the N2 Domain Decrease the Editing Activity—G145D and S308F were located in the second half of the editing domain (N2 domain). G145D induced a severe growth defect compared with native enzyme, whereas S308F induced complete lethality. When comparing the ThrRS sequences, it appeared that Gly<sup>145</sup> is only a semiconserved residue and is often an Ala residue in other eukaryotic ThrRSs. On the other hand, Ser<sup>308</sup> is a highly conserved residue (Fig. 1C). To explore the effect of the mutations on the catalytic properties of the enzyme, the genes of two mutated His-tagged proteins were overexpressed in E. coli, and the mutants were purified by Ni<sup>2+</sup> affinity chromatography. The Thr activation parameters of ScThrRS-G145D and -S308F showed unmodified <i>K<sub>m</i></sub> and a slight decrease in <i>k<sub>cat</sub></i> (2.6 ± 0.2 and 2.4 ± 0.2 s<sup>-1</sup>, respectively) compared with that of the wild-type <i>k<sub>cat</sub></i> (3.2 ± 0.1 s<sup>-1</sup>; Table 2). In the tRNA<sub>Thr</sub>-charging reaction, the <i>k<sub>cat</sub></i> values of both mutants were significantly decreased, retaining only 11 and 5% of the value for the native enzyme. However, the <i>k<sub>catalytic</sub></i> and <i>K<sub>m</sub></i> values of the two mutants for tRNA<sub>Thr</sub> were slightly increased. Their catalytic efficiencies (<i>k<sub>cat</sub>/K<sub>m</sub></i>) were 11.4- and 32.5-fold lower, respectively, than the wild-type (Tables 3 and 4). These results showed that both mutations had little effect on Thr activation and the affinity for tRNA<sub>Thr</sub> but obviously decreased the rate of tRNA aminoacylation, despite their presence in the N2 editing domain. Notably, the mutations are located at the interface between the editing and aminoacylation domains (Fig. 2D), suggesting a distal effect on the synthetic active site. The mutations may disturb the subtle structure of the active site and thus affect the rate of aminoacylation. Moreover, Western blot showed that in vivo, the amounts of ScThrRS-G145D and -S308F were significantly decreased compared with that of native enzyme (Fig. 2, B and C). Taken together, the results suggested that the deleterious effect of the mutations might act both at the catalytic level and on the enzyme concentration in the cell.

Gly<sup>145</sup> and Ser<sup>308</sup> are located in the N2 domain; therefore, the editing activities of ScThrRS-G145D and -S308F were monitored. In the absence of tRNA<sub>Thr</sub> and in the presence of Ser as the non-cognate amino acid, the <i>k<sub>obs</sub></i> of ScThrRS-G145D in...
AMP formation was \((3.1 \pm 0.2) \times 10^{-3} \text{ s}^{-1}\), which was similar to the \((2.8 \pm 0.8) \times 10^{-3} \text{ s}^{-1}\) of the native enzyme. For ScThrRS-S308F, the \(k_{\text{obs}}\) for AMP formation was significantly decreased to \((0.5 \pm 0.1) \times 10^{-3} \text{ s}^{-1}\) (Table 5 and Fig. 3A). Therefore, S308F might be the first identified mutation of eukaryotic ThrRSs to affect tRNA-independent pretransfer editing. In the presence of tRNAThr, which measured the total editing reaction, the \(k_{\text{obs}}\) values of ScThrRS-G145D and -S308F in AMP formation reaction were \((4.1 \pm 0.4) \times 10^{-3} \text{ s}^{-1}\) and \((2.9 \pm 0.4) \times 10^{-3} \text{ s}^{-1}\), respectively, representing only 27.5 and 19.4% of the wild-type activity (Table 5 and Fig. 3A). Moreover, a deacylation assay of preformed mischarged Ser-tRNAThr was performed. The deacylation curves showed that both mutants were unable to hydrolyze mischarged Ser-tRNAThr (Fig. 3B).

Thus, the results showed that the G145D mutation impacted the tRNA-dependent editing activity but not the tRNA-independent editing. However, both the tRNA-independent and -dependent editing activities of the ScThrRS-S308F were impaired. Although the two residues are not located in the previously identified editing pocket (17), their mutations have a profound effect on the editing activity of eukaryotic ThrRS.

**Mutations G356N and P627L Impair Dimer Formation**—Amino acid activation and tRNA aminoacylation assays showed that ScThrRS-G356N and -P627L had completely lost their synthetic activity (Table 2 and 3). Moreover, the \(k_{d}\) values for tRNA mostly increased (Table 4). However, the crystal structure of EcThrRS showed that Gly\(^{275}\) and Pro\(^{539}\) of EcThrRS, the corresponding residues of Gly\(^{276}\) and Pro\(^{627}\) in EcThrRS. The protein modules are colored green (editing domain), cyan (catalytic domain), and orange (anti-codon binding domain). tRNA\(^{\text{Thr}}\) is shown in pink.

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**FIGURE 2.** Twelve mutants exhibited lethal or growth-deficient phenotypes. A, ScThrRS-R52F, -G145D, -S308F, -G349N, -S457F, -G458D, -R461H, -G604E, -P627L, -G685D, -Q704stop, and -Q719stop were cloned into p425TEF(His) and introduced in strain Sc\(\Delta\)thrS, where the growth phenotypes were confirmed. Wild-type ScThrRS and empty p425TEF(His) were used as positive and negative controls, respectively. Drop tests were performed on SD/Leu\(^{-}\)/5-FOA plate with an equal number of cells. B, Western blot (WB) showing the amounts of different ScThrRS mutants. GAPDH was used as a control. C, quantification of the Western blot. The amounts of ScThrRS mutants were normalized according to the endogenous GAPDH. The amount of wild-type ScThrRS was designed as 100%. Relative protein amounts were calculated based on at least two independent Western blot assays and shown in parentheses with S.D. values omitted for clarity. D, corresponding residues (shown as red spheres) are shown on the crystallographic structure of EcThrRS-tRNA\(^{\text{Thr}}\) complex (Protein Data Bank code 1QF6). The protein modules are colored green (editing domain), cyan (catalytic domain), and orange (anti-codon binding domain). tRNA\(^{\text{Thr}}\) is shown in pink. Error bars, S.D.
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### TABLE 2
Kinetic parameters of ScThrRS and its variants for Thr in amino acid activation reaction

| Enzyme | $k_a$ | $K_m$ | $k_{cat}/K_m$ | Relative $k_{cat}/K_m$ | Loss of catalytic efficiency |
|--------|-------|-------|---------------|------------------------|-----------------------------|
| WT     | 3.2 ± 0.1 | 0.22 ± 0.02 | 14.6 | 100 | 100 |
| R52F   | 1.3 ± 0.1 | 0.23 ± 0.02 | 5.7 | 391 | 2.6 |
| R52A   | 1.8 ± 0.1 | 0.24 ± 0.04 | 7.5 | 51.7 | 1.9 |
| R52D   | 1.6 ± 0.2 | 0.26 ± 0.05 | 6.1 | 42.0 | 2.4 |
| R52K   | 3.3 ± 0.1 | 0.21 ± 0.01 | 15.5 | 106.4 | 1.2 |
| G145D  | 2.6 ± 0.2 | 0.22 ± 0.05 | 12.0 | 81.9 | 1.2 |
| S308F  | 2.4 ± 0.2 | 0.24 ± 0.03 | 9.9 | 67.7 | 1.5 |
| G356N  | 0.1 ± 0.1 | 0.1 ± 0.01 | 0.2 | 0.2 | 0.2 |
| S457F  | 0.1 ± 0.1 | 0.1 ± 0.01 | 0.2 | 0.2 | 0.2 |
| G356N  | 0.1 ± 0.1 | 0.1 ± 0.01 | 0.2 | 0.2 | 0.2 |
| S457F  | 0.1 ± 0.1 | 0.1 ± 0.01 | 0.2 | 0.2 | 0.2 |

### TABLE 3
Kinetic parameters of ScThrRS and its variants for tRNA^{Thr} (AGU) in aminoacylation reaction

| Enzyme | $k_a$ | $K_m$ | $k_{cat}/K_m$ | Relative $k_{cat}/K_m$ | Loss of catalytic efficiency |
|--------|-------|-------|---------------|------------------------|-----------------------------|
| WT     | 2.8 ± 0.1 | 1.1 ± 0.1 | 24.7 | 100 | 100 |
| R52F   | 0.2 ± 0.1 | 2.0 ± 0.3 | 1.0 | 4.3 | 23.1 |
| R52A   | 1.7 ± 0.1 | 1.2 ± 0.2 | 13.6 | 54.8 | 1.8 |
| R52D   | 1.7 ± 0.1 | 1.2 ± 0.2 | 13.6 | 54.8 | 1.8 |
| R52K   | 0.3 ± 0.02 | 1.4 ± 0.2 | 2.2 | 8.7 | 11.4 |
| G145D  | 0.14 ± 0.01 | 1.8 ± 0.1 | 0.8 | 3.1 | 32.5 |
| S308F  | 0.15 ± 0.06 | 1.2 ± 0.1 | 1.2 | 5.0 | 20.0 |
| G356N  | 0.15 ± 0.06 | 1.2 ± 0.1 | 1.2 | 5.0 | 20.0 |
| S457F  | 0.15 ± 0.06 | 1.2 ± 0.1 | 1.2 | 5.0 | 20.0 |
| G458D  | 0.15 ± 0.06 | 1.2 ± 0.1 | 1.2 | 5.0 | 20.0 |
| R461H  | 0.15 ± 0.06 | 1.2 ± 0.1 | 1.2 | 5.0 | 20.0 |
| G685D  | 0.15 ± 0.06 | 1.2 ± 0.1 | 1.2 | 5.0 | 20.0 |
| C-Δ3   | 1.5 ± 0.2 | 0.4 ± 0.1 | 10.4 | 42.1 | 2.4 |
| C-Δ5   | 1.2 ± 0.2 | 0.3 ± 0.2 | 5.6 | 37.3 | 4.4 |
| C-Δ10  | 1.0 ± 0.1 | 0.5 ± 0.2 | 1.5 | 26.2 | 4.9 |

### TABLE 4
$k_0$ of ScThrRS for SctRNA^{Thr} (AGU) in filter binding reaction at 4 °C

| Enzyme | $k_0$ (s$^{-1}$) | $k_{cat}/K_m$ |
|--------|-----------------|--------------|
| WT     | 3.3 ± 0.3       | 3.3 ± 0.3    |
| R52F   | 3.0 ± 0.2       | 3.0 ± 0.2    |
| R52A   | 3.1 ± 0.5       | 3.1 ± 0.5    |
| G145D  | 3.0 ± 0.8       | 3.0 ± 0.8    |
| S308F  | 13 ± 3          | 13 ± 3      |
| S457F  | 3.8 ± 0.5       | 3.8 ± 0.5    |
| G458D  | 3.1 ± 0.5       | 3.1 ± 0.5    |
| R461H  | 3.2 ± 0.5       | 3.2 ± 0.5    |
| P627L  | 19 ± 5          | 19 ± 5      |
| G685D  | 3.9 ± 0.9       | 3.9 ± 0.9    |

### FIGURE 3
Editing properties of mutants ScThrRS-G145D and -S308F. A, quantification of AMP formation in the presence of Ser and absence of tRNA by ScThrRS-WT (○), ScThrRS-G145D (□), and -S308F (△) or in the presence of tRNA by ScThrRS-WT (●), ScThrRS-G145D (□), ScThrRS-S308F (△), and ScThrRS-G145D and -S308F (◆). Spontaneous deacylation of Ser-tRNA^{Thr} (○) was included as a control. Error bars, S.D.

instance, residue Trp$^{271}$ (adjacent of Gly$^{270}$) is located at only 2.71 Å from Glu$^{292}$ of another subunit (Fig. 4C). Therefore, the flexible residue Gly at position 270 might control the conformation of the interface, and the introduction of a bulky and more rigid Asn residue at this site probably caused a local structural rearrangement and influenced the dense network of interactions of the interface.

The second mutated residue, Pro$^{627}$ in ScThrRS, the equivalent of Pro$^{279}$ in EcThrRS, is located in the linker loop connecting the aminoacylation and anticodon binding domains (Figs. 2D and 4A) (14). When a Leu residue replaced the rigid Pro in the P627L mutant, the loop was probably able to become more flexible and adopt a different conformation, which influenced the dimer interface.

Therefore, we assumed that mutations G356N and P627L might be damaging for dimerization of ScThrRS. This could also explain the low amount of protein found in yeast cells, because the isolated subunits might be unstable and subjected to degradation (Fig. 2B). To test this assumption, the genes of FLAG- and c-Myc-tagged wild-type ScThrRS or ScThrRS-G356N or -P627L were co-expressed in HEK293T cells. Cell lysates were immunoprecipitated with anti-FLAG antibodies. Western blot showed that c-Myc-tagged wild-type ScThrRS could be readily co-immunoprecipitated by the anti-FLAG antibody (Fig. 4D), suggesting that c-Myc-ScThrRS and FLAG-ScThrRS subunits could form "heterodimers," as expected. In sharp contrast, neither c-Myc-tagged ScThrRS-G356N nor ScThrRS, located far from the catalytic center (Fig. 4A) (14). In the case of EcThrRS, Gly$^{270}$ is located in a α-helix at the interface of the subunits of the EcThrRS dimer, just before specific motif 1 of class II aaRSs (14). Hydrogen bonds, salt bonds, and hydrophobic interactions connect the α-helix of one subunit and a peptide loop of another subunit (Fig. 4B). For
P627L could be co-immunoprecipitated by the anti-FLAG antibody, despite the fact that both FLAG-tagged ScThrRS-G356N and -P627L were well immunoprecipitated by the anti-FLAG antibodies (Fig. 4D). Thus, FLAG/c-Myc-tagged ScThrRS-G356N and -P627L were unable to form “heterodimers.” These results suggested that the failure to dimerize might be the cause of the lethal phenotype observed with the two mutants. In vivo, the single subunits are probably subjected to a rapid degradation, as observed by the low content of mutated ScThrRS found in the yeast cells (Fig. 2, B and C).

Lethal Mutations in the Synthetic Active Site Domain—One lethal mutation (G604E) was found in the class II-defining synthetase motif 3. In class II synthetases, Gly residues and other small residues are often found in the β-strand preceding the invariant Arg residue (Arg618 of ScThrRS) (24). These small residues form the ATP-binding cavity and replacement of a Gly in a class II synthetase by a charged residue affected the enzyme activity to alter the enzyme conformation (24). In the case of EcThrRS, the equivalent residue Gly516 (Gly604 of ScThrRS) is only at 3.01 Å from the AMP moiety of activated Thr-AMP (Fig. 5, A and B). Furthermore, its adjacent residue, Ser517, directly contacts the AMP moiety by hydrogen bonding (14, 33). Therefore, the introduction of a negatively charged Glu residue might create a spatial hindrance with the base moiety of AMP with consequences for Thr-AMP activation kinetics. Indeed, amino acid activation kinetics showed that ScThrRS-G604E was completely deficient in Thr activation and tRNAThr charging (Tables 2 and 3). Western blot also showed that the lethal effect observed in the yeast was not the consequence of protein insta-
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bility because the mutation had little impact on the protein quantity (Fig. 2C).

Interestingly, three mutations (S457F, G458D, and R461H) were clustered in the motif 2 loop. The motif 2 loop (residues 449–463 in ScThrRS) is part of signature motif 2, connecting two antiparallel β-strands. In class II synthetases (34–36), this loop plays a critical role during catalysis by participating in the binding of amino acid, ATP, and the acceptor end of the tRNA.

Gly372 in EcThrRS (corresponding to Gly458 in ScThrRS) is far from AMP and does not contact the substrates (Fig. 5B) (14). However, here we showed that the lethal mutant ScThrRS-G458D nearly abolished Thr activation, with only 3.4% of remaining catalytic efficiency compared with the wild-type ScThrRS. Both the $K_m$ and $k_{cat}$ values for Thr were responsible for this drop and were obviously increased and decreased, respectively (Table 2). The severe decrease in amino acid activation was accompanied by a total loss of tRNA aminoacylation activity, which became non-measurable (Table 3). However, the $k_d$ for tRNA Thr was unchanged, suggesting that a loss of tRNA binding was not responsible for this inactivation (Table 2). In EcThrRS, Gly372 ends the loop connecting the two β-strands of motif 2 (14). Adding the negatively charged side chain of Asp at this position might have modified the geometry and flexibility of the loop and following β-strand with consequent effects on the catalytic properties. Nevertheless, the structural effect probably remained local because the mutant was stable in yeast cells (Fig. 2, B and C).

EcThrRS uses the strictly conserved basic residue Arg275 in motif 2 loop to recognize nucleotide C74 of tRNA Thr by two base-specific hydrogen bonds (14). Arg275 also stabilizes the γ-phosphate of ATP during the amino acid activation step and then switches to C74 after completion of amino acid activation and pyrophosphate departure (33). A lethal mutation of Arg461 (R461H) of ScThrRS, corresponding to Arg275 from EcThrRS, was isolated in our selection. The amino acid activation assay showed that R461H induced a 30-fold decrease in catalytic efficiency, with a 10-fold increase of $K_m$ for Thr and a 3-fold decrease in $k_{cat}$ (Table 2). tRNA charging of ScThrRS-R461H was also undetectable; however, the $k_d$ value for tRNA Thr was similar to that of the wild type, which indicated that the tRNA binding site was essentially intact in the mutant (Tables 3 and 4). Western blot showed that the quantity of ScThrRS-R461H was close to that of wild-type ScThrRS, suggesting that the mutation did not affect the protein stability (Fig. 2, B and C).

The third mutation selected at position 457 (S457F) of the motif 2 loop in ScThrRS slightly damaged the catalytic efficiency in amino acid activation, indicated by the slightly increased $K_m$ for Thr (Table 2). The corresponding His371 residue of EcThrRS is more than 4 Å from nucleotides 68–69 of tRNA Thr (Fig. 5A and C). Here, this mutation induced a 20-fold drop of the catalytic efficiency in tRNA aminoacylation, mainly caused by a decrease of $k_{cat}$, whereas the $K_m$ and $k_d$ for tRNA Thr were almost unchanged (Tables 3 and 4). However, despite this severe reduction of aminoacylation activity, slight growth of the ScΔthrS strain could be observed (Fig. 2A), suggesting that the threshold of cell lethality was close to, but below, this value of remaining activity.

Mutations in the C-terminal Domain—In the crystal structure of EcThrRS, Gly597 (the counterpart of Gly685 of ScThrRS, selected as a lethal mutation, G685D) is located in the anticodon binding module of EcThrRS, in close proximity of the anticodon of tRNA Thr, which contains G35 and U36, two major identity elements of EcThrRS (14, 37). The distance between the carboxyl group of Gly597 and G35 is only 2.96 Å (Fig. 5D).

Therefore, the presence of the Asp side chain in the selected ScThrRS-G685D mutant might have created steric hindrance, charge repulsion, and/or loss of flexibility, potentially leading to tRNA rejection or weaker accommodation of the anticodon nucleotides. Remarkably, ScThrRS-G685D exhibited intact amino acid-activating properties but was unable to aminoacylate tRNA Thr (Tables 2 and 3). However, the total loss of tRNA-charging activity was not caused by a reduction of tRNA Thr affinity (Table 4). The quantity of ScThrRS-G685D was the same as the native ScThrRS in the ScΔthrS strain (Fig. 2C), suggesting that the protein was correctly folded and stable in vivo.

Deletions of the C terminus of ScThrRS (mutants Q704stop and Q719stop, –31 and –16 residues, respectively) failed to complement the growth of ScΔthrS strain and induced very low amounts compared with the wild-type enzyme (Fig. 2, A and C). These observations suggested that the last residues play a crucial role in the activity of ScThrRS and in protein stability. In addition, mutants ScThrRS-Q704stop and -Q719stop formed inclusion bodies in E. coli and could not be purified for further studies. Therefore, to study the function of the very end residues of the C terminus, we used site-directed mutagenesis to delete the 3, 5, or 10 last residues of ScThrRS. In the amino acid activation reaction, the three mutants, ScThrRS-C-Δ3, -C-Δ5, and -C-Δ10, exhibited virtually unchanged properties except for a moderate increase in the $K_m$ for Thr (Table 2). In the tRNA aminoacylation reaction, the three mutants displayed progressive decreases in their aminoacylation properties of 2.4-, 4.4-, and 4.9-fold, respectively, compared with wild-type ScThrRS.

The catalytic constant $k_{cat}$ was mainly responsible for the decrease, and the $K_m$ for tRNA Thr was almost unchanged (Table 3). Despite the loss of tRNA-charging activity, the three mutants were equally able to rescue the ScΔthrS strain as well as wild-type ScThrRS (Fig. 6A), suggesting that partial activity of ScThrRS is sufficient to sustain yeast growth. Thus, the C-terminal truncations harbor lower and lower enzymatic activities as the length of C terminus diminished; a 16-residue truncation (Q719stop), being inactive for normal physiological growth, more severely impaired the enzymatic activities.

The C-terminal domain of ThrRS binds the anticodon loop of tRNA (14). Because tRNA binding is responsible for tRNA-dependent pretransfer editing and post-transfer editing, we checked whether deletions of the C-terminal residues might affect the editing properties of ScThrRS. We measured AMP formation in the presence of Ser and performed mischarged tRNA deacylation assays. The three mutants showed a decrease in both tRNA-independent pretransfer editing and total editing activity, especially C-Δ5 and C-Δ10 (Table 5). However, deacylation of Ser-tRNA Thr by post-transfer editing was unaffected by the deletions (Fig. 6B), suggesting that the three mutants could still bind charged tRNA, and truncations of C-terminal amino acid base-specific hydrogen bonds (14). Arg375 also stabilizes the γ-phosphate of ATP during the amino acid activation step and then switches to C74 after completion of amino acid activation and pyrophosphate departure (33). A lethal mutation of Arg461 (R461H) of ScThrRS, corresponding to Arg275 from EcThrRS, was isolated in our selection. The amino acid activation assay showed that R461H induced a 30-fold decrease in catalytic efficiency, with a 10-fold increase of $K_m$ for Thr and a 3-fold decrease in $k_{cat}$ (Table 2). tRNA charging of ScThrRS-R461H was also undetectable; however, the $k_d$ value for tRNA Thr was similar to that of the wild type, which indicated that the tRNA binding site was essentially intact in the mutant (Tables 3 and 4). Western blot showed that the quantity of ScThrRS-R461H was close to that of wild-type ScThrRS, suggesting that the mutation did not affect the protein stability (Fig. 2, B and C).

The third mutation selected at position 457 (S457F) of the motif 2 loop in ScThrRS slightly damaged the catalytic efficiency in amino acid activation, indicated by the slightly increased $K_m$ for Thr (Table 2). The corresponding His371 residue of EcThrRS is more than 4 Å from nucleotides 68–69 of tRNA Thr (Fig. 5A and C). Here, this mutation induced a 20-fold drop of the catalytic efficiency in tRNA aminoacylation, mainly caused by a decrease of $k_{cat}$, whereas the $K_m$ and $k_d$ for tRNA Thr were almost unchanged (Tables 3 and 4). However, despite this severe reduction of aminoacylation activity, slight growth of the ScΔthrS strain could be observed (Fig. 2A), suggesting that the threshold of cell lethality was close to, but below, this value of remaining activity.
acid residues had negligible influence on tRNA binding and subsequent editing by deacylating misaminoacylated tRNA.

Arg52 Library Screening Revealed Pleiotropic Effects on ScThrRS Properties—Like many other eukaryotic aaRSs, eukaryotic ThrRSs have additional N-extensions whose functions are not yet understood. Our previous study on ScThrRS showed that mutants deleted of the first 40 and 65 residues (ScThrRS-Δ40 and ScThrRS-Δ65, respectively) could rescue the thrS knock-out strain; however, ScThrRS-Δ40 mutant exhibited only 25% of the wild-type charging activity, mainly because of loss of tRNA affinity (21). Examining the N-extensions of eukaryotic ThrRSs revealed the presence of several conserved or invariant residues. Arg52 in ScThrRS is one of two invariant residues (Fig. 1A). Positively charged residues are potentially able to interact with the negatively charged phosphate groups of tRNA and/or form hydrogen bond interaction with other amino acid residues (especially acid amino acid residues); therefore, we decided to study this residue using a degenerated library containing all possible amino acids at position 52. The library was constructed by site-directed mutagenesis with a randomly mutagenized primer. The Arg52 library was then introduced into the ScthrS strain harboring the rescuing plasmid pRS426-ScThrRS. 160 colonies (5 times the theoretical size library) were tested on 5-FOA-containing plates. 10 clones that could not grow on these plates contained a non-sense mutation at position 52 causing premature translation arrest. Three other clones shared a missense mutation (R52F).

The gene of ScThrRS-R52F was cloned into p425TEF(His) vector and reintroduced into ScΔthrS, which confirmed its lethality for yeast (Fig. 2A).

To further analyze the lethal effect of ScThrRS-R52F, we overexpressed its gene in E. coli and purified it. An amino acid activation assay showed that the mutant had an unchanged $K_m$ for Thr (0.23 ± 0.02 mM; wild-type, 0.22 ± 0.02 mM). However, the catalytic rate constant ($k_{cat}$) of the mutant was lower (1.3 ± 0.1 s$^{-1}$) compared with that of the wild-type enzyme (3.2 ± 0.1 s$^{-1}$), leading to a 2.6-fold drop in the catalytic efficiency ($k_{cat}/K_m$) (Table 2). In contrast to amino acid activation, tRNA$^{Thr}$ charging by mutant ScThrRS-R52F was too low to measure (Table 3). This result suggested that a deficiency of tRNA charging was responsible for R52F lethality.

Arg52 is located in the N-extension that is appended to the editing domain; therefore, we checked if the mutation affected the editing activity. In the absence or presence of tRNA$^{Thr}$, the observed rate constants ($k_{obs}$) of ScThrRS-R52F for AMP formation were (0.3 ± 0.1) × 10^{-3} s$^{-1}$ and (0.6 ± 0.2) × 10^{-3} s$^{-1}$, respectively (Table 5). The latter actually reflected whole editing activities, including tRNA-independent and -dependent pretransfer editing and post-transfer editing. These results revealed an obvious decrease in the tRNA-dependent and tRNA-independent editing properties. Further deacylation assay of mischarged tRNA$^{Thr}$ (Ser-tRNA$^{Thr}$) confirmed that ScThrRS-R52F was defective in post-transfer editing (Fig. 7A). Post-transfer editing requires proper binding of mischarged tRNA$^{Thr}$ and the presence of active editing residues. Arg52 is located in the N-extension, whose structure has not been solved; therefore, it would be highly speculative to try explaining the molecular effect of the mutation. Nevertheless, we cannot exclude the possibility that the conformation of ScThrRS-R52F has been altered by the mutation. Indeed, the amount of R52F in the yeast cells was nearly undetectable by Western blot (Fig. 2, B and C), indicating that the mutation had an effect on protein stability or conformation or both, together with significant effects on aminoacylation and editing activities.

For a better understanding of the effect of R52F, and despite the fact that the Arg52 library theoretically covered all possible mutations, we performed additional site-directed mutagenesis at the Arg52 position. We substituted Arg52 for Ala (smaller side chain), Asp (negatively charged), and Lys (positively charged).
Several aARSs, such as LysRS (38) and MetRS (39), have N-extensions that are rich in basic residues and can bind nonspecifically tRNA elements. To determine if the ThrRS N-extension was similarly involved in tRNA binding, we measured the $k_d$ values of the two lethal mutants identified at position 52. The analysis showed that both ScThrRS-R52F and ScThrRS-R52D had full tRNA binding capacity (Table 4), indicating that this residue does not contribute significantly to tRNA binding. Altogether, the effects induced by R52A, R52D, and R52F strongly suggest that this part of the N-extension of ThrRS plays a critical role in catalysis, structure, and/or the stability of this enzyme.

**The Yeast thrS Knock-out Strain as a Useful Tool to Analyze Mutations in Human Cytoplasmic ThrRS**—Human cytoplasmic ThrRS comprises 723 amino acid residues and shows 53.8% sequence identity to ScThrRS. Here, 12 mutations were found in the two mutated ScThrRS libraries, and eight of the mutated residues were conserved in HsThrRS and ScThrRS (Fig. 1, A and C). This high homology between the two proteins allows test point mutations experimentally introduced into HsThrRS. An increasing number of point mutations causing various types of human diseases have been identified in aARSs (40); therefore, the availability of a cellular system designed to test new mutations would be helpful.

To verify that the complementation assay of the thrS knock-out strain assay can be used with confidence to analyze mutated HsThrRS, we introduced the eight conserved missense mutations and one truncation in ScThrRS into HsThrRS. The eight mutations were single substitutions (R57F, S301F, G349N, G451D, R454H, G595E, P618L, and G677D) (Fig. 1, A and C), and the truncation was a deletion of the 11 residues of the C terminus (L711stop). Genes corresponding to these mutants were cloned into p425TEF(His) and introduced into HsThrRS. All transformants grew well on SD/Leu−/Ura− plates, but none could grow in the SD/Leu−/Ura−/5-FOA (Fig. 8A). The synthesis of the mutated proteins was confirmed by Western blot (Fig. 8B). Overall, the gene expression patterns of the HsThrRS mutants were very similar to those of the ScThrRS mutants (Figs. 2C and 8C). Some mutants, such as HsThrRS-G451D, -R454H, -G595E, and -G677D, were found in the same amounts as native HsThrRS, despite being lethal. However, mutants HsThrRS-R57F, -S301F, -G349N, -P618L, and -L711stop were present at about 5% of the relative amounts of either ScThrRS or HsThrRS, suggesting that these amino acid residues play similar functional and structural roles in both ThrRSs (Figs. 2C and 8C).

Considering that G145D, S457F, and C-Δ3 of ScThrRS were not lethal, we further confirmed whether the ScThrRS strain could be used to test the function of HsThrRS mutations by introducing A149D, T450F, and C-Δ3 (corresponding to G145D, S457F, and C-Δ3 of ScThrRS, respectively) into HsThrRS. We found that yeast containing HsThrRS-C-Δ3 was growth-deficient and the other two mutations were lethal (data not shown). ScThrRS-C-Δ3 was viable (Fig. 6A), despite exhibiting a 2.4-fold loss of aminocacylation activity (Table 2), but cognate HsThrRS-C-Δ3 led to a significant deficiency in the

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| TABLE 6 |
| --- |
| Catalytic kinetic parameters of ScThrRS-WT and -R52A in the tRNA aminocacylation reaction |
| | Kinetic parameters$^a$ | WT | R52A |
| Transcript CGU $k_{cat}$ (×10$^{-3}$ s$^{-1}$) | 3.4 ± 0.2 | 0.2 ± 0.03 |
| | $k_m$ (μM) | 1.3 ± 0.2 | 1.2 ± 0.2 |
| | Relative $k_{cat}/k_m$ (%) | 100 | 5.7 |
| Transcript UGU $k_{cat}$ (×10$^{-3}$ s$^{-1}$) | 4.5 ± 0.2 | 0.3 ± 0.1 |
| | $k_m$ (μM) | 1.4 ± 0.2 | 1.3 ± 0.1 |
| | Relative $k_{cat}/k_m$ (%) | 100 | 6.7 |

$^a$ Results are shown as the average of three independent trials ± S.D.

In the amino acid activation reaction, ScThrRS-R52K, -R52A, and -R52D displayed $K_m$ values similar to that of the wild-type ScThrRS and catalytic efficiencies of 106.4, 51.7, and 42.0%, respectively (Table 2). Surprisingly, tRNA-charging assays showed that mutant ScThrRS-R52D was totally unable to aminocylate tRNAThr. Mutants ScThrRS-R52A and -R52K displayed unchanged $K_m$ values, but the $k_{cat}$ of ScThrRS-R52A for tRNA charging was dramatically decreased, leading to a global loss of catalytic efficiency of nearly 25.7-fold (Table 3). The catalytic efficiency of ScThrRS-R52K decreased by 1.8-fold compared with that of wild-type (Table 3). Further hydrolysis of mischarged Ser-tRNAThr showed that R52K moderately decreased the post-transfer editing activity (Fig. 7A). These biochemical data confirmed that Arg$^{22}$ is important for the aminocylation and editing activities, although the residue was replaced by the same charge Lys. When the three ScThrRS-R52D, -R52A, and -R52K mutants were tested for complementation of the ScΔthrS strain, it appeared that ScThrRS-R52D was lethal to yeast, whereas ScThrRS-R52A and -R52K could rescue ScΔthrS similarly to the wild-type ScThrRS (Fig. 7B). Western blot analysis of yeast extracts showed that the amount of ScThrRS-R52K was equal to the wild-type ScThrRS (Fig. 7, C and D). In contrast, the amounts of ScThrRS-R52A and -R52D were significantly decreased, to 2.7 and 4.6%, respectively (Fig. 7, C and D). This observation supported the hypothesis that Arg$^{22}$ is critical for protein stability. It is also remarkable that ScThrRS-R52A for CGU and UGU reached 5.7 and 4.3% respectively, compared with the wild-type enzyme ScThrRS-C-Δ3 was viable (Fig. 6A), despite exhibiting a 2.4-fold loss of aminocacylation activity (Table 2), but cognate HsThrRS-C-Δ3 led to a significant deficiency in the
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Mutations in the Editing Site Simultaneously Affected the tRNA Proofreading and Aminoacylation—In the editing domain, mutations G145D and S308F induced a severe defect of editing activities. The crystal structure of EcThrRS shows that the two residues do not directly interact with the tRNA acceptor arm and CCA sequence into the editing site (14). Thus, an indirect effect would probably be responsible for the decrease of editing activity. Gly67 (Gly145 in ScThrRS) is located in helix H3 containing the signature sequence 72HXXXH77, which is involved in the hydrolytic process of mischarged tRNA (41). Therefore, the G145D mutation might have modified the flexibility and conformation of the catalytic helix H3, which might explain its loss of post-transfer editing. Similarly, Ala222 in EcThrRS, corresponding to Ser308 in yeast, is located before the α-helix bridging the editing and aminoacylation domains (14). The insertion of a bulky Phe residue in the S308F mutant might have modified the relative orientations of the two domains, with pleiotropic effects on synthetic, editing activities and mutant stability. Furthermore, G145D and S308F strongly decreased tRNA aminoacylation, despite being more than 30 Å from the synthetic site (14). The decrease of tRNA charging observed in the two mutants might also result from an inefficient editing activity. Actually, a full cycle of aminoacylation comprises the tRNA-charging step and, after translocation of the CCA acceptor end, the proofreading step in the editing site. Alteration of one step will definitely affect the rate of the next cycle, which might explain the parallel effect on tRNA charging and post-transfer editing.

Mutations in the Synthetic Active Site—Three mutations were isolated in the loop of class II-defining motif 2 and one in motif 3. According to the structural data, three of these mutations (S457F, G458D, and G604E) do not alter direct contacts with the substrates (14). However, the location of the residues and nature of the substitutions indicated that the damaging effects could have resulted from local structural changes resulting from steric hindrance with other side chains. It resulted in a loss of the synthetic activities, despite good protein stability. A fourth mutant, R461H, is remarkable in that it modifies an Arg residue that directly binds ATP during amino acid activation and then, after pyrophosphate departure, interacts with nucleos-
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otide C74 of tRNA during the transfer step (33). A conservative mutation was isolated during the screening procedure (R461H), confirming that this residue is critical and could not tolerate any change.

Lethal Mutations Also Target Dimerization of ThrRS—Class II aaRSs share three conserved motifs, one of which is involved in dimer formation (4, 42). Indeed, dimerization is essential for efficient formation of the active sites of AspRS (43), LysRS (44), and ProRS (45). However, dimerization is not strictly required for class II aaRS activity, and natural monomeric PheRSs exist in mitochondria (46). Similarly, a truncated monomeric E. coli AlaRS could complement in vivo in cells (47). ThrRS is a dimer with a typical class II interface between the two aminocytosine sites (14). Here, the analyses of G356N and P627L showed that dimerization is also a critical prerequisite for ScThrRS activity, as already observed with other class II synthetases. For the closely related LysRS, the dimeric character of LysRS was essential to stabilize the subunits in a productive conformation (44). The properties of the lethal mutants studied here fully support this view.

Binding of the Anticodon Controls tRNA Aminoacylation Activity—The C-terminal residues of aaRSs are sometimes essential for the enzymatic activity (48, 49). In E. coli tRNAThr, all of the bases from 32 to 37 interact through at least one hydrogen bond to the protein, and bases 35–38 are splayed out from the anticodon loop (14), indicating that the tRNA recognition cleft is a mobile region. This is consistent with the observation that almost identical Km values were determined for E. coli tRNAThr isoacceptors having C, G, or U at position 34 (37), despite only C34 directly binding to the enzyme and interacting with N575 (14). It is also interesting to note that a hydrogen bond was found between N2 of G35 and O4 of U36 (14). In addition, a highly conserved acidic residue Glu600 of EcThrRS lying adjacent to Gly597 (analogous to Gly685 of ScThrRS) makes a direct hydrogen bond with N1 of G35 (14). In brief, the conformational flexibility of the tRNA binding site, the novel base interaction between G35 and U36, and the mutation of Gly685 into acidic Asp might explain why the selected G685D lethal mutation did not affect the initial binding of tRNA to the synthetase, as shown by the kcat measurement.

An N-terminal Appended Domain with Critical Residues—One distinct feature in the structural evolution of aaRSs is the appending of sequences at the amino or C terminus. These new domains are generally thought to be dispensable for aminoacylation but are responsible for a number of new noncanonical functions, such as multisynthetase complex formation, transcription regulation, translation control, and neurodegeneration (50). For example, in the multisynthetase complex, the C-terminal extension of LeuRS is dispensable for enzymatic activity but critical for the interaction with the N-terminal of ArgRS (32). Eukaryotic ThrRS has evolved an N-extension harboring several highly conserved residues, such as Arg52. We found that R52F and R52D mutations are lethal, inhibiting aminoacylation and reducing protein stability in vivo. Appended domains were reported to enhance tRNA binding through nonspecific tRNA binding interactions (51–53), and no critical residue has been described in these extensions. The Arg52 mutant of ScThrRS and the equivalent mutant in HsThrRS are the first examples of lethal mutants described in the N-extension that reduced both aminoacylation and editing properties. This shows that the appended domains can play key roles in regulating the enzymatic activities of the catalytic core.

Interestingly, ScThrRS-R52A could support the growth of the ScΔthr5 strain with a 23-fold loss of aminoacylation activity plus 33-fold lower protein amount, as compared with the native enzyme. However, ScThrRS-G145D and -S457F, which caused less severe aminoacylation and expression defects, led to growth deficiency. Mutation of the residue Ser457 of the motif 2 loop into Phe probably impedes aminoacylation directly, because the motif 2 loop is crucial for the correct positioning of the tRNA acceptor end and amino acid binding (33). ScThrRS-G145D exhibited a severe decrease in post-transfer editing; its low enzymatic activity probably resulted from the impaired aminoacylation cycle of tRNA charging and proofreading. In contrast to the direct impact on tRNA charging and editing caused by S457F and G145D mutations, it seems that R52A indirectly weakened the catalytic efficiency through impairing protein stability or conformation/structure. The indirect influence of R52A mutation might be diminished in vivo in unidentified manners, such as assistance of chaperones, product release, or interaction with other translation factors. As a consequence, ScThrRS-R52A is more efficient in producing Thr-tRNAThr and is viable enough to complement the ScΔthr5 strain.

Analyzing and Characterizing Human aaRS/tRNA Pathogenic Mutations—Since the first example of human genetic disease-related mutation in an aaRS was reported in 2003, when GlyRS was implicated in Charcot-Marie-Tooth disease (10), an increasing number of aaRSs has been revealed to cause human diseases (40). We show that the high sequence conservation between HsThrRS and ScThrRS is suitable to perform complementation assays of the yeast ThrRS knock-out strain by HsThrRS or mutated derivatives. Loss of function mutations selected in yeast ThrRS and transplanted in human cytoplasmic ThrRS induce very similar defects, indicating that the yeast strain can be used to gain insights into the pathological mechanisms induced by human ThrRS mutations in the future, although no mutations have currently been linked pathologically with the human cytoplasmic ThrRS gene. Recently, a spontaneous mutation of human mitochondrial ThrRS P282L was reported to cause mitochondrial encephalopathies (54). The ThrRS knock-out system designed to test cellular function might also be helpful to examine the effects of this P282L mutation and establish an in vivo functional diagnosis.

Six different mutations in human mitochondrial tRNAThr (see the Compilation of Mammalian Mitochondrial tDNA Genes Web site, Université de Strasbourg) have been identified to induce human diseases, including mitochondrial myopathies, lethal infantile mitochondrial myopathy, Alzheimer and Parkinson disease, and Leber hereditary optic neuropathy. Yeast was reported as a platform to understand the cellular role of human pathogenetic mitochondrial tRNAs, because mitochondrial tRNA sequence and function between yeast and humans are general conservative (55). For example, the A14G substitution of yeast mitochondrial tRNAArg(UUR) caused equivalent aminoacylation defects and structure alterations to...
the A3243G human pathogenetic tRNA mutation (56). A similar yeast tRNA<sup>Thr</sup> knock-out system may be easily constructed and adapted to investigate in vivo the impact of tRNA<sup>Thr</sup> mutations and also provide insights into the cellular mechanism of tRNA-associated mitochondrial diseases.

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