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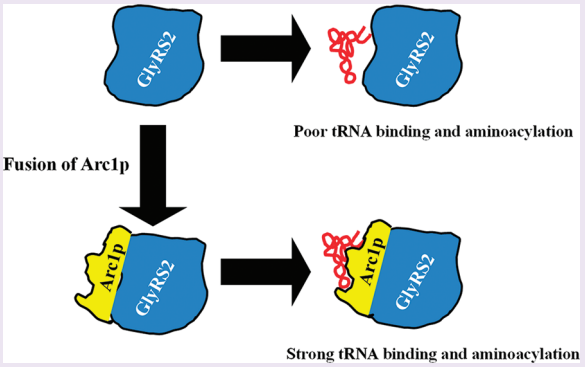
Rescuing a Dysfunctional Homologue of a Yeast Glycyl-tRNA Synthetase Gene

Shun-Jia Chen, Chih-Ying Lee, Szu-Ting Lin, and Chien-Chia Wang*

Department of Life Sciences, National Central University, 300 Jung-da Rd., Jung-li 32001, Taiwan

ABSTRACT: The yeast *Saccharomyces cerevisiae* contains two distinct nuclear glycyl-tRNA synthetase (GlyRS) genes, *GRS1* and *GRS2*. *GRS1* is dual functional in that possesses both cytoplasmic and mitochondrial activities, whereas *GRS2* is pseudogene-like. GlyRS1 and GlyRS2 are highly similar on the whole but are distinguished by a lysine-rich insertion domain of 44 amino acid residues, present only in GlyRS1. We herein present evidence that whereas the insertion domain is dispensable for the complementary activity of *GRS1 in vivo*, deletion of this domain from GlyRS1 reduced its aminoacylation activity by up to 9-fold. On the other hand, fusion of a constitutive *ADH* promoter to *GRS2* failed to confer a functional phenotype to the gene, but further fusion of *ARC1* (a yeast gene encoding a tRNA-binding protein, Arc1p) to this hybrid gene successfully rescued its activity. Most intriguingly, purified GlyRS2 retained a substantial level of aminoacylation activity. Fusion of Arc1p to this enzyme further enhanced its activity and stability. These findings highlight not only the structural integrity of the pseudogene-encoded enzyme but also the necessity of obtaining an auxiliary tRNA-binding domain for functioning of a yeast tRNA synthetase.



Faithful decoding of mRNA into protein depends on accurate aminoacylation of tRNA by aminoacyl-tRNA synthetases (aaRSs) and specific codon/anticodon base pairings. AaRSs are a structurally diverse group of enzymes, each of which catalyzes the attachment of a specific amino acid to its cognate tRNA. The resultant aminoacyl-tRNA is then delivered to ribosomes for protein translation. Typically, there are 20 aaRSs in prokaryotes, one for each amino acid.[1−3] In contrast, eukaryotes, such as yeast, possess two distinct sets of aaRSs, one localized to the cytoplasm and the other to mitochondria. Each set aminoacylates the isoaccepting tRNAs within its respective cellular compartment and is sequestered from isoacceptors confined in other compartments. Cytoplasmic and mitochondrial forms of a given tRNA synthetase are nearly always encoded by two distinct nuclear genes, regardless of the cellular compartment in which they are active. However, four *Saccharomyces cerevisiae* genes, *ALA1* (which encodes alanyl-tRNA synthetase),[4] *GRS1* (which encodes glycyl-tRNA synthetase (GlyRS)),[5] *HTS1* (which encodes histidyl-tRNA synthetase),[6] and *VAS1* (which encodes valyl-tRNA synthetase),[7] specify both the mitochondrial and cytosolic forms through alternative initiation of translation from two in-frame initiator codons.

Many yeast cytoplasmic aaRSs possess an N- or C-terminal polypeptide extension, known as an appended domain, which is absent from their prokaryotic counterparts.[8] Many of these domains are rich in lysine residues and are involved in tRNA binding, examples of which include glutaminyl-,[9] arginyl-,[10] and valyl-tRNA synthetases.[11] These domains act in *cis* as an auxiliary tRNA-binding domain and enhance the tRNA-binding affinity of the enzymes. In contrast, appended domains of some yeast cytoplasmic aaRSs participate in protein−protein interactions, examples of which include glutamyl-, methionyl-, and seryl-tRNA synthetases. Glutamyl- and methionyl-tRNA synthetases form a ternary complex with an aaRS cofactor, Arc1p (a nonspecific tRNA-binding protein encoded by *ARC1*), through their N-terminal appended domains,[12] while seryl-tRNA synthetase forms a binary complex with the peroxisome biogenesis-related factor, Pex21p, through its C-terminal appended domain.[13] These interactions were also shown to enhance the tRNA-binding and aminoacylation activities of the associated enzymes. Moreover, many of the appended domains contain one or more nuclear localization signals,[14] which are believed to target synthetases to the nucleus. It was shown that nuclear aminoacylation serves as a functional checkpoint for the maturation or integrity of tRNAs to be exported to the cytosol.[15,16]

Pseudogenes are dysfunctional homologues of functional genes that have lost their protein-coding ability or are otherwise no longer expressed in cells.[17] Some pseudogenes are devoid of an active promoter and introns, while others have mutations in their coding sequences leading to stop codons or frameshifts. In *S. cerevisiae*, two distinct nuclear GlyRS genes were identified. The first, *GRS1*, is dual functional in that encodes both cytoplasmic and mitochondrial forms of GlyRS through alternative initiation of translation, whereas the second, *GRS2*, is dispensable

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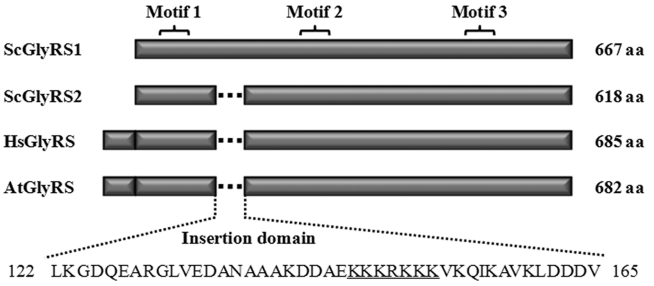

Figure 1. Comparison of various eukaryotic GlyRS sequences. Although the catalytic cores of GlyRS1 and GlyRS2 were considerably similar to each other, an insertion domain of 44 amino acid residues was present in GlyRS1 but not in GlyRS2 or other eukaryotic homologues. Relative positions of the class II-defining sequence motifs are marked at the top of the schematic: motifs 1 (amino acid residues 48−80), 2 (amino acid residues 251−279), and 3 (amino acid residues 503−522). Embedded inside the insertion domain of yeast GlyRS1 is a highly conserved sequence motif, KKKRKKK (underlined). ScGlyRS1, GlyRS1 of *S. cerevisiae*; HsGlyRS, GlyRS of *Homo sapiens*; AtGlyRS, GlyRS of *Arabidopsis thaliana*.

for growth.[5,18,19] Both genes encode an α_2-type protein. *GRS1* is constitutively expressed, whereas *GRS2* is essentially silent under normal growth conditions. It was thus hypothesized that *GRS2* is a pseudogene.[18] These findings prompted us to ask whether this dysfunctional gene can be converted to a functional copy *via* certain genetic modifications or recombinations. Results presented herein show that *GRS2* is poor in both transcription and translation and therefore cannot act as a functional gene even under the control of a constitutive *ADH* promoter. However, fusion of *ARC1* to this *ADH* promoter-driven gene successfully rescued its activity. Surprisingly, the purified recombinant GlyRS2 was fairly active *in vitro*. These data suggest that, like GlyRS1, GlyRS2 contains a functional active site for aminoacylation. The biological relevance of this finding to discovery of the true functions of a hypothetical pseudogene is further discussed.

Results and Discussion. *A Yeast-Specific Insertion Domain.* Despite obvious differences in functional potentials, yeast GlyRS1 and GlyRS2 share considerable sequence similarity on the whole (64.3% identity), and both possess a class II-defining architecture. Further analysis of these two proteins revealed an insertion domain of 44 amino acid residues (N-terminal amino acid residues 122−165), which was present in GlyRS1 but not in GlyRS2. Moreover, this domain appeared to be yeast-specific. It was conserved in almost all yeast GlyRS sequences, such as those from *Candida albicans*, *Schizosaccharomyces pombe*, and *Yarrowia lipolytica*, but was absent from other eukaryotic GlyRS sequences, such as those from human and *Arabidopsis* (Figure 1). This domain was highly enriched in charged amino acid residues (~50%), including 10 negatively charged (7 aspartate and 3 glutamate residues) and 13 positively charged residues (2 arginine and 11 lysine residues). In particular, lysine residues contributed up to 25% of its total amino acid composition. As a result of the high density of lysine residues, the insertion domain is expected to act as an auxiliary tRNA-binding domain. Embedded inside this insertion domain was a short motif, KKKRKKK, which was highly conserved in the insertion domains of yeast GlyRSs. In contrast to the yeast glycine enzymes, cytoplasmic GlyRSs of human and *Arabidopsis* possessed an N-terminal appended domain, instead of an insertion domain (Figure 1).

a

	Construct	Target gene	Cloning vector	Complementing activity	
				Cyt	Mit
①	pADH	None	pADH	-	-
②	pKJ111	*GRS1*	pADH	+	+
③	pSJ433	*GRS2*	pADH	-	-
④	pBC94	*GRS1(ΔID)*	pADH	+	+
⑤	pSJ471	*GRS2(ID)*	pADH	-	-
⑥	pSJ435	*ARC1-GRS2*	pADH	+	-
⑦	pSJ517	*MTS-ARC1-GRS2*	pADH	-	+
⑧	pSJ530	*MTS-GRS2*	pADH	-	-
⑨	pSJ436	*ID-GRS2*	pADH	-	-

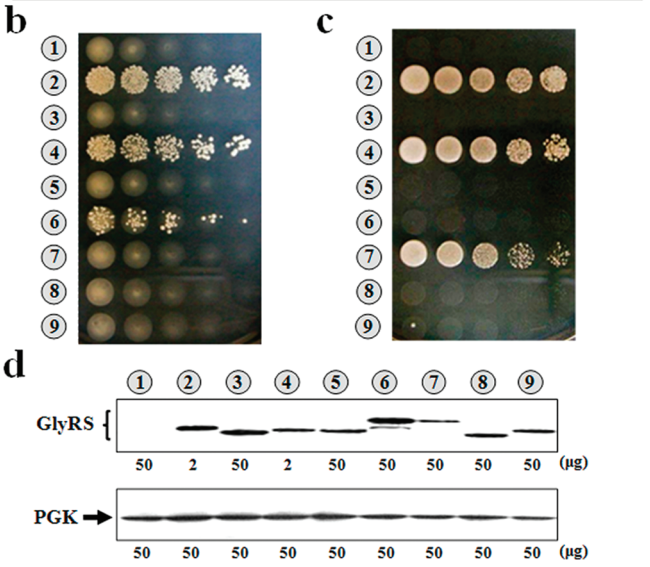

Figure 2. Complementation assays for yeast GlyRS genes and their derivatives. Constructs bearing yeast GlyRS genes were transformed into a *grs1*$^-$ strain of *S. cerevisiae*, and the ability of the transformants to grow on 5-FOA and YPG was tested. (a) Summary of constructs and their complementary activities. The symbols "+" and "−" denote positive and negative complementation, respectively. *mit*, mitochondrial; *cyt*, cytoplasmic. (b) Complementation assays for cytoplasmic activity on a 5-FOA plate. (c) Complementation assays for mitochondrial activity on a YPG plate. (d) Assay of protein expression by Western blotting. Upper panel, GlyRS; lower panel, PGK (as a control). Indicated at the bottom of the blots are the amounts of protein extracts loaded into the gel (2 μg for GlyRS1 and GlyRS1(ΔID); 50 μg for GlyRS2 and its derivatives). The numbers 1−9 (circled) in panels b−d represent the constructs shown in panel a.

Converting GRS2 into a Functional Gene. As *GRS2* is essentially silent under the control of its native promoter,[18] all GlyRS genes and their derivatives used in this assay were cloned in a pADH vector (a high-copy-number yeast shuttle vector with a constitutive *ADH* promoter and a short sequence coding for a His_6 tag) and expressed under the control of a constitutive *ADH* promoter. To investigate whether the insertion domain is essential for the complementary activity of *GRS1 in vivo*, a DNA sequence encoding this domain was deleted from *GRS1*, and the ability of the resultant construct to rescue growth defects of a *grs1*$^-$ strain of *S. cerevisiae*, RJT3/II-1, was tested. Figure 2 shows that deletion of the insertion domain from *GRS1* had little effect

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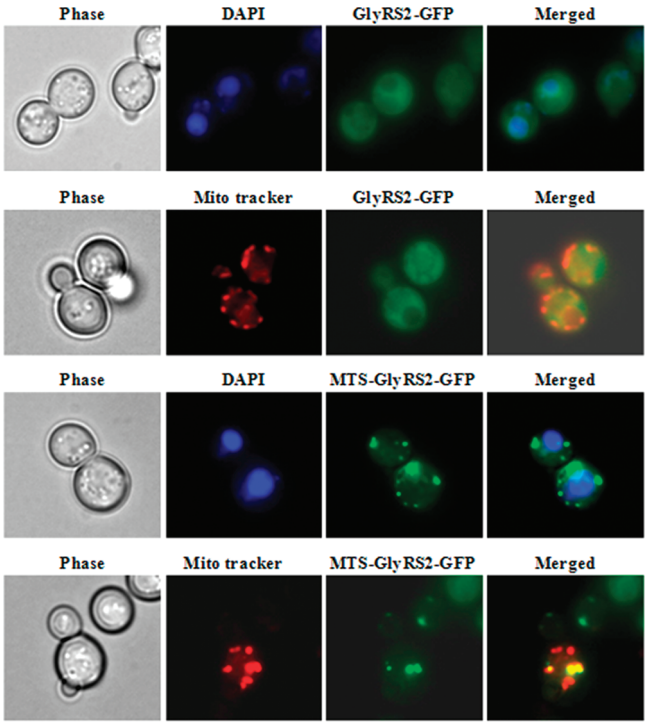

Figure 3. Analysis of the cellular localization of GlyRS2-GFP and MTS-GlyRS2-GFP by fluorescence microscopy. *GRS2-GFP* and *MTS-GRS2-GFP* were first transformed into INVSc1. The resultant transformants were then treated with a mitochondrial tracker dye or DAPI and visualized under fluorescence microscopy. A mitochondrial tracker and DAPI were used to label mitochondria and nuclei, respectively.

on its complementary activity. The deletion construct rescued the growth defects of the knockout stain on both 5-FOA and YPG with an efficiency comparable to that of the wild-type (see *GRS1(ΔID)*). Thus, regardless of whether the insertion domain is actually involved in tRNA binding, removal of this domain did not eliminate the enzyme's aminoacylation activity. On the other hand, insertion of this domain into the corresponding position or the 5′ end of *GRS2* (see *GRS2(ID)* and *ID-GRS2*) failed to convert the pseudogene into a functional gene. Neither hybrid constructs supported the growth of the knockout strain on 5-FOA or YPG. Note that because a yeast cell cannot survive on glycerol, a nonfermentable carbon source, without functional mitochondria, the cotransformants did not grow on the YPG (yeast extract-peptone-glycerol) plates unless a functional mitochondrial GlyRS was generated from the test plasmid.

We next tested whether *ARC1*, a yeast gene encoding a non-specific tRNA-binding protein, Arc1p, could rescue the complementary activity of *GRS2*. Pursuant to this objective, *ARC1* was fused in-frame to the 5′ end of *GRS2*, and the complementary activity of the resultant construct was assayed. Remarkably, *ARC1-GRS2* successfully rescued the growth defect of the knockout strain on 5-FOA, albeit to a lesser extent than that of *GRS1* (see *ARC1-GRS2* in Figure 2). This result argues that GlyRS2 *per se* retained a competent core active site, and its activity could somehow be enhanced by fusion to Arc1p. Just as remarkable was the finding that the *ARC1* fusion construct could rescue the growth defect of the knockout strain on YPG upon further fusion of a sequence encoding a mitochondrial targeting signal (see *MTS-ARC1-GRS2*). Taken together, these data suggest that Arc1p-GlyRS2 can recognize both cytoplasmic and mitochondrial isoaccepting $tRNAs^{Gly}$. As Arc1p is itself a nonspecific tRNA-binding protein, it is conceivable that the GlyRS2 moiety determined the tRNA specificity (k_{cat} discrimination) of the fusion enzyme.

To examine the relative protein expression levels of *GRS1* and *GRS2* under the control of a constitutive *ADH* promoter, a Western blotting analysis was carried out. The aforementioned constructs were first transformed into a *S. cerevisiae* strain, INVSc1, and protein extracts were prepared from the resultant transformants and analyzed by Western blotting using an anti-His_6 tag antibody. As shown in Figure 2d, the protein expression level of *ARC1-GRS2* was slightly higher (∼1.5-fold) than that of *GRS2* but much lower (∼16-fold) than that of *GRS1* or *GRS1-(ΔID)*. Hence, an *ADH* promoter did improve the expression of *GRS2*, but only to a relatively limited level. Overall, *GRS2* and its fusion constructs had protein expression levels 16- to 25-fold lower than that of *GRS1*. It was truly amazing to find that *ARC1-GRS2* could rescue the growth defect of the null allele with such a low level of protein expression (Figure 2b and c).

Cellular Localization of GlyRS2. To explore the cellular localization of GlyRS2 and MTS-GlyRS2, a DNA sequence encoding the green fluorescence protein (GFP) was inserted in-frame at the 3′ end of *GRS2* and *MTS-GRS2*, resulting in *GRS2-GFP* and *MTS-GRS2-GFP*, respectively. Note that these constructs were also expressed by a constitutive *ADH* promoter. The resultant constructs were transformed into INVSc1, and the cellular localization of these two fusion proteins was examined under fluorescence microscopy. As shown in Figure 3, GlyRS2-GFP was exclusively localized in the cytoplasm, while MTS-GlyRS2-GFP was colocalized with a mitochondrial tracker dye in mitochondria. Thus, GlyRS2, when expressed, existed in the cytoplasm but could be targeted to mitochondria by fusion to a mitochondrial targeting signal. This result is essentially consistent with the observation that *ARC1-GRS2* and *MTS-ARC1-GRS2* complemented the cytoplasmic and mitochondrial defects, respectively, of the null allele (Figure 2b and c).

Protein Stability of GlyRS2 and Arc1p-GlyRS2. To investigate whether Arc1p enhanced the protein stability of GlyRS2, which in turn increased its steady-state level and complementary activity, a cycloheximide-chase assay was performed. *GRS2* and *ARC1-GRS2* were first cloned into pGAL1, a high-copy-number yeast shuttle vector with an inducible *GAL1* promoter and a short sequence coding for a His_6 tag. Constructs were transformed into INVSc1, and cultures of the transformants were then induced with galactose for 2 h, followed by the addition of cycloheximide to terminate protein synthesis. Cells were harvested at various intervals following cycloheximide treatment and prepared for Western blotting analyses using an anti-His_6 tag antibody. As shown in Figure 4a, Arc1p-GlyRS2 was much more stable than GlyRS2. Protein levels of GlyRS2 dropped sharply after cycloheximide treatment compared to those of the Arc1p fusion protein under similar conditions. Up to ∼50% of GlyRS2 was degraded after 4 h of cycloheximide treatment, but only 10% of Arc1p-GlyRS2 was degraded within the same time period. Thus, Arc1p significantly enhanced the protein stability of GlyRS2, which may at least partially account for the positive complementary activity of Arc1p-GlyRS2.

The protein stability of GlyRS1 and GlyRS1(ΔID) was analyzed following a similar approach. As shown in Figure 4a, GlyRS1 was more stable than GlyRS1(ΔID). In fact, GlyRS1 was

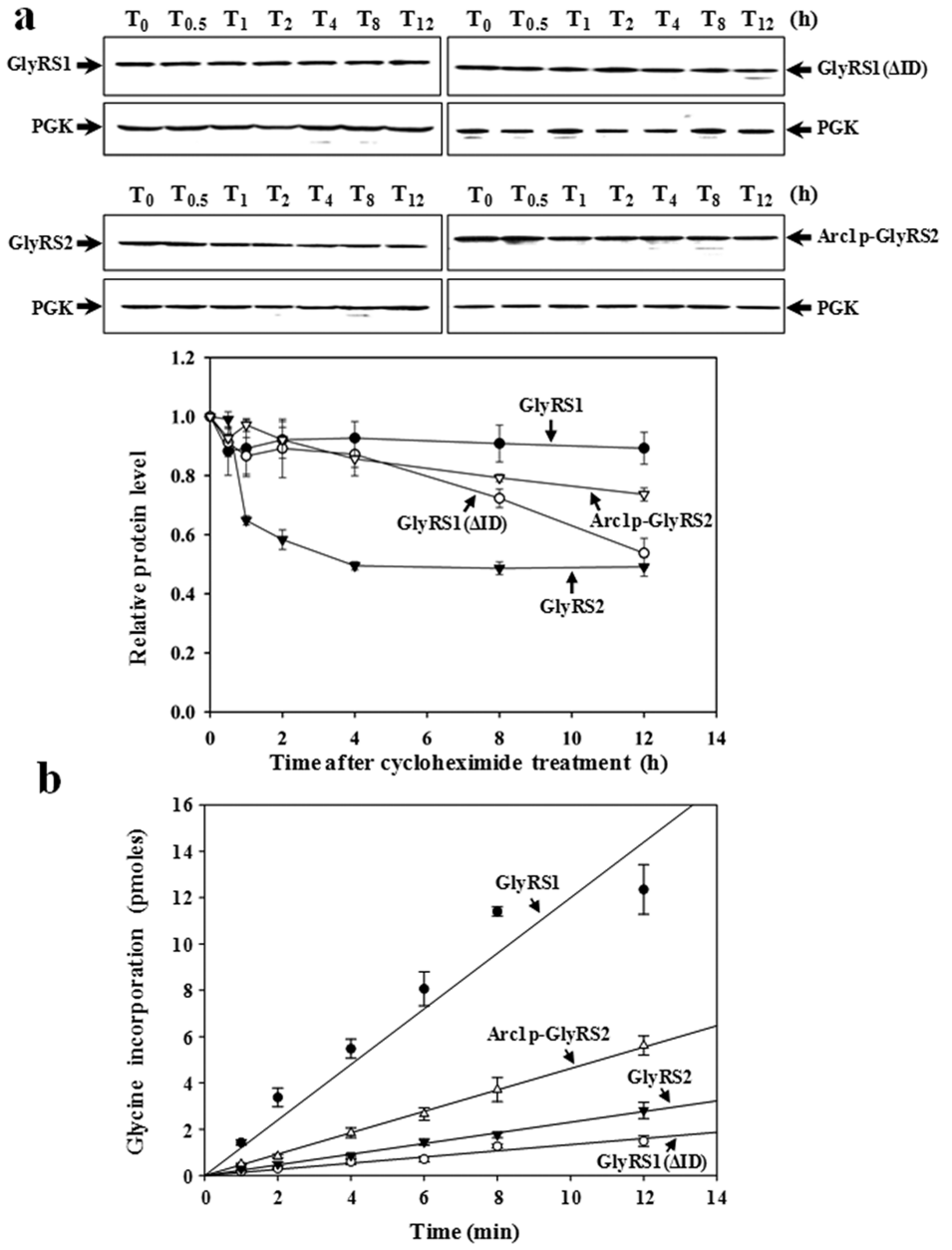

Figure 4. Degradation and aminoacylation assays for GlyRS1, GlyRS2, and their derivatives. a) Degradation assay. T_0, $T_{0.5}$, T_1, T_2, T_4, and T_8 denote 0, 0.5, 1, 2, 4, and 8 h, respectively, after the addition of cycloheximide. Upper panel, GlyRS; lower panel, PGK (as a loading control). Quantitative data for relative levels of GlyRSs are shown as a separate diagram below the Western blots. (b) Aminoacylation assay. Aminoacylation activities of the purified recombinant GlyRSs were determined by measuring the relative amounts of ^{3}H-glycine that were incorporated into tRNA using a liquid scintillation counter. The final concentration of GlyRS used in the reactions was 50 nM.

the most stable one among the four proteins examined. Its protein levels remained almost constant (~90%) throughout the entire time period tested. In contrast, GlyRS1(ΔID) was considerably stable in the early hours (0−4 h) but became less stable at later time points (8−12 h). This result suggests that deletion of the insertion domain impaired the protein stability of GlyRS1 to some extent. Nonetheless, such a deletion had little effect on the enzyme's complementary activity *in vivo* (Figure 2).

Aminoacylation Activity of Purified GlyRSs. To compare the aminoacylation activity of these enzymes, His_6-tagged GlyRS1, GlyRS1(ΔID), GlyRS2, and Arc1p-GlyRS2 were purified to homogeneity using Ni-NTA column chromatography[11] or in combination with SP-Sepharose column chromatography. To this end, the aforementioned pGAL1-based constructs were individually transformed into INVSc1, and cultures of the resultant transformants were then induced with galactose for 4 h. After induction, cells were harvested, and His_6-tagged enzymes were purified according to standard protocols. As shown in Figure 4b, deletion of the insertion domain from GlyRS1 significantly reduced its aminoacylation activity (~9-fold) (compare GlyRS1 and GlyRS1(ΔID)). Surprisingly, GlyRS2, which was expected to lose all or at least the majority of its biochemical activity, retained a substantial level of aminoacylation activity *in vitro*, ~20% relative to that of GlyRS1. Moreover,

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GlyRS2 appeared to have aminoacylation activity slightly higher than that of GlyRS1(ΔID) when they were tested at the same enzyme concentration *in vitro* (compare GlyRS2 and GlyRS1-(ΔID)). Conceivably, GlyRS2 is not as "inactive" as previously anticipated, and the "pseudogene-like" phenotype of *GRS2* is actually caused by its poor protein expression *in vivo*. Consistent with our assumption, fusion of Arc1p to GlyRS2 enhanced its aminoacylation activity by ~2-fold (compare GlyRS2 and Arc1p-GlyRS2). Thus, Arc1p increased the protein stability and also the catalytic activity of GlyRS2 (Figure 4), which might provide a rational basis for the positive complementary activity of *ARC1-GRS2 in vivo* (Figure 2).

Many yeast cytoplasmic aaRSs contain an N- or C-terminal appended domain. These domains act through different mechanisms to help the synthetases recruit tRNA to their vicinity for aminoacylation. Although GlyRS1 lacks such a domain, it contains a lysine-rich insertion domain (Figure 1). As this domain is located at a position that was predicted to interact with the acceptor stem of its cognate tRNA,[20] it might help the enzyme overcome differences between the acceptor stems of mitochondrial and cytoplasmic isoaccepting $tRNAs^{Gly}$ by providing more electrostatic interactions. Evidence presented here suggests that the insertion domain is important, but not essential, for the catalytic activity of the enzyme (Figure 4b). It remains unclear whether this deletion impaired recognition of the cytoplasmic and mitochondrial $tRNAs^{Gly}$ to similar extents. Paradoxically, deletion of such an important domain had little effect on the enzyme's complementary activity *in vivo*. The deletion mutant rescued the growth defects of the knockout strain with a fairly high efficiency (Figure 2). It appears that a relatively low level of glycylation activity is sufficient to confer a near wild-type growth phenotype to the null allele. A similar scenario was observed for yeast *ALA1* [11] and *VAS1* genes,[21] indicative of a common feature of yeast aaRSs. Such a feature may also account for the observation that *ARC1-GRS2*, while expressed at a considerably low level, could rescue the growth defect of the knockout strain (Figure 2). These and other findings led us to propose that *GRS2* is defective in both transcription and translation under normal conditions.

E. coli glutaminyl-tRNA synthetase *per se* cannot substitute for its yeast homologue *in vivo*, but can do so when it is fused to a nonspecific tRNA-binding domain, such as Arc1p or the appended domain of yeast glutaminyl- or valyl-tRNA synthetase.[9,11,22] Likewise, *E. coli* valyl-tRNA synthetase can be converted into a functional yeast enzyme.[21] In these instances, Arc1p and the appended domains act as an auxiliary tRNA-binding domain and enhance the tRNA-binding affinity of the associated enzymes. It appears that acquiring a *cis*-acting auxiliary tRNA-binding domain is crucial for functioning of yeast cytoplasmic aaRSs.[21,23] In accordance with this hypothesis, nearly all yeast cytoplasmic aaRSs possess an appended or insertion domain. It is noteworthy that while *E. coli* enzymes used in these studies poorly recognized yeast tRNA, they are fully functional wild-type enzymes responsible for protein translation in their native host. From this perspective, results presented herein are of particular interest. *GRS2*, as a pseudogene, can be readily converted to a functional copy *via* a similar approach (Figure 2). Most intriguingly, the protein product encoded by this otherwise silent gene retained a substantial level of aminoacylation activity *in vitro* (Figure 4b). Perhaps, *GRS2* is not as inactive as previously anticipated and plays a role under certain special conditions.

METHODS

Plasmid Construction. Cloning of the wild-type *GRS1* and *GRS2* genes of *S. cerevisiae* into pADH (a high-copy-number yeast shuttle vector with a constitutive *ADH* promoter, followed by multiple cloning sites and a short sequence coding for a His_6 tag) followed a protocol described earlier.[5] Briefly, a DNA sequence containing the open reading frame of *GRS1* (−75 to +2001 bp relative to ATG1) was amplified by a polymerase chain reaction (PCR) as an EagI-*Sal*I fragment (~2100 bp) using a pair of gene-specific primers. The forward primer (with an inserted EagI site) was annealed to −90 to −60 bp of this gene, while the reverse primer (with an inserted *Sal*I site) was annealed to +1986 to +2015 bp. After restriction enzyme digestion, the PCR fragment was cloned into the EagI/*Sal*I site of pADH. Cloning of *GRS2* followed a similar protocol.

To delete the insertion domain from GlyRS1, the DNA sequences flanking the insertion domain of *GRS1* were amplified as two independent fragments (an EagI-SpeI fragment containing −75 to +366 bp and an SpeI-*Sal*I fragment containing +493 to +2001 bp) and were cloned together into pADH, resulting in *GRS1(ΔID)* (pBC69). To introduce the insertion domain of GlyRS1 into the corresponding position in GlyRS2, a SpeI site was first created in *GRS2* between +369 and +370 bp, resulting in *GRS2-SpeI*. The DNA sequence encoding the insertion domain of GlyRS1 (+364 to +495 bp) was then amplified by PCR as a SpeI−SpeI fragment and cloned into the SpeI site of *GRS2-SpeI*, resulting in *GRS2(ID)* (pSJ471). To fuse the DNA sequence coding for Arc1p, the mitochondrial targeting signal of yeast valyl-tRNA synthetase (+1 to +138 bp of *VAS1*), or the insertion domain (+364 to +495 bp of *GRS1*) to the 5′ end of *GRS2*, these sequences were independently amplified by PCR as EagI−EagI fragments and cloned into the EagI site at the 5′ end of *GRS2*, resulting in *ARC1-GRS2* (pSJ435), *MTS-GRS2* (pSJ530), and *ID-GRS2* (pSJ436), respectively.

To fuse the green fluorescence protein (GFP) to GlyRS2, a DNA sequence encoding the GFP was PCR-amplified as a *Sal*I-*Sal*I fragment and isolated. After restriction enzyme digestion, the PCR-amplified DNA fragment was inserted in-frame at the 3′ end of *GRS2*, resulting in *GRS2-GFP*. Construction of *MTS-GRS2-GFP* followed a similar approach. GFP assays followed a protocol described elsewhere.[12] Western blotting followed a protocol described earlier.[11] Complementation assays for the cytoplasmic and mitochondrial functions of *GRS1* were previously described.[5]

Degradation Assay. To determine the turnover of proteins of interest, *GRS2* and *ARC1-GRS2* were first cloned in pGAL1 (a high-copy-number yeast shuttle vector with an inducible *GAL1* promoter and a short sequence coding for a His_6 tag), and the resultant constructs were then transformed into INVSc1 (*MATa*, *his3Δ1*, *leu2*, *trp1-289*, *ura3-52*, *MATα*, *his3Δ1*, *leu2*, *trp1-289*, and *ura3-52*) (Invitrogen, Carlsbad, CA). Transformants containing these constructs were grown in medium lacking leucine with 2% raffinose to a cell density of ~1.0 A_{600} and were then induced with 2% galactose for 2 h. Afterward, cells were washed twice and then grown in medium containing 2% glucose and 100 $\mu g\ mL^{-1}$ cycloheximide but lacking leucine. Cells were harvested at 0, 0.5, 1, 2, 4, and 8 h after the addition of cycloheximide and lysed. Samples (40 μg) of the cell extracts were resolved on 10% polyacrylamide and electrophoresed at 100 V for ~1 h, and the proteins were transferred to a polyvinylidene fluoride (PVDF) membrane and immunoblotted with a horseradish peroxidase (HRP)-conjugated anti-His_6 tag antibody (Sigma, St. Louis, MO). Quantitative data were obtained from three independent experiments and normalized by phosphoglycerate kinase (PGK).

Aminoacylation Assay. Aminoacylation reactions were carried out at 25 °C in a buffer containing 50 mM HEPES (pH 7.5), 50 mM KCl, 15 mM $MgCl_2$, 5 mM dithiothreitol, 10 mM ATP, 0.1 mg mL^{-1} bovine serum albumin (BSA), 100 μM unfractionated yeast tRNA (Boehringer

Mannheim, Germany), and 20 μM glycine (4 μM ^{3}H-glycine; Moravek Biochemicals, Brea, CA). The specific activity of ^{3}H-glycine used was 35.0 Ci mmol^{-1}. Purification of His_6-tagged GlyRS or its derivatives was as previously described.[24] Determination of active protein concentrations by active site titration was as previously described.[25] Reactions were quenched by spotting 10-μL aliquots of the reaction mixture onto Whatman filters (Maidstone, U.K.) soaked in 5% trichloroacetic acid and 1 mM glycine. Filters were washed three times for 15 min each in ice-cold 5% trichloroacetic acid before liquid scintillation counting. Data were obtained from three independent experiments and averaged. Error bars indicate ($\pm$2 $\times$ standard deviation).

AUTHOR INFORMATION

Corresponding Author
*E-mail: dukewang@cc.ncu.edu.tw.

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