WHEP domains exist in certain eukaryotic aminoacyl-tRNA synthetases and play roles in tRNA or protein binding. We present evidence herein that cytoplasmic and mitochondrial forms of Caenorhabditis elegans glycyl-tRNA synthetase (CeGlyRS) are encoded by the same gene (CeGRS1) through alternative initiation of translation. The cytoplasmic form possessed an N-terminal WHEP domain, whereas its mitochondrial isoform possessed an extra N-terminal sequence consisting of an mitochondrial WHEP domain, and an appended domain. Cross-species complementation assays showed that CeGRS1 effectively rescued the cytoplasmic and mitochondrial defects of a yeast GRS1 knock-out strain. Although both forms of CeGlyRS efficiently charged the cytoplasmic tRNAGly\textsuperscript{Cy} of C. elegans, the mitochondrial form was much more efficient than its cytoplasmic counterpart in charging the mitochondrial tRNAGly isoacceptor, which carries a defective U\textsuperscript{YC} hairpin. Despite the WHEP domain per se lacking tRNA binding activity, deletion of this domain reduced the catalytic efficiency of the enzyme. Most interestingly, the deletion mutant possessed a higher thermal stability and a somewhat lower structural flexibility. Our study suggests a role for the WHEP domain as a regulator of the dynamic structure and activity of the enzyme.

Aminoacyl-tRNA synthetases (aaRSs)\textsuperscript{3} belong to a ubiquitous and ancient family of enzymes that establish their genetic codes by attaching specific amino acids to their cognate tRNAs. The resultant aminoacyl-tRNAs are then delivered to ribosomes to decipher mRNA codons through base pairing with the anticodon of the aminoacyl-tRNA (1). Because protein translation takes place in both the cytoplasm and mitochondria in eukaryotes, two distinct sets of aaRSs are required: one functioning in the cytoplasm and the other functioning in mitochondria (1–4). In most cases, the cytoplasmic and mitochondrial forms of an aaRS are encoded by two different nuclear genes. Occasionally, both isoforms of an aaRS are encoded by the same nuclear gene through alternative initiation of translation, examples of which include genes encoding yeast alanyl-, glycyl-, histidyl-, and valyl-tRNA synthetases (5–9). As a result, cytoplasmic and mitochondrial forms of an aaRS, for example yeast glycyl-tRNA synthetase (GlyRS), possess essentially the same polypeptide sequence, except for a cleavable mitochondrial targeting signal (MTS) attached at the N terminus of the mitochondrial precursor form.

GlyRS is one of the most intriguing aaRSs because of its divergent quaternary structure and evolutionary origin. Two distinct oligomeric structures of GlyRS exist: one with an α\textsubscript{2} structure and the other with an α\textsubscript{2}β\textsubscript{2} structure (10, 11). These two forms are divergent not only in subunit composition but also in molecular size and protein sequence (12–14). Even so, they possess the same signature motifs and are thus assigned to the same class (class II). To date, α\textsubscript{2}β\textsubscript{2} enzymes exist only in bacteria and chloroplasts, but α\textsubscript{2} enzymes are spread over all three domains of life. The major identity elements of tRNA\textsuperscript{Gly} reside in the discriminator base (N73), the top three base pairs of the acceptor stem (1:72, 2:71, and 3:70), and C35/C36 in the anticodon loop (4). Among these identity elements, the discriminator base is of particular interest. It is a U in bacteria and an A in eukaryotic cytoplasm. In general, U73-containing tRNA\textsuperscript{Gly} pairs with an α\textsubscript{2}β\textsubscript{2}-type GlyRS enzyme, whereas A73-containing tRNA\textsuperscript{Gly} pairs with an α\textsubscript{2}-type GlyRS enzyme (15). However, this once-tight rule was broken by the discovery that Thermus thermophilus possesses a U73/α\textsubscript{2} pair (15).

GlyRS has attracted enormous attention over the past decade because of its implication in Charcot-Marie-Tooth (CMT) disease, one of the most common inherited neurological disorders (16). To date, 13 missense mutations of human GlyRS were shown to cause a dominant axonal form of CMT, also known as CMT type 2D (17, 18). However, there is no direct causal relationship between loss of the primary function of the enzyme and CMT disease, because not all CMT-causing mutants possess impaired aminoacylation activity (19). Recent studies suggested that GlyRS mutations also cause a CMT-like syndrome in other animals such as mice (20) and flies (21).

There is only one GlyRS gene in the nuclear genome of Caenorhabditis elegans, namely, CeGRS1. Because mitochondrial tRNAs in this organism typically possess a defective T\textsuperscript{YC} hairpin, the question arose as to how enzyme(s) encoded by this gene can efficiently charge such a tRNA species. Our study shows that CeGlyRS acquired two functional domains, a mitochondrial appended domain (MAD) and a WHEP domain, during evolution. The MAD enabled the enzyme to charge its cognate tRNA (containing a defective T\textsuperscript{YC} hairpin) with a higher efficiency, whereas the WHEP domain contributed to regulat-

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\*This work was supported by Ministry of Science and Technology Grants MOST 103-2311-B-008-003-MY3, MOST 103-2923-B-008-001-MY3, and NSC 102-2311-B-008-004-MY3 (to C.-C. W.) and National Central University and Landsseed Hospital Joint Research Program Grant NCU-LSH-103-A-003 (to C.-C. W.). The authors declare that they have no conflicts of interest with the contents of this article.

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3 The abbreviations used are: aaRS, aminoacyl-tRNA synthetase; CHX, cycloheximide; DAPI, 4',6-diamidino-2-phenylindole; 5-FOA, 5-fluoroorotic acid; GlyRS, glycyl-tRNA synthetase; MAD, mitochondrial appended domain; MTS, mitochondrial targeting signal; YPG, yeast extract peptone glycero; CMT, Charcot-Marie-Tooth.
ing the dynamic structure of the enzyme. As a result, deletion of the WHEP domain altered the thermal stability, structural flexibility, and catalytic rate of the enzyme. These results provide new insights into understanding the structure-function relationships of CeGlyRS and its homologues.

Results

*C. elegans* GRS1 Is a Dual Functional Gene—Analysis of the ORF of CeGRS1 suggested that this gene is dual functional, with ATG1 and ATG65 being the respective initiator codons of the mitochondrial and cytoplasmic forms of CeGlyRS (Fig. 1A). The mitochondrial form of CeGlyRS (CeGlyRS<sub>m</sub>) possessed a 64-residue polypeptide extension with a mitochondrial matrix-processing peptidase cleavage site in between N-terminal residues 20 and 21. The sequence containing N-terminal residues 1–20 was rich in positively charged and hydroxylated residues and was devoid of acidic residues, a feature characteristic of an MTS, and the sequence containing N-terminal residues 21–64 was specific to the mitochondrial form; this sequence is herein referred to as the MAD. The MAD was absent from all other eukaryotic GlyRS homologues shown herein. In contrast, the N-terminal domain (containing residues 1–66) of the cytoplasmic form of CeGlyRS (CeGlyRS<sub>c</sub>) shared high sequence identity (57–70% identity) with the WHEP domains of GlyRSs of *Drosophila melanogaster*, *Bombyx mori*, and *Homo sapiens* (Fig. 1B).

Because only one GlyRS gene exists in the genome of *C. elegans*, one would normally assume that the identity elements must be strictly conserved between its nuclear and mito-
chondrial encoded tRNAs\textsuperscript{Gly} (CetRNA\textsubscript{n}\textsuperscript{Gly} and CetRNA\textsubscript{m}\textsuperscript{Gly}, respectively) so that CeGlyRS can efficiently charge both tRNA isoacceptors. As expected, the discriminator base A73 and C35/C36 in the anticodon loop were conserved in yeast and \textit{C. elegans} tRNAn\textsuperscript{Gly} and tRNAm\textsuperscript{Gly} (Fig. 1C). However, contrary to our anticipation, the first three base pairs in the acceptor stem highly diverged among these tRNA isoacceptors, suggesting that they are not critical for recognition in these two eukaryotic organisms. In addition to differences identified in the acceptor stem, CetRNAm\textsuperscript{Gly} possessed a defective T\textsuperscript{Ψ}C hairpin, which prompted us to ask how CeGlyRS can effectively charge this tRNA.

\textit{C. elegans GRS1 Can Rescue Growth Defects of a Yeast GRS1 Knock-out Strain}—To test the functional potential of CeGRS1, we cloned the ORF of this gene or its derivatives into pADH and tested the ability of the resultant transfectants to grow on 5-FOA and YPG. As shown in Fig. 2, CeGlyRS\textsubscript{m} conferred a positive growth phenotype to the null allele on both 5-fluoroorotic acid (5-FOA) and YPG, suggesting that this construct can functionally substitute for the yeast \textit{GRS1} gene (row 3 in Fig. 2, A and B). This result also implied that a minor portion of the CeGlyRSm protein was retained in the cytoplasm for functioning, a scenario often seen in yeast aaRS complementation (22). Deletion of the MTS alone or both the MTS and MAD specifically impaired its mitochondrial rescue activity; MTS-deleted mutants restored the growth phenotype of the knock-out strain on 5-FOA but not YPG (rows 4 and 5). Thus, the MTS was required for mitochondrial targeting of CeGlyRS\textsubscript{m}, and the MAD was dispensable for efficient aminoacylation of yeast tRNAn\textsuperscript{Gly} by CeGlyRS. Further deletion of the WHEP domain impaired both the cytoplasmic and mitochondrial rescue activities (rows 6 and 8). As expected, attaching a heterologous MTS to CeGlyRSc yielded a fusion construct that could restore the growth phenotype of the knock-out strain on
Deletion of the MTS from CeGlyRS_m redirected the protein to the cytoplasm. Because of the imaging technology resolution constraints, we could not rule out the possibility that a minor portion of the GFP fusion proteins targeted other unintended cellular compartments.

The WHEP Domain-deleted CeGlyRS Variant Possesses Higher Thermal Stability—Because deletion of the WHEP domain impaired the cross-species rescue activity of CeGlyRS_c (Fig. 2), we next investigated whether and to what extent this deletion alters the protein’s thermal stability in vitro. Pursuant to this objective, genes encoding CeGlyRS variants were transformed into Escherichia coli, and His6-tagged CeGlyRS proteins were purified to homogeneity through nickel-nitrioltriacetic acid affinity chromatography (Fig. 4A). Purified CeGlyRS_m and ΔWHEP proteins were then subjected to CD spectrometry at 200–250 nm. As shown in Fig. 4B, CeGlyRS_c retained most of its secondary structure (with high molar ellipticity values at 222 and 208 nm) at temperatures below 40 °C but lost much of its secondary structure when the temperature reached 50 °C or higher. In contrast, ΔWHEP retained most of its secondary structure even when the temperature reached 50 °C. The deletion mutant did not lose much of its secondary structure until the temperature reached 60 °C or higher (Fig. 4B). As a result, CeGlyRS_c and ΔWHEP, respectively, had melting temperatures of ~60 and ~68 °C (Fig. 4C). Thus, deletion of the WHEP domain increased the thermal stability of the protein.

The WHEP Domain-deleted CeGlyRS Variant Possesses Slightly Lower Structural Flexibility—To further study the effect of the deletion on the structural flexibility of CeGlyRS_c, limited proteolysis was carried out with CeGlyRS_c/trypsin in a ratio of 100:1. Limited proteolysis is often used to probe the structure and dynamics of proteins. Exposed regions such as loops and other flexible regions are more susceptible to the prolific protease. As shown in Fig. 4D, ΔWHEP was somewhat more resistant to the protease than was the WT. Approximately 80% of the WT protein was hydrolyzed after 2 h of protease treatment, and only ~10% protein remained after 4 h of treatment. In contrast, ~40% of the ΔWHEP protein remained after 4 h of treatment. This result suggests that the WT protein possessed a slightly more-flexible structure than did the deletion mutant.

The MAD and WHEP Domain Respectively Play Roles in Aminoacylation of CetRNA_m^{Gly} and CetRNA_c^{Gly}—As mentioned above, CeGlyRS possesses two distinctive domains at its N terminus: MAD and WHEP. We were prompted to ask whether these two domains are involved in tRNA aminoacylation. Aminoacylation reactions were carried out at ambient temperature with 20 or 200 nM of enzyme and 5 μM of in vitro transcribed tRNA^{Gly}. Note that purified CeGlyRS_m represents a mature form of CeGlyRS_m (without MTS). As shown in Fig. 5A, CeGlyRS_m and CeGlyRS_c charged CetRNA_m^{Gly} with a similarly high efficiency, suggesting that the MAD is dispensable for aminoacylation of CetRNA_m^{Gly}. On the other hand, deletion of the WHEP domain from CeGlyRS_c reduced its glycylation activity ~3-fold, suggesting that the WHEP domain is somehow involved in this reaction (Fig. 5A). Although in vitro transcribed CetRNA_m^{Gly} (lacking the entire T-arm) was a very poor sub-

![FIGURE 3. Cellular localization of C. elegans glycyl-tRNA synthetase (CeGlyRS) variants in yeast. Constructs encoding various GFP fusion proteins were individually transformed into a yeast GRS1 knockout strain. The resultant transformants were treated with MitoTracker and visualized under fluorescence microscopy.](image-url)
strate for glycylation (presumably because of a lack of modification at position 9 with 1-methyladenosine) (24), CeGlyRSm was more efficient (at least 5-fold) than CeGlyRSc (and WHEP) in charging this T-armless tRNA (Fig. 5B). These results together suggest that the MAD enhances aminoacylation of CetRNAmGly by CeGlyRSm and that the WHEP domain contributes to aminoacylation of CetRNAnGly by CeGlyRSc.

Relative aminoacylation activities of CeGlyRSc and WHEP at various temperatures were determined and shown in Fig. 5C. To take a closer look at the role of the WHEP domain in tRNA aminoacylation, kinetic parameters for aminoacylation of CetRNAnGly by CeGlyRSc and WHEP were determined. As shown in Table 1, CeGlyRSc had a $K_m$ value of 0.24 M for CetRNAnGly and a $k_{cat}$ value of 1.73 s$^{-1}$, whereas WHEP had a $K_m$ value of 0.21 M for CetRNAnGly and a $k_{cat}$ value of 0.62 s$^{-1}$. Thus, deletion of the WHEP domain had almost no effect on the apparent affinity of the enzyme for CetRNAnGly but reduced its catalytic rate 2.8 times. That is, CeGlyRSc possessed a catalytic efficiency ($k_{cat}/K_m$) 2.4 times higher than that of WHEP. Similar results were obtained using yeast tRNAnGly as the substrate (Table 1).

The WHEP Domain per se Does Not Bind tRNA—To test whether the WHEP domain itself binds tRNA, a DNA sequence encoding the WHEP domain was cloned into pET21, and the resultant construct was transformed into an E. coli strain, BL21(DE3), for expression. The recombinant WHEP domain (with a C-terminal His$_6$ tag) was purified through nickel-nitrilotriacetic acid affinity chromatography, and an affinity co-electrophoresis assay was carried out using CetRNAnGly as the ligand. Affinity co-electrophoresis is particularly useful for determining weak protein-nucleic acid interactions. As shown in Fig. 5D, whereas CeGlyRSc effectively bound tRNAnGly (with a $K_d$ value of $\sim$4 M), the purified WHEP domain failed to bind the tRNA even at high protein concentrations (up to 64 M). This result is in agreement with the findings of the kinetic study shown in Table 1.

Overexpression of the WHEP Domain-deleted CeGlyRS Variant Can Rescue the Yeast Knock-out Strain—Because deletion of the WHEP domain only reduced the aminoacylation efficiency ($k_{cat}/K_m$) of CeGlyRS for yeast tRNA$^{Gly}$ 3.5-fold (Table 1), we wondered whether the deletion mutant could be made a functional yeast enzyme in vivo by overexpression from a stronger promoter. To this end, a DNA sequence encoding WHEP was cloned into pTEF1 (with a strong TEF1 promoter), and the ability of the resultant construct to rescue the growth defects of the yeast GRS1 knock-out strain was tested. As shown in Fig. 6A, WHEP, overexpressed from a TEF1 promoter, successfully restored the growth phenotype of the knock-out strain on 5-FOA, but not on YPG (row 3). Attaching a heterologous MTS to WHEP failed to yield a functional yeast mitochondrial
Western blotting results showed that MTS-H9004WHEP had an expression level much lower than that of its counterpart without an MTS, i.e., H9004WHEP, in yeast (Fig. 6B, left panel), regardless of the promoters used. This may have been the reason why MTS-H9004WHEP failed to provide sufficient glycylation activity in yeast mitochondria (Figs. 2, 6). We also found that the TEF1 promoter used was ~12-fold stronger than the ADH promoter used (Fig. 6B, right panel).

TABLE 1
Kinetic parameters for aminoacylation of tRNA_Gly by C. elegans GlyRS variants

| GlyRS variant | tRNA   | Km (µM) | k_cat (µM·s⁻¹) | k_cat/K_m (µM⁻¹·s⁻¹) |
|---------------|--------|---------|----------------|-----------------------|
| CeGlyRS       | CetRNA_Gly | 0.24 ± 0.09 | 1.73 ± 0.61 | 7.2                   |
| ΔWHEP         | CetRNA_Gly | 0.21 ± 0.10 | 0.62 ± 0.28 | 3.0                   |
| CeGlyRS       | ScetRNA_Gly | 0.39 ± 0.13 | 1.92 ± 0.75 | 4.9                   |
| ΔWHEP         | ScetRNA_Gly | 0.37 ± 0.14 | 0.51 ± 0.20 | 1.4                   |

Discussion

Based on the modular organization of aaRSs, it is widely accepted that modern aaRSs descended from successive additions of new domains to the catalytic core (25). According to this hypothesis, primitive aaRSs possessed only a minimal core capable of activating amino acids. New domains were later recruited to the catalytic core, forming a larger aaRS capable of recognizing an ancient tRNA with a structure mimicking the existing tRNA acceptor stem. These early tRNA-aaRS pairs continued to grow through additions of the anticodon stem-loop structure and anticodon-binding domain to yield contemporary tRNAs and aaRSs. Other functional domains, such as the editing, protein-interacting, and auxiliary tRNA-binding domains, were later recruited by aaRSs to facilitate their translation efficiencies (25). The MAD and WHEP domain of CeGlyRS are probably among such examples (Fig. 5). However, some domains recruited by aaRSs are unrelated to their primary functions. Such domains often confer noncanonical functions, such as transcriptional regulation, translational regulation, mitochondrial RNA splicing, and cytokine-like activity, to the aaRSs (26).

In addition to serving as an intracellular translation enzyme, human GlyRS also circulates in serum and acts as a component of the innate defense system against ERK-activated tumorigen-
Amino acid residues 1–46 of the mitochondrial precursor form of yeast valyl-tRNA synthetase was amplified by a PCR as an EagI-EagI fragment and was inserted at the 5’ end of the ORF of CeGlyRS<sub>c</sub>. To fuse the GFP to CeGlyRS<sub>c</sub> (or its derivatives), a DNA sequence encoding the GFP was amplified by a PCR as an XhoI-XhoI fragment and then inserted at the 3’ end of the ORF of CeGlyRS<sub>c</sub>.

**Reduction of Inflammatory Response**—The yeast <i>GRS1</i> knock-out strain RJT3/II-1 was previously described (32). We carried out a reduction of inflammatory response assay by introducing a test plasmid carrying the target gene and a <i>LEU2</i> marker into RJT3/II-1 and determining the ability of the transformants to grow in the presence of 5-FOA. Starting from a cell density of 1.0 × 10<sup>5</sup> cells/mL, cell cultures were 3-fold serially diluted, and 10-μL aliquots of each dilution were spotted onto designated plates containing 5-FOA. The plates were incubated at 30 °C for 3 days. Transformants expressing the maintenance plasmid (which carried the WT ScGlyRS1 gene) with a <i>URA3</i> marker in the presence of 5-FOA and thus could not grow on 5-FOA plates unless the test plasmid encoded a functional cytoplasmic GlyRS.

**Complementation Assay for Mitochondrial Activity**—The yeast <i>GRS1</i> knock-out strain RJT3/II-1 was cotransformed with a test plasmid (which carried a <i>LEU2</i> marker) and a second maintenance plasmid (which carried a <i>TRP1</i> marker and only expressed the cytoplasmic form of ScGlyRS). In the presence of 5-FOA, the first maintenance plasmid (which carried a <i>URA3</i> marker) was evicted from the cotransformants, and the second maintenance plasmid was retained. Thus, all cotransformants could survive 5-FOA selection because of the presence of the cytoplasmic ScGlyRS derived from the second maintenance plasmid. The mitochondrial phenotypes of the cotransformants were further tested on YPG plates at 30 °C, with results documented on day 3 following plating. Because a yeast cell cannot survive on glycerol (a nonfermentable carbon source) without functional mitochondria, the cotransformants did not grow on the YPG plates unless the test plasmid encoded a functional mitochondrial GlyRS.
**WHEP Domain**

**Fluorescence Microscopy**—Yeast cells were grown to ~0.6 A600 in SD/−Leu selective medium. We pre-treated cells with DAPI (0.5 μg/ml) or MitoTracker (300 nm) for 30 min. Fluorescence microscopy (Axioobserver.A1; Carl Zeiss, Oberkochen, Germany) was then used to examine samples with a 100× objective at 25 °C, and images were captured with a CCD camera (Axioimager MRm; Carl Zeiss). Nuclear and mitochondrial tracks and merged images were generated with Axiovision Rel. 4.8 software and then subjected to two-dimensional deconvolution with AutoQuant X2.

**Aminoacylation Assay**—Aminoacylation reactions were carried out at ambient temperature in a buffer containing 50 mM HEPES (pH 7.5), 50 mM KCl, 15 mM MgCl2, 5 mM dithiothreitol, 10 mM ATP, 0.1 mg/ml bovine serum albumin, 5 μM tRNA Gly, and 20 μM glycine (2 μM [3H]glycine; Moravek Biochemicals, Brea, CA). The specific activity of [3H]glycine used was 35.0 Ci/mmol. The yeast VAS1 gene encodes both the mitochondrial and cytoplasmic valyl-tRNA synthetases. Wide divergence of primary structure from bacterial counterparts and species-specific aminoacylation. J. Biol. Chem. 269, 5017–5035.

Kinetic parameters for aminoacylation of tRNA by purified enzymes were determined by directly fitting the data points to the Michaelis-Menten equation. Initial rates of aminoacylation were determined at 25 °C with tRNA Gly concentrations ranging 1–20 μM and enzyme concentrations ranging 10–200 nM. The data were obtained from three independent experiments and averaged.

**Polyacrylamide Affinity Co-electrophoresis**—In vitro transcribed CtRNA Gly was labeled with [32P] using polynucleotide kinase (New England Biolabs, Beverly, MA) after dephosphorylation with calf intestine phosphatase. The recombinant WHEP domain was 2-fold serially diluted and mixed with a 5% protein gradient of 1–64 WHEP domain was 2-fold serially diluted and mixed with a 5%

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