Thio-modification of Yeast Cytosolic tRNA Requires a Ubiquitin-related System That Resembles Bacterial Sulfur Transfer Systems*

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The wobble uridine in yeast cytosolic tRNA^{\text{cyt}}^{\text{u}} \text{UUC} and tRNA^{\text{Glu}}^{\text{u}} \text{UUC} undergoes a thio-modification at the second position (s^2 modification) and a methoxycarbonylmethyl modification at the fifth position (mcm^5 modification). We previously demonstrated that the cysteine and mitochondrial iron-sulfur (Fe/S) cluster assembly machineries termed CIA and ISC, including a cysteine desulfurase called Nfs1, were essential for the s^2 modification. However, the cystosolic component that directly participates in this process remains unclear. We found that ubiquitin-like protein Urm1 and ubiquitin-activating enzyme-like protein Uba4, as well as Tuc1 and Tuc2, were strictly required for the s^2 modification. The carboxyl-terminal glycine residue of Urm1 was critical for the s^2 modification, indicating direct involvement of the unique ubiquitin-related system in this process. We also demonstrated that the s^2 and mcm^5 modifications in cytosolic tRNAs influence each other’s efficiency. Taken together, our data indicate that the s^2 modification of cytosolic tRNAs is a more complex process that requires additional unidentified components.

Many modified nucleotides are found in tRNAs of various organisms, and the post-translational modification of tRNA molecules is thought to be necessary to maintain their structure and thereby to exert their proper function in translation (1). In yeast, uridine of the first position of anticodon, in cytosolic tRNA for glutamate (cy-tRNA^{\text{Glu}}^{\text{u}} \text{UUC}) contains sulfur instead of oxygen at the second position (the s^2 modification) and 5-methoxycarbonylmethyl at the fifth position (the mcm^5 modification). These cy-tRNAs read split codon boxes; they decode the general NAA-type codon and can wobble into the NAG codon (2). Thus, the s^2 and mcm^5 modifications in U_{34} are thought to be important in maintaining stable codon-anticodon pairing during decoding of these cy-tRNAs on the ribosome.

For the s^2 modification of cy-tRNA^{\text{cyt}}^{\text{UUC}} and cy-tRNA^{\text{Glu}}^{\text{u}} \text{UUC}, the cysteine desulfurase Nfs1 located in the mitochondria was essential, indicating that a sulfur atom used for the s^2 modification should originate from the cysteine sulfur atom located inside the mitochondria (3). Nfs1 is also known to provide sulfur for the iron-sulfur (Fe/S) cluster biosynthesis, which involves the mitochondrial ISC and cytosolic CIA machineries (4–6). We previously demonstrated that the s^2 modification of cy-tRNAs was dependent not only on Nfs1 but also on other ISC and CIA proteins such as Cfd1 (7). Because the cytosolic Fe/S cluster assembly mediated by CIA must precede Fe/S cluster biosynthesis in the mitochondria, which is mediated by ISC (8), our previous observation suggests that at least one cytosolic Fe/S cluster-containing protein plays an indispensable role in the s^2 modification of cy-tRNAs (7). It may also be possible that the sulfur atom forming an Fe/S cluster may itself be directly used for the s^2 modification. Besides the mitochondrial Nfs1 and Fe/S cluster assembly machineries, ISC and CIA, other cystosolic components directly involved in the s^2 modification remain to be elucidated. However, for the mcm^5 modification of U_{34} in cy-tRNA^{\text{cyt}}^{\text{UUC}} and cy-tRNA^{\text{Glu}}^{\text{u}} \text{UUC}, several cytosolic proteins, including the Elongator proteins (Elp1–6) and Kti11–13 proteins, have been shown to be involved (9). Among them, Elp3 is an Fe/S cluster-containing protein (10), but its knock-out mutant was reported to retain the s^2-modified uridine derivatives in their nucleoside pool (9).

Here we identify Tuc1 (previously named Ncs6) as an indispensable factor for the s^2 modification. In addition, we found that Tuc2 (previously named Ncs2), Urm1, and Uba4 were also strictly required for the s^2 modification of cy-tRNAs. All of these factors are somehow genetically linked to Cla4, whose depletion causes a synthetic lethality with the tuc1 null mutation (11). Although Urm1 and Uba4 were initially identified as a ubiquitination-like protein modifier system (12), the exact function of this system remains unclear. We propose here that a cystosolic sulfur transfer system mediated by Urm1 and Uba4 plays a key role in the s^2 modification of cy-tRNAs.

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§1 The abbreviations used are: cy-tRNA, cytosolic tRNA; cy-tRNA^{\text{cyt}}^{\text{UUC}}; tRNA^{\text{Glu}}^{\text{u}} \text{UUC} and tRNA^{\text{cyt}}^{\text{UUC}}; cytosolic tRNA for lysine; cy-tRNA^{\text{cyt}}^{\text{UUC}}; tRNA^{\text{Glu}}^{\text{u}} \text{UUC} for glutamate; s^2 modification, thio-modification at the second position; s^2U, 2-thiouridine; mcm^5 modification, 5-methoxycarbonylmethyl modification at the fifth position; mcm^5U, 5-methoxycarbonylmethyl uridine; cm^5U, 5-carboxymethyl uridine; Fe/S, iron-sulfur; ISC machinery, iron-sulfur cluster biogenesis machinery; CIA machinery, cytosolic iron-sulfur cluster assembly machinery; TAP, tandem affinity purification tag; AP, [N-acryloylamino]phenyl mercuric chloride; U_{34} uridine at the first anticodon sequence in tRNA; E1, ubiquitin-activating enzyme; HPLC, high pressure liquid chromatography.
**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Growth Conditions**—BY4742 (MATa his3Δ1 leu2Δ0 ura3Δ0) and all gene knock-out strains were purchased from OpenBiosystems (Huntsville, AL). Cells were grown with YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) or with synthetic complete media containing glucose lacking leucine (SD-Leu) or histidine (SD-His) for plasmid maintenance. Synthetic medium supplemented with 2% galactose and 3% glycerol (SGGly-Leu or SGGly-His) was used for maintenance. Synthetic medium supplemented with 2% galactose and 3% glycerol (SGGly-Leu or SGGly-His) was used for maintenance. Synthetic medium supplemented with 2% galactose and 3% glycerol (SGGly-Leu or SGGly-His) was used for maintenance.

The **TUC1** gene was amplified by PCR and subcloned into the plasmid pESC-LEU (Stratagene, La Jolla, CA), generating the plasmid pESC-Tuc1-myc, which expressed Tuc1 followed by a c-Myc epitope under the GAL1 promoter. The plasmid was introduced into Δtuc1 cells to generate the recombinant strain Δtuc1/Tuc1-myc. Cells grown on SD-Leu plates were cultivated with SGGly-Leu medium to express the recombinant protein, and recovery of the s² modification was confirmed with the APM-Northern analysis. The **TUC2** gene was also amplified by PCR and subcloned into the plasmid pESC-HIS to generate pESC-Tuc2-FLAG, generating Tuc2 followed by a FLAG epitope sequence under the GAL10 promoter. The plasmid was introduced into Δtuc2 cells to generate Δtuc2/Tuc2-FLAG cells. Cells grown on SD-His plates were cultivated with SGGly-His to express the recombinant protein, and recovery of the s² modification was confirmed. The Tuc2-TAP strain was also purchased (Open Biosystems, the yeast TAP strain).

The **URM1** gene was amplified with the same 5’-primer (5’-AAGGATCCGATGGTAAACGTGAAAGTGGA-3’) and the 3’-primer designed to contain the stop codon (5’-CCGTCGGACTTAACCACCATGTAATGTTGAA-3’) or the primer to construct the in-frame fusion of Urm1-myc (5’-CCGTCGACAACCACATGTAATGTTGAA-3’). Amplified fragments were subcloned into pESC-LEU to construct the pESC-Urm1 or pESC-Urm1-myc, respectively. These plasmids were then introduced into Δurm1 cells.

To examine temperature-sensitive and/or caffeine-sensitive growth, each strain grown to the mid-log phase in appropriate liquid media was harvested and transferred to SD medium. After a 4-h incubation at 30 °C, each cell suspension that measured an A₆₀₀ = 0.1 unit was serially diluted, spotted onto agar plates, and incubated at 30 °C or 37 °C.

**Gel Retardation Analysis Using APM Coupling with Northern Hybridization**—The presence of thiouridine in the prepared tRNA can be verified by the retardation of electrophoretic mobility on polyacrylamide gels containing [(N-acryloylaminophenyl)mercuric chloride (APM). Equal amounts (2 μg) of total RNA were separated with a denaturing gel containing 7 M urea and 24 μM of APM and analyzed with a specific DNA probe against cy-tRNA⁰⁺⁰ subcell, cy-tRNA⁰⁺⁰ subcell, and cy-tRNA⁰⁺⁰ subcell as shown previously (3). The relative proportion of the s² modification in each tRNA was determined with three independent experiments as described previously (7).

**Detection of the Adenylation Intermediate of Tuc1**—The TUC1 gene was inserted into pET28a (Novagen) and used to express a His₆-Tuc1 protein in *Escherichia coli* BL21(DE3)Star (Invitrogen). E. coli soluble cell extracts containing His₆-Tuc1 were incubated with a mixture of 4 μg of tRNA prepared from Δtuc1 cells at 37 °C for 30 min in a 10-μl reaction mixture with 50 mM Tris-HCl buffer (pH 7.5) containing 50 mM KCl, 12 mM Mg(OAc)₂, 1 mM dithiothreitol, and 5 μM of [α⁻³²P]ATP or [γ⁻³²P]ATP (~300 Ci/mmol, BD Biosciences). After quenching the reaction by adding 90 μl of 5% formic acid, tRNA was extracted and precipitated and then subjected to PAGE under denaturing conditions. The gel was stained with ethidium bromide and also exposed on an imaging plate to visualize the radioactivity using the BAS2500 system (FujiFilm).

**Sulfur Incorporation into the Recombinant Uba4 Protein in Vitro**—URM1 and UBA4 genes were cloned into pACYC-Duet1 (Novagen) to express Urm1 and Uba4 with a carboxyl-terminal S-tag (Uba4-S-tag either separately or simultaneously). Expression of the recombinant proteins in *E. coli* cells was induced by adding 0.5 mM isopropyl galactopyranoside for 3 h at 37 °C. When grown to A₆₀₀ = 0.5, *E. coli* cells were collected and resuspended in Luria Bertani medium containing half-concentration of salts, followed by further incubation for 30 min at 37 °C in the presence of 50 μg/ml blasticidin-S HCl (Invitrogen). The cells were then collected and suspended in 20 mM Tris-HCl (pH 7.5) with 0.1% Triton X-100. After disruption by freeze-thawing, cells were incubated with 1-[³⁵S]cysteine (1075 Ci/mmol, Tokyo Medical CRO, Japan) for 30 min at room temperature. The reaction mixtures were spun down, and then the supernatants were subjected to acrylamide gel electrophoresis under nonreducing conditions. ³⁵S-Labeled protein bands were detected by contacting the gel to the imaging plate and analyzed with BAS2500 (FujiFilm).

**HPLC Analysis of the Total tRNA Nucleosides**—Total tRNAs prepared were digested with 4 units of ribonuclease P1 (Yuasa Shou Co., Ltd.) in 100 μl of 10 mM sodium acetate (pH 5.2) for 1 h. After digestion with ribonuclease P1, the tRNA was loaded onto a chromatography column (COS-MOSIL 5C₁₈ column, 4.5 mm × 25 cm, Nakalai Tesque, Japan) and then subjected to a gradient of 25 mM sodium acetate and 50% acetonitrile as described previously (1). The eluted nucleosides were monitored at 256 nm.

**Gel Filtration Chromatography with Yeast Cytosol Coupling Immunological Detection of Proteins**—Cytosolic fractions were prepared from TAP-Tuc1 and TAP-Tuc2 cells that expressed Tuc1 and Tuc2 proteins with TAP tags at their carboxyl termini, as described previously. They were fractionated using Superdex-200 gel filtration column chromatography using SMART System (GE Healthcare) with a running buffer of 150 mM NaCl and 50 mM HEPES-NaOH (pH 7.5). Aliquots of each fraction were separated using electrophoresis on a 12.5% SDS-polyacrylamide gel followed by Western blotting with an anti-TAP antibody (Open Biosystems) and an anti-Urm1 antibody (Invitrogen).
Tuc1 and Tuc2 as Well as Urm1 and Uba4 Are Strictly Required for the s\textsuperscript{2} Modification of cy-tRNAs

During a search for possible cytosolic proteins that are directly involved in the s\textsuperscript{2} modification of cy-tRNAs, we noticed that Ncs6 (YGL211W gene product, now termed Tuc1), which is likely to be a cytosolic protein, contained a PP-loop-type ATPase domain as well as some conserved cysteine residue-containing domains. Because proteins containing this domain arrangement have been shown to be involved in some kinds of tRNA modifications (13), and because Cfd1 and Nbp35, which participate in both the s\textsuperscript{2} modification of cy-tRNAs and the cytosolic Fe/S cluster assembly, also possess the P-loop motif and can transfer certain sulfur-containing compounds, we regarded Tuc1 (Ncs6) as a likely candidate that could be directly involved in the s\textsuperscript{2} modification of cy-tRNAs. The s\textsuperscript{2} modification of total tRNAs prepared from \(\Delta\text{tuc1}\) cells was examined to observe the retardation of tRNA during migration in the APM-containing gel (see “Experimental Procedures” for details). We analyzed three cytosolic tRNAs as follows: cy-tRNA\textsubscript{Lys}\textsuperscript{2} UUU and cy-tRNA\textsubscript{Glu}\textsuperscript{3} UUC undergo s\textsuperscript{2} modification in addition to mcm\textsuperscript{5} modification, whereas cy-tRNA\textsubscript{Arg}\textsuperscript{UCU} undergoes only mcm\textsuperscript{5} modification. As shown in Fig. 1, the migration of cy-tRNA\textsubscript{Arg}\textsuperscript{UCU} was not altered in either wild-type BY4742 or \(\Delta\text{tuc1}\) cells. In contrast, the migration of cy-tRNA\textsubscript{Lys}\textsuperscript{2} UUU and cy-tRNA\textsubscript{Glu}\textsuperscript{3} UUC prepared from wild-type cells was significantly retarded as compared with those prepared from \(\Delta\text{tuc1}\) cells, the latter of which corresponded well to the migration of cy-tRNA\textsubscript{Arg}\textsuperscript{UCU}. These data indicate that the s\textsuperscript{2} modification of both cy-tRNA\textsubscript{Lys}\textsuperscript{2} UUU and cy-tRNA\textsubscript{Glu}\textsuperscript{3} UUC was severely impaired in \(\Delta\text{tuc1}\) cells.

Tuc1 was originally designated Ncs6 (need Cla4 to survive 6) because depletion of Tuc1 causes a synthetic lethality in a \(\Delta\text{cla4}\) strain (11). Cla4 is known as a Ste20-like protein kinase involved in signal transduction and whose deletion exhibits multiple defects (14). NCS2 (now termed TUC2), URM1, and UBA4 genes were also demonstrated to be essential in a \(\Delta\text{cla4}\) strain, but their exact functions still remain unclear (15). Therefore, we examined whether deletion of TUC2, URM1, or UBA4 causes any defect in the s\textsuperscript{2} modification of cy-tRNAs. As shown in Fig. 1, we observed that the s\textsuperscript{2} modification of both cy-tRNA\textsubscript{Lys}\textsuperscript{2} UUU and cy-tRNA\textsubscript{Glu}\textsuperscript{3} UUC was almost completely impaired in \(\Delta\text{tuc2}\), \(\Delta\text{urm1}\), or \(\Delta\text{uba4}\) cells. However, in contrast to these stains, \(\Delta\text{cla4}\) did not exhibit any such defects in the s\textsuperscript{2} modification of both cy-tRNA\textsubscript{Lys}\textsuperscript{2} UUU and cy-tRNA\textsubscript{Glu}\textsuperscript{3} UUC, indicating that Tuc2, Urm1, and Uba4, but not Cla4, are necessary for the s\textsuperscript{2} modification of cy-tRNAs. To know whether these novel factors involved in the s\textsuperscript{2} modification of cy-tRNAs form a complex in the cytosol, we performed gel filtration chromatography of cytosolic fractions prepared from yeast cells expressing Tuc1-TAP and Tuc2-TAP proteins followed by Western analysis using an anti-TAP tag and anti-Urm1 antibodies (Fig. 2A). Note that expression of these tagged proteins in the respective deletion strains was able to restore the cytosolic s\textsuperscript{2} modification (data not shown), indicating that they are functional. Although the calculated molecular mass of

\[\text{FIGURE 1. Gel retardation analysis coupled with Northern hybridization. The s}\textsuperscript{2} \text{modification of total tRNAs was examined by APM-Northern analysis with specific DNA probes against cy-tRNA\textsubscript{Lys}\textsuperscript{2} UUU (cy-K), cy-tRNA\textsubscript{Glu}\textsuperscript{3} UUC (cy-E), and cy-tRNA\textsubscript{Arg}\textsuperscript{UCU} (cy-R) (upper panels). Parenthesis and arrowhead indicate corresponding tRNAs with and without the s}\textsuperscript{2} \text{modification. The brace indicates smaller degradation products of the unmodified form. Proportions of the s}\textsuperscript{2} \text{modified tRNAs were plotted (lower panels) as a bar graph representing the average values obtained from three independent experiments. Error bars represent the ranges of values of the three independent experiments.}\]

\[\text{RESULTS}\]

Tuc1 and Tuc2 as Well as Urm1 and Uba4 Are Strictly Required for the s\textsuperscript{2} Modification of cy-tRNAs—During a search for possible cytosolic proteins that are directly involved in the s\textsuperscript{2} modification of cy-tRNAs, we noticed that Ncs6 (YGL211W gene product, now termed Tuc1), which is likely to be a cytosolic protein, contained a PP-loop-type ATPase domain as well as some conserved cysteine residue-containing domains. Because proteins containing this domain arrangement have been shown to be involved in some kinds of tRNA modifications (13), and because Cfd1 and Nbp35, which participate in both the s\textsuperscript{2} modification of cy-tRNAs and the cytosolic Fe/S cluster assembly, also possess the P-loop motif and can transfer certain sulfur-containing compounds, we regarded Tuc1 (Ncs6) as a likely candidate that could be directly involved in the s\textsuperscript{2} modification of cy-tRNAs. The s\textsuperscript{2} modification of total tRNAs prepared from

\[\text{FIGURE 2A. Gel filtration chromatography of cytosolic fractions prepared from yeast cells expressing Tuc1-TAP and Tuc2-TAP proteins followed by Western analysis using an anti-TAP tag and anti-Urm1 antibodies (Fig. 2A). Note that expression of these tagged proteins in the respective deletion strains was able to restore the cytosolic s}\textsuperscript{2} \text{modification (data not shown), indicating that they are functional. Although the calculated molecular mass of}\]
Tuc1-TAP protein is 60,523 Da, the protein was eluted as a large molecular weight complex (>250 kDa) (fraction 4 in Fig. 2A). However, Tuc2-TAP, whose calculated molecular mass is 77,008 Da, eluted at ~140 kDa, which most likely corresponds to a homodimer (fraction 6 in Fig. 2A). Urm1 protein was eluted at ~24 kDa, which was also most likely a homodimer. Taken together, Urm1, Tuc1, and Tuc2 exist as distinct entities in the yeast cytosol.

Tuc1 Belongs to the Family of PP-loop Type ATPases and Can Catalyze Adenylation of tRNAs—MnmA, a PP-loop ATPase required for the s2 modification of U34 in E. coli tRNAs, exhibits pyrophosphatase activity in vitro, and it can activate U34 by adenylation just prior to sulfur incorporation (16). Tuc1 is also characterized as a PP-loop ATPase, whereas the unique cysteine motif that is lacking in MnmA protein exists at the amino-terminal region of Tuc1 (2). We therefore investigated whether Tuc1 possesses similar adenylation activity. When the non-s2 modified tRNAs prepared from Δtuc1 cells were incubated with E. coli extracts containing His6-Tuc1 protein in the presence of [α-32P]ATP, radiolabeled tRNAs were seen. However, when [γ-32P]ATP was used, no such radiolabeled species were detected (Fig. 2B). Therefore, like MnmA in E. coli, yeast Tuc1 can form an adenylated tRNA intermediate that is likely required for the s2 modification of yeast cy-tRNAs.

Carboxyl-terminal Glycine of Urm1 Is Important for the s2 Modification of cy-tRNAs—Urm1 and Uba4 were initially identified as a novel protein conjugation system, where Urm1 acts as a ubiquitin-related modifier, and Uba4 functions as a ubiquitin-activating enzyme (E1)-like protein (12). Later it was proposed that this protein conjugation system be termed urmylation, although the target proteins that undergo urmylation remain unclear (15). In parallel with the ubiquitin system, Urm1 forms a thioester with the target proteins that undergo urmylation remain unclear (15). In parallel with the ubiquitin system, Urm1 forms a thioester with the target proteins that undergo urmylation remain unclear (15). In parallel with the ubiquitin system, Urm1 forms a thioester with the target proteins that undergo urmylation remain unclear (15). In parallel with the ubiquitin system, Urm1 forms a thioester with the target proteins that undergo urmylation remain unclear (15). In parallel with the ubiquitin system, Urm1 forms a thioester with the target proteins that undergo urmylation remain unclear (15). In parallel with the ubiquitin system, Urm1 forms a thioester with the target proteins that undergo urmylation remain unclear (15). In parallel with the ubiquitin system, Urm1 forms a thioester with the target proteins that undergo urmylation remain unclear (15).
modification of cy-tRNAs and that this unique ubiquitin-related system involving Urm1 and Uba4 plays a critical role in the s² modification.

Uba4 Can Bind the Cysteine-derived Sulfur with the Aid of Urm1—In bacteria, sulfur that is incorporated into thiamine is derived from L-cysteine, and this sulfur incorporation reaction is known to be catalyzed, at least in part, by ThiS and ThiF, both of which were demonstrated to bind cysteine-derived sulfur (17). Interestingly, Urm1 and Uba4 show significant sequence similarities to ThiS and ThiF, respectively. We therefore asked if Urm1 and/or Uba4 can bind cysteine-derived sulfur. To achieve this, Urm1 and Uba4 (with S-tag) proteins were expressed in E. coli cells either separately or simultaneously (Fig. 4

A, expression of Urm1 and Uba4-Stag in E. coli cells was confirmed by Western analysis using an anti-Urm1 antibody and S-protein-horseradish peroxidase conjugate, respectively. Whole cell extracts were prepared from E. coli cells that expressed (+) or did not express (−) Urm1 and/or Uba4-Stag protein(s). B, whole cell extracts prepared as described in A were incubated with L-[³⁵S]cysteine and subjected to SDS-PAGE under nonreducing conditions. After electrophoresis, ³⁵S-labeled protein bands were detected by autoradiography. An arrowhead indicates the position of ³⁵S-labeled Uba4-Stag.

mcm⁵ Modification of U₃₄ Influences the Efficiency of s² Modification of cy-tRNAs—As described above, U₃₄ of cy-tRNA⁰⁰⁰ and cy-tRNA⁰⁰³ undergoes both mcm⁵ and s² modifications. Trm9 is a methyltransferase for the mcm⁵ modification (18), and both Kti11 and Epl3 are involved in the early steps of this modification (9, 19). In Δktt11, Δepl3, and Δtrm9 cells, the mcm⁵ modification of cy-tRNA was almost completely inhibited (9, 19). In those mutants, although the s² modification of cy-tRNAs was reported to occur, the impact on the efficiency of s² modification was not investigated. As shown in Fig. 1, the s² modification was significantly, but not totally, inhibited in Δktt11, Δepl3, and Δtrm9 mutants, suggesting that impairment of mcm⁵ modification has some inhibitory effects on the s² modification in cy-tRNAs. In contrast, to examine the impact on efficiency of mcm⁵ modification by eliminating the s² modification in cy-tRNAs, we compared HPLC elution profiles of nucleosides of bulk tRNA prepared from yeast strains of wild-type BY4742, Δepl3, Δtuc2, and Δepl3 Δtuc2 cells. As shown in Fig. 5A, we observed a common major nucleoside peak in Δepl3, Δtuc2, and Δepl3 Δtuc2 samples but not with the wild-type sample. The nucleoside was purified, and its molecular mass was determined to be 302.03, which shows good agreement with that of 5-carboxymethyluridine (cm⁵U) (302.24). This may indicate that impairment of s² modification also affects the efficiency of methylation of cm⁵U₃₄.
DISCUSSION

This study identifies the following four additional yeast cytosolic components that are strictly required for the $s^2$ modification of U$_{34}$ of cy-tRNAs: ubiquitin-like protein Urm1, ubiquitin-activating enzyme E1-like protein Uba4, cytosolic PP-loop ATPase Tuc1, and unknown function protein Tuc2 (Fig. 7). Therefore, the $s^2$ modification of cy-tRNAs requires more protein factors than initially anticipated. While preparing this manuscript, studies were published suggesting that Tuc1 and Tuc2 (previously called Ncs6 and Ncs2, respectively) are involved in the $s^2$ modification of cy-tRNAs because their mutants exhibited no detectable amounts of 2-thiouridine nucleoside derivatives (2, 19). Our experimental data are in agreement with this previous observation.

Both Yeast Tuc1 and bacterial MnmA are classified as part of the PP-loop ATPase family (2). We showed here that Tuc1 is truly required for the $s^2$ modification of cy-tRNAs (Fig. 1) and also that it can catalyze the formation of the adenylated form of yeast tRNAs (Fig. 2B). MnmA was previously demonstrated to recognize the tRNA nucleoside U$_{34}$ to form an adenylated intermediate that is thought to be a prerequisite for sulfur incorporation at that position (16). A similar reaction was also found in another N-type ATP pyrophosphatase, TilS and MesJ, which facilitates adenylation of C$_{34}$ of isoleucine tRNA to incorporate L-lysine to form lysidine (21). Therefore, by analogy with MnmA and TilS/MesJ, Tuc1 is likely to function in the adenylation of U$_{34}$ of cy-tRNA$_{2\text{ys2}}$ and cy-tRNA$_{\text{Glu3}}$ that should be necessary for sulfur incorporation (Fig. 7).

Another MnmA-like protein, Mtu1, is localized in yeast mitochondria and functions in $s^2$ modification of U$_{34}$ of mitochondrial tRNAs (22). We previously demonstrated that the $s^2$ modification of cy-tRNAs is Fe/S protein-dependent but that the $s^2$ modification of mt-tRNAs is Fe/S cluster-independent, although both require the mitochondrial cysteine desulfurase Nfs1 (7). Thus, yeast cytosolic and mitochondrial $s^2$ modifications of tRNAs are mechanically distinct from each other, at least in part, but appear to require two homologous PP-loop ATPases localized separately in each compartment. Interestingly, Tuc1 more closely resembles bacterial TtcA, which is required for the $s^2$ modification of C$_{34}$ (2), than MnmA or Mtu1, whereas no $s^2$ modification has been found in yeast so far. This may indicate different evolutionary histories for cytosolic and mitochondrial $s^2$ modification of U$_{34}$ of tRNAs.

Tuc1 can activate U$_{34}$ to be $s^2$-modified by preceding adenylation, but subsequent $s^2$-modification itself requires additional cytosolic components. Tuc2 might be one of these cytosolic components, although the exact function of Tuc2 remains unclear at this time. Very recently, Tuc1 and Tuc2 orthologues (termed Ctu1 and Ctu2, respectively) identified in the nematode and fission yeast were found to form a complex (23). However, our data demonstrated in Fig. 2A indicate that, in budding yeast, the two proteins exist as distinct entities in the cytosol. In this context, it is noteworthy that Tuc1 appears to form a complex larger than 250 kDa. Identification and characterization of other constituents of the complex may help to elucidate a detailed molecular mechanism for the final $s^2$ modification step.

The strict requirement of Urm1 and Uba4 for the $s^2$ modification of cy-tRNAs is surprising because the two proteins were originally identified as part of a ubiquitin-related protein modifier system (12, 15). Urm1 is conserved among eukaryotes and shows a weak homology to ubiquitin (24). More intriguingly, Urm1 resem-
bacterial sulfur carrier proteins, ThiS and MoaD, both in their sequence and structure (25, 26). Correspondingly, Uba4 shows significant sequence similarity to E1 as well as ThiF and MoeB, which are involved in thiamine and molybdenum cofactor biosyntheses in cooperation with ThiS and MoaD, respectively (12). The carboxyl-terminal glycine of ThiS is activated by ThiF to form an acyl-adenylated intermediate (27). Such a reaction is mechanistically similar to that seen between ubiquitin and the E1 enzyme (Fig. 7). Different from the ubiquitin system that converts the acyl-adenylate to acyl-thioester linkage with the active site cysteine residue of E1, ThiS undergoes an acyl-disulfide linkage with an active site cysteine of ThiF (28), where the additional sulfur donated by the bacterial cysteine desulfurase IscS is supplied for the s2 modification (3). The sulfur atom is then transferred somehow via the sulfur-transfer system involving Urm1 and Uba4 and also with Tuc2 and as-yet-unidentified cytosolic Fe/S protein to the activated tRNA by adenylation where Tuc1 functions in this process (C). Dark gray arrows shown in B and C indicate proposed sulfur transfer pathways.

By analogy, the critical roles of Urm1 and Uba4 in the s2 modification of cy-tRNAs can be explained as follows (Fig. 7). The carboxyl-terminal glycine residue of Urm1, whose importance is shown in this study (Fig. 3), is activated by Uba4 to form acyl-adenylate using ATP, followed by the formation of an acyl-disulfide linkage between Urm1 and Uba4 (Fig. 7). Although we could not detect a complex between Urm1 and Uba4 when expressed in E. coli, such a complex was previously identified in yeast (12). Thus, the s2 modification of cy-tRNAs involving Urm1 and Uba4 may resemble the sulfur-transfer system found in bacterial thiamine biosynthesis more than the eukaryotic ubiquitination system (Fig. 7). That recombinant Uba4 can bind sulfur derived from cysteine in E. coli when Urm1 was co-expressed, as shown in Fig. 4B, may suggest the formation of such an acyl-thioester-linked Urm1-Uba4 conjugate, although this conjugate might be unstable during our assay system so that only Uba4 carrying a cysteinyl persulfide could be observed.

Recently, Schmitz et al. (30) reported that yeast Uba4 had the sulfur transferase activity in vitro. They also demonstrated that persulfurated Uba4 was able to form a thiocarboxylate group of Urm1 in vitro. Therefore, their experimental data are in good agreement with our observations and our working model.

In this study, we also demonstrated that the s2 and mcms modifications in cy-tRNAs influence each other. As shown in Fig. 1, the defect in the mcms modification of cy-tRNA could not totally inhibit the s2 modification (only about 50% reduction was seen). Actually, total tRNA nucleoside pool purified from ∆elp3 cells was reported to contain at least s2U (9). However, we successfully observed the accumulation of cm5U, a possible direct precursor of mcmsU group in ∆elp3 or in ∆tuc1 or ∆tuc2 cells (Fig. 5). Because ELP3 is a key protein that mediates the mcmsU formation in yeast cells, the observation that the degree of cm5U accumulation in ∆tuc1 or ∆tuc2 seems to be comparable with that found in ∆elp3 cells strongly suggests that loss of an s2 modification does affect the mcms modification. Although yeast mutants that abolish either modification exhibit slight growth defects, the complete absence of both modifications is lethal because ∆elp3∆tuc1 or ∆elp3∆tuc2 double knock-out mutants are not viable (2). Therefore, the s2 and mcms modifications in cy-tRNAs are probably important for the stability of these tRNAs and/or to maintain translation efficiency, although they may have interrelated but complementary effects. Indeed, a significant number of smaller tRNA fragments are accumulated when either modification was inhibited (Fig. 1).

Despite the identification of the novel cytosolic components shown in this study, it remains unclear how a sulfur atom is provided in yeast cytosol for the process of the s2 modification of cy-tRNAs (Fig. 7). One sulfur atom should presumably be supplied as a protein-bound persulfide that originated from cysteine desulfuration activity of mitochondrial Nfs1, and such incorporated sulfur is somehow exported to the cytosol and finally used for the s2
modification of cy-tRNAs. We found that both mitochondrial ISC and cytosolic CIA proteins for the Fe/S protein maturation system in yeast are involved in the s² modification of cy-tRNAs (7). Therefore, currently unidentified cytosolic Fe/S proteins may be involved somewhere in this process. At present, it remains unclear whether the newly identified Tuc1, Tuc2, or Uba4 proteins are Fe/S proteins. Further experiments are needed to elucidate molecular details on the involvement of this unique ubiquitin-related system in the s² modification of cy-tRNAs.

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