Inhibition of selenocysteine tRNA\[^{\text{Ser}}\text{Sec}\]\ aminoacylation provides evidence that aminoacylation is required for regulatory methylation of this tRNA

Jin Young Kim\(^a,1\), Bradley A. Carlson\(^b,1\), Xue-Ming Xu\(^b\), Yu Zeng\(^c\), Shawn Chen\(^c\), Vadim N. Gladyshev\(^d\), Byeong Jae Lee\(^a,1\),* Dolph L. Hatfield\(^b,1\),*

\(^a\) Laboratory of Molecular Genetics and Genomics, School of Biological Sciences, Institute of Molecular Biology and Genetics, Seoul National University, Seoul 151-742, Republic of Korea

\(^b\) Laboratory of Cancer Prevention, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

\(^c\) Department of Biological Sciences, Ohio University, Athens, OH 45701, USA

\(^d\) Division of Genetics, Department of Medicine, Brigham and Women’s Hospital and Harvard Medical School, Boston, MA 02115, USA

**A R T I C L E   I N F O**

Article history:
Received 6 May 2011
Available online 23 May 2011

Keywords:
Selenium status
Selenocysteine
Selenocysteine tRNA
Selenoproteins
Selenoprotein hierarchy
Um34 tRNA methylation

**A B S T R A C T**

There are two isoforms of selenocysteine (Sec) tRNA\[^{\text{Ser}}\text{Sec}\]\ that differ by a single methyl group, Um34. The non-Um34 isoform supports the synthesis of a subclass of selenoproteins, designated housekeeping, while the Um34 isoform supports the expression of another subclass, designated stress-related selenoproteins. Herein, we investigated the relationship between tRNA\[^{\text{Ser}}\text{Sec}\]\ aminoacylation and Um34 synthesis which is the last step in the maturation of this tRNA. Mutation of the discriminator base at position 73 in tRNA\[^{\text{Ser}}\text{Sec}\]\ dramatically reduced aminoacylation with serine, as did an inhibitor of seryl-tRNA synthetase, SB-217452. Although both the mutation and the inhibitor prevented Um34 synthesis, neither precluded the synthesis of any of the known base modifications on tRNA\[^{\text{Ser}}\text{Sec}\]\ following microinjection and incubation of the mutant tRNA\[^{\text{Ser}}\text{Sec}\]\ transcript, or the wild type transcript along with inhibitor, in Xenopus oocytes. The data demonstrate that Sec tRNA\[^{\text{Ser}}\text{Sec}\]\ must be aminoacylated for Um34 addition. The fact that selenium is required for Um34 methylation suggests that Sec must be attached to its tRNA for Um34 methylation. This would explain why selenium is essential for the function of Um34 methylase and provides further insights into the hierarchy of selenoprotein expression.

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1. Introduction

The biosynthesis of selenocysteine (Sec) was recently established in eukaryotes and archaea, and unlike any other known amino acid in eukaryotes, Sec synthesis occurs on its tRNA (reviewed in [1]). Sec tRNA, designated tRNA\[^{\text{Ser}}\text{Sec}\]\, is initially aminoacylated with serine in the presence of seryl-tRNA synthetase (SerRS) that is in turn phosphorylated on the seryl moiety to form phosphoseryl-tRNA\[^{\text{Ser}}\text{Sec}\]\ by phosphoseryl-tRNA\[^{\text{Ser}}\text{Sec}\]\ kinase (PSTK) [2]. Phosphoseryl-tRNA\[^{\text{Ser}}\text{Sec}\]\ serves as a substrate for Sec synthase (SecS), wherein the active selenium donor, monoselenophosphate, that is synthesized by selenophosphate synthetase 2 (SPS2) in eukaryotes, replaces the phosphate group in phosphoseryl to yield selenocysteyl-tRNA\[^{\text{Ser}}\text{Sec}\]\ [1].

There are two isoforms of tRNA\[^{\text{Ser}}\text{Sec}\]\ that differ from each other by a single methyl group, Um34, and are designated 5-methoxycarbonyl-2\,-O-methyluridine (mcm\(^5\)Um) and 5-methoxycarbonylmethyl-2\,-O-methyluridine (mcm\(^5\)Um) [3]. Addition of Um34 is a highly specialized step in the maturation of tRNA\[^{\text{Ser}}\text{Sec}\]\ in that this methylation step is stringently dependent on an intact primary and tertiary structure [4] and requires selenium [5]. When mammalian cells or tissues are deficient in selenium, the level of the mcm\(^5\)Um isoform is enriched and the mcm\(^5\)Um isoform is reduced, while cells and tissues sufficient in selenium have the ratios of these two isoforms reversed [5,6]. Interestingly, the expression of stress-related selenoproteins, such as glutathione peroxidase 1 (GPx1), is dependent on the presence of selenium and their abundance correlates with the presence of mcm\(^5\)Um [6,7]. On the other hand, the expression of housekeeping selenoproteins, such as thioredoxin reductase 1 and 3 (TR1 and TR3), is less dependent on selenium status and their expression occurs in the presence of mcm\(^5\)Um [7–9]. As expected, mice and organs lacking the mcm\(^5\)Um isoform synthesize only housekeeping selenoproteins [9,10]. In addition to the mcm\(^5\)Um modification at position 34, there are only three other modified bases in tRNA\[^{\text{Ser}}\text{Sec}\]\ which are N\(^6\)-isopentenyladenosine i6A at position 37, pseudouridine (\(\text{pU}\)) at position 55 and 1-methyladenosine (i\(^1\)A) at position 58 [3].

The purpose of the present study was to determine the relationship between synthesis of Um34 and aminoacylation of tRNA\[^{\text{Ser}}\text{Sec}\]\. To elucidate the aminoacylation status of tRNA\[^{\text{Ser}}\text{Sec}\]\ prior to Um34 methylation, we introduced a mutation at position 73, the
discriminator base, in the synthetic gene of tRNA<sup>Ser[Sec]</sup>. This discriminator base is essential in the recognition of tRNA by its corresponding aminoacyl-tRNA synthetase [11] and is, in fact, critical in the aminoacylation of tRNA<sup>Ser[Sec]</sup> with serine by SerRS [12,13]. The G73 → A73 mutant tRNA<sup>Ser[Sec]</sup> transcript was microinjected into Xenopus oocytes, then isolated after overnight incubation and the base modification status of the resulting products analyzed. The data show that the mcm<sup>U</sup> is the substrate for Um34 methylase.

2. Materials and methods

2.1. Materials

| [α-<sup>32</sup>P]ATP and | [α-<sup>32</sup>P]UTP (specific activity, 3000 Ci/mmol) and | [1)<sub>H</sub> serine (specific activity, 29.5 Ci/mmol) were purchased from Perkin Elmer. All other materials and methods were used as given below.

2.2. Preparation of tRNA<sup>Ser[Sec]</sup> mutant and tRNA<sup>Ser[Sec]</sup> and tRNA<sup>Ser</sup> wild type transcripts

The wild type tRNA<sup>Ser[Sec]</sup> and tRNA<sup>Ser</sup> vectors were prepared as described [2]. The templates for producing mutant tRNA<sup>Ser[Sec]</sup> transcripts were generated by PCR using forward primer T7 (5'-TAATACGACTCACTATAGGG-3') and reverse primers containing the desired mutation(s) at the 3'-end of tRNA<sup>Ser[Sec]</sup>. For in vitro aminoacylation studies, transcription of tRNA<sup>Ser[Sec]</sup> was performed using the T7 Ribomax Express Large Scale RNA Production System as described [2].<sup>1</sup> Labeled transcripts were generated using 1 μg of template, 50 μCi of [α-<sup>32</sup>P]ATP or [α-<sup>32</sup>P]UTP, 100 units of T7 RNA polymerase (Stratagene), 40 units of RNase inhibitor (Promega), the other components and this mixture incubated, subsequently treated with Dnase 1 (Alboin), the resulting transcripts isolated and stored until used exactly as described [2,4].

2.3. Isolation of the naturally-occurring tRNA<sup>Ser[Sec]</sup> and serine (Ser) tRNA<sup>Ser</sup> isoforms and tRNA aminoacylation

The naturally-occurring tRNA<sup>Ser[Sec]</sup>,<sup>5</sup> and Ser tRNA<sup>Ser</sup> isoforms [15] were isolated from bovine liver and purified as described in these studies. Aminoacylation of these isoforms and the corresponding tRNA<sup>Ser[Sec]</sup> and tRNA<sup>Ser</sup> transcripts were aminoacylated with [1)<sub>H</sub>serine in the presence of rabbit reticulocyte synthetases as described [16].

2.4. Xenopus oocyte microinjection and RPC-5 chromatography

Preparation of Xenopus oocytes and microinjection of tRNA<sup>Ser[Sec]</sup> transcripts into oocytes were performed as described [17,18]. After overnight incubation, tRNAs were extracted and chromatographed on a RPC-5 column [19] as described [4,7,20].

2.5. Minor base analysis

tRNAs (approximately 1 × 10<sup>5</sup> cpm) within pooled samples of each peak from the RPC-5 column were digested with nuclease P1 in 30 mM ammonium acetate (pH 5.3). Half of the digests were subjected to two-dimensional chromatography on cellulose TLC plates using solvents A and C ([21] and see also [4,18]), and the radioactivity was detected by autoradiography.

3. Results

3.1. Aminoacylation status of wild type and mutant tRNA<sup>Ser[Sec]</sup>

Three mutant tRNA<sup>Ser[Sec]</sup> isoforms were prepared as described in Materials and Methods to investigate the aminoacylation status of the mcm<sup>U</sup> isoform prior to Um34 synthesis. All mutations were made at position 73, the discriminator base and were UCCA, GCCA and ACCA (mutations are shown in bold).

The ability of the UCCA, GCCA and ACCA mutant isoforms to be aminoacylated by SerRS was compared to wild type tRNA<sup>Ser[Sec]</sup>, GCCA, is shown in Fig. 1. The mutant isoforms were poorly aminoacylated.

3.2. Microinjection of mutant and wild type tRNA<sup>Ser[Sec]</sup> isoforms into Xenopus oocytes and identification of base modifications

Transcripts of wild type and each mutant tRNA were prepared with either [α-<sup>32</sup>P]UTP or [α-<sup>32</sup>P]ATP, microinjected individually into Xenopus oocytes, isolated after overnight incubation and chromatographed on an RPC-5 column. The reason for the use of only [α-<sup>32</sup>P]UTP and [α-<sup>32</sup>P]ATP as labels is that the base modifications only occur on U or A nucleosides. The elution profiles of the wild type, GCCA, and discriminator base mutant, ACCA, isoforms labeled with [α-<sup>32</sup>P]ATP are shown in Fig. 2A. GCCA and ACCA had similar elution profiles that eluted primarily as two peaks, a smaller, front-running peak and a larger, late-running peak, designated as Peaks I and II, respectively. The corresponding [α-<sup>32</sup>P]UTP-labeled isoforms had similar elution profiles from the RPC-5 column as the [α-<sup>32</sup>P]ATP-labeled isoforms (compare Supplementary Fig. 1 to Fig. 2A).

The fractions within each peak were pooled as shown in Figs. 2A and Supplementary Fig. 1, tRNA within each peak precipitated, collected, digested with nuclease and the resulting 32P-labeled nucleotides separated by 2-D chromatography as given in Materials and Methods. The 32P-labeled U and A modified bases and 32P-labeled Um34 nucleoside from GCCA and ACCA isoforms are shown in Fig. 2B. As expected, the greater hydrophobicity of Peak II was due to the highly modified base at position 37, i6A [3], that was found in this peak of the [α-<sup>32</sup>P]ATP-labeled isoforms. m1A was found in Peaks I and II in each of the isoforms examined. γΨU and mcm<sup>U</sup> were also found in each of the [α-<sup>32</sup>P]UTP-labeled isoforms, while mcm<sup>U</sup> was observed in the wild type, GCCA, isoform but not in the mutant, ACCA, isoform.
The other two $^{32}$P-labeled discriminator base mutant tRNA\textsubscript{Ser}\textsubscript{Sec} isoforms, U\textsubscript{CCA} and C\textsubscript{CCA}, were microinjected into Xenopus oocytes, incubated overnight, isolated and chromatographed over the RPC-5 column (Supplementary Fig. 2A). The resulting two major peaks were then isolated and analyzed for their minor base and Um34 compositions (Supplementary Fig. 2B) as described above for the other tRNA\textsubscript{Ser}\textsubscript{Sec} isoforms. Both these discriminator base mutant isoforms yielded virtually identical chromatographic and base analysis results as observed above for the A\textsubscript{CCA} isoform (compare Supplementary Fig. 1A,B to Fig. 3A,B, respectively, and see Table 1).

3.3. Inhibition of SerRS in vitro and in Xenopus oocytes

We examined the effect of the reported inhibitor of SerRS in yeast, SB-217452 \cite{14}, on the aminoacylation of Ser tRNA using total calf liver tRNA and mammalian SerRS at varying concentrations of inhibitor (Fig. 3A). Since the maximal level of SerRS inhibition occurred at 20 $\mu$M SB-217452, incubated overnight, extracted and chromatographed on a RPC-5 column as described in Section 2. The graphs show the elution profiles of $[^{32}$P]ATP-labeled tRNAs and the $[^{32}$P]UTP elution profiles are shown in Supplementary Fig. 1). X and Y axis represent fraction number and CPM, respectively. Peaks I and II were pooled as shown by the hatched areas in the figures, collected, digested with nuclease, and in (B), the digests of the two peaks of $[^{32}$P]UTP- or $[^{32}$P]ATP-labeled were resolved by two-dimensional TLC as described in Section 2 and the modified bases and Um34 detected in the digests by autoradiography as described (see text and \cite{4}). The films were exposed for 12–16 h.

The wild type tRNA\textsubscript{Ser}\textsubscript{Sec} transcript, labeled with $[^{32}$P]ATP or $[^{32}$P]UTP was microinjected into Xenopus oocytes with or without SB-217452, incubated overnight, isolated, chromatographed on the RPC-5 column and the resulting peaks isolated (Fig. 4A and Supplementary Fig. 3, respectively) and minor base and Um34 analyses determined (Fig. 4B). As shown in the figure, all modifications occurred in tRNA\textsubscript{Ser}\textsubscript{Sec} that was not exposed to the inhibitor but the Um34 modification was more than 90% reduced in the presence of the inhibitor (see Table 1). These data are further considered in the Discussion.

4. Discussion

An important question regarding the function and regulation of tRNA\textsubscript{Ser}\textsubscript{Sec} is whether this tRNA is aminoacylated prior to its
methylated on the ribosyl moiety at position 34 or does the methylation occur independently of aminoacylation. If the Um34 methylase requires Sec-tRNA\[Ser\][Sec] as a substrate, this would provide further insight into why selenium is required for Um34 synthesis [3,5,6], and in turn, for the requirement of the element for stress-related selenoprotein expression (e.g., GPx1) [2,7–9]. We used two different approaches to elucidate the aminoacylation status of tRNA\[Ser\][Sec]. A G73 → A73 mutation was prepared wherein the discriminator base of tRNA\[Ser\][Sec] was changed resulting in poor aminoacylation. Interestingly, all the known base modifications, mcm5U, i6A, and m1A at positions 34, 37, 55 and 58, respectively, occurred in tRNA\[Ser\][Sec] following microinjection and incubation of the mutant transcript into Xenopus oocytes. However, the nucleoside Um34 modification did not occur. The other two discriminator base mutants, UCCA and CCCA, also yielded the same results.

We also used a reported inhibitor of SerRS, SB-217452, which is an enzymatically processed product of albomycin δ2 [14]. We examined the ability of this compound to inhibit the aminoacylation of the wild type tRNA\[Ser\][Sec] and tRNA\[Ser\][Sec] transcripts and the naturally-occurring tRNA\[Ser\][Sec] and tRNA\[Ser\][Sec] isoforms in vitro. The data showed that SB-217452 was a relatively potent inhibitor of mammalian SerRS in vitro and likely in Xenopus oocytes since all base modifications in tRNA\[Ser\][Sec] occurred in oocytes with the exception of the Um34 modification that supported our findings with the discriminator base mutations.

The above studies demonstrate that tRNA\[Ser\][Sec] is aminoacylated for Um34 synthesis to occur. However, these studies in themselves do not demonstrate whether any one specific amino acid or all of the known amino acid attachments to tRNA\[Ser\][Sec], serine, phosphoserine, Sec and Cys [22], could serve as substrates for the Um34 methylase. The following observations suggest that Sec-tRNA\[Ser\][Sec], and only Sec attached to tRNA\[Ser\][Sec], serves as a substrate for the Um34 methylase: (1) the Um34 methylation step requires selenium [3,5–7] suggesting that the methylation either requires this element as a cofactor or it requires selenium in the form of Sec as its substrate. Since eukaryotes are not known to utilize selenium as a cofactor (whereas its use as Sec explains biological functions of dietary selenium), the more likely possibility is that the Um34 methylase requires Sec as its substrate; (2) the synthesis of GPx1 is dependent on the mcm\(^5\)Um isoform [8,9], and

Interestingly, thioephosphate can replace selenophosphate in the Sec biosynthetic pathway yielding Cys-tRNA\[Ser\][Sec] and the resulting Cys is inserted into TR1 and TR3 but apparently not into GPx1 [22] and see also [3]) the Sec tRNA transcript serves as a substrate for seryl-tRNA synthetase, phosphoseryl-tRNA\[Ser\][Sec] kinase, Sec synthase and selenophosphate synthetase [1,23], but the Um34 methylase has a requirement for the fully modified tRNA\[Ser\][Sec] [4] that must be aminoacylated (this study); although this observation in itself does not demonstrate that Sec must be attached to its tRNA, in light of the above considerations, the most logical conclusion is that indeed Sec must be attached for Um34 methylation to occur.

In the event Sec-tRNA\[Ser\][Sec] is the only substrate for the Um34 methylase, this final step in the maturation of Sec-tRNA\[Ser\][Sec] would provide an important autoregulatory step in the synthesis...
of the stress-related selenoprotein subclass and may account, at least in part, for the hierarchy of selenoprotein expression. It has been known for many years that the expression of some selenoproteins in mammalian cells and tissues, such as TR1 (designated as housekeeping selenoproteins), is fairly insensitive to selenium status while others, such as GPx1 (designated as stress-related selenoproteins), are highly sensitive and are poorly expressed under conditions of selenium deficiency, which is a phenomenon known as selenoprotein hierarchy [24,25]. Stress-related selenoproteins are not essential to the animal’s survival and are synthesized by the Um34 containing isoform [8,9]. However, housekeeping selenoproteins are essential to the animal’s survival and are synthesized by the precursor, non-Um34 isoform. Whether housekeeping selenoproteins can also be synthesized by the mcm5Um isoform has not be resolved.

Interestingly, mice maintained on a selenium deficient diet contain about a 1:1 ratio of Sec/Cys in liver TR1 and about a 9:1 ratio in mice fed a selenium adequate diet [22]. Although further studies are required to show unequivocally that Cys cannot be inserted into GPx1 by this de novo synthetic pathway for synthesizing Cys, the available data suggest that this is the case as noted above. Clearly, additional work has to be done to fully elucidate how selenium status regulates the two selenoprotein subclasses and hierarchy of selenoprotein expression, but the observation that the tRNA[Ser]Sec isoform likely has Sec attached provides an important step in the complete understanding of these processes.

Acknowledgments

This work was supported by the Intramural Research Program at the Center for Cancer Research, National Cancer Institute, National Institutes of Health (to D.L.H.); the Priority Research Centers Program and Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (Grant Nos. 2009-0094020 and 2011-0012947 to B.J.L.); National Institutes of Health (Grant Nos. GM061603, GM065204 and CA080946 to V.N.G.), and American Heart Association (Grant No. 09BGIA2070029 to S.C.);
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JYK was supported by Brain Korea 21 Research Fellowship from the Korea Ministry of Education and Human Resources Development.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.05.096.

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