Biochemical and Biophysical Research Communications 409 (2011) 814–819

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Inhibition of selenocysteine tRNA[Ser]Sec aminoacylation provides evidence that aminoacylation is required for regulatory methylation of this tRNA

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ARTICLE INFO

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

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

ABSTRACT

There are two isoforms of selenocysteine (Sec) tRNA[Ser]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[Ser]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[Ser]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 other of the known base modifications on tRNA[Ser]Sec following microinjection and incubation of the mutant tRNA[Ser]Sec transcript, or the wild type transcript along with inhibitor, in *Xenopus* oocytes. The data demonstrate that Sec tRNA[Ser]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[Ser]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[Ser]Sec by phosphoseryl-tRNA[Ser]Sec kinase (PSTK) [2]. Phosphoseryl-tRNA[Ser]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 phosphoserine to yield selenocysteyl-tRNA[Ser]Sec [1].

There are two isoforms of tRNA[Ser]Sec that differ from each other by a single methyl group, Um34, and are designated 5-methoxycarbonylmethyluridine ($mcm^5U$) and 5-methoxycarbonylmethyl-2′-*O*-methyluridine ($mcm^5Um$) [3]. Addition of Um34 is a highly specialized step in the maturation of tRNA[Ser]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 $mcm^5U$ isoform is enriched and the $mcm^5Um$ 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 $mcm^5Um$ [6,7]. On the other hand, the expression of housekeeping selenoproteins, such as the thioredoxin reductase 1 and 3 (TR1 and TR3), is less dependent on selenium status and their expression occurs in the presence of $mcm^5U$ [7–9]. As expected, mice and organs lacking the $mcm^5Um$ isoform synthesize only housekeeping selenoproteins [9,10]. In addition to the $mcm^5U$ modification at position 34, there are only three other modified bases in tRNA[Ser]Sec which are $N^6$-isopentenyladenosine (i6A) at position 37, pseudouridine (ψU) at position 55 and 1-methyladenosine ($m^1A$) at position 58 [3].

The purpose of the present study was to determine the relationship between synthesis of Um34 and aminoacylation of tRNA[Ser]Sec. To elucidate the aminoacylation status of tRNA[Ser]Sec prior to Um34 methylation, we introduced a mutation at position 73, the

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0006-291X/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.bbrc.2011.05.096

discriminator base, in the synthetic gene of tRNA$^{[Ser]Sec}$. 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$^{[Ser]Sec}$ with serine by SerRS [12,13]. The G73 → A73 mutant tRNA$^{[Ser]Sec}$ 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$^5$Um isoform must be aminoacylated prior to Um34 synthesis. To confirm this observation, a potent inhibitor of SerRS, SB-217452 [14], was co-microinjected with the wild type tRNA$^{[Ser]Sec}$ transcript into oocytes. The mcm$^5$U isoform was synthesized without SerRS activity but not the mcm$^5$Um isoform, providing further evidence that an aminoacylated tRNA$^{[Ser]Sec}_{mcm^5U}$ is the substrate for Um34 methylase.

## 2. Materials and methods

### 2.1. Materials

[α-$^{32}$P]ATP and [α-$^{32}$P]UTP (specific activity, 3000 Ci/mmol) and [$^3$H] serine (specific activity, 29.5 Ci/mmol) were purchased from Perkin Elmer. All other commercial products were purchased and used as given below.

### 2.2. Preparation of tRNA$^{[Ser]Sec}$ mutant and tRNA$^{[Ser]Sec}$ and tRNA$^{Ser}$ wild type transcripts

The wild type tRNA$^{[Ser]Sec}$ and tRNA$^{Ser}$ vectors were prepared as described [2]. The templates for producing mutant tRNA$^{[Ser]Sec}$ 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$^{[Ser]Sec}$. For *in vitro* aminoacylation studies, transcription of tRNA$^{[Ser]Sec}$ was performed using the T7 RiboMAX Express Large Scale RNA Production System as described [2]. $^{32}$P-Labeled transcripts were generated using 1 μg of template, 50 μCi of [α-$^{32}$P]ATP or [α-$^{32}$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 I (Alboin), the resulting transcripts isolated and stored until used exactly as described [2,4].

### 2.3. Isolation of the naturally-occurring tRNA$^{[Ser]Sec}$ and serine (Ser) tRNA$_1^{Ser}$ isoforms and tRNA aminoacylation

The naturally-occurring tRNA$^{[Ser]Sec}_{mcm^5U}$ [3] and Ser tRNA$_1$ isoforms [15] were isolated from bovine liver and purified as described in these studies. Aminoacylation of these isoforms and the corresponding tRNA$^{[Ser]Sec}$ and tRNA$^{Ser}$ transcripts were aminoacylated with [$^3$H]-serine in the presence of rabbit reticulocyte synthetases as described [16].

### 2.4. Xenopus oocyte microinjections and RPC-5 chromatography

Preparation of *Xenopus* oocytes and microinjection of tRNA$^{[Ser]Sec}$ 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 $1 \times 10^5$ cpm) within pooled samples of each peak from the RPC-5 column were digested with nuclease P1 in 50 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$^{[Ser]Sec}$

Three mutant tRNA$^{[Ser]Sec}$ isoforms were prepared as described in Materials and Methods to investigate the aminoacylation status of the mcm$^5$U isoform prior to Um34 synthesis. All mutations were made at position 73, the discriminator base and were **U**CCA, **C**CCA and **A**CCA (mutations are shown in bold).

The ability of the **U**CCA, **C**CCA and **A**CCA mutant isoforms to be aminoacylated by SerRS was compared to wild type tRNA$^{[Ser]Sec}$, GCCA, is shown in Fig. 1. The mutant isoforms were poorly aminoacylated.

### 3.2. Microinjection of mutant and wild type tRNA$^{[Ser]Sec}$ isoforms into Xenopus oocytes and identification of base modifications

Transcripts of wild type and each mutant tRNA were prepared with either [α-$^{32}$P]UTP or [α-$^{32}$P]ATP, microinjected individually into *Xenopus* oocytes, isolated after overnight incubation and chromatographed on a RPC-5 column. The reason for the use of only [α-$^{32}$P]UTP and [α-$^{32}$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, **A**CCA, isoforms labeled with [α-$^{32}$P]ATP are shown in Fig. 2A. GCCA and **A**CCA 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-$^{32}$P]UTP-labeled isoforms had similar elution profiles from the RPC-5 column as the [α-$^{32}$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 $^{32}$P-labeled nucleotides separated by 2-D chromatography as given in Materials and Methods. The $^{32}$P-labeled U and A modified bases and $^{32}$P-labeled Um34 nucleotide from GCCA and **A**CCA isoforms are shown in Fig. 2B. As expected, the greater hydrophobicity of Peak II was due to the highly modified base at position 37, i$^6$A [3], that was found in this peak of the [α-$^{32}$P]ATP-labeled isoforms. m$^1$A was found in Peaks I and II in each of the isoforms examined. ψU and mcm$^5$U were also found in each of the [α-$^{32}$P]UTP-labeled isoforms, while mcm$^5$Um was observed in the wild type, GCCA, isoform but not in the mutant, **A**CCA, isoform.

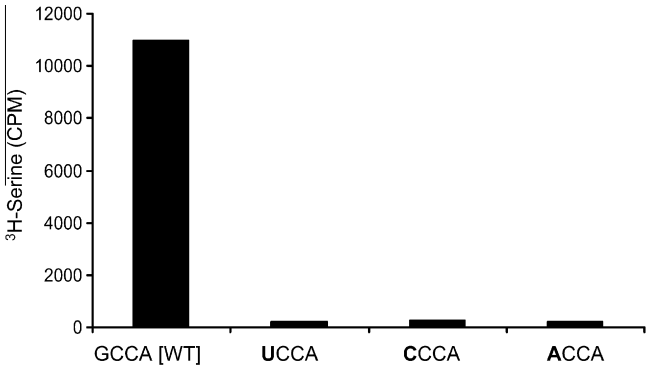


**Fig. 1.** Aminoacylation of wild type and mutant tRNA$^{[Ser]Sec}$ isoforms with serine. Two micrograms of wild type and mutant (**U**CCA, **C**CCA or **A**CCA) tRNA$^{[Ser]Sec}$ transcripts were aminoacylated with $^3$H-serine in the presence of SerRS as described in Section 2. Results shown are counts per minute (CPM) of $^3$H-serine.