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A factor protecting mammalian [^{75}Se]SeCys-tRNA is different from EF-1α

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Abstract

In *Escherichia coli*, an elongation factor (EF-Tu-like) specific to SeCys-tRNA, SELB, has been identified; however, a mammalian counterpart of SELB has not been reported to date. We searched for and found this factor in bovine liver extracts using the assay of [^{75}Se]SeCys-tRNA protecting activity against alkaline hydrolysis (SePF activity). We found SePF activity in the protein extracts of the precipitate (microsomal fraction) collected at 150,000 × *g* from bovine liver. The proteins were separated by Sephacryl S-300 chromatography, and the SePF and EF-1α activities were found in the same fraction, indicating that SePF and EF-1α have the same molecular mass (approximately 50 kDa). We then chromatographed this active fraction using CM-Sephadex C-25 columns. The SePF activity was eluted after the peak of EF-1α activity. This result indicated that this SePF activity was not dependent on EF-1α. In addition to performing these two chromatographies, we investigated pure EF-1α from *Bombyx mori* but could not detect any SePF activity in *B. mori* EF-1α. Thus we showed that the SePF activity in bovine liver differs from that of EF-1α in eukaryotes. Therefore the factor protecting [^{75}Se]SeCys-tRNA in bovine liver is not EF-1α and must be a SELB-like factor.

Key words: Selenocysteine; Selenium; tRNA; Elongation factor; SELB

1. Introduction

Selenium is found as selenocysteine (SeCys) in mammalian glutathione peroxidase (GSHPx) [1], type-1 iodothyronine deiodinase [2], and selenoprotein P [3]. SeCys plays an important role at these active sites and shares a UGA codon with a stop codon. Using [^{75}Se]HSe$^-$ we have studied the mechanism of mammalian SeCys-tRNA synthesis [4,5]. The tRNA corresponding to UGA can accept serine, and this serine moiety is converted to SeCys on the tRNA. Then SeCys-tRNA is co-translationally incorporated into selenoproteins at the position of UGA [4]. However, the mechanisms of incorporating SeCys into selenoproteins in mammals are still unclear.

On the other hand, SeCys is also found in the active site of *Escherichia coli* formate dehydrogenase (FDH) [6]. For FDH, the SeCys incorporation mechanism has been clarified by A. Böck et al. [7] as follows. The stem–loop structure located at the 3′ side of the SeCys UGA codon on mRNA is essential for incorporation of SeCys on to the SeCys UGA codon. However, this stem–loop structure at the 3′ side of the SeCys UGA codon is not found in mammalian selenoprotein mRNA; therefore, the mammalian UGA-SeCys decoding system may differ from that in *E. coli*. The stem–loop structure on FDH mRNA is recognized by SELB, which is an elongation factor differing in function to EF-Tu and transports only SeCys-tRNA^{SeCys} to ribosomes [8]. SELB has a higher molecular mass (68 kDa) relative to that of EF-Tu (43 kDa) and is composed of two regions, i.e. the region for stem–loop structure recognition and the EF-Tu-like region [9]. tRNA^{SeCys} in *E. coli* has an unusual structure: a long variable arm and an 8 bp acceptor stem to which EF-Tu cannot bind; thus this is the discriminating factor rendering SeCys-tRNA^{SeCys} specific to SELB [10]. Although it is known that SELB plays a key role in decoding UGA-SeCys in *E. coli*, the UGA-SeCys decoding mechanisms in mammals are still unclear.

In order to clarify the mechanisms of mammalian SeCys incorporation, we searched for a mammalian SELB-like factor. In this report we show that the activity protecting [^{75}Se]SeCys-tRNA against alkaline hydrolysis was found and separated from EF-1α activity by chromatography.

2. Experimental

2.1. [^{75}Se]SeCys-tRNA

tRNA^{SeCys} was prepared from bovine liver and chromatographed using a BD-cellulose column, as described in [11]. The final fraction (1.5 M NaCl) eluting from the column, which was rich in tRNA^{SeCys}, did not contain major tRNA^{Ser} and was further chromatographed using a Sephadex A-50 column. The obtained tRNA^{SeCys} fraction had a purity of approximately 10%, as measured by hybridization with a DNA probe specific to tRNA^{SeCys}. SerRS was purified from bovine liver, as described in [12]. [^{75}Se]HSe$^-$ was prepared from [^{75}Se]selenite by the enzymatic method of Ganther [13], described in detail in [4]; the final specific radioactivity of [^{75}Se]HSe$^-$ was 1.6 Ci/mmol (6.0 μM).

[^{75}Se]SeCys-tRNA was prepared as described in [5]. tRNA^{SeCys} (0.02 nmol) in 20 μl of 0.2 M Tris-HCl (pH 7.4), 20 mM $MgCl_2$, 20 mM KCl, 20 mM mercaptoethanol, 0.4 mM serine, 10 mM ATP, and 5 μg SerRS was mixed with 10 μl of the above [^{75}Se]HSe$^-$, 5 μg of bovine SeCys

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Abbreviations: SeCys, selenocysteine; SELB, elongation factor specific to SeCys-tRNA in *Escherichia coli*; SePF, SeCys-tRNA protecting factor; SerRS, seryl-tRNA synthetase; PheRS, phenylalanyl-tRNA synthetase.

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synthase (10 mg/ml) and 10 μl of a selenide-activating protein (1 mg/ml) and incubated at 30°C for 2 h. [^{75}Se]SeCys-tRNA was collected by ethanol precipitation, after the addition of 1 μl acetic acid to protect [^{75}Se]SeCys-tRNA.

2.2. [^{14}C]Phe-tRNA

tRNA^{Phe} was extracted from bovine liver according to methods described in [11]. tRNA^{Phe} was partially purified by chromatography using BD-cellulose and DEAE Sephadex A-50 columns and used as tRNA^{Phe}. PheRS was prepared from bovine liver as follows. Bovine liver extracts in 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 10 mM mercaptoethanol, were centrifuged at 150,000 × *g*, and the supernatant was chromatographed using DEAE-cellulose columns. The PheRS activity eluted at 0.1 M NaCl was used as PheRS. Preparation of [^{14}C]Phe-tRNA was performed as described in [14] with some modifications. tRNA^{Phe} (8 A_{260} units per 10 μl) in 10 μl of 1.0 M imidazole-HCl (pH 8.0), 750 mM KCl, 15 mM ATP, 40 mM $MgCl_2$ and 2.5 mM DTT was mixed with 10 μl of [^{14}C]Phe (50 Ci/ml) and PheRS for a final volume of 50 μl. The mixture was incubated at 33°C for 30 min. After the incubation, [^{14}C]Phe-tRNA was collected by ethanol precipitation.

2.3. Assay of EF-1α activity

Ribosomes were prepared from bovine brain according to methods described in [15]. The EF-1α activity assay was performed by measuring the binding of [^{14}C]Phe-tRNA to ribosomes according to methods described in [14] and as follows. The standard assay mixture (final volume of 50 μl) contained 50 mM Tris-HCl (pH 7.5), 75 mM KCl, 0.2 mM DTT, 5 μg of BSA, 0.1 mM GMP-P(CH_2)P (unhydrolyzable GTP analogue; Sigma), 5 μg of poly(U), 0.5 A_{260} units of ribosomes, 16 pmol of [^{14}C]Phe-tRNA (382 Ci/mol) and an appropriate amount of each fractionated protein. Incubation was performed for 5 min at 37°C, and the reaction was terminated by the addition of 3 ml of cold wash buffer which contained 20 mM Tris-HCl (pH 7.5), 5 mM $Mg(CH_3COO)_2$ and 100 mM NH_4Cl. Finally, the reaction mixture was poured onto a nitrocellulose membrane filter (0.45 μm pore size; Millipore), and the membrane was washed twice with 3 ml of wash buffer and dried. The radioactivity of the membrane was measured with a liquid scintillation counter.

2.4. Assay of [^{75}Se]SeCys-tRNA protecting activity against alkaline hydrolysis (SePF assay)

The SePF assay was performed as follows. The standard assay mixture (final volume of 50 μl) contained 50 mM Tris-HCl (pH 7.5), 75 mM KCl, 0.2 mM DTT, 0.1 mM GMP-P(CH_2)P, 5 μg BSA, [^{75}Se]SeCys-tRNA (20×10^{-12} Ci) and an appropriate amount of each fractionated protein. Incubation was carried out for 10 min at 30°C. Generally, aminoacyl-tRNAs were labile and easily hydrolysed under these conditions (pH 7.5 for 30 min). Alkaline hydrolysis was terminated by ethanol precipitation, and free [^{75}Se]SeCys liberated from tRNA was removed by centrifugation. [^{75}Se]SeCys-tRNA protected in the precipitate was hydrolysed in 2 N ammonia for 60 min at 30°C. The solution was mixed with 2 vols. of ethanol and the mixture was re-centrifuged. The supernatant was immediately analyzed by TLC on silica-gel G plates with (*n*-butanol:acetic acid:water, 4:1:1). After development, [^{75}Se]SeCys on the silica plate was detected and measured with a Bioimage Analyzer BAS 2000 (Fujix). Authentic SeCys was co-chromatographed and colored by the ninhydrin reaction. The substrate [^{75}Se]HSe^- was co-precipitated with tRNA and appeared at the top of the TLC plates.

2.5. Chromatography conditions

Chromatography on Sephacryl S-300 column (3.6 × 67 cm) was performed with standard buffer composed of 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 10 mM mercaptoethanol and 5% (v/v) glycerol, and 7 ml fractions were collected. Standard buffer with 0–0.8 M KCl (linear gradient) was used as the eluent for the CM-Sephadex C-25 column (0.6 × 25 cm), and 300 μl fractions were collected. Chromatography and preparation of proteins were done in a cold room at 4°C.

3. Results

Active proteins were prepared from a precipitate of bovine liver extracts collected at 150,000 × *g*, according to the same method for *E. coli* SELB preparation [8] with some modifications. Bovine liver (500 g) was homogenized and mixed with 500 ml of extraction buffer composed of 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose and 10 mM mercaptoethanol. The extract was centrifuged at 600 × *g* for 10 min, and the supernatant was further centrifuged at 24,000 × *g* for 10 min to remove mitochondria. The resultant supernatant was centrifuged at 150,000 × *g* for 60 min to precipitate microsomes, and the precipitate was washed with 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 10 mM KCl, 10 mM mercaptoethanol and 0.15 M NH_4Cl and centrifuged at 150,000 × *g* for 60 min. This precipitate was extracted with high-salt buffer composed of 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 10 mM KCl, 10 mM mercaptoethanol and 1.0 M NH_4Cl, the extract was centrifuged at 150,000 × *g* for 60 min, and the supernatant was collected. SePF activity was not detected in the 0.15 M NH_4Cl wash but was found in this extract using 1 M NH_4Cl (data not shown). Proteins in the supernatant were precipitated with ammoniumsulfate, at a final saturation of 66.7%.

These proteins (180 mg of protein was obtained from 500 g bovine liver) were chromatographed using a Sephacryl S-300 column, and the results are shown in Fig. 1. The highest main EF-1α activity, indicated by open circles, was found in fractions 27–30 and weak activity was found in fraction 20. This EF-1α activity represents the binding of [^{14}C]Phe-tRNA to ribosomes. [^{75}Se]SeCys-tRNA protecting activity (SePF activity) was searched for in several eluted fractions and found in

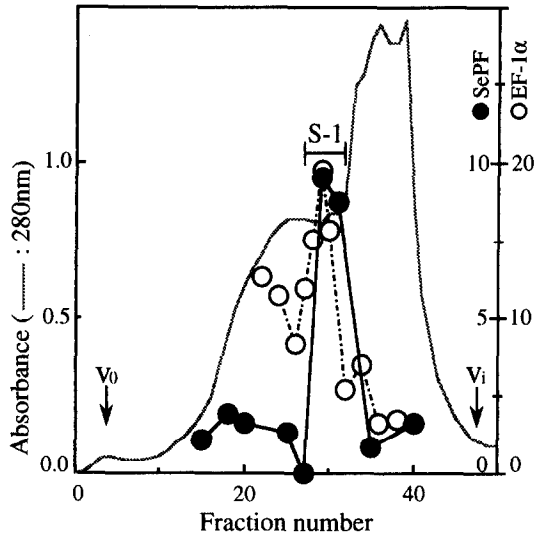

Fig. 1. A chromatography pattern of bovine liver extracts using Sephacryl S-300. ○, EF-1α activity (pmol/mg protein) which was measured based on the binding of [^{14}C]Phe-tRNA to ribosomes in the presence of poly(U). ●, SePF activity (pmol/mg protein) which is the activity protecting [^{75}Se]SeCys-tRNA against alkaline hydrolysis. The passed-through fractions were omitted.

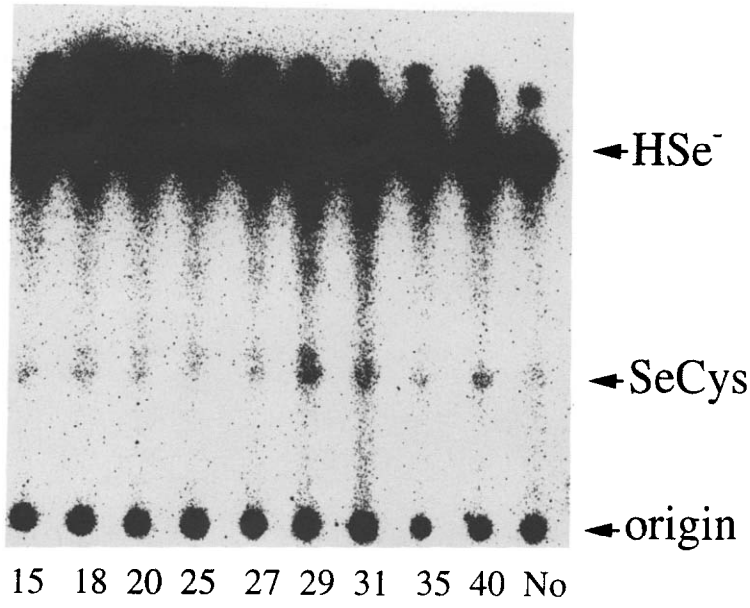

Fig. 2. [^{75}Se]SeCys-tRNA protecting activity (SePF activity) of the eluates shown in Fig. 1. The results of autoradiography with the Bioimage Analyzer BAS2000 are shown. Numbers indicate fraction numbers as plotted in Fig. 1. 'No' indicates control without added protein.

fractions 29–31 based on the TLC [^{75}Se]SeCys autoradiography results using BAS2000, as shown in Fig. 2. The SeCys bands in Fig. 2 indicate [^{75}Se]SeCys protected with SePF. The identity of [^{75}Se]SeCys on the TLC plates was confirmed by co-chromatography with authentic SeCys. We detected dense bands in autoradiographs of fractions 29 and 31, and concluded that these fractions contained SePF activity. The value of SePF activity was estimated based on the radioactivity of protected [^{75}Se]SeCys minus that of [^{75}Se]SeCys of the control lane ('No'; no protein was added to the assay mixture). The relative values of SePF activity are plotted in Fig. 1 as closed circles; SePF activity was eluted in fractions of approximately 50 kDa proteins (S-1 in Fig. 1). EF-1α activity, indicated by open circles, was found in the same fractions. Since both SePF and EF-1α activities were detected in fraction S-1, it may be concluded that this EF-1α has SePF activity. However, we demonstrate a difference between SePF and EF-1α in the next section.

We chromatographed this S-1 fraction using CM-Sephadex C-25 columns. The separation pattern is shown in Fig. 3, in which the passed-through fractions are omitted. Fig. 4 shows the TLC [^{75}Se]SeCys autoradiography results, indicating dense bands for fractions 30–38. The relative values of SePF activity are plotted in Fig. 3. EF-1α activity was eluted in a pattern similar to that of the protein elution (absorbance at 280 nm); however, SePF activity was found in fractions 30–38, after the protein peak. Also, SePF activity was eluted at 0.4 M KCl, compared with 0.3 M KCl for EF-1α. Although we could not completely separate SePF activity from EF-1α activity, this result strongly suggests that the factor in fractions 30–38, and not EF-1α, protected [^{75}Se]SeCys-tRNA against alkaline hydrolysis. One mg of protein in fraction 35 protected 4.2 pmol [^{75}Se]SeCys-tRNA in the SePF assay system.

In order to confirm that SePF and EF-1α are two distinct factors, we measured SePF activity in a sample of pure EF-1α from *Bombyx mori* [16]. Fig. 5 shows the TLC [^{75}Se]SeCys autoradiography results. Lane 1 is the result of fraction 35 of CM-Sephadex C-25 chromatography, and lane 2 is that of EF-1α from *B. mori*. We detected a dense band of [^{75}Se]SeCys only in lane 1, while lane 2 showed the same level as that in lane 'No'

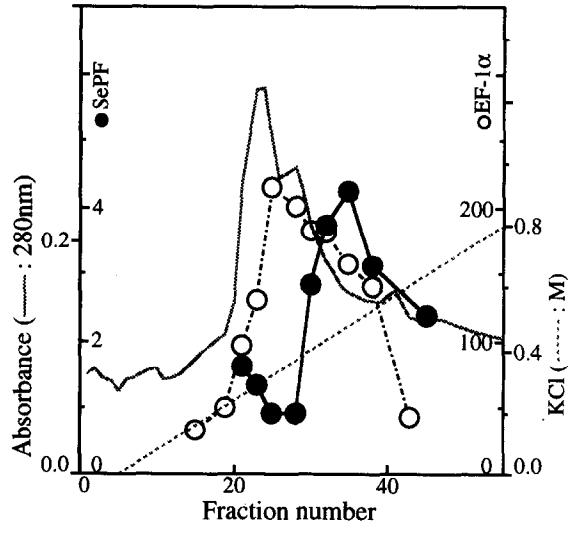

Fig. 3. A chromatography pattern of the S-1 fraction indicated in Fig. 1, using CM-Sephadex C-25. ○, EF-1α activity (pmol/mg protein); ●, SePF activity (pmol/mg protein). Dashed line shows linear gradient of KCl.

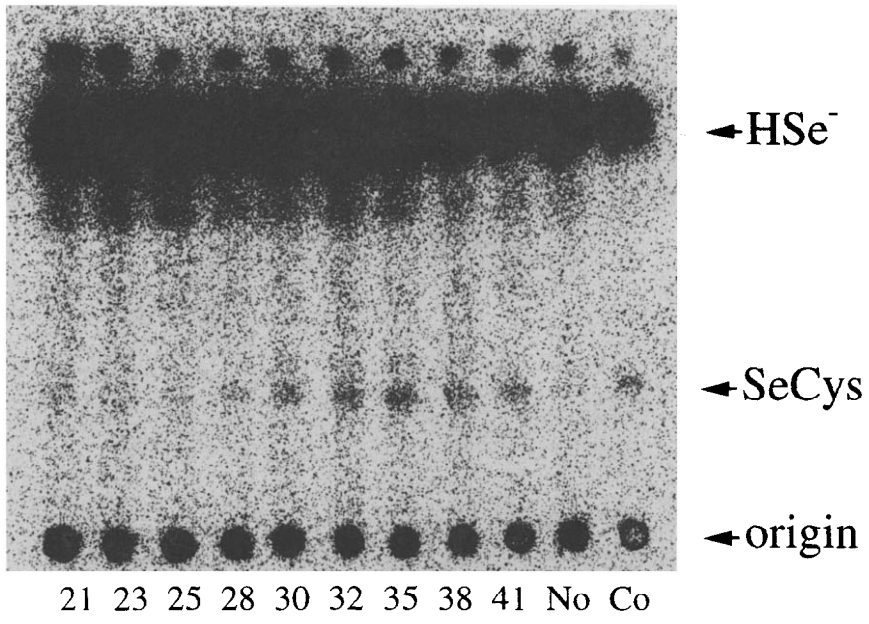

Fig. 4. [^{75}Se]SeCys-tRNA protecting activity of the eluates shown in Fig. 3. The results of autoradiography with the Bioimage Analyzer BAS2000 are shown. Numbers indicate fraction numbers as plotted in Fig. 3. 'No' indicates control without added protein. 'Co' indicates control without alkaline hydrolysis.

(no-protein control), and pure EF-1α (lane 2) did not show any SePF activity. Pure EF-1α from *B. mori* had very strong EF-1α activity (about 1,000-fold higher than that of fraction 25 from CM-Sephadex C-25 chromatography) in this bovine EF-1α assay system, and bovine and *B. mori* EF-1α are structurally and functionally homologous. Since EF-1α from *B. mori* had no SePF activity, we concluded that the factor protecting [^{75}Se]SeCys-tRNA against alkaline hydrolysis is not EF-1α.

4. Discussion

We searched for a SELB-like factor in bovine liver extracts and found a [^{75}Se]SeCys-tRNA protecting activity against alkaline hydrolysis. We showed that this activity did not depend upon EF-1α, based on two main results. This activity was eluted after the peak of EF-1α activity from a CM-Sephadex C-25 column (Fig. 3) and was found in fractions different from those containing the highest level of EF-1α activity. We used EF-1α from *B. mori* as an EF-1α control in the SePF assay, and found that EF-1α from *B. mori* had little SePF activity (Fig. 5). EF-1α's among eukaryotes are highly similar, both in amino acid sequence (80–90% homology) [17] and in function. Since EF-1α from *B. mori* shows very strong EF-1α activity in our bovine EF-1α assay system, we concluded that EF-1α from *B. mori* is a valid control in our assay system.

Although SePF activity (protecting activity) cannot be compared directly with EF-1α activity (binding activity), based on our results we estimate that SePF may occur as less than 2% of total EF-1α in microsomal bovine liver extracts. We used microsomal fractions as a source of SePF, and it is possible that SePF may be present in cytosol, although this was not investigated in the present study.

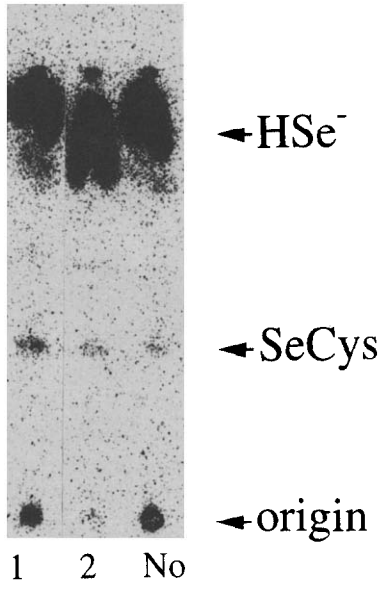

Fig. 5. [^{75}Se]SeCys-tRNA protecting activity of pure EF-1α from *Bombyx mori*. The results of autoradiography with the Bioimage Analyzer BAS2000 are shown. Lane 1 is the active fraction of SePF obtained from CM-Sephadex C-25 chromatography. Lane 2 is that of EF-1α from *B. mori*. 'No' indicates control without added protein.

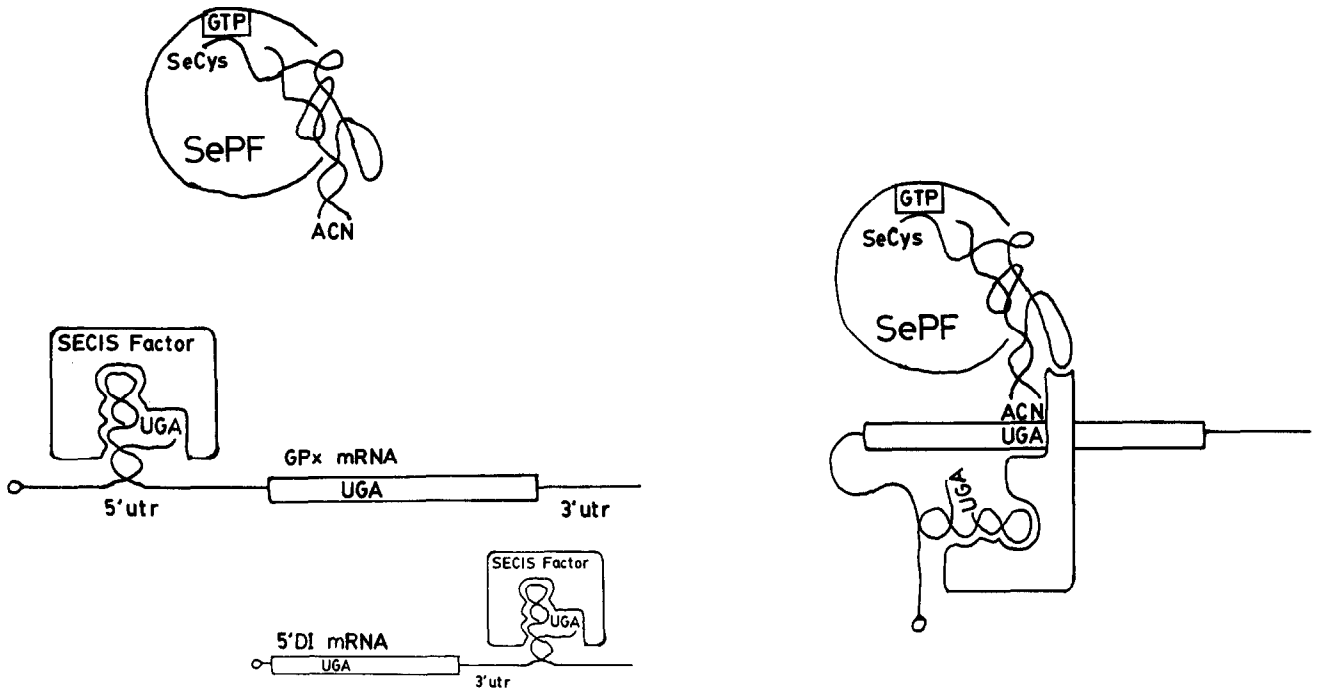

Fig. 6. Model for selenocysteine incorporation in eukaryotes. The SECIS factor is not identified.

It is possible that this SePF activity is dependent upon other proteins related to SeCys-tRNA. These enzymes, such as SerRS, SeCys synthase and tRNA kinase, have little affinity for CM-Sephadex, although we found SePF activity in the high-affinity fraction (0.4 M KCl) after CM-Sephadex chromatography. Moreover, the molecular mass of SePF is not identical to that of these enzymes (65, 70 and 450 kDa, respectively); therefore, we can exclude the possibility that SePF activity is dependent upon these enzymes.

The isoelectric point of EF-1α is 9–10 [16] due to a high content of basic amino acids, and therefore EF-1α has a high affinity for CM-Sephadex (cation exchanger). Due to this basic property, EF-1α easily binds aminoacyl-tRNA. The SePF described in this paper also has a high affinity for CM-Sephadex and may have a basic isoelectric point, allowing it to bind [^{75}Se]SeCys-tRNA. However, tRNA^{SeCys} has an abnormally long 9 bp acceptor stem [18] which may not be protected by EF-1α itself and may require an additional elongation factor for protection. The SePF described in the present report is a possible candidate. A model for SeCys incorporation in eukaryotes is shown in Fig. 6.

Gelpi et al. reported a protein binding tRNA^{SeCys} in humans [19] This protein was precipitated by the antibodies from autoimmune disease (chronic active hepatitis) patients, and has a 48 kDa molecular mass. The SePF described in the present report has a similar molecular mass (approximately 50 kDa), and it is possible that SePF is the same protein as that identified by Gelpi et al.

On *E. coli* FDH mRNA, the information for UGA-SeCys decoding is present on the stem–loop structure downstream of the UGA codon. In mammals, it has been proposed that the selenocysteine insertion sequence (SECIS) elements are present in the 3′ untranslated region of type-1 iodothyronine deiodinase (5′ DI) and selenoprotein P [20]. Since these elements are located far from the SeCys-UGA codon, a mechanism linking SECIS elements with the SeCys-UGA codon is necessary. SELB in *E. coli* play a role in this mechanism and consists of two regions. One is the EF-Tu-like region, and the other is the region involved in stem–loop recognition; thus, SELB has a larger molecular mass (68 kDa) than EF-Tu (42 kDa) [9]. SePF described in this report has a similar size to EF-1α and most likely does not contain a region for SECIS element recognition. Therefore, we conclude that a factor (SECIS factor in Fig. 6) in addition to SePF is needed for recognition of the unknown UGA-SeCys decoding information in eukaryotes.

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