The selenocysteine-specific elongation factor contains a novel and multi-functional domain

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*Running Title: Selenocysteine-specific translation elongation

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Keywords: ribosome, translation elongation, selenocysteine

Background: Selenocysteine incorporation requires the function of a unique translation elongation factor, eEFSec.

Results: The novel Domain IV of eEFSec is required for at least three functions.

Conclusion: Domain IV of eEFSec is the key site for dictating specificity in the conversion of the UGA stop codon into a Sec codon.

Significance: Understanding elongation factor function is critical to deciphering the mechanism of Sec incorporation.

SUMMARY

The amino acid selenocysteine (Sec) is incorporated into a small group of proteins that are involved in preventing cellular oxidative stress, which has been implicated in several human pathologies such as cancer, cardiomyopathy, osteoarthritis, and neurodegeneration (reviewed in 1). The incorporation of Sec is unique because it requires the recoding of UGA from a stop codon to one that specifies Sec. This recoding event is dependent on a stem-loop structure found at the 3' untranslated region (3' UTR) called the Sec Insertion Sequence (SECIS) element. The proposed function of the SECIS element is to form a complex with two essential trans-acting factors: The SECIS binding protein 2 (SBP2) and the Sec-specific elongation factor eEFSec. However, it remains unresolved how SBP2 and the SECIS element activate eEFSec for the insertion of Sec into nascent peptides.

The elongation factor eEFSec is a GTP binding protein with similar affinity to GDP, thus it likely does not require a guanine nucleotide exchange factor (GEF) (2,3). As for its tRNA-binding properties, eEFSec can only interact with Sec-tRNA^{Sec}, but not the serylated-tRNA^{Sec} precursor or the canonical aminoacyl-tRNAs (2,3). The crystal structure of archaeal EFSec revealed a “chalice-like” structure consisting of Domains I, II, and III forming the cup of the chalice, with a unique fourth domain that is separated from the first three (4). The function of each eEFSec domain remains untested, but based on sequence conservation, the first three domains in eEFSec may have similar properties to the canonical eukaryotic translation elongation factor, eEF1A, which itself is composed of three domains. Domain I is required for GTPase activity and ribosome interactions. Domain II is mainly involved in aminoacyl-tRNA binding, and Domain III is proposed to be involved in interactions with the T arm of aminoacyl-tRNAs (reviewed in 5).

The function of Domain IV in eEFSec is not well understood. In bacteria, Sec incorporation is achieved by functional interactions between the bacterial...
SECIS (bSECIS) and SelB, the bacterial homolog of eEFSec. Physical contacts of this complex were mapped to the apical loop of the bSECIS and the C-terminus of Domain IV (6). Mutations in the apical loop of bSECIS or deletion of Domain IV in SelB abolished the interaction of these two factors and, as a consequence, depleted Sec incorporation activity (7,8). The simple model for eukaryotic Sec incorporation is that a SBP2/SECIS/eEFSec/GTP/Sec-tRNA^Sec complex is required for delivery of Sec-tRNA^Sec to the A site of the ribosome. However, the existence of this complex has not been verified, and a physical interaction between eEFSec and SBP2 is highly condition-dependent. eEFSec and SBP2 were first demonstrated to interact by a co-immunoprecipitation experiment in transiently transfected mammalian cells, forming a complex that was RNase sensitive (2). Further studies, also in transiently transfected mammalian cells, showed that eEFSec and SBP2 interactions were enhanced by over-expression of the tRNA^Sec gene (9).

Paradoxically, however, the two factors could not be co-immunoprecipitated from rabbit reticulocyte lysate, despite this being an active Sec incorporation system. In contrast, a native electrophoretic mobility shift assay (EMSA) demonstrated that eEFSec can form a complex with SBP2 in the presence of a SECIS element without the requirement of Sec-tRNA^Sec (10). This report also showed that the RNA binding domain (RBD) of SBP2 and the SECIS element are sufficient for eEFSec recruitment. This suggests that the RBD of SBP2 and the SECIS element may together create a binding interface that is favorable for eEFSec interaction. Overall it is clear that the eEFSec/SBP2 interaction is not highly stable and likely consists of several intermediate conformations.

In the present article, we provide a thorough examination of eEFSec-Domain IV for Sec incorporation, SBP2/SECIS binding, Sec-tRNA^Sec binding and GTPase activity. Our results have revealed that Domain IV of eEFSec is required for Sec incorporation by being involved in Sec-tRNA^Sec binding, GTPase regulation and interactions with SBP2 in a SECIS-dependent manner.

**EXPERIMENTAL PROCEDURES**

**Constructs** - The coding region of murine eEFSec (gift of M. Berry, University of Hawaii) was amplified with a 5′ primer that included the FLAG tag sequence and the product was TA cloned into pTrcHis2 (Invitrogen). Penta-alanine mutants at Domain IV of eEFSec were created with the Quickchange kit (Stratagene). The construct of FLAG-eEFSec without Domain IV (aa 1-440) was PCR amplified and TA cloned into pTrcHis2. FLAG-eEF1A was created with the rat coding region and prepared as the eEFSec constructs. All constructs were confirmed by DNA sequence analysis. SBP2 constructs were made as previously described (10,11).

**Recombinant protein expression and purification** - Recombinant Xpress/His-tagged C-terminal SBP2 (XH-CTSBP2) was purified as previously described (12). All of our FLAG-eEFSec and eEF1A recombinant proteins were expressed and purified from E. coli BL21. The transformed bacteria was grown at 37°C in LB medium with 100 µg/ml of Ampicillin to a density of ~1.0 OD600. Induction was performed with 0.1 mM of isopropyl−β−D−thiogalactoside for 12–16 hours at 18°C. Each liter of cell culture was pelleted and resuspended in 40 ml of Buffer A (20 mM Tris-HCl pH 7.5, 20 mM KCl, 0.1 mM EDTA, 25% Glycerol, 500 mM NaCl, 1% Tween, and 0.5 mM PMSF). Solutions were sonicated four times at 2 sec/ml with 1-2 min resting periods on ice and then centrifuged at 15,000 x g for 15 min at 4°C. Purification was performed by incubating 1 ml of anti-FLAG M2 magnetic beads (Sigma-Aldrich) with a total of 80 ml of protein extract, in 40ml aliquots, for 2 h each at 4°C. After the binding step, the beads were washed 5 times with Buffer A without PMSF followed by 5 times with Buffer B (20 mM Tris-HCl pH 7.5, 20 mM KCl, 0.1 mM EDTA, 25% Glycerol). Elution was done in 1 ml of Buffer B with 250 µg/ml of 3X FLAG peptide for 30 min at 4°C. Purified protein fractions were concentrated with Amicon Ultra 30K, brought to 1 mM DTT, aliquoted and stored at -80°C. The FLAG purification method yielded ~0.25 mg of purified wild-type eEFSec and ~0.05 mg of recombinant eEF1A and Domain IV mutant proteins per liter of bacterial culture.

**In vitro translation and Sec incorporation assay** - Sec incorporation activity in cell-free extracts was measured with a luciferase mRNA reporter containing a UGA-Sec codon at position 258 of the coding region and the rat GPX4 SECIS element at the 3′UTR (13). The luciferase reporter was also used to measure the translation activity by having an UGU-Cys codon instead of the UGA-Sec codon. Insect cell extract (ICE; Promega) made from Sf21 cells was used for our in vitro translation reactions according to the manufacturer’s protocol. PC-3 cells were grown in RPMI medium supplemented with 50 nM of sodium selenite for 7 days. Cells were scraped in translation buffer (20 mM Tris-HCl pH 7.5, 100 mM KCl, 0.25 mM MgCl2, 2 mM DTT, 0.4 mM GTP, 0.25 mM spermidine, 20% glycerol and Roche EDTA-free protease inhibitors) and lysed by passing them through a 30 1/2 g syringe needle. Cellular lysates were centrifuged for 10 min at 17,000 x g and the supernatant was aliquoted and stored at -80°C. The in
vitro translation assays were 12.5 µl reactions that contained 5.5 µl of each Sf21 and PC-3 lysates, 100 ng of luciferase mRNA reporter, 160 nM of SBP2 and wild-type or mutant eEFSec recombinant protein. Translation and Sec incorporation reactions were incubated for 1h at 30°C and measured for luminescence in a 96-well plate luminometer (Berthold Tristar).

Electrophoretic mobility shift assay and UV cross-linking - EMSA experiments were carried out as described but with the following modifications (10). 32P-labeled GPX4 SECIS probe was heated for 3 min at 95°C and cooled down to 25°C for 5 min. Probe was incubated at 30°C for 30 min with the indicated proteins in binding buffer (20 mM Tris- HCl pH 7.5, 100 mM KCl, 2.5 mM MgCl2, 0.5 mM GTP, 10 mM DTT, 10% glycerol, 250 ng/µl bovine tRNA, and 52.5 ng/µl soy trypsin inhibitor). Complexes were resolved in 6% acrylamide Tris-glycine gels and visualized by PhosphorImager. UV cross-linking experiments were performed in conditions described above for EMSA. Reactions were cross-linked for 15 min in Spectrolinker XL-1000 (Spectronics), followed by RNase A treatment for 15 min at 30°C. Samples were separated in 12% SDS gels and visualized by PhosphorImager.

Preparation of [75Se]-Sec-tRNA<Sup>sec</Sup> and isolation of total aminoacylated tRNA - Sec-tRNA<Sup>sec</Sup> was radio-labeled with 50 nM of Na2[75Se]O3 (926 Ci/g, Missouri University Research Reactor) in 10 ml of crude rabbit reticulocyte (Green Hectares) and incubated for 30 min at 30°C. Incubation was followed by adding 6 ml of H2O, 4 ml of 5X Buffer T (50 mM NaOAc, 3.25 M NaCl, 50 mM MgCl2, 5 mM EDTA) and 20 ml of phenol pH 4. The solution was briefly vortexed and centrifuged at 12,000 x g for 5 min at 4°C. The aqueous phase was transferred to a water bath at 95°C and cooled down to 25°C for 5 min. The solution was centrifuged at 95°C for 30 min with the indicated proteins in binding buffer (20 mM Tris- HCl pH 7.5, 100 mM KCl, 2.5 mM MgCl2, 0.5 mM GTP, 10 mM DTT, 10% glycerol, 250 ng/µl bovine tRNA, and 52.5 ng/µl soy trypsin inhibitor). Complexes were resolved in 6% acrylamide Tris-glycine gels and visualized by PhosphorImager.

RESULTS

A novel functional assay for eEFSec - The development of a functional assay to study eEFSec for Sec incorporation has remained one of the hurdles in the field. The addition of recombinant His-tagged eEFSec to rabbit reticulocyte lysate, a system limiting for SBP2, did not increase Sec incorporation activity (13). Efforts to deplete eEFSec from reticulocyte lysate by immunoprecipitation have also been unsuccessful (data not shown). Since translationally competent lysates from mammalian cells are generally inefficient, we sought to provide general translation factors from a robust in vitro translation system that lacks Sec incorporation factors. Genomic in silico analysis indicates that the organisms in the Lepidopteran order, including the Spodoptera genus, do not possess selenoproteins and are missing Sec incorporation machinery (16). Cells from Spodoptera frugiperda (Sf21) have been extensively used for protein expression, and a commercially available in vitro translation lysate from Sf21 cells provides robust translation activity (8). Since the addition of purified SBP2, eEFSec and rabbit aminoacyl tRNA to Sf21 lysates did not result...
in Sec incorporation activity (Gupta and Copeland unpublished observation), we combined mammalian cell lysate (supplying Sec incorporation factors) and S21 lysate (supplying ample general translation factors) in order to identify conditions where eEFSec or SBP2 may be limiting. To analyze Sec incorporation in this system, we used a luciferase reporter consisting of the luciferase coding region with a single in-frame UGA codon at position 258, followed by the rat GPX4 SECIS element and a 100 nucleotide poly(A) tail (Figure 1A). This method has been demonstrated to faithfully indicate Sec incorporation since the production of full-length and active luciferase is strictly SECIS and SBP2-dependent (13).

Previously we have shown that PC-3 prostate cancer cells had reduced levels of the selenoprotein GPX1 compared to LNCaP prostate cancer cells and RWPE-1 non-tumorigenic prostate cells (even when selenium levels were sufficient) suggesting that eEFSec and/or SBP2 may be limiting (17). We therefore first tested PC-3 lysates for Sec incorporation activity in the S21 system. As shown in Figure 1B (lane 1), we observed easily detectable Sec incorporation that was about 9-fold higher than the background obtained from the mRNA with a mutant SECIS element. Upon the addition of FLAG-eEFSec, a 2-fold enhancement was observed while the addition of the fully active Xpress/His tagged C-terminal fragment of SBP2 (XH-CTSGBP2) alone or XH-CTSBP2 plus FLAG-eEFSec resulted in activity that was 3.5 and 7-fold greater than unsupplemented extract, respectively. The addition of FLAG-eEFSec and XH-CTSBP2 proteins did not enhance non-specific UGA readthrough from mRNAs that harbored a mutant SECIS element (black bars) or total translation activity (data not shown). Importantly, these data clearly show a ~2-fold enhancement in Sec incorporation upon the addition of recombinant FLAG-eEFSec, thus allowing the functional analysis of eEFSec mutants as described below.

Domain IV in eEFSec is critical for Sec incorporation - The overall structure and sequence of the first three domains of eEFSec are analogous to their counterparts in elongation factor eEF1A, while a C-terminal extension known as Domain IV is only found in eEFSec. This implies that eEFSec contains most of the canonical translation functions (e.g. nucleotide binding, tRNA recognition) within the first three domains and Sec-specific activities (e.g. SBP2-SECIS binding) may be located in Domain IV. However, it is still unknown if Domain IV of eEFSec is required for Sec incorporation.

The reported crystal structure of archaeal EFSec and sequence analysis placed Domain IV of mouse eEFSec at residues 441 through 583 (Figure 2A). Multiple sequence alignments from species ranging from archaebacteria to human were used to determine conserved residues in Domain IV of eEFSec (Figure 2A). The conserved residues that were analyzed in this study are boxed and identified by the mouse sequence. Each group of five residues was changed to alanine and analyzed for function, and each mutated protein is referred to by the residues that were changed (e.g. VERVM466-470). We successfully employed a similar method to identify critical residues in SBP2 (10,11). A structural model of archaeal eEFSec is shown in Figure 2B (4), illustrating the overall domain structure and the positions of the conserved residues within Domain IV.

Each of the mutant proteins, plus a version lacking all of Domain IV (ΔIV) were expressed in bacteria, purified on anti-FLAG beads and analyzed for Sec incorporation activity in our novel eEFSec-dependent assay. The experiment shown in Fig. 2C (right panel) demonstrates that most of the penta-alanine mutants tested were defective for Sec incorporation, with the exception of VERVM466-470, which had near wild-type activity levels at ~1.8 fold above background. This result indicates that most of Domain IV in eEFSec is essential for Sec incorporation.

eEFSec-Domain IV is involved in SBP2 and SECIS element interactions - One explanation for the lack of Sec incorporation activity from Domain IV mutants could be the disruption of functional contacts between eEFSec and the SBP2/SECIS complex. We have previously described an electrophoretic mobility shift assay (EMSA) that revealed a direct tRNA-independent but SECIS-dependent interaction between SBP2 and eEFSec (10). Using this assay, we sought to determine whether the Domain IV mutant proteins are able to form a stable complex with SBP2/SECIS. As shown in Figure 3, the addition of recombinant XH-CTSBP2 results in a mobility shift of the GPX4 probe and the further addition of FLAG-eEFSec to the SBP2/SECIS binding reaction results in a “super-shift” of the probe (Figure 3, lanes 1-3). The complete deletion of eEFSec Domain IV eliminates binding to SBP2-SECIS (Figure 3, compare lane 3 to 4). In addition, the mutation at KKRSR536-540 also eliminates super-shifting activity, but the other mutations in Domain IV do not affect stable SBP2/SECIS binding. This data establishes that Domain IV, specifically the region at KKRSR536-540, is required for the interaction between eEFSec and the SBP2/SECIS complex. This implies that disruption of SBP2-SECIS binding in eEFSec could inhibit Sec incorporation. However, the rest of the Domain IV mutants that are defective for Sec incorporation retained SBP2/SECIS binding, suggesting other functional roles in Domain IV of eEFSec.
The SECIS core and the RBD domain of SBP2 form the eEFSec recognition site - As mentioned above, we have thus far been unable to isolate a complex between eEFSec and SBP2 that lacks the SECIS element, suggesting that the SBP2/SECIS complex presents a unique conformation of either the SECIS element or SBP2 (or both) to eEFSec. The RBD of SBP2 was previously shown to be the site of eEFSec binding, thus making EMSA analysis of SBP2 mutants that lack SECIS binding limited to the two mutations within the SBP2 RBD that were previously shown to be inactive for Sec incorporation but retain SECIS binding (RFQDR647-651 and DGAQD746-750) (11). An analysis of those mutations in the context of the RDB protein fragment did not show defects in eEFSec binding (data not shown). We therefore chose to focus on determining whether we could identify a region of the SECIS element that is required for the SBP2/SECIS/eEFSec complex. Eukaryotic SECIS elements are stem-loop structures that are members of the kink-turn family of RNAs (see Figure 4) (18,19). They consist of an internal loop of 4-18 nucleotides flanked by two helices (20). Within this loop is the highly conserved “AUGA” motif, which together with GA on the 3' side forms the GA quartet that is a hallmark of kink-turn RNAs (21). At the terminal loop there lies an apical “AAR” motif comprised of two highly conserved unpaired adenosines followed by either an A or G (20,22,23). While the AUGA motif has been identified as the SBP2 binding site (24,25), the basis for the AAR motif requirement has not been determined. In addition, Helix 2 and the internal loop of the SECIS elements were found to be involved in Sec incorporation efficiency (26). We therefore set out to determine whether the AAR motif and/or helix 2 are involved in eEFSec binding to the SBP2/SECIS complex. GPX4 SECIS RNAs harboring mutations were created and tested for XH-CTSBP2 and FLAG-eEFSec binding. As expected, the deletion of the AUGA motif disrupted SBP2 binding and, as a consequence, FLAG-eEFSec binding was undetectable (Figure 4, lanes 5-8). As expected, the mutation of the AAR motif to UUC did not affect SBP2 binding or eEFSec super-shifting. In the case where the entire helix 2 and terminal loop were deleted, SBP2 binding was reduced, (see lane 15 of Figure 4 where only about 50% of the probe was shifted), but eEFSec was still able to super-shift all of the SBP2/SECIS complex. Together, these results strongly suggest that eEFSec recognizes SBP2 residues and/or SECIS bases in the vicinity of the L7Ae/core interaction, making the identification of mutants that retain the ability to form an SBP2/SECIS complex but that lack eEFSec binding very difficult or even impossible.

Domain IV in eEFSec is crucial for Sec-tRNA interactions - Early studies on eEFSec revealed that it specifically bound the Sec-tRNA^Sec and not the precursor Ser-tRNA^Sec or the canonical Ser-tRNA^Sec (2,3). Itoh et al., recently published the human tRNA^Sec crystal structure and suggested a tRNA recognition model for eEFSec that differs from the canonical elongation factor eEF1A (27). The archaeal EFSec and human tRNA^Sec docking model, based on the EF-Tu and Phe-tRNA^Phe co-crystal, locates Domain IV in proximity to the variable arm of tRNA^Sec. This argues that Domain IV of eEFSec might be used to stabilize interactions by creating extra contacts with the Sec-tRNA^Sec.

In order to test the involvement of Domain IV in Sec-tRNA^Sec binding, we employed nitrocellulose filter binding assay with 75Se radiolabeled total rabbit
aminoacyl-tRNA. Rabbit reticulocyte lysate was incubated with 50 nM of Na$_2$[75Se]O$_3$, followed by total aa-tRNA purification (see methods). Preparation of radio-labeled Sec-tRNA$_{Sec}$ was verified by acid urea gel electrophoresis and scintillation counting (data not shown). Background filter retention of [75Se]-Sec-tRNA$_{Sec}$ was subtracted from all data and binding was normalized to wild-type eEFSec. Purified recombinant FLAG-tagged eEF1A was used as a negative control, and it showed only ~13% binding relative to eEFSec (Figure 6A compare lanes 1-2). Interestingly, the deletion of Domain IV resulted in a nearly complete loss of Sec-tRNA$_{Sec}$ binding activity, and three of the five penta-alanine mutations in Domain IV had lost most of the Sec-tRNA$_{Sec}$ binding activity (Figure 6A). Mutations in VERVM$^{466-470}$ and LFKKE$^{480-484}$ showed only moderate defects with 77% and 76% of wild-type binding, respectively. Since the residues in Domain IV that are required for Sec-tRNA$_{Sec}$ binding are also required for SBP2 binding, we examined the effect of adding SBP2 and an SBP2/SECIS complex to the tRNA binding assay. Figure 6B shows that the addition of SBP2 and SBP2/SECIS does not disrupt but may slightly enhance the interactions between eEFSec and Sec-tRNA$_{Sec}$. This data establishes the critical role of Domain IV in Sec-tRNA$_{Sec}$ recognition and illustrates that the critical determinants for tRNA binding are in the C-terminal portion of the domain. Interestingly, SBP2 and Sec-tRNA$_{Sec}$ binding do not appear to be exclusive, but our attempts to observe a quaternary SBP2/SECIS/eEFSec/tRNA complex by EMSA have been unsuccessful.

Having established a specific Sec-tRNA$_{Sec}$ binding assay, we next determined whether, as in the case of eEF1A, stabilization of aa-tRNA binding is GTP-dependent. Figure 6C shows that in the absence of nucleotide, or in the presence of GDP, Sec-tRNA$_{Sec}$ binding was at background levels. Interestingly, the addition of GDPNP, a non-hydrolyzable analog of GTP, resulted in only 38% binding. One explanation for this result could be found in the fact that the conformation of some translation factors bound to GDPNP is more similar to the GDP-bound state rather than to the GTP conformational state (28,29). This data therefore may suggest that the GDPNP nucleotide induces eEFSec into a GDP-like conformation that is unfavorable for stable interactions with Sec-tRNA$_{Sec}$.

**eEFSec GTPase activity is regulated by Domain IV** -The activation of translation GTPases, such as eEF1A, is achieved through conformational changes induced by the ribosome (30,31). Previous work with SelB, the Sec-specific elongation factor in bacteria, demonstrated that 70S ribosomes increased the GTPase activity only when a bacterial SECIS element was present (32). This finding implies that the bSECIS/SelB complex is a better ligand for the ribosome. Considering that eEFSec might have an analogous mechanism on the ribosome that could be triggered by SBP2 and the SECIS element, we sought the determinants that could increase the eEFSec GTP hydrolytic activity.

A colorimetric GTPase assay was used to test the intrinsic GTP hydrolysis of wild-type eEFSec and Domain IV mutants. In order to validate the assay, we again used recombinant FLAG-eEF1A, which showed significant intrinsic GTPase activity that was stimulated about 3-fold in the presence of purified salt-washed rabbit ribosomes (Figure 7A). In contrast, FLAG-eEFSec showed about 4 times less intrinsic GTPase activity than eEF1A, and this was also true for all of the penta-alanine mutants except KRYV$^{569-573}$, which surprisingly showed almost 2-fold higher activity than eEF1A (Figure 7B). Since these results suggest that the C-terminus of Domain IV may be involved in regulating GTPase activity, we analyzed the effect of adding SBP2 and/or aa-tRNA to the reaction with the idea that these other factors that depend on Domain IV may also be revealed to play a role in GTP hydrolysis. Figure 7C shows that the low intrinsic GTPase activity for wild-type eEFSec is not enhanced by the addition of any combination of factors including aa-tRNA, ribosomes, GPX4 SECIS RNA or CTSBP2. In addition, Figure 7C also shows that the high intrinsic GTPase activity of the KRYV$^{569-573}$ mutant is not suppressed or further activated by CTSBP2 or CTSBP2/SECIS complex. Together these data suggest that eEFSec may not display GTP hydrolysis activity that is comparable to eEF1A, potentially suggesting a different or additional role for GTP hydrolysis in Sec incorporation. In addition, these data reveal the unexpected possibility that Domain IV may play a role in regulating GTP hydrolysis activity.

**DISCUSSION**

The Sec-specific elongation factor eEFSec was first identified by searching for homologous sequences in the expressed sequence tag (EST) database with the *M. jannaschii* SelB amino acid sequence (2,3). From its discovery, eEFSec was shown to interact with SBP2 in co-transfected mammalian cells by co-immunoprecipitation (co-IP) experiments, but no further mechanistic or functional details have been revealed (2,9). The lack of a comprehensive functional study of eEFSec has been one of the barriers to understanding the molecular mechanism of eukaryotic Sec incorporation, and one of the key barriers to studying eEFSec function has been the lack of an in vitro functional assay. Here we have described the first such assay, which is comprised of
two basic components: a source of highly efficient general translation factors from S21 cell extracts and a source of limiting Sec incorporation factors from prostate cancer cells. The requirement for exogenous translation factors is likely due to the low efficiency of Sec incorporation, which has been reported to be around 10% \textit{in vitro} and even lower in transfected mammalian cells (13). The success of this method has opened the door to further experiments that will establish whether or not the known Sec incorporation factors are sufficient or whether other essential factors have yet to be discovered, but for our purposes here, this system has been critical to deepening our understanding of the structure/function relationships within eEFSec.

**Domain IV in SBP2 and SECIS binding** - The first study that addressed the mechanism by which eEFSec may interact with SBP2 was performed by Zavacki et al (2003). In this report a fragment corresponding to Domain IV was able to co-IP SBP2 in the absence of a SECIS element from rabbit reticulocyte lysate. However, co-IP experiments with full-length eEFSec didn’t appear to stably bind SBP2 under the same conditions. It was suggested that this lack of interaction was due to limiting Sec-tRNA\textsuperscript{Sec} in rabbit reticulocyte, since eEFSec and SBP2 were successfully co-IPed when all three components were transiently transfected into cells. In contrast, our group reported EMSA conditions for the study of full-length eEFSec, SBP2 and SECIS interactions without the addition of Sec-tRNA\textsuperscript{Sec} (10), and we have used that assay here to show that the residues within Domain IV that are required for SBP2 binding are apparently limited to the KKRSR\textsuperscript{536-540} region. Whether the SBP2/SECIS complex physically interacts with Domain IV at this location is a subject for future study, but we have here established a clear link between subcellular and eukaryotic Sec incorporation since Domain IV for both elongation factors is involved in recruiting the SECIS element, and it is likely that this complex is the signal that allows access to the ribosomal either as a complex or as an induced conformation the permits ribosomal recognition.

**Novel allosteric Sec-tRNA\textsuperscript{Sec} interactions found in Domain IV** - Physical interactions of Domain IV with the Sec-tRNA\textsuperscript{Sec} were proposed previously for SelB and eEFSec after examination from their respective crystal structures (27,33,34). Biochemical evidence to test this hypothesis was curtailed due to mutations in Domain IV of SelB rendering the protein insoluble and difficult to express and purify (35). Interestingly, we also encountered this problem with our recombinant eEFSec Domain IV mutants, but we have managed to optimized their expression and purification in quantities sufficient for this study. This observation suggests that Domain IV in both eEFSec and SelB contains key determinants for protein folding and/or stability.

Domain IV mutant analysis by our Sec-tRNA\textsuperscript{Sec} filter binding assay provided the first biochemical evidence that Domain IV of eEFSec is required for Sec-tRNA\textsuperscript{Sec} binding activity. Residues in Domain IV involved in Sec-tRNA\textsuperscript{Sec} interactions were found within its C-terminal region (KKRSR\textsuperscript{536-540} and KRYVF\textsuperscript{569-573}), which overlaps with the residues required for SBP2/SECIS binding (KKRSR\textsuperscript{536-540}). A possibility exists that the SBP2/SECIS complex could also regulate tRNA binding, but we have thus far been unable to develop an assay to study how the eEFSec/GTP/Sec-tRNA\textsuperscript{Sec} ternary complex interacts with the SBP2/SECIS complex. Nevertheless, addition of SBP2 and the SECIS element did not alter Sec-tRNA\textsuperscript{Sec} binding to eEFSec, providing good evidence for the existence of a complex composed of all four components. The verification for the existence of an eEFSec/GTP/Sec-tRNA\textsuperscript{Sec}/SBP2/SECIS complex is a critical piece of evidence in elucidating the mechanistic details of eukaryotic Sec incorporation. Further structural and biochemical studies are needed to understand the complicated functional interplay of SBP2/SECIS binding and Sec-tRNA\textsuperscript{Sec} binding within eEFSec Domain IV.

**Domain IV regulates the G-domain of eEFSec** - To prevent the Sec-tRNA\textsuperscript{Sec} from acting as a suppressor tRNA, eEFSec must be denied general access to the ribosomal A-site. Unlike eEFSec, eEF1A can obtain ribosomal A-site access without the requirement of additional factors. Near the ribosomal A-site is found the elongation factor binding site that is composed of two elements: 1) the GTPase associated center (GAC) and 2) the sarcin-ricin loop (SRL). The GAC and SRL main functions are to activate GTP hydrolysis in elongation factors, such as eEF1A and eEF2 (36). It was shown in a previous study that SelB achieved ribosome-dependent GTP hydrolysis activation only when the bacterial SECIS element (bSECIS) was added (32). This evidence suggests that the bSECIS induces a conformational change within SelB to promote functional interactions with the ribosome. Further structural analysis indicated that the C-terminal region in Domain IV of SelB was involved in specific interactions with the bSECIS (37,38). An analogous activating mechanism has been proposed for eEFSec where functional interactions with the ribosome could be driven by SBP2 and the SECIS element (15). By using selective 2-hydroxyl acylation analyzed by primer extension (SHAPE), it was recently discovered that SBP2 causes conformational change specifically at the Helix 89 on the 28S ribosomal RNA. Helix 89, located near the A-site, is involved in the proofreading and accommodation of aa-tRNA (39).
and GTPase activation of factors such as IF2/eIF5B (40-42). In this study, a model was proposed where SBP2 rearranges Helix 89 near eEFSec for GTP hydrolysis activation through Domain IV.

Although this model puts GTP hydrolysis as a key part of the mechanism, the full-length crystal structures of SelB/eEFSec from *Methanococcus maripaludis* did not show appreciable conformational differences in the GDP versus GDPNP state (4). Our study in combination with others suggests that this apparent lack of G-domain induced conformational flexibility may be due to the fact that GDPNP is not a true GTP mimetic in the case of eEFSec or SelB. Interestingly, kinetic studies of SelB with Sec-tRNA^Sec^ with different nucleotides revealed that dissociation of Sec-tRNA^Sec^ was considerably higher with GDP-NP but much tighter when incubated with GTP (43). In accordance to this finding, the GDP-NP nucleotide was also a poor GTP analog for eEFSec in our Sec-tRNA^Sec^ filter binding assays. In addition, a recent study using a isothermal titration calorimetry found that SelB underwent small conformational changes when incubated with GDP and GDP-NP, but dramatic rearrangements were found in the SelB-GTP complex (29). This means that the SelB/eEFSec crystal structure with GDP-NP doesn’t represent an accurate view of GTP driven conformations, of which Domain IV could possibly be part, and it is therefore likely that eEFSec undergoes significant conformational changes upon GTP hydrolysis.

Despite good evidence for GTPase induced conformational changes, one apparently significant difference between eEFSec and eEF1A is its lack of intrinsic and ribosome-stimulated GTPase activity. In addition, unlike SelB, eEFSec didn’t acquire significant GTPase stimulation upon the addition of SBP2 and the SECIS element. Surprisingly, however, the KKRKR^536-540^ and KRYVF^569-573^ Domain IV mutants respectively have about 2 and 7 times greater intrinsic GTPase activity than wild-type eEFSec. We speculate that the KKRKR^536-540^ and KRYVF^569-573^ mutants represent eEFSec intermediates that have an “activated” conformation state with enhanced GTP hydrolysis. In this context the question becomes what the mechanism might be that induces the C-terminal of Domain IV in eEFSec to undergo an active conformation state? We propose that Sec-tRNA^Sec^ will “prime” eEFSec into the proper conformation to be readily activated by both SBP2/SECIS and the ribosome. In other words, quantitative amounts of stable eEFSec/GTP/Sec-tRNA^Sec^ ternary complex will have a fully-occupied and pre-activated Domain IV. When eEFSec ternary complex gets near the ribosomal A-site, the SBP2/SECIS complex in conjunction with Helix 89 will interact with Domain IV to dissociate and deliver the Sec-tRNA^Sec^ by

**Other function for Domain IV?** - All together, we have here described three functions for eEFSec Domain IV: SBP2 binding, Sec-tRNA^Sec^ binding and regulation of GTP hydrolysis. The mutational analysis presented in this work shows that four conserved segments within Domain IV are essential for Sec incorporation activity in vitro. One of those is defective for SBP2 binding (KKRKR) and three are defective for tRNA binding (FGQSG, KKRKR and KRYVF). Interestingly, one mutant, LFKKE, lacks Sec incorporation activity but does not have a discernible defect in any of the known functions. This opens up the possibility that this region of Domain IV is directly involved in another aspect of Sec incorporation such as ribosome binding. Deciphering the function of these residues as well as those in SBP2 that have a similar unexplained defect (10) in Sec incorporation will require significant further effort and likely the development of a completely reconstituted Sec incorporation system where each step of the process can be monitored with purified components.

Acknowledgements -

*FOOTNOTES*

*This work was supported by Public Health Service Grant GM077073 (PRC) and the graduate fellowship programs of IGERT Biointerfaces NSF DGE 0333196 and Rutgers-UMDNJ Biotechnology NIH 2T32GM008339-21 (JNGF).*

1 The abbreviations used are: SBP2, SECIS binding protein 2; eEFSec, eukaryotic elongation factor for selenocysteine; XH, Xpress/His
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FIGURE LEGENDS

Figure 1. Novel Sec incorporation assay for eEFSec function. (A) Diagram of the monocistronic luciferase mRNA reporter with an in-frame UGA codon at position 258, the rat GPX4 SECIS element and a 100 nucleotide poly(A) tail. (B) Sec incorporation activity from reactions containing both PC-3 and Sf21 cell-free lysates. In vitro translation of the Sec incorporation reporter in lysates containing 50% Sf21 lysate and 50% PC-3 cell lysate either in the presence or absence of 160 nM of recombinant XH-CTSBP2 and/or FLAG-eEFSec protein as indicated. The gray and black bars represent luciferase activity from wild-type GPX4 SECIS element and mutant GPX4 SECIS element, respectively. The data represents the mean and standard deviation of three independent experiments.

Figure 2. Sec incorporation activity of Domain IV penta-alanine mutants. (A) Multiple sequence alignment of eEFSec Domain IV from Methanococcus maripaludis (Mm), Drosophila melanogaster (Dm), Caenorhabditis elegans (Ce), Danio rerio (Dr), Bos taurus (Bt), Mus musculus (Mmus), and Homo sapiens (Hs). Sequence alignments were performed with complete eEFSec sequences from each species with the Muscle multiple sequence alignment algorithm. Only Domain IV sequence is shown beginning with murine residue  (top). The domain structure of eEFSec (bottom) is shown with an expanded view of domain IV. This model was derived from the Protein Data Bank entry 1WB1. The mutated sequences are (B) In vitro translation of the Sec incorporation reporter mRNAs as described in Figure 1B in the presence of 160 nM wild-type or Domain IV mutant versions of eEFSec as indicated. Data was normalized for no addition of recombinant FLAG-eEFSec. The data shown are the averages (± standard errors) of results from at least three independent experiments.

Figure 3. Domain IV of eEFSec is involved in SBP2/SECIS interactions. 20 fmol of 32P-labeled wild-type GPX4 SECIS element was incubated with 400 nM XH-CTSBP2 in the presence or absence of wild-type or mutant FLAG-eEFSec, as indicated. Complexes were resolved on a 6% nondenaturing polyacrylamide gel. Lane 1 contains the GPX4 SECIS probe alone.

Figure 4. The SECIS apical loop region not required for eEFSec binding. 20 fmol of 32P-labeled wild-type and mutant GPX4 SECIS elements, as indicated, were incubated in the presence or absence of 400 nM of XH-CTSBP2 and FLAG-eEFSec and resolved on 6% nondenaturing polyacrylamide gel.

Figure 5. The RNA binding domain of SBP2 recruits eEFSec at the SECIS element. (A) EMSA analysis as described in Figure 4 of FLAG-eEFSec with XH-CTSBP2, XH-SID and XH-RBD domains with 32P-labeled wild-type GPX4 SECIS element probe. (B) Samples from (A) were also subjected to UV-crosslinking. UV-crosslinked samples were treated with RNase A and labeled proteins were resolved on a 12% SDS-PAGE gel and analyzed by Phosphorimaging.

Figure 6. Novel interactions between Domain IV and Sec-tRNA^{Sec}. (A) Filter binding assay of 1 µM wild-type FLAG-eEFSec and Domain IV mutants with 33 µg of aa-tRNA containing 10000 cpm[75Se]-Sec-tRNA^{Sec}. Data
was normalized as a percentage of total cpm retained on the filters with wild-type FLAG-eEFSec. eEF1A was used as a negative control. (B) Sec-tRNA\textsuperscript{Sec} from 1 µM of wild-type FLAG-eEFSec in the absence or presence of 1 µM of GPX4 SECIS RNA and XH-CTSBP2. (C) Interactions of Sec-tRNA\textsuperscript{Sec} and wild-type FLAG-eEFSec in the presence of 0.5 mM GTP, GDP-NP, GDP and no nucleotides. The data shown are the averages (± standard errors) of results from at least three independent experiments.

**Figure 7.** Domain IV regulates G-domain activity in eEFSec. (A) Malachite green GTPase assays containing 1 µM eEF1A alone or with the addition of 0.12 mM 80S rabbit ribosomes (rib) and/or 100 ng/µl rabbit aa-tRNA as indicated. GTP hydrolysis was measured as pmol of inorganic phosphate (Pi) released per pmol of eEF1A. (B) GTPase analysis of eEF1A, eEFSec and Domain IV mutants as described in (A). (C) eEFSec GTPase activity was assessed in the presence or absence of aa-tRNA (100 ng/µl), rabbit reticulocyte 80S ribosomes (0.12 µM), equimolar amounts (1 µM) of XH-CTSBP2 and GPX4 SECIS RNA. (D) Intrinsic GTPase activity of Domain IV KRYVF\textsuperscript{569-573} mutant was analyzed in the presence of 1 µM of XH-CTSBP2 and GPX4 SECIS RNA. The data shown are the averages (± standard errors) of results from at least three independent experiments.
Figure 1

A

UGA (stop or Sec)  GPX4 SECIS

5' Cap  Luciferase coding region  3'UTR  A(100)

Luciferase reporter mRNA

B

Luciferase activity (x10^3)

- LUC/Sec/wtGPX4
- LUC/Sec/mutGPX4

no additions  +eEFSec  +SBP2  +SBP2 + eEFSec
Figure 3

[Image of a gel electrophoresis diagram showing bands labeled SECIS, SECIS/SBP2, and SBP2 under different conditions indicated by '+' and '-' symbols for eEFSec, DIV deletion, VERVM, LFK4E, FGQSG, KKRSR, and KRYVF.]
Figure 4
Figure 5

A

| Probe | eEFSec | SID | RBD | SID + eEFSec | RBD + eEFSec | SID + RBD | SID+RBD+eEFSec | CT-SBP2 | CT-SBP2 + eEFSec |
|-------|--------|-----|-----|--------------|--------------|-----------|-----------------|---------|------------------|

B

1 2 3 4 5 6 7 8 9 10
Figure 6

A

B

C

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