THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 46, pp. 38936–38945, November 9, 2012
© 2012 by The American Society for Biochemistry and Molecular Biology, Inc. Published in the U.S.A.

The Selenocysteine-specific Elongation Factor Contains a Novel and Multi-functional Domain*

Received for publication, August 31, 2012, and in revised form, September 18, 2012 Published, JBC Papers in Press, September 19, 2012, DOI 10.1074/jbc.M112.415463

Jonathan N. Gonzalez-Flores, Nirupama Gupta, Louise W. DeMong, and Paul R. Copeland[1]

From the Department of Biochemistry and Molecular Biology, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, Piscataway,NewJersey 08854

Background: Selenocysteine (Sec) 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.

The selenocysteine (Sec)-specific eukaryotic elongation factor (eEFSec) delivers the aminoacylated selenocysteine-tRNA (Sec-tRNA^{Sec}) to the ribosome and suppresses UGA codons that are upstream of Sec insertion sequence (SECIS) elements bound by SECIS-binding protein 2 (SBP2). Multiple studies have highlighted the importance of SBP2 forming a complex with the SECIS element, but it is not clear how this regulates eEFSec during Sec incorporation. Compared with the canonical elongation factor eEF1A, eEFSec has a unique C-terminal extension called Domain IV. To understand the role of Domain IV in Sec incorporation, we examined a series of mutant proteins for all of the known molecular functions for eEFSec: GTP hydrolysis, Sec-tRNA^{Sec} binding, and SBP2/SECIS binding. In addition, wild-type and mutant versions of eEFSec were analyzed for Sec incorporation activity in a novel eEFSec-dependent translation extract. We have found that Domain IV is essential for both tRNA and SBP2 binding as well as regulating GTPase activity. We propose a model where the SBP2/SECIS complex activates eEFSec by directing functional interactions between Domain IV and the ribosome to promote Sec-tRNA^{Sec} binding and accommodation into the ribosomal A-site.

The amino acid selenocysteine (Sec)[2] 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 (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 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 eukaryotic 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 (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 properties similar 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 (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 dem-

* This work was supported, in whole or in part, by Rutgers-University of Medicine and Dentistry of New Jersey Biotechnology National Institutes of Health 2T32GM008339-21, Public Health Service Grant GM077073, and the graduate fellowship programs of Integrative Graduate Education and Research Traineeship Biointerfaces National Science Foundation Directorate of Graduate Education 0333196.
[1] To whom correspondence should be addressed: Department of Biochemistry and Molecular Biology, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, 675 Hoes Lane, Piscataway, NJ 08854. Tel.: 732-235-4670; Fax: 732-235-5223; Email: paul.copeland@umdnj.edu.
[2] The abbreviations used are: Sec, selenocysteine; eEFSec, eukaryotic elongation factor for selenocysteine; SECIS, Sec insertion sequence; SBP2, SECIS-binding protein 2; bSECIS, bacterial SECIS; RBD, RNA-binding domain; SID, Sec incorporation domain; aa, amino acid; XH, Xpress/His.

onstrated 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 overexpression 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 study 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 this study, 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 QuikChange 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 described previously (10, 11).

Recombinant Protein Expression and Purification—Recombinant Xpress/His-tagged C-terminal SBP2 (XH-CTSBP2) was purified as described previously (12). All of our FLAG-eEFSec and eEF1A recombinant proteins were expressed and purified from *Escherichia coli* BL21. The transformed bacteria were grown at 37 °C in LB medium with 100 μg/ml ampicillin to a density of ~1.0 A_{600}. Induction was performed with 0.1 mM isopropyl-β-D-thiogalactoside for 12–16 h 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 s/ml with 1–2-min resting periods on ice and then centrifuged at 15,000 × *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 40-ml 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, and 25% glycerol). Elution was done in 1 ml of Buffer B with 250 μg/ml 3× 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′ untranslated region (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 (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 1640 medium supplemented with 50 nM sodium selenite for 7 days. Cells were scraped in translation buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.25 mM $MgCl_2$, 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 30½-gauge syringe needle. Cellular lysates were centrifuged for 10 min at 17,000 × *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 each of *Sf*21 and PC-3 lysates, 100 ng of luciferase mRNA reporter, and 160 nM SBP2 and wild-type or mutant eEFSec recombinant protein. Translation and Sec incorporation reactions were incubated for 1 h 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). ^{32}P-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 $MgCl_2$, 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 analyzed by phosphoimaging. 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 phosphoimaging.

Preparation of [^{75}Se]Sec-$tRNA^{Sec}$ and Isolation of Total Aminoacylated tRNA—Sec-$tRNA^{Sec}$ was radiolabeled with 50 nM $Na_2[^{75}Se]O_3$ (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 H_2O, 4 ml of 5× Buffer T (50 mM NaOAc, 3.25 M NaCl, 50 mM $MgCl_2$, and 5 mM EDTA), and 20 ml of phenol, pH 4. The solution was briefly vortexed and centrifuged at 12,000 × *g* for 5 min at 4 °C. The aqueous phase was transferred to another tube and re-extracted with one volume of phenol, pH 4 to remove remaining protein contamination. RNA was precipitated with 2.5 volumes of 100% ethanol and stored at −80 °C for 5 min. RNA was pelleted at 12,000 × *g* for 15 min at 4 °C and resuspended in 800 μl of 1× Buffer T. RNA was re-pelleted by

ethanol precipitation, washed once with 70% ethanol, and air-dried for 10 min. Pellet was resuspended in aa-tRNA storage solution (5 mM NaOAc, pH 4.5 and 2 mM DTT), aliquoted, and stored at −80 °C. Agarose gel analysis showed little or no ribosomal RNA contamination in our aa-tRNA preparations. [^{75}Se]Sec-tRNA^{Sec} was quantified by liquid scintillation counting and yielded ~300 cpm per μg of total aa-tRNA. Sec-tRNA^{Sec} integrity was examined by acid urea gel electrophoresis (14) followed by PhosphorImager analysis.

[^{75}Se]Sec-tRNA^{Sec} Filter-binding Assay—Binding reactions (20 μl) were performed in Buffer C (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 0.25 mM $MgCl_2$ 0.5 mM GTP, 1 mM DTT, and 10% glycerol), 33 μg of aa-tRNA containing 10,000 cpm of [^{75}Se]Sec-tRNA^{Sec}, and 1 μM eEFSec for 30 min at 30 °C. After incubation, samples were pipetted onto nitrocellulose filters (Millipore 0.45-μm HA) that were prewashed with translation buffer. Samples were washed three times with 500 μl of Buffer C on a Millipore vacuum manifold. Membrane filters were air-dried and subjected to liquid scintillation counting.

GTP Hydrolysis Assay—GTP hydrolysis activity was measured by using a colorimetric GTPase assay kit (Innova Biosciences) according to the manufacturer's protocol. Reactions were assayed in Buffer C and incubated for 1 h at 30 °C. FLAG-eEF1A, FLAG-eEFSec, XH-CTSBP2, and the SECIS element were each added at a final concentration of 1 μM. Isolation of total aa-tRNA was performed as above but without the addition of $Na_2[^{75}Se]O_3$. Purification of salt washed 80 S ribosomes from rabbit reticulocyte was performed as described (15). Concentrations of total aa-tRNA and ribosomes in the GTPase activation reactions were at 100 ng/μl and 0.12 μM, respectively. Measurements of the hydrolyzed free phosphate were performed at a wavelength of 600 nm, and absorbance values were compared with a standard curve to determine the free phosphate concentration.

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). Because 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* (*Sf*21) have been extensively used for protein expression, and a commercially available *in vitro* translation lysate from *Sf*21 cells provides robust translation activity (8). Because the addition of purified SBP2, eEFSec, and rabbit aminoacyl tRNA to *Sf*21 lysates did not result in Sec incorporation activity,[3] we combined mammalian cell lysate (supplying Sec incorporation factors) and *Sf*21 lysate (supplying ample general translation factors) 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 (Fig. 1*A*). This method has been demonstrated to faithfully indicate Sec incorporation because the production of full-length and active luciferase is strictly SECIS- and SBP2-dependent (13).

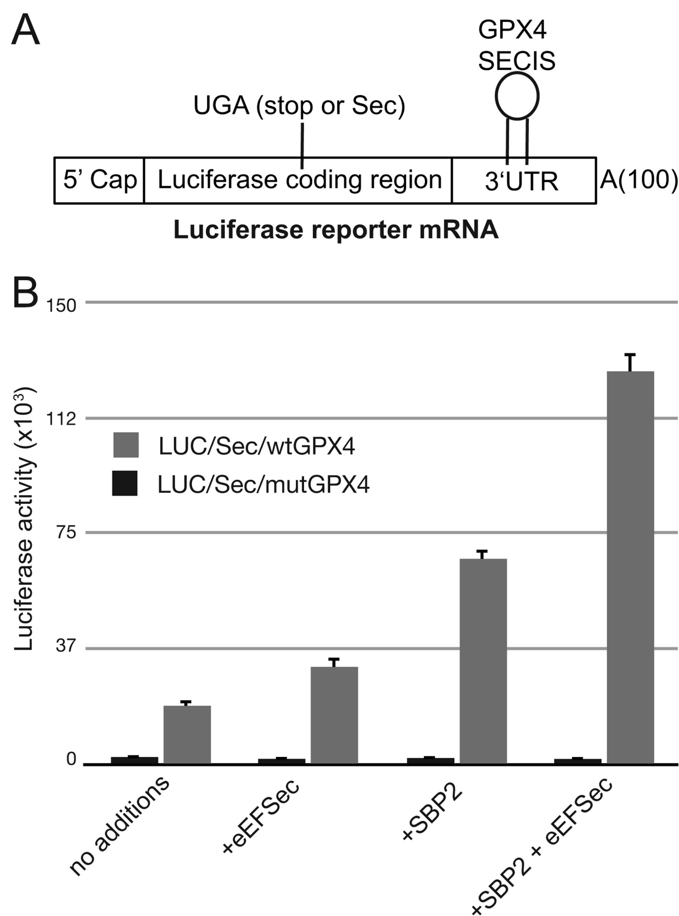

FIGURE 1. **Novel Sec incorporation assay for eEFSec function.** *A*, diagram of the monocistronic luciferase mRNA reporter with an in-frame UGA codon at position 258, the rat GPX4 SECIS element, and a 100-nucleotide poly(A) tail. *B*, Sec incorporation activity from reactions containing both PC-3 and 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 recombinant XH-CTSBP2 and FLAG-eEFSec protein as indicated is shown. The *gray* and *black bars* represent luciferase activity from wild-type GPX4 SECIS element and mutant GPX4 SECIS element, respectively. The data represent the mean ± S.D. of three independent experiments.

Previously we have shown that PC-3 prostate cancer cells had reduced levels of the selenoprotein GPX1 compared with LNCaP prostate cancer cells and RWPE-1 nontumorigenic prostate cells (even when selenium levels were sufficient) suggesting that eEFSec and SBP2 may be limiting (17). We therefore first tested PC-3 lysates for Sec incorporation activity in the *Sf*21 system. As shown in Fig. 1*B* (*lane 1*), we observed easily detectable Sec incorporation that was approximately 9-fold higher than the background obtained from the mRNA with a mutant SECIS element. Upon the addition of FLAG-eEFSec, a

[3] N. Gupta and P. R. Copeland, unpublished observation.

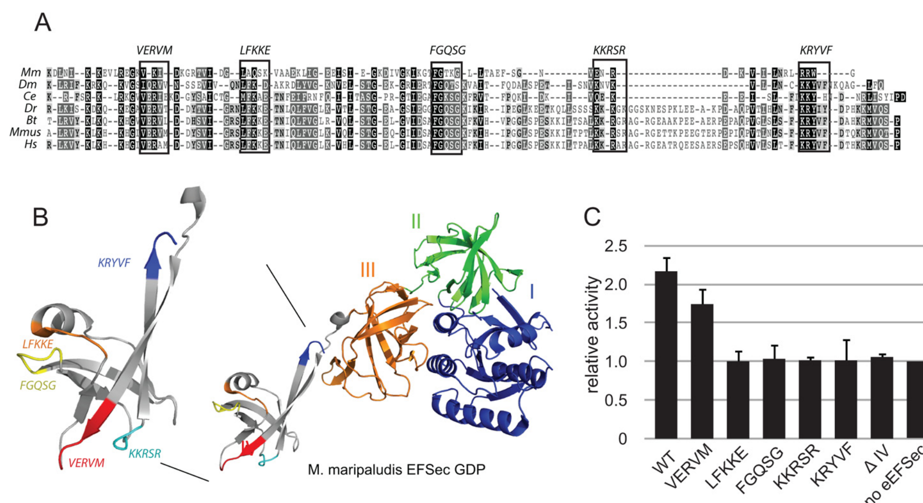

FIGURE 2. **Sec incorporation activity of Domain IV penta-alanine mutants.** *A*, multiple sequence alignment of eEFSec Domain IV from *Methanococcus maripaludis* (*Mm*), *Drosophila melanogaster* (*Dm*), *Caenorhabditis elegans* (*Ce*), *Danio rerio* (*Dr*), *Bos taurus* (*Bt*), *Mus musculus* (*Mmus*), and *Homo sapiens* (*Hs*) is shown. 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 4AC9. *B*, mutated sequences are *in vitro* translation of the Sec incorporation reporter mRNAs as described in the legend to Fig. 1*B* in the presence of 160 nM wild-type or Domain IV mutant versions of eEFSec as indicated. Data were normalized for no addition of recombinant FLAG-eEFSec. The data shown are the averages (± S.E.) of results from at least three independent experiments.

2-fold enhancement was observed whereas the addition of the fully active Xpress/His-tagged C-terminal fragment of SBP2 (XH-CTSBP2) 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 nonspecific UGA readthrough from mRNAs that harbored a mutant SECIS element (*black bars*) or total translation activity (data not shown). Importantly, these data clearly show an ~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, whereas 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 (Fig. 2*A*). Multiple sequence alignments from species ranging from archaebacteria to human were used to determine conserved residues in Domain IV of eEFSec (Fig. 2*A*). 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.* VERVM$^{466-470}$). We successfully employed a similar method to identify critical residues in SBP2 (10, 11). A structural model of archaeal eEFSec is shown in Fig. 2*B* (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 (Δ*IV*) 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. 2*C* (*right panel*) demonstrates that most of the penta-alanine mutants tested were defective for Sec incorporation, with the exception of VERVM$^{466-470}$, 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 described previously an 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 Fig. 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 "supershift" of the probe (Fig. 3, *lanes 1–3*). The complete deletion of eEFSec Domain IV eliminates binding to SBP2/SECIS (Fig. 3, compare *lane 3* with *4*). In addition, the mutation at KKRSR$^{536-540}$ also eliminates supershifting activity, but the other mutations in Domain IV do not affect stable SBP2/SECIS binding. These data establish that Domain IV, specifically the region at KKRSR$^{536-540}$, 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.

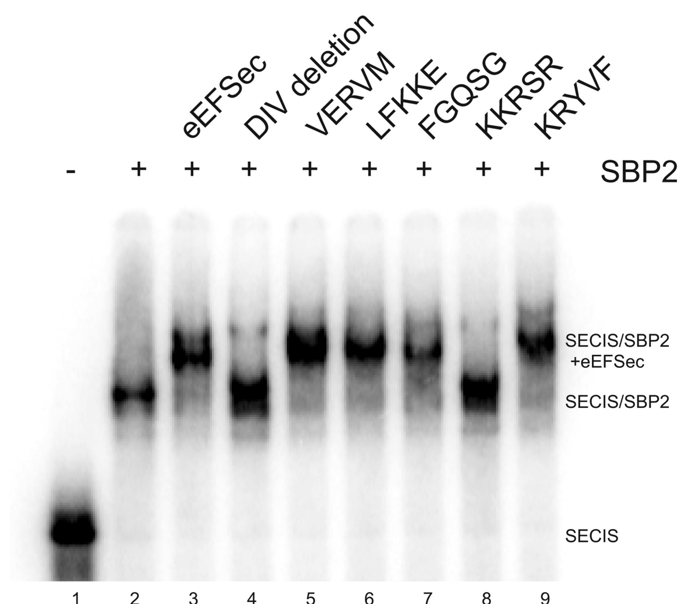

FIGURE 3. **Domain IV of eEFSec is involved in SBP2/SECIS interactions.** 20 fmol of ^{32}P-labeled wild-type GPX4 SECIS element was incubated with 400 nM XH-CTSBP2 in the presence or absence of wild-type or mutant FLAG-eEFSec, as indicated. Complexes were resolved on a 6% nondenaturing polyacrylamide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

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 shown previously 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 (RFQDR$^{647-651}$ and DGAQD$^{746-750}$) (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 Fig. 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 composed of two highly conserved unpaired adenosines followed by either an A or G (20, 22, 23). Whereas 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 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 (Fig. 4, *lanes 5–8*). As expected, the mutation of the AAR motif to UUC did not affect SBP2 binding or eEFSec supershifting. In the case where the entire helix 2 and terminal loop were deleted, SBP2 binding was reduced (see *lane 15* of Fig. 4 where only about 50% of the probe was shifted), but eEFSec was still able to supershift all of the SBP2/SECIS complex. Together, these results strongly suggest that eEFSec recognizes SBP2 residues and 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.

To address the question of whether eEFSec is making direct contacts with the SECIS element when bound to the SBP2/SECIS complex, UV cross-linking was performed on the EMSA reactions identical to those shown in Fig. 5*A*. Because CTSBP2 and FLAG-eEFSec migrate at the same molecular weight in SDS-PAGE, we took advantage of the fact that CTSBP2 retains its function when physically split into its two domains, the Sec incorporation domain (SID) and RBD (10). As expected, when recombinant Xpress/His-tagged SID (XH-SID) protein is added to an EMSA, no SECIS or eEFSec binding is observed (Fig. 5*A*, *lanes 3 and 4*). However, upon addition of the XH-RBD protein, the GPX4 probe is shifted, and the addition of eEFSec causes a super-shift (Fig. 5, *lanes 5 and 6*). A similar result is obtained when both the XH-SID and XH-RBD proteins are added (*lanes 7 and 8*), although the extent of super-shift appears to be greater than with the XH-RBD alone. These results confirm that the XH-RBD is sufficient for eEFSec binding but that the inclusion of the XH-SID may result in a more stable complex. When these samples were subjected to UV cross-linking, no cross-linked band was observed when the reaction contained eEFSec or the SID alone or in combination (Fig. 5*B*, l*anes 3 and 4*). The RBD alone, however, was able to cross-link to the GPX4 probe, but no band corresponding to the size of eEFSec was observed either in this case or in the presence of both XH-SID and XH-RBD (*lanes 5–8*). We also did not observe any increase in cross-linking efficiency when eEFSec was included in the reaction, suggesting that eEFSec does not affect the ability of the XH-RBD or XH-CTSBP2 to cross-link to the GPX4 probe (Fig. 5*B*, *lanes 7–10*). Together these data confirm that the SBP2 RNA-binding domain is the site of eEFSec interaction but that we are unable to detect direct interactions between eEFSec and the SECIS element in the context of an SBP2/SECIS/eEFSec complex.

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^{Ser} (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-tRNAPhe 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}.

To test the involvement of Domain IV in Sec-tRNA^{Sec} binding, we employed nitrocellulose filter-binding assay with

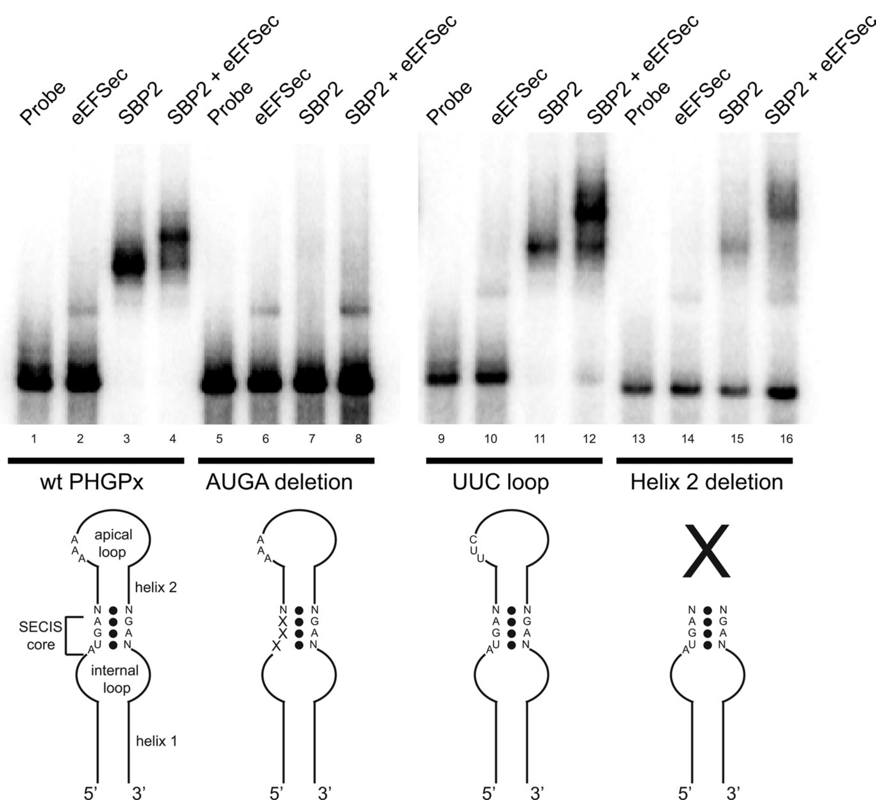

FIGURE 4. **The SECIS apical loop region not required for eEFSec binding.** 20 fmol of ^{32}P-labeled wild-type and mutant GPX4 SECIS elements, as indicated, was incubated in the presence or absence of 400 nM XH-CTSBP2 and FLAG-eEFSec and resolved on 6% nondenaturing polyacrylamide gel.

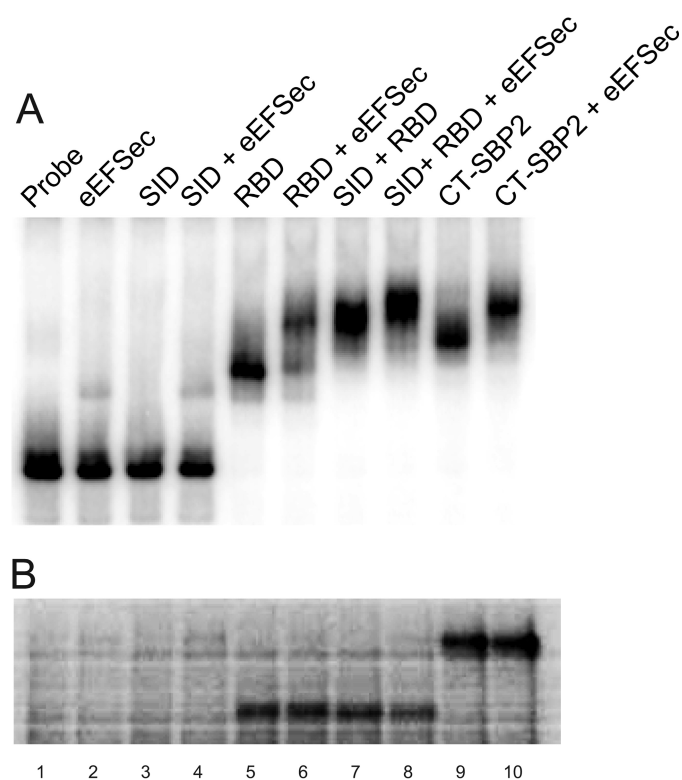

FIGURE 5. **The RNA-binding domain of SBP2 recruits eEFSec at the SECIS element.** *A*, EMSA analysis as described in the legend to Fig. 4 of FLAG-eEFSec with XH-CTSBP2, XH-SID, and XH-RBD domains with ^{32}P-labeled wild-type GPX4 SECIS element probe is shown. *B*, samples from *A* were also subjected to UV-cross-linking. UV-cross-linked samples were treated with RNase A, and labeled proteins were resolved on a 12% SDS-PAGE gel and analyzed by phosphorimaging.

^{75}Se-radiolabeled total rabbit aminoacyl-tRNA. Rabbit reticulocyte lysate was incubated with 50 nM $Na_2[^{75}Se]O_3$, followed by total aa-tRNA purification (see "Experimental Procedures"). Preparation of radiolabeled Sec-tRNA^{Sec} was verified by acid urea gel electrophoresis and scintillation counting (data not shown). Background filter retention of [^{75}Se]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 (Fig. 6*A*, compare *lanes 1* and *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 (Fig. 6*A*). Mutations in VERVM$^{466-470}$ and LFKKE$^{480-484}$ showed only moderate defects with 77 and 76% of wild-type binding, respectively. Because 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. Fig. 6*B* shows that the addition of SBP2 and SBP2/SECIS does not disrupt but may slightly enhance the interactions between eEFSec and Sec-tRNA^{Sec}. These data establish the critical role of Domain IV in Sec-tRNA^{Sec} recognition and illustrate 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.

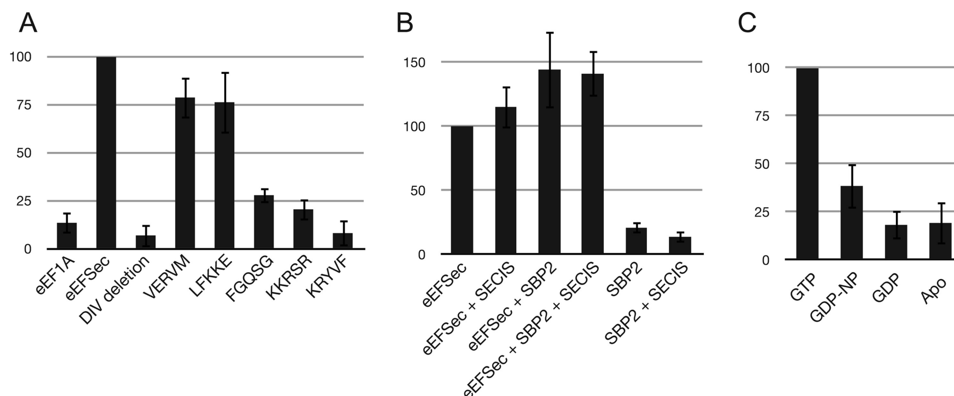

FIGURE 6. **Novel interactions between Domain IV and Sec-tRNA^{Sec}.** *A*, filter-binding assay of 1 μM wild-type FLAG-eEFSec and Domain IV mutants with 33 μg of aa-tRNA containing 10,000 cpm [^{75}Se]Sec-tRNA^{Sec}. Data were normalized as a percentage of total cpm retained on the filters with wild-type FLAG-eEFSec. eEF1A was used as a negative control. *B*, Sec-tRNA^{Sec} from 1 μM wild-type FLAG-eEFSec in the absence or presence of 1 μM GPX4 SECIS RNA and XH-CTSBP2. *C*, interactions of Sec-tRNA^{Sec} and wild-type FLAG-eEFSec in the presence of 0.5 mM GTP, GDPNP, GDP, and no nucleotides. The data shown are the averages (± S.E.) of results from at least three independent experiments.

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. Fig. 6*C* 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 nonhydrolyzable 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). These 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 70 S 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. 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 (Fig. 7*A*). 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 KRYVF$^{569-573}$, which surprisingly showed almost 2-fold higher activity than eEF1A (Fig. 7*B*). Because 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 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. Fig. 7*C* 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, Fig. 7*C* also shows that the high intrinsic GTPase activity of the KRYVF$^{569-573}$ 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 with 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 database with the *Methanocaldococcus 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 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 composed of two basic components: a source of highly efficient general translation factors from *Sf*21 cell extracts and a source of limiting Sec incorporation factors from prostate cancer cells. The requirement for exogenous translation factors is likely because of the low efficiency of Sec incorporation, which has been reported to be approximately 10% *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

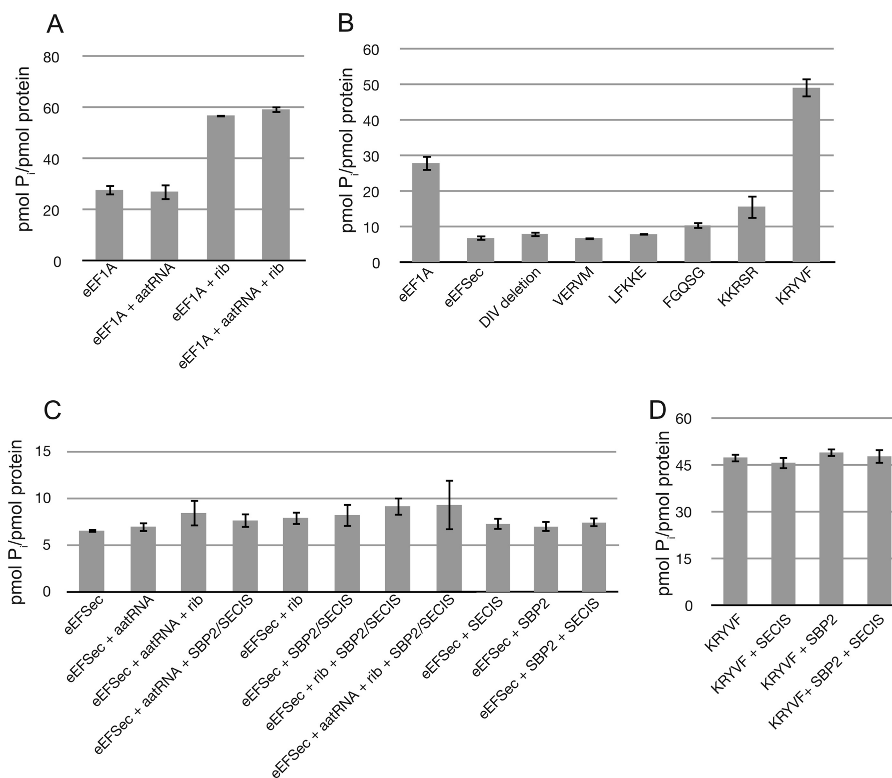

FIGURE 7. **Domain IV regulates G-domain activity in eEFSec.** *A*, malachite green GTPase assays containing 1 μM eEF1A alone or with the addition of 0.12 μM 80 S rabbit ribosomes (*rib*) and 100 ng/μl rabbit aa-tRNA as indicated. GTP hydrolysis was measured as pmol of inorganic phosphate (*Pi*) released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

SBP2 was performed by Zavacki *et al.* (9). In this study, a fragment corresponding to Domain IV was able to co-immunoprecipitate SBP2 in the absence of a SECIS element from rabbit reticulocyte lysate. However, co-immunoprecipitation experiments with full-length eEFSec did not appear to stably bind SBP2 under the same conditions. It was suggested that this lack of interaction was because of limiting Sec-tRNA^{Sec} in rabbit reticulocyte, because eEFSec and SBP2 were successfully co-immunoprecipitated 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^{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$^{536-540}$ region. Whether the SBP2/SECIS complex physically interacts with Domain IV at this location is a subject for future study, but here we have established a clear link between eubacterial and eukaryotic Sec incorporation because 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^{Sec} Interactions Found in Domain IV—Physical interactions of Domain IV with the Sec-tRNA^{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 because of 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 optimize 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 stability.

Domain IV mutant analysis by our Sec-tRNA^{Sec} filter-binding assay provided the first biochemical evidence that Domain IV of eEFSec is required for Sec-tRNA^{Sec}-binding activity. Residues in Domain IV involved in Sec-tRNA^{Sec} interactions were found within its C-terminal region (KKRSR$^{536-540}$ and KRYVF$^{569-573}$), which overlaps with the residues required for SBP2/SECIS binding (KKRSR$^{536-540}$). 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^{Sec} ternary complex interacts with the SBP2/SECIS complex. Nevertheless, addition of SBP2 and the SECIS element did not alter Sec-tRNA^{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^{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^{Sec} binding within eEFSec Domain IV.

Domain IV Regulates the G-domain of eEFSec—To prevent the Sec-tRNA^{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, the elongation factor binding site is composed of two elements: 1) the GTPase associated center and 2) the sarcin-ricin loop. The GTPase-associated center and sarcin-ricin loop 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, it was recently discovered that SBP2 causes a conformational change specifically at the Helix 89 on the 28 S 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 because 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 GDPNP but much tighter when incubated with GTP (43). In accordance to this finding, the GDPNP nucleotide was also a poor GTP analog for eEFSec in our Sec-tRNA^{Sec} filter-binding assays. In addition, a recent study using an isothermal titration calorimetry found that SelB underwent small conformational changes when incubated with GDP and GDPNP, but dramatic rearrangements were found in the SelB-GTP complex (29). This means that the SelB/eEFSec crystal structure with GDPNP does not 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 did not acquire significant GTPase stimulation upon the addition of SBP2 and the SECIS element. Surprisingly, however, the KKRSR$^{536-540}$ 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 KKRSR$^{536-540}$ 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 preactivated 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 inducing eEFSec in a KKRSR$^{536-540}$-KRYVF$^{569-573}$-like conformation.

Other Functions for Domain IV?—Altogether, 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 study shows that four conserved segments within Domain IV are essential for Sec incorporation activity *in vitro*. One of those is defective for SBP2 binding (KKRSR), and three are defective for tRNA binding (FGQSG, KKRSR, 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.

REFERENCES

1. Steinbrenner, H., and Sies, H. (2009) Protection against reactive oxygen species by selenoproteins. *Biochim. Biophys. Acta* **1790,** 1478–1485
2. Tujebajeva, R. M., Copeland, P. R., Xu, X. M., Carlson, B. A., Harney, J. W., Driscoll, D. M., Hatfield, D. L., and Berry, M. J. (2000) Decoding apparatus for eukaryotic selenocysteine insertion. *EMBO Rep.* **1,** 158–163
3. Fagegaltier, D., Hubert, N., Yamada, K., Mizutani, T., Carbon, P., and Krol, A. (2000) Characterization of mSelB, a novel mammalian elongation factor for selenoprotein translation. *EMBO J.* **19,** 4796–4805
4. Leibundgut, M., Frick, C., Thanbichler, M., Böck, A., and Ban, N. (2005) Selenocysteine tRNA-specific elongation factor SelB is a structural chimaera of elongation and initiation factors. *EMBO J.* **24,** 11–22
5. Noble, C. G., and Song, H. (2008) Structural studies of elongation and release factors. *Cell Mol. Life Sci.* **65,** 1335–1346
6. Yoshizawa, S., Rasubala, L., Ose, T., Kohda, D., Fourmy, D., and Maenaka, K. (2005) Structural basis for mRNA recognition by elongation factor SelB. *Nat. Struct. Mol. Biol.* **12,** 198–203
7. Baron, C., Heider, J., and Böck, A. (1993) Interaction of translation factor SELB with the formate dehydrogenase H selenopolypeptide mRNA. *Proc. Natl. Acad. Sci. U.S.A.* **90,** 4181–4185
8. Ezure, T., Suzuki, T., Higashide, S., Shintani, E., Endo, K., Kobayashi, S., Shikata, M., Ito, M., Tanimizu, K., and Nishimura, O. (2006) Cell-free protein synthesis system prepared from insect cells by freeze-thawing. *Biotechnol. Prog.* **22,** 1570–1577
9. Zavacki, A. M., Mansell, J. B., Chung, M., Klimovitsky, B., Harney, J. W., and Berry, M. J. (2003) Coupled tRNA(Sec)-dependent assembly of the

selenocysteine-decoding apparatus. *Mol. Cell* **11,** 773–781

10. Donovan, J., Caban, K., Ranaweera, R., Gonzalez-Flores, J. N., and Copeland, P. R. (2008) A novel protein domain induces high affinity selenocysteine insertion sequence binding and elongation factor recruitment. *J. Biol. Chem.* **283,** 35129–35139
11. Caban, K., Kinzy, S. A., and Copeland, P. R. (2007) The L7Ae RNA-binding motif is a multi-functional domain required for the ribosome-dependent Sec incorporation activity of Sec insertion sequence-binding protein 2. *Mol. Cell. Biol.* **27,** 6350–6360
12. Kinzy, S. A., Caban, K., and Copeland, P. R. (2005) Characterization of the SECIS binding protein 2 complex required for the co-translational insertion of selenocysteine in mammals. *Nucleic Acids Res.* **33,** 5172–5180
13. Mehta, A., Rebsch, C. M., Kinzy, S. A., Fletcher, J. E., and Copeland, P. R. (2004) Efficiency of mammalian selenocysteine incorporation. *J. Biol. Chem.* **279,** 37852–37859
14. Köhrer, C., and Rajbhandary, U. L. (2008) The many applications of acid urea polyacrylamide gel electrophoresis to studies of tRNAs and aminoacyl-tRNA synthetases. *Methods* **44,** 129–138
15. Caban, K., and Copeland, P. R. (2012) Selenocysteine insertion sequence (SECIS)-binding protein 2 alters conformational dynamics of residues involved in tRNA accommodation in 80 S ribosomes. *J. Biol. Chem.* **287,** 10664–10673
16. Donovan, J., and Copeland, P. R. (2009) Evolutionary history of selenocysteine incorporation from the perspective of SECIS-binding proteins. *BMC Evol. Biol.* **9,** 229
17. Rebsch, C. M., Penna, F. J., 3rd, and Copeland, P. R. (2006) Selenoprotein expression is regulated at multiple levels in prostate cells. *Cell Res.* **16,** 940–948
18. Caban, K., and Copeland, P. R. (2006) Size matters: a view of selenocysteine incorporation from the ribosome. *Cell Mol. Life Sci.* **63,** 73–81
19. Cléry, A., Bourguignon-Igel, V., Allmang, C., Krol, A., and Branlant, C. (2007) An improved definition of the RNA-binding specificity of SECIS-binding protein 2: an essential component of the selenocysteine incorporation machinery. *Nucleic Acids Res.* **35,** 1868–1884
20. Chapple, C. E., Guigó, R., and Krol, A. (2009) SECISaln: a web-based tool for the creation of structure-based alignments of eukaryotic SECIS elements. *Bioinformatics* **25,** 674–675
21. Walczak, R., Westhof, E., Carbon, P., and Krol, A. (1996) A novel RNA structural motif in the selenocysteine insertion element of eukaryotic selenoprotein mRNAs. *RNA* **2,** 367–379
22. Berry, M. J., Banu, L., Harney, J. W., and Larsen, P. R. (1993) Functional characterization of the eukaryotic SECIS elements which direct selenocysteine insertion at UGA codons. *EMBO J.* **12,** 3315–3322
23. Korotkov, K. V., Novoselov, S. V., Hatfield, D. L., and Gladyshev, V. N. (2002) Mammalian selenoprotein in which selenocysteine (Sec) incorporation is supported by a new form of Sec insertion sequence element. *Mol. Cell. Biol.* **22,** 1402–1411
24. Copeland, P. R., Fletcher, J. E., Carlson, B. A., Hatfield, D. L., and Driscoll, D. M. (2000) A novel RNA-binding protein, SBP2, is required for the translation of mammalian selenoprotein mRNAs. *EMBO J.* **19,** 306–314
25. Fletcher, J. E., Copeland, P. R., Driscoll, D. M., and Krol, A. (2001) The selenocysteine incorporation machinery: Interactions between the SECIS RNA and the SECIS-binding protein SBP2. *RNA* **7,** 1442–1453
26. Latrèche, L., Jean-Jean, O., Driscoll, D. M., and Chavatte, L. (2009) Novel structural determinants in human SECIS elements modulate the translational recoding of UGA as selenocysteine. *Nucleic Acids Res.* **37,** 5868–5880
27. Itoh, Y., Chiba, S., Sekine, S., and Yokoyama, S. (2009) Crystal structure of human selenocysteine tRNA. *Nucleic Acids Res.* **37,** 6259–6268
28. Hauryliuk, V., Zavialov, A., Kisselev, L., and Ehrenberg, M. (2006) Class-1 release factor eRF1 promotes GTP binding by class-2 release factor eRF3. *Biochimie* **88,** 747–757
29. Paleskava, A., Konevega, A. L., and Rodnina, M. V. (2012) Thermodynamics of the GTP-GDP-operated conformational switch of selenocysteine-specific translation factor SelB. *J. Biol. Chem.* **287,** 27906–27912
30. Villa, E., Sengupta, J., Trabuco, L. G., LeBarron, J., Baxter, W. T., Shaikh, T. R., Grassucci, R. A., Nissen, P., Ehrenberg, M., Schulten, K., and Frank, J. (2009) Ribosome-induced changes in elongation factor Tu conformation control GTP hydrolysis. *Proc. Natl. Acad. Sci. U.S.A.* **106,** 1063–1068
31. Voorhees, R. M., Schmeing, T. M., Kelley, A. C., and Ramakrishnan, V. (2010) The mechanism for activation of GTP hydrolysis on the ribosome. *Science* **330,** 835–838
32. Hüttenhofer, A., and Böck, A. (1998) Selenocysteine inserting RNA elements modulate GTP hydrolysis of elongation factor SelB. *Biochemistry* **37,** 885–890
33. Soler, N., Fourmy, D., and Yoshizawa, S. (2007) Structural insight into a molecular switch in tandem winged-helix motifs from elongation factor SelB. *J. Mol. Biol.* **370,** 728–741
34. Ose, T., Soler, N., Rasubala, L., Kuroki, K., Kohda, D., Fourmy, D., Yoshizawa, S., and Maenaka, K. (2007) Structural basis for dynamic interdomain movement and RNA recognition of the selenocysteine-specific elongation factor SelB. *Structure* **15,** 577–586
35. Kromayer, M., Wilting, R., Tormay, P., and Böck, A. (1996) Domain structure of the prokaryotic selenocysteine-specific elongation factor SelB. *J. Mol. Biol.* **262,** 413–420
36. Sergiev, P. V., Bogdanov, A. A., and Dontsova, O. A. (2005) How can elongation factors EF-G and EF-Tu discriminate the functional state of the ribosome using the same binding site? *FEBS Lett.* **579,** 5439–5442
37. Fourmy, D., Guittet, E., and Yoshizawa, S. (2002) Structure of prokaryotic SECIS mRNA hairpin and its interaction with elongation factor SelB. *J. Mol. Biol.* **324,** 137–150
38. Selmer, M., and Su, X. D. (2002) Crystal structure of an mRNA-binding fragment of *Moorella thermoacetica* elongation factor SelB. *EMBO J.* **21,** 4145–4153
39. Jenner, L., Demeshkina, N., Yusupova, G., and Yusupov, M. (2010) Structural rearrangements of the ribosome at the tRNA proofreading step. *Nat. Struct. Mol. Biol.* **17,** 1072–1078
40. La Teana, A., Gualerzi, C. O., and Dahlberg, A. E. (2001) Initiation factor IF 2 binds to the α-sarcin loop and Helix 89 of *Escherichia coli* 23S ribosomal RNA. *RNA* **7,** 1173–1179
41. Unbehaun, A., Marintchev, A., Lomakin, I. B., Didenko, T., Wagner, G., Hellen, C. U., and Pestova, T. V. (2007) Position of eukaryotic initiation factor eIF5B on the 80S ribosome mapped by directed hydroxyl radical probing. *EMBO J.* **26,** 3109–3123
42. Burakovskiĭ, D. E., Smirnova, A. S., Lesniak, D. V., Kiparisov, S. V., Leonov, A. A., Sergiev, P. V., Bogdanov, A. A., and Dontsova, O. A. (2007) Interaction of 23S ribosomal RNA Helices 89 and 91 of *Escherichia coli* contributes to the activity of IF2 but is insignificant for elongation factors functioning. *Mol. Biol.* **41,** 1031–1041
43. Paleskava, A., Konevega, A. L., and Rodnina, M. V. (2010) Thermodynamic and kinetic framework of selenocysteyl-tRNA^{Sec} recognition by elongation factor SelB. *J. Biol. Chem.* **285,** 3014–3020