A Novel Protein Domain Induces High Affinity Selenocysteine Insertion Sequence Binding and Elongation Factor Recruitment

Jesse Donovan, Kelvin Caban, Ruchira Ranaweera, Jonathan N. Gonzalez-Flores, and Paul R. Copeland

From the Department of Molecular Genetics, Microbiology and Immunology, University of Medicine and Dentistry of New Jersey Robert Wood Johnson Medical School, Piscataway, New Jersey 08854

Selenocysteine (Sec) is incorporated at UGA codons in mRNAs possessing a Sec insertion sequence (SECIS) element in their 3′-untranslated region. At least three additional factors are necessary for Sec incorporation: SECIS-binding protein 2 (SBP2), Sec-tRNA^{Sec}, and a Sec-specific translation elongation factor (eEFSec). The C-terminal half of SBP2 is sufficient to promote Sec incorporation in vitro, which is carried out by the concerted action of a novel Sec incorporation domain and an L7Ae RNA-binding domain. Using alanine scanning mutagenesis, we show that two distinct regions of the Sec incorporation domain are required for Sec incorporation. Physical separation of the Sec incorporation and RNA-binding domains revealed that they are able to function in trans and established a novel role of the Sec incorporation domain in promoting SECIS and eEFSec binding to the SBP2 RNA-binding domain. We propose a model in which SECIS binding induces a conformational change in SBP2 that recruits eEFSec, which in concert with the Sec incorporation domain gains access to the ribosomal A site.

Selenocysteine (Sec) is co-translationally incorporated into proteins by recoding the opal stop codon (UGA). In eukaryotes, Sec incorporation requires the cis-acting Sec insertion sequence (SECIS) element in the 3′-untranslated region of sel-enoprotein mRNAs. In addition, three trans-acting factors are known to be required: SECIS-binding protein 2 (SBP2), a Sec-specific translation elongation factor (eEFSec), and Sec-tRNA^{Sec} (reviewed in Ref. 1). eEFSec and Sec-tRNA^{Sec} do not suppress UGA termination codons, indicating that SBP2 and the SECIS work in concert to provide specificity for the appropriate UGA (Sec) codons. eEFSec and SBP2 have been shown to interact in what may be a Sec-tRNA^{Sec}-dependent fashion by co-immunoprecipitation from transfected cells (2, 3). Interestingly, this interaction is not detectable in rabbit reticulocyte lysate (4), despite this system being fully competent for Sec incorporation (5). This apparent discrepancy remains unresolved.

Mammalian SBP2 has been ascribed three functions: 1) Sec incorporation, 2) SECIS binding, and 3) ribosome binding (6). Amino acids (aa) 1–398 (rat numbering is used throughout) are dispensable for Sec incorporation in vitro, whereas the C-terminal half of SBP2 (CT-SBP2; aa 399–864) comprises the minimum fully functional protein (7). Indeed, all eukaryotes more “primitive” than echinoderms lack the N-terminal domain of SBP2 (8). In addition, truncation analysis provided evidence that CT-SBP2 is comprised of an N-terminal “functional domain” (aa 399–516) and a C-terminal RNA-binding domain (aa 517–777). In terms of ribosome binding, both regions were shown to be required (6).

The SBP2 RNA-binding domain (RBD) contains an L7Ae RNA-binding domain that mediates SECIS interactions (6, 8–10). Two lines of evidence suggest that the SBP2 RBD is also important for ribosome binding. First, inclusion of SECIS elements in ribosome binding assays inhibits SBP2-ribosome interaction, suggesting that SBP2 may bind a kink-turn motif in the rRNA (10, 11). Further, mutations in the conserved core region of the L7Ae RBD eliminate ribosome binding (10). Mutation of residues 647RFQDR651 upstream of the core L7Ae RNA-binding motif, has been reported to compete with SBP2 for ribosomal protein L30, which also contains an L7Ae RNA-binding domain (10, 11). In contrast to the RNA-binding domain, little is known of the SBP2 functional domain (hereafter referred to as the Sec incorporation domain (SID)). Structure/function studies of SBP2 showed that a truncation mutant comprised of residues 459–
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846 had reduced Sec incorporation activity but no loss in SECIS binding (6). Deletion of residues 399–516 from CT-SBP2 ablated Sec incorporation, did not affect SECIS binding, but resulted in a significant decrease in ribosome binding as assayed by gradient centrifugation (6), suggesting that residues within the SID are also required for ribosome binding, thus adding further complexity to the SBP2-ribosome interaction.

In this study, we sought to identify critical residues within the SBP2 SID using homology-driven scanning mutagenesis. This allowed us to identify two SID regions important for Sec incorporation: one contributing solely to Sec incorporation and another required for all three SBP2 functions. We also show that the SID and RBD, when expressed as separate proteins, are able to provide wild-type levels of Sec incorporation in vitro, thus indicating that these regions are independently folding domains. Together these data allowed us to redefine the domain boundaries of the SID (aa 399–535) and RBD (aa 615–777). SECIS binding studies indicated that the SID functions, in part, by modulating SECIS affinity. Further, in a novel interaction assay, we show that the RBD forms a stable complex with eEFSec.

EXPERIMENTAL PROCEDURES

Constructions—Penta-alanine mutant constructs were made by site-directed mutagenesis using the coding region corresponding to the C-terminal half of rat SBP2 (aa 399–846) in pCR3.1 (Invitrogen; for in vitro translated protein) or pTrcHis (Invitrogen; for recombinant protein) as a template. Constructs for N-terminally Xpress-His (XH)-tagged recombinant proteins were generated by TOPO-TA cloning the coding regions for rat and mouse eEFSec were made by TOPO-TA cloning the coding regions for rat and mouse eEFSec containing an additional 0.5 mM MgCl2, and 40 mM NaCl. Eighty percent of this reaction was layered onto a 20% sucrose cushion at 30,000 g for 45 min at 4 °C. Five percent of supernatant and pellet fractions was resolved by SDS-PAGE and quantitated by PhosphorImager analysis.

SECIS Probes and EMSA—Labeled wild-type and mutant GPX4 SECIS elements were transcribed as described (10). SECIS binding of in vitro translated proteins was assayed by incubating equal saturating amounts (16 fmol) with 20 fmol of wild-type or mutant (ΔAUGA) SECIS probes in 1× PBS containing an additional 0.5 mM MgCl2, and 40 mM NaCl. Eighty percent of this reaction was layered onto a 20% sucrose cushion containing the same salt concentrations plus 2 mM DTT and spun at 300,000 × g for 45 min at 4 °C. Five percent of supernatant and pellet fractions was resolved by SDS-PAGE and quantitated by PhosphorImager analysis.

In Vitro Translation—Plasmids encoding wild-type CT-SBP2 or penta-alanine mutants in pCR3.1 were linearized with XhoI and transcribed with T7 RNA polymerase using the mMessage kit (Applied Biosystems). mRNAs were translated in nuclease-treated rabbit reticulocyte lysate (RRL; Promega) in the presence of [35S]Met as directed by the manufacturer. Protein quantitation was performed by PhosphorImager analysis as described previously (10).

Sec Incorporation Assay—Sec incorporation assays with in vitro translated protein were performed by adding 2 μl of in vitro translated CT-SBP2 to a luciferase reporter translation as described (10). Sec incorporation assays with recombinant protein were 12.5–μl reactions containing 50 ng of luciferase reporter mRNA and the appropriate amount of 1.5 μM or 0.1 μM recombinant protein to achieve the concentrations indicated in the figures. All of the Sec incorporation reactions were incubated for 1 h at 30° and processed as previously described (10).

Sucrose Cushion Assay—Ribosome purification and ribosome binding of in vitro translated proteins was assayed as described (10). Briefly, in vitro translated proteins were incubated with 4 pmol of salt-washed rat testes ribosomes in 1× PBS containing an additional 0.5 mM MgCl2, and 40 mM NaCl. Eighty percent of this reaction was layered onto a 20% sucrose cushion containing the same salt concentrations plus 2 mM DTT and spun at 300,000 × g for 45 min at 4 °C. Five percent of supernatant and pellet fractions was resolved by SDS-PAGE and quantitated by PhosphorImager analysis.

Co-immunoprecipitation—[32P]-Labeled wild-type and mutant GPX4 SECIS elements used the same protocol as for EMA SECIS elements but were transcribed with the T7 Ribomax kit (Promega) supplemented with 1 μl of 3000 Ci/mmol [α-32P]UTP. SECIS elements were phenol:chloroform-extracted, ethanol-precipitated twice, resuspended in water, and quantitated by UV spectrophotometry. Equimolar amounts (40 pmol) of XH-tagged SID, FLAG-RBD, and SECIS elements as...
indicated in Fig. 5 were mixed in a final volume of 60 μl of PBS and incubated for 10 min at 30 °C. Following incubation, all of the reactions were diluted with 120 μl of PBS and 1 mM DTT and 40 μl of a 50% slurry of M2 α-FLAG beads (Sigma) in the same buffer and mixed for 1 h at 4 °C. Supernatants (50%) from SECIS-containing reactions were phenol:chloroform-extracted, precipitated, resolved on a 6 M urea 6% denaturing polyacrylamide gel, and visualized by PhosphorImaging. The pellets were washed four times with 1 ml PBS + 0.5% Tween. After the final wash all liquid was removed with a needle, and bound proteins were eluted in 30 μl of 1× SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.01% bromphenol blue). Prior to boiling the samples for SDS-PAGE, 1 μl from SECIS-containing reactions was used for liquid scintillation counting. Amounts equal to 7.5% of the supernatants and 50% of the pellets were resolved by SDS-PAGE followed by Western blotting against SBP2.

Formaldehyde Cross-linking—Messenger RNAs encoding CT-SBP2, SID, or RBD were in vitro translated in a 12.5-μl RRL reaction in the presence of [35S]Met as described above. After 30 min of translation at 30 °C, 1.4 μl of 2.5% formaldehyde (diluted in water) was added to the reactions followed by incubation at 30 °C for 10 min. The reactions were then supplemented with 14 μl of 4% SDS, 100 mM Tris, pH 6.8, and layered over a 178-μl cushion containing 20% sucrose, 2% SDS, and 50 mM Tris-HCl, pH 6.8, followed by a 30-min spin at 300,000 g at 18 °C in a Sorvall M20 ultracentrifuge. The supernatants were removed, and the pellets were resuspended in 1 μl SDS sample buffer. The pellet fraction (20%) and the supernatant fraction (5%) were resolved by SDS-PAGE, and the radioactive proteins were detected by PhosphorImaging. Band quantitation was performed using ImageQuant software (GE Healthcare).

RESULTS

**The Sec Incorporation Domain Possesses Two Regions Critical for Sec Insertion**—To identify the critical determinants within the rat SBP2 SID, we utilized a conservation-based approach to identify potentially important residues. Within blocks of high conservation, groups of five consecutive amino acids were changed to alanine in groups of five (boxed) are indicated above the alignment. The SBP2 arrow also indicates the stop and start positions of the recombinant SID and RBD, respectively.
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![Image](83x627 to 217x701)

FIGURE 3. Analysis of SECIS and ribosome binding for penta-alanine mutants. A, 16 fmol of [35S]Met labeled in vitro translated wild-type or mutant CT-SBP2, as indicated, was incubated with 32P-labeled wild-type GPX4 SECIS elements and resolved on 4% nondenaturing polyacrylamide gels (the last four mutants were evaluated in a separate experiment). B, [35S]Met-labeled in vitro translated CT-SBP2 was assayed for ribosome binding by pelleting complexes through a 20% sucrose cushion. Five percent of supernatants (S) and pellets (P) were resolved by SDS-PAGE (top panel) and visualized by PhosphorImaging. To control for nonspecific binding, the amount of pelleting observed for hnrNP F was subtracted and data were normalized to CT-SBP2. C, summary of Sec incorporation (black bars), ribosome binding (light gray bars), and SECIS binding (dark gray bars) data for the penta-alanine mutants expressed as the amount of each activity relative to that obtained with wild-type CT-SBP2 (means ± S.E., n = 3).

normalized first to the amount of mutant protein added and then to the amount of activity obtained with wild-type CT-SBP2. Although most of the mutant proteins retained significant levels of Sec incorporation activity, five of the fourteen penta-alanine mutations resulted in Sec incorporation defects below 20% of wild-type CT-SBP2 (Fig. 2B). These included 443LGGML447 in the N-terminal portion of the SID, which resulted in a reduction of Sec incorporation activity to ~18% of wild-type CT-SBP2. In the C-terminal portion, 504PLMKK508, 511QREIP515, 521TSLKK525, and 526IILKE530 had Sec incorporation activities of 5, 12.5, 4.5, and 16%, respectively. These results indicate that only a small subset of the conserved residues within the SBP2 SID are required for Sec incorporation activity and that the important residues lie mainly in the C-terminal region of this domain between residues 504 and 530.

The C Terminus of the SID Affects SECIS Binding—Previous deletion analysis implicated residues in the C-terminal portion of the SID in SECIS element binding (6). To determine the extent to which SECIS binding defects could account for the loss of Sec incorporation activity observed in Fig. 2, we screened CT-SBP2 mutants for defects in SECIS binding by incubating 16 pmol of in vitro translated wild-type or mutant proteins with 32P-labeled GPX4 SECIS elements and complexes resolved by EMSA. Although this method generated complexes that were more difficult to quantitate than those from recombinant proteins, it allowed us to rapidly screen the mutants for severe defects in SECIS binding and then perform detailed analysis of interesting mutants as recombinant proteins. None of the mutants N-terminal to 514KAKPK520 displayed a significant SECIS binding defect (Fig. 3A). At the C-terminal end of the SID, mutations 516KAKPK520, 521TSLKK525, and 526IILKE530 resulted in a nearly complete loss of detectable SECIS binding activity relative to wild-type CT-SBP2 (Fig. 3, A and C). None of the CT-SBP2 mutants were able to bind a mutant SECIS element in which the AUGA core motif is deleted (data not shown). These data show a clear correlation between SECIS binding and Sec incorporation for the residues downstream of Pro515. Interestingly, however, the nearly complete loss of Sec incorporation activity for the 504PLMKK508 and 514QREIP515 mutant proteins cannot be attributed to a concomitant loss in SECIS element binding.

SID Regions Affecting Sec Incorporation Activity and SECIS Binding Also Affect Ribosome Binding—To explore the potential role of ribosome binding defects in causing Sec incorporation defects in the penta-alanine mutant proteins, we analyzed their ability to bind ribosomes in a sucrose cushion assay where [35S]Met-labeled wild-type or mutant CT-SBP2 was incubated with excess salt-washed rat testis ribosomes, and protein-ribo-
some complexes were resolved by ultracentrifugation through a sucrose cushion. Supernatant and pellet fractions (5% of each) were resolved by SDS-PAGE. After normalizing to wild-type CT-SBP2 and subtracting the amount of pelleting observed with a nonspecific RNA-binding protein (hnRNP F), the mutants 516KAKKP520, 521-TSLKK525, and 526ILLKE530 showed 5, 0, and 0% pelleting relative to wild type, respectively (Fig. 3, B and C). These data establish a strong correlation between SECIS element binding and ribosome binding, but the mutations upstream of 516KAKKP520 do not allow a clear correlation to be made between Sec incorporation activity, SECIS element binding, and ribosome binding. A summary of these three parameters for all of the penta-alanine mutations is shown in Fig. 3C. Interestingly, 511QREIP515, which is greatly impaired for Sec incorporation but not SECIS binding, retained ~87% ribosome binding activity. Similarly, 504PLMKK508 retained over 60% ribosome binding activity, retained wild-type levels of SECIS element binding, but displayed a loss of over 90% of its Sec incorporation activity. In addition, 478KDASS482, 485RGRRS489, and 495PHNPL499, all had wild-type levels of SECIS binding and 80% ribosome binding but showed a decreasing trend in Sec incorporation. Further, mutants such as 473VPVLS477 had wild-type SECIS and ribosome binding but showed only 44% Sec incorporation. The mutation at 443LGGML447 was unique in that it displayed slightly higher than wild-type levels of ribosome binding and slightly lower levels of SECIS binding, resulting in an ~80% decrease in Sec incorporation activity. Together the analysis of these mutant proteins confirms the tight correlation between SECIS element binding and Sec incorporation activity and establishes the role of amino acids 504–515 in promoting Sec incorporation without contributing substantively to SECIS element binding or ribosome binding.

The SID and RBD Are Independently Folding Domains—Having identified residues within the SID that are required for Sec incorporation but not SECIS and ribosome binding, we hypothesized that the primary function of the SID is distinct from any of the known biochemical activities of SBP2. In addition, two lines of evidence suggest that the SID and RBD may form independent domains. First, the sequence alignments clearly show two regions of conservation with a ~70-amino acid spacer. Second, the RBD contains the entirety of a conserved L7Ae domain, which has been shown to bind SECIS RNA in the absence of the SID (6). To address this question directly, we subcloned the SID and RBD and expressed them as individual recombinant proteins with an N-terminal Xpress-His tag (XH-SID, aa 399–585; XH-RBD, aa 586–846) and tested their ability promote Sec incorporation in trans. We tested limiting (8 nM) or saturating (120 nM) amounts of either domain alone in rabbit reticulocyte lysate using the luciferase reporter described above. Individual domains were inactive (Fig. 4A, compare columns 2–5 with column 1). When combined, however, the domains were able to support Sec incorporation to 14% at 8 nM (versus 9% for limiting CT-SBP2) and 86% at 120 nM. To determine whether one of the domains could act as a transient activator of the other, we mixed limiting and saturating amounts of the SID and RBD in the Sec incorporation assay. When saturating levels of the SID were mixed with limiting amounts of RBD, Sec incorporation activity was ~30%. Similarly, when saturating levels of the RBD are mixed with limiting amounts of SID, Sec incorporation activity was 36%. Thus, when compared with the 14% activity obtained when both domains were limiting, a ~2-fold enhancement was observed particularly in the case when the RBD was saturating. We next sought to determine whether a similar enhancement could be obtained when saturating amounts of the each domain were mixed with a limiting amount of CT-SBP2. In this case, the RBD, but not the SID, was able to enhance the activity of limiting CT-SBP2 from 9% to 21% (Fig. 4A, column 12), suggesting that steric hindrance within the complete CT-SBP2 molecule prevents enhancement of Sec incorporation by the SID in trans (Fig. 4A, column 11). This finding allowed us to predict that two molecules of CT-SBP2, each lacking function in either the SID or RBD because of mutation, would be unable to promote Sec incorporation. To test this hypothesis, we combined 120 nM each of recombinant CT-SBP2 bearing a G669R mutation in the RBD together with CT-SBP2 bearing the 504PLMKK508 penta-alanine mutation. The G669R mutation has previously been shown to prevent SECIS element binding as well as Sec incor-

![Figure 4. The domains of CT-SBP2 function in trans.](image-url)
poration activity (7), and as shown above, the 504PLMKK508 mutation lacks Sec incorporation activity but retains SECIS binding activity. As shown in Fig. 4B, the mutant proteins are unable to complement each other and are not dominant negative when added to a reaction containing wild-type CT-SBP2, providing evidence that intact CT-SBP2 is unlikely to form a functional dimer. Together these data establish that SBP2 is comprised of two independently folding domains that when combined in stoichiometric amounts retain nearly full Sec incorporation activity.

### The SECIS Element Is Required for Interaction of the SID and RBD

The ability of the SID and RBD to function in trans suggests that they may form a stable complex. To test whether the SID and RBD physically interact, we determined whether the SID could be co-immunoprecipitated by the RBD. Recombinant (XH-tagged) wild-type or mutant SID was incubated in the presence or absence of recombinant FLAG-tagged RBD (FLAG-RBD). These reactions also contained either wild-type or H9004AUGA mutant 32P-trace-labeled GPX4 SECIS elements. Complexes were precipitated with M2 α-FLAG beads. As shown in Fig. 5, wild-type and mutant XH-SID alone did not cross-react with α-FLAG beads (lanes 1–4). Wild-type XH-SID was co-immunoprecipitated by FLAG-RBD in the presence of wild-type SECIS elements (lane 6), as were the penta-alanine XH-SID mutants 443LGGML447 and 504PLMKK508 (lanes 7 and 8, respectively). Interestingly, the XH-SID mutant 526IILKE530 could not be co-immunoprecipitated under any of the conditions tested (lanes 9 and 13), and none of the recombinant XH-SID, either wild-type or mutant, co-immunoprecipitated in the presence of the ΔAUGA mutant SECIS elements (lanes 10–13). SECIS RNA was purified from 50% of the supernatants and analyzed on 6% urea denaturing polyacrylamide gel to verify that the SECIS elements remained intact during the incubation periods (Fig. 5, bottom panel). These data establish that a physical SID-RBD interaction is mediated by SECIS binding, suggesting that a SECIS-induced conformational change is required to promote Sec incorporation.

### The SID Enhances SECIS Binding

Although the co-immunoprecipitation data show the formation of a SECIS-dependent SID-RBD complex, they do not address SECIS affinity for the SBP2 domains in trans. We therefore assessed the SECIS binding activity of the domains in trans by EMSA. As shown in Fig. 6A, there is a CT-SBP2 dose-dependent increase in shifted GPX4 SECIS elements with greater than 50% of the probe shifted at 100 nM CT-SBP2, consistent with the published dissociation constant of 94 nM (13). In contrast, no binding is seen with the ΔAUGA mutant SECIS at any concentration of CT-SBP2 (data not shown). Complex formation was not seen even at the highest concentration of SID. This, together with the fact that the SID cannot be UV-cross-linked to the GPX4 SECIS element (data not shown), suggests that SID alone may not

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**FIGURE 5.** The SECIS element promotes formation of a stable SID-RBD complex. Wild-type or penta-alanine mutant recombinant XH-SID (SID) was incubated with equimolar amounts of FLAG-RBD and 32P trace-labeled wild-type (wt) or mutant (mt) SECIS elements. The proteins in the supernatant (top panel) and pellet (middle panel) fractions were resolved by SDS-PAGE and detected by Western blot using an anti-SBP2 antibody. Fifty percent of the supernatants from SECIS containing reactions were removed, and RNA was extracted, precipitated, and resolved on a 6% denaturing gel (bottom panel). Lanes 4 and 5 of the bottom panel show the starting RNA.

**FIGURE 6.** The SID enhances SECIS affinity. A–D, 2-fold serial dilutions of the recombinant proteins (6–400 nM) were incubated with 32P-labeled wild-type GPX4 SECIS elements, and complexes were resolved on 4% nondenaturing polyacrylamide gels and visualized by PhosphorImaging. E–G, same as D but with the SID mutant proteins indicated, and lanes 9–11 contain 400 nM each CT-SBP2, RBD, and SID + RBD, respectively.
contact SECIS RNA (Fig. 6B). The affinity of the RBD for SECIS elements was dramatically lower than that of CT-SBP2 as evidenced by the lack of shifting until a concentration of 400 nM was reached (Fig. 6C, lane 8). Consistent with the wild-type levels of Sec incorporation obtained by combining the SID and RBD in trans, wild-type SECIS binding activity was also restored when the SID and RBD were incubated together (Fig. 6D). Interestingly, at the lower concentrations of the SID-RBD combination, the size of the shifted complex is smaller than that obtained with CT-SBP2, migrating at the same position as XH-RBD alone (Fig. 6, compare C, lane 8, with D, lane 5). This suggests that the SID is able to induce a SECIS binding conformation in the RBD, which may permit stable SECIS binding in the absence of a stable SID-RBD complex.

To determine whether high affinity SECIS binding by the RBD and SID-RBD-SECIS ternary complex formation represents distinct steps in forming active SBP2, we tested combinations of mutant XH-SID and wild-type XH-RBD for SECIS binding activity (Fig. 6, E–G). CT-SBP2, XH-RBD, and XH-SID + XH-RBD were included as controls (Fig. 6, E–G; lanes 9–11). As shown in Fig. 6D, wild-type XH-SID promoted high affinity SECIS binding (i.e. similar to CT-SBP2) and resulted in a ternary complex “supershift” at 100–400 nM of each domain (Fig. 6D, lanes 5–8). In contrast, XH-SID-526ILKE530 promoted high affinity SECIS binding by the XH-RBD, but did not promote the supershifted ternary complex even at the highest concentration (Fig. 6E, lane 8). Interestingly, XH-SID-504PLMKK508 was also able to promote high affinity SECIS binding by the RBD, but its ability to promote ternary complex formation was reduced such that a minimum of 400 nM of each domain was required (Fig. 6F, lane 8). In the case of XH-SID-443LGGML447, high affinity SECIS binding and ternary complex formation were observed (Fig. 6G). Interestingly, at 200 nM XH-SID-443LGGML447, two discrete complexes formed, whereas the same concentration of wild-type domains appeared to have only one complex (Fig. 6, compare G, lane 7, and D, lane 7). It is also worth noting that the shift associated with the wild-type SID-RBD-SECIS complex is notably more diffuse than that of either CT-SBP2 or RBD alone (Fig. 6, E–G, compare lane 11 with lanes 9 and 10), suggesting that the ternary complex contains multiple conformational states or unstable SID-RBD interaction. Interestingly, we found no correlation between ternary complex formation and Sec incorporation, indicating that a ternary complex of SID-RBD-SECIS appears to be necessary but not sufficient for Sec incorporation (data not shown), but activity does correlate with the production of the diffuse supershifted complex, suggesting that active CT-SBP2 is able to adopt multiple conformation states. Overall, these data indicate that the SID regulates the ability of the RBD to bind SECIS elements independently of its ability to form a stable SID-RBD-SECIS complex.

The SID-RBD Complex Does Not Stably Bind Ribosomes—The above EMSA studies demonstrated that the SID and RBD in trans are a powerful tool for assessing SBP2 function. We therefore tested in vitro translated SID and RBD for ribosome binding as described for the penta-alanine mutants in Fig. 3. Messenger RNAs corresponding to CT-SBP2, SID, and RBD were in vitro translated in the presence of [35S]Met and incubated with 4 pmol of rat testis ribosomes. SBP2-ribosome complexes were pelleted through a 20% sucrose cushion, and 5% of supernatant and pellet fractions were resolved by SDS-PAGE. As expected, ~80% of CT-SBP2 was in the pellet (Fig. 7, lanes 1 and 2). Surprisingly, neither the SID nor the RBD, either alone or combined, showed ribosome binding above the hnRNP F background (compare lanes 4, 12, and 14). Because this ribosome binding assay did not show stable binding by either the SID or RBD, we asked whether the ribosome binding of the separated domains is transient and difficult to observe under these conditions. Thus, we employed cross-linking to determine whether the SID and RBD could be trapped in transient SBP2-ribosome interactions. For this experiment, we performed formaldehyde cross-linking, which has been used previously to study the interaction of proteins with ribosomes (14, 15). Messenger RNAs encoding CT-SBP2, SID, RBD, luciferase, and hnRNP F were translated in the presence of [35S]Met. After translation, formaldehyde was added to 0.25% and incubated for 10 min. Ribosome bound proteins were pelleted by centrifugation through a 20% sucrose cushion containing 2% SDS to remove non-cross-linked proteins. Supernatant fractions (5%) and 20% of pellet fractions were resolved by SDS-PAGE. After subtracting background cross-linking observed for hnRNP F and luciferase, the ratio of in vitro translated protein in the pellet relative to that in the supernatant was calculated as 14% for CT-SBP2 (Fig. 7B). Background cross-linking to the ribosome was negligible as shown by a reaction lacking formaldehyde (Fig. 7B, lane 2). The penta-alanine mutant 526ILKE530 in CT-SBP2, which displayed a ~90% reduction in the ribosome binding assay describe above (Fig. 3), showed ~50% reduction in cross-linking in this assay (see Fig. 7B, compare lanes 1 and 3). Wild-type SID was also cross-linked to the ribosome at ~4%, and 526ILKE530 SID showed a slight increase to 6% (Fig. 7B, lanes 4 and 5, respectively). In contrast, The RBD alone showed only ~1% cross-linking (Fig. 7B, lane 6). The extent of cross-linking for the domains was not altered when the SID and RBD were combined (Fig. 7B, lanes 7 and 8). These data indicate that a transient ribosome interaction can be stabilized by formaldehyde cross-linking and that the stable ribosome binding observed in Fig. 3 may not be essential for Sec incorporation in vitro, thus suggesting that that the SID likely functions directly on the ribosome in a fashion that is biochemically distinguishable from the stable ribosome binding observed for CT-SBP2. In addition, the fact the 526ILKE530 mutant is defective for ribosome binding in the context of CT-SPB2 but slightly more active than wild-type in the context of the domains in trans is consistent with that same observation regarding SECIS binding for this mutation.

eEFSec Stably Interacts with the SBP2 RBD—We have generated mutants in both the SID and RBD that retain SECIS and ribosome binding yet lack Sec incorporation activity (see Fig. 3 and Ref. 10). One of the possible functions for these regions within CT-SBP2 is an interaction with the Sec-specific elongation factor eEFsec. As mentioned above, eEFsec cannot be co-immunoprecipitated with SBP2 in reticulocyte lysate (4), and it has been reported that they do not form a supershifted complex when combined in mobility shift assays (16). We chose to investigate this potential interaction further by determining whether
eEFSec would form a stable complex with either the SECIS element or a SECIS-SBP2 complex in our assays. Fig. 8 shows an EMSA where 8 pmol of wild-type or mutant XH-CT-SBP2 was incubated with 32P-labeled GPX4 SECIS elements either in the absence or presence of eEFSec. In each case the lane labeled “probe” contains the wild-type 32P-labeled GPX4 SECIS element alone.

**FIGURE 7.** The SID interacts transiently with the ribosome. A, [35S]Met-labeled in vitro translated proteins as indicated were assayed for ribosome binding as described for Fig. 3. The asterisk denotes a band that likely corresponds to the product of internal translation initiation at Met 629. B, [35S]Met-labeled in vitro translated proteins as indicated were cross-linked to the ribosome in 0.25% formaldehyde and spun through a 20% sucrose cushion containing 2% SDS. Amounts equal to 5% of supernatants and 20% of pellets were resolved by SDS-PAGE, visualized by PhosphorImaging, and quantitated using ImageQuant software as described for ribosome binding assays in Figs. 2 and 5.

**FIGURE 8.** eEFSec forms a stable complex with SBP2. A, 32P-labeled wild-type GPX4 SECIS elements were incubated with 8 pmol each of wild-type or mutant XH-CT-SBP2, FLAG-eEFSec, ovalbumin, or yeast eEF1A as indicated. B, same as in A except using 8 pmol each of XH-RBD, wild-type, or mutant XH-SID and FLAG-eEFSec as indicated. C, same as in A and B except using 8 pmol of wild-type or mutant XH-SID in the presence of increasing amounts of FLAG-eEFSec as indicated. In each case the lane labeled “probe” contains the wild-type 32P-labeled GPX4 SECIS element alone.
the presence or the absence of 8 pmol of FLAG-tagged recombinant eEFSec. FLAG-eEFSec alone shifts only a small amount of the GPX4 probe, consistent with previous results that have revealed weak, nonspecific SECIS binding activity for eEFSec (lane 3) (17). However, the addition of FLAG-eEFSec to the reactions containing wild-type CT-SBP2 resulted in a quantitative supershift (Fig. 8A, compare lanes 2 and 6). This supershift was not observed with the same amount of either ovalbumin or eEF1A (lanes 7–9). When eEFSec was added to reactions containing the XH-CT-SBP2 mutants 443LGGML447 and 504PLMKK508, a supershift was still observed (lanes 16 and 17). No shift was observed for the 526IILKE530 mutant, confirming that SECIS binding is necessary to observe the shift in this assay (lane 18).

To determine how eEFSec might interact with the SBP2 domains in trans, we assessed the ability of eEFSec to cause a supershift in the presence of XH-SID and/or XH-RBD. Fig. 8B shows that although XH-SID alone does not shift the GPX4 probe, it appears to enhance the ability of FLAG-eEFSec to bind the SECIS element (compare lanes 2 and 8 with lane 5). In the case of both XH-RBD and XH-RBD plus XH-SID, FLAG-eEFSec causes a quantitative supershift (compare lanes 3 and 4 with lanes 6 and 7). Similar to the test of SID mutations within CT-SBP2 shown in Fig. 8A, we also tested the effect of using Sec incorporation-defective mutants versions of XH-SID on the eEFSec supershift. Fig. 8B (lanes 9–15) shows a GPX4 EMSA of wild-type and mutant XH-SID combined with XH-RBD in the presence or absence of FLAG-eEFSec. In each case, eEFSec is able to cause a quantitative supershift, suggesting that mutations in the SID do not directly affect eEFSec recruitment. Notably, the XH-SID-526IILKE530-RBD-eEFSec complex is indistinguishable from the XH-RBD-eEFSec complex (Fig. 8B, lanes 6 and 16), indicating that the RBD is the primary target for an eEFSec interaction. To further analyze the role of the SID in apparently promoting eEFSec SECIS element binding, we tested a range of FLAG-eEFSec concentrations in a GPX4 EMSA containing wild-type XH-SID or XH-SID-526IILKE530. Fig. 8C shows that with increasing concentrations of eEFSec, a shifted complex gradually increases in size, pointing to the assembly of a multi-component complex as a function of eEFSec concentration. This is also observed with the XH-SID-526IILKE530 mutant protein, but the amount of shifting is substantially reduced, suggesting a role for these residues in stabilizing the overall SBP2-eEFSec complex. Interestingly, we and others have thus far been unable to observe an SBP2-eEFSec interaction by co-immunoprecipitation of in vitro assembled complexes (data not shown) (4). As such, we cannot definitively determine whether the SBP2-eEFSec interaction is SECIS element-dependent but stained EMSA gels do not show quantitative complex formation, suggesting that only the probe-bound proteins are present in the supershifted complex (data not shown). Together these data describe an assay that reveals a stable interaction between eEFSec and SBP2 that appears to be SECIS element-dependent.

**DISCUSSION**

Selenocysteine incorporation into a growing polypeptide presents a unique challenge to the protein synthetic machinery because it must recode UGA from a termination signal to a Sec codon. In eukaryotes three components are known to be required: the SECIS element, eEFSec ternary complex (eEFSec-Sec-tRNA^{Sec}-GTP), and SBP2. Although the role of eEFSec in Sec-tRNA^{Sec} delivery is likely straightforward, the mechanism by which SBP2 and the SECIS element promote Sec incorporation is unclear. In this work we have functionally defined two
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independently folding domains within SBP2 that are required for Sec incorporation: a central SID and a C-terminal L7Ae RBD. To decipher the mechanism of SBP2 function, we have identified the key residues within the SID that are essential for promoting Sec incorporation but not SECIS binding or ribosome binding, thus uncovering a novel function for SBP2 proximal to the Sec incorporation event.

SECIS Binding—Work from several groups has characterized the SBP2 RNA-binding domain and its interaction with the SECIS element (8, 10, 9, 13). Here we used scanning mutagenesis to identify the residues within the SID that are required for any of the three known functions ascribed to SBP2: SECIS binding, ribosome binding, and Sec incorporation. With regard to SECIS binding, we have found that any mutation N-terminal to 516KAKKP520 has no significant effect on SECIS binding activity. This is consistent with the truncation data that originally placed the N terminus of the fragment required for SECIS binding between residues 517 and 535 (6) and more recent refinement to between residues 520 and 535 (18). The latter study termed the SBP2 RBD as bipartite, but based on the data presented here, we propose to redefine the SBP2 RBD as beginning at position 615, because the 586–846 fragment is sufficient for wild-type levels of GPX4 SECIS element binding by virtue of the fact that the XH-SID524ILKE530 mutant was able to induce high affinity SECIS element binding in the RBD without forming a stable SID-RBD-SECIS ternary complex. Note that this boundary definition does not include the nonconserved region (aa 536–614) because it has been shown to be dispensable for Sec incorporation activity (18). This indicates that one of the roles of the SID is to promote a conformation within the RBD that allows high affinity SECIS binding. In addition, phylogenetic analysis clearly demarcates the two domains within CT-SBP2, and the ability to perform Sec incorporation complementation with the two domains in trans lends biochemical support for our domain definition.

Phylogenetic analysis also indicates that the SBP2 SID is conserved throughout eukaryotic evolution, except for fungi and higher plants, which lack selenoproteins. Interestingly the C-terminal portion (equivalent to rat SBP2 residues 508–530) appears to be more highly conserved than sequences in the N-terminal portion (such as rat 438PVQLD442 and 443LGGML447), which are present in vertebrate SBP2 but absent from SBP2 of lower eukaryotes. It is possible that these more recent N-terminal regions of the SID could add another layer of regulation to SBP2 that is not required in early organisms and may explain why the mutations in much of the SID resulted in only modest reductions in Sec incorporation in vitro.

The EMSA data shown in Fig. 6 suggest that the 504PLMKK508 residues are required to achieve maximal SECIS affinity, whereas the nearby 526ILKE530 residues are required for a downstream step that involves SID-RBD complex formation, which in the context of wild-type CT-SBP2 likely reflects a substantial conformation shift upon SECIS element binding. Interestingly, the 526ILKE530 and 504PLMKK508 mutations in the context of CT-SBP2 produce the opposite result where the former shows a significant SECIS binding defect, whereas the latter has none. This suggests that when the SID is physically attached to the RBD, steric constraints regulate SECIS element binding in a fashion that is not observable when the domains are tested in trans. The loss of SECIS binding when 526ILKE530 is mutated in CT-SBP2 specifically suggests that the role of 504PLMKK508 in inducing a high affinity conformation within the RBD is somehow blocked in a dominant fashion when 526ILKE530 is mutated. The appearance of the shifted complex is also of interest because a wild-type SID-RBD-SECIS complex does not form a tight band, whereas SID504PLMKK508 and SID443LGGML447 do, indicating a distinct difference in conformation or a fundamental instability associated with a wild-type ternary complex. This assay may thus reveal a distinct step downstream of SECIS element binding that is required for Sec incorporation.

Ribosome Binding—Previously, we examined the interaction of SBP2 with the ribosome and hypothesized that ribosome-bound SBP2 confers a conformational change that allows eEFSec to deliver the Sec-tRNAsec (10). That work also demonstrated that the ribosome interaction was mediated by the RNA-binding domain, which contains a core L7Ae region required for ribosome and SECIS binding as well as an auxiliary domain required for ribosome binding that correlated more with SEC incorporation activity than SECIS binding. Based on having isolated Sec incorporation-defective mutants that had reduced ribosome binding but wild-type levels of SECIS binding, we concluded that ribosome binding was likely required for Sec incorporation. In the present work, we have found that mutations in any of the residues between 516 and 530 results in a dramatic loss of ribosome binding activity. These defects are also correlated with a loss of SECIS binding. Although some of the mutations upstream of Lys516 showed slight ribosome binding defects, they cannot be clearly linked to Sec incorporation activity. Indeed, the region spanning residues 504–515 display little or no ribosome binding defects and yet display sharply reduced Sec incorporation activity, suggesting a novel function for these residues, similar to that observed for 648FQ649 in the RBD (10).

The most surprising result with regard to ribosome binding is that when assessed as domains in trans, the SID and RBD, despite being active in Sec incorporation, do not display stable ribosome binding. Based on this result we can conclude that stable ribosome binding is not absolutely required for Sec incorporation in vitro. There are two likely explanations for this apparent discrepancy: 1) the conformation of intact CT-SBP2 is such that stable ribosome binding is required, whereas the domains in trans are not restrained, and/or 2) stable ribosome binding may be required for in vivo Sec incorporation when there is significant competition among translationally competent mRNAs. Future cell-based work will be required to substantiate or refute these hypotheses. Despite the caveat that the domains in trans do not stably bind the ribosome, a ribosome-based function for SBP2 remains an attractive model for Sec incorporation. To analyze potential transient interactions, we employed formaldehyde cross-linking to trap ribosome bound intermediates. Under the conditions used, we were able to cross-link CT-SBP2 and the SID to ribosomes to a significantly greater extent than RBD and the control proteins, hnRNP F and luciferase. Not surprisingly, CT-SBP2 was more efficiently
cross-linked to ribosomes than the SID alone, likely reflecting the effect of its stable ribosome binding capacity. Although SID cross-linking was about 70% less than that of CT, it was well above the background level, suggesting that this assay may be trapping a functional but transient interaction between the SID and the ribosome. Interestingly, this ribosome cross-linking activity was increased when SID-526IILE530 was used, suggesting that this region is not directly involved in ribosome contacts but may be required to adopt a specific conformation for a functional ribosome interaction.

eEFSec—Here we provide the first evidence that SBP2 and eEFSec form a stable complex in vitro. Previous work has clearly shown SBP2 and eEFSec co-immunoprecipitation from transiently transfected cells, but a similar experiment in reticulocyte lysate showed no interaction (4). This discrepancy is further convoluted by the fact that previous studies have failed to reveal a stable interaction by EMSA (16). One of the major differences in the assay described here is the use of FLAG-tagged eEFSec instead of the His-tagged version used by Lescurie et al. (16). In addition, translation elongation factors are also notoriously unstable (19, 20), and it may be that scrutiny of the details of how the proteins were handled would reveal a critical difference. In the conditions used here, we have demonstrated a stable eEFSec-SBP2 complex that appears to be SECIS element-dependent. This interaction is driven by the RBD, and the three SID mutants that are defective in Sec incorporation show no impairment in eEFSec binding. Interestingly, however, the SID does apparently stimulate eEFSec SECIS binding and may form a SID-SECIS-eEFSec complex, which is less efficiently formed when 526IILE530 is mutated. This result suggests that the SID may function downstream of the initial eEFSec-RBD interaction, thus further implicating the SID in a ribosome-proximal role. These data, combined with our previous finding that SECIS elements effectively prevent CT-SBP2 ribosome-binding, suggest that SECIS element binding triggers a switch from ribosome bound RBD to eEFSec-bound RBD. The inclusion of this hypothesis into our model of Sec incorporation is discussed below.

A Model for Sec Incorporation—Taken together, the data presented here suggest that the SID likely plays a role both in SECIS binding and a subsequent step as evidenced by the identification of mutations that affect Sec incorporation but not SECIS binding or ribosome binding (i.e. 511QREIP515). Diamond et al. (21) demonstrated that the addition of 5 μg of Ser-tRNA Sec can suppress a UGA codon in β-globin mRNA translated in rabbit reticulocyte lysate. Because Ser-tRNA Sec cannot bind eEFSec (2, 17), it is likely that in the case of β-globin UGA suppression, Ser-tRNA Sec was bound by eEF1A. These results indicate that the inherent structure of the tRNA Sec itself does not preclude binding at the ribosomal A site and that Sec codon specificity is driven primarily by eEFSec. The data in this report suggest that the SID may serve as a two-way switch that first induces high affinity SECIS binding by the RBD, which then transmits a signal back to the region of 526IILE530 that modifies eEFSec, the ribosome, or both. Based on this we propose a model in which SBP2 is bound to the ribosome and upon encountering a UGA codon in a selenoprotein mRNA, the SECIS element makes a low affinity interaction with the RBD that is stabilized by the SID, which in turn promotes eEFSec recruitment (Fig. 9). Based on the transient interaction of the SID with the ribosome, it is tempting to speculate that the SID additionally increases affinity of the factor-binding site for eEFSec (or vice versa) or affects GTP hydrolysis.

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