RESEARCH PAPER

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Characterization of the UGA-recoding and SECIS-binding activities of SECIS-binding protein 2

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Abbreviations: Sec, selenocysteine; SECIS, Selenocysteine Insertion Sequence; SBP2, SECIS binding protein 2; RRL, rabbit reticulocyte lysate; PHGPx, phospholipid hydroperoxide glutathione peroxidase; GPx1, glutathione peroxidase 1; Dio1, deiodinase 1; Dio2, deiodinase 2; REMSA, RNA electrophoretic mobility shift assay; DTT, dithiothreitol.

Selenium, a micronutrient, is primarily incorporated into human physiology as selenocysteine (Sec). The 25 Sec-containing proteins in humans are known as selenoproteins. Their synthesis depends on the translational recoding of the UGA stop codon to allow Sec insertion. This requires a stem-loop structure in the 3' untranslated region of eukaryotic mRNAs known as the Selenocysteine Insertion Sequence (SECIS). The SECIS is recognized by SECIS-binding protein 2 (SBP2) and this RNA:protein interaction is essential for UGA recoding to occur. Genetic mutations cause SBP2 deficiency in humans, resulting in a broad set of symptoms due to differential effects on individual selenoproteins. Progress on understanding the different phenotypes requires developing robust tools to investigate SBP2 structure and function. In this study we demonstrate that SBP2 protein produced by in vitro translation discriminates among SECIS elements in a competitive UGA recoding assay and has a much higher specific activity than bacterially expressed protein. We also show that a purified recombinant protein encompassing amino acids 517-777 of SBP2 binds to SECIS elements with high affinity and selectivity. The affinity of the SBP2:SECIS interaction correlated with the ability of a SECIS to compete for UGA recoding activity in vitro. The identification of a 250 amino acid sequence that mediates specific, selective SECIS-binding will facilitate future structural studies of the SBP2:SECIS complex. Finally, we identify an evolutionarily conserved core cysteine signature in SBP2 sequences from the vertebrate lineage. Mutation of multiple, but not single, cysteines impaired SECIS-binding but did not affect protein localization in cells.

Introduction

Selenium is an essential dietary micronutrient that contributes to several different cellular functions. As a result, selenium has been shown to participate in multiple aspects of human health including inflammation,[1] cardiovascular disease,[2] male infertility,[3] and muscle myopathies.[4] The biological effects of selenium are predominantly mediated through its use in the amino acid selenocysteine (Sec). Members of the protein family that contain selenocysteine are known as selenoproteins, of which there are 25 in humans.[5] While frank selenium deficiency is rare, even mild insufficiency can perturb the expression of the selenoproteome. Selenoproteins display differential sensitivity to selenium levels in the cell, with expression of some family members being preserved at the expense of others.[6] This hierarchy of expression differs among tissues in vivo and it is apparent that the cell must be able to distinguish between the various selenoprotein mRNAs. However, the mechanism(s) that controls the hierarchy remains poorly understood.

In order to accommodate selenocysteine insertion, multiple specialized alterations to the translational process must be made at the molecular level. The first challenge to selenoprotein expression is that selenocysteine is encoded by the UGA stop codon and therefore requires a translational recoding event to synthesize the protein. This also appears to place selenoprotein synthesis in competition with translation termination. The UGA recoding is strictly dependent on an RNA stem-loop structure, the Selenocysteine Insertion Sequence (SECIS), which is found in the 3' untranslated region of eukaryotic selenoprotein messages.[7] It also requires a specific elongation factor (EFSec),[8,9] a Sec-tRNA $^{(Sec)}$ that is unusual both in its structure and synthesis,[10,11] and SECIS-binding protein 2 (SBP2), an RNA-binding protein that mediates interactions of the selenocysteine insertion machinery with the selenoprotein mRNAs.[12,13] Several other proteins involved in tRNA synthesis, selenocysteine recycling and regulation of recoding efficiency have also been identified, reviewed in.[14,15] Thus, a complex cellular network is required to maintain the ability to incorporate selenocysteine into proteins.

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Within this network, SBP2 is recognized as the master regulator of selenoprotein mRNA translation. The interaction between the SECIS element and SBP2 is absolutely critical for selenocysteine incorporation and mutations of either the RNA or protein can have pathological implications. A point mutation in the SECIS of Selenoprotein N (SepN1) mRNA inhibits SBP2 binding, causing the loss of SepN1 expression that results in a muscle myopathy.[16] Over the past several years, the presence of SBP2 mutations in several different families has been detected.[17-21] In general, affected patients usually have a combination of heterozygous mutations that are not completely functionally null, resulting in a partial SBP2 deficiency. This is not surprising given that the mouse knockout model of SBP2 is embryonically lethal,[22] as are several selenoprotein knockouts [23,24] supporting the essential nature of some selenoproteins. The most common clinical phenotype is an unusual thyroid hormone profile, as the deiodinase proteins, which are integral to thyroid hormone signaling, are selenoproteins. However, patients can display a complex phenotype with a broad range of symptoms of varying severity depending on the nature of the mutation and its location within SBP2.

In humans, the SBP2 protein is 854 amino acids in length. All known functions of SBP2 can be ascribed to the C-terminal half of the protein (SBP2-CT). This includes the selenocysteine insertion, ribosome interaction and SECIS-binding activities.[25] Across the protein only a single conserved region of homology is identifiable, which corresponds to the L7Ae family of RNA-binding proteins.[25] The RNA-binding domain of SBP2 is a conserved bipartite motif that encompasses the L7Ae region, but also extends to include additional sequences.[26,27] Disruption of SECIS-binding activity can negatively impact human health. We previously investigated the effect of a naturally occurring patient mutation within the RNA-binding domain and found that it impaired SECIS-binding activity.[26] Amino-terminal to the RNA-binding domain is an additional region that is required for UGA recoding. The precise mechanism of selenocysteine insertion, and the molecular interactions mediated by this region are still unclear, however this region is strictly required for selenoprotein synthesis. SBP2 is also a nucleo-cytoplasmic shuttling protein, and several import and export signals have been predicted across the protein,[12,28,29] a subset of which been functionally validated.[28,29] SBP2-CT is capable of shuttling,[28] suggesting signals in this region are sufficient to preserve the import and export activities. Currently, the role of the N-terminal region of the SBP2 is an area that remains largely unexplored.

Other than the L7Ae region, SBP2 demonstrates little homology to other proteins but does contain several short conserved motifs distributed across the length of protein.[30] The role of these motifs is unknown, although they distinguish SBP2 from its paralogue SBP2L.[30] The presence of several conserved regions outside of the RNA binding domain, as well as the discovery of multiple different patient mutations in SBP2 that impair selenoprotein synthesis provides a broad landscape that should inform our understanding of SBP2 structure and function. A mutation in a single allele is not generally sufficient to cause clinical phenotypes.[16-21] The patients generally have different mutations on each of their SBP2 alleles such that they are compound heterozygous at their SBP2 locus. The presence of 2 independently acquired mutations confounds the analysis of the SBP2 defects in these patients. The contribution of each allele to the overall deficit in SBP2 function needs to be individually examined to understand the mechanistic defect in the mutant protein and as well as to understand their relative impacts on patient health.

In order to lay the framework for studies into mechanistic effects of SBP2 mutations found in patients, we wished to establish the most effective approach for analyzing the mutant proteins. Both bacterial expression and in vitro translation methods have been previously used to study SBP2-CT, but a direct comparison between the 2 approaches has not been done.

In this study, we performed a comparative analysis between SBP2-CT produced by cell-free expression using rabbit reticulocyte lysate (RRL) and bacterially expressed recombinant SBP2-CT. We demonstrate that the in vitro translated SBP2-CT has a higher specific activity than recombinant SBP2-CT, which will inform future experimental design. Previously, we showed that the RNA-binding domain of SBP2 produced by in vitro translation had the ability to discriminate between SECIS elements, a property known as selectivity.[26] The influence of other proteins in the in vitro translation reaction on the binding affinity or selectivity could not be excluded. In here, we show that a bacterially expressed protein comprised of amino acids 517-777 of the rat SBP2 are sufficient to bind a SECIS element with high affinity, and to discriminate between SECIS elements. Currently, the mechanism by which this occurs is not understood and any information derived from a successful structural analysis would significantly advance our understanding of selenoprotein synthesis. Our result that the RNA-binding domain is sufficient for a high-affinity, selective interaction with the SECIS also lays the groundwork for future structural studies on the SBP2:SECIS interaction. Finally, we explored the contribution of the cysteine-rich region to the RNA-binding activity of SBP2. Initial bioinformatic analysis in vertebrates revealed the presence of 5 highly conserved cysteine residues, which we define as the vertebrate cysteine signature. We examined the RNA-binding activity of proteins with individual or multiple cysteine to serine mutations across this motif. While this region is resilient to individual mutations, multiple mutations impact the ability of protein to bind to the SECIS. Thus, our results show that the evolutionarily conserved cysteine signature contributes to the RNA-binding activity of SBP2.

Results

In vitro translated SBP2-CT is more active than recombinant SBP2-CT

Many laboratories, including ours, have used the SBP2-CT (amino acids 399-846 in the rat protein) for functional assays.[25,27,31,32] SBP2-CT is sufficient to support selenocysteine incorporation in transfected cells and cell-free translation systems. Both in vitro translation and bacterial expression systems have been used to generate SBP2-CT for mechanistic studies. However, the potential influence of the production method on the functional activities of SBP2 has not been examined and differences in experimental approaches across studies do not allow

for direct comparisons to be made. Therefore, we concurrently assessed the UGA recoding activity of both bacterially expressed and in vitro translated rat SBP2-CT proteins.

The Sec insertion activity of these proteins was analyzed using a luciferase reporter assay that detects translational readthrough of the UGA/Sec codon. The reporter construct contains a UGA at position 258 in the luciferase coding region and the Phospholipid Hydroperoxide Glutathione Peroxidase (PHGPx) SECIS element in the 3' UTR (luc/UGA/PHGPx) (**Fig. 1A**). The luciferase reporter RNAs were translated in RRL, which contains very little SBP2. Thus UGA recoding, and luciferase activity are stimulated by the addition of exogenous SBP2. This assay, which is widely used in the field, has been validated to be specific for selenocysteine incorporation.[33,34]

As shown in **Figure 1B**, maximal luciferase activity was obtained using 80 nM purified bacterially expressed SBP2-CT, which is comparable to reports in the literature that range from 40-300 nM.[35-39] In contrast, 0.4 nM in vitro translated SBP2-CT generates an equivalent signal to 40 nM of recombinant protein (compare Figs. 1B and C), suggesting that the in vitro translated protein is significantly more active than the bacterially expressed protein.

In addition to measuring luciferase activity, we performed UGA recoding assays in the presence of ^{35}S-methionine to visualize the translation products by SDS-PAGE and autoradiography (**Fig. 1D**). In the absence of exogenous SBP2, only the truncated luciferase was detected due to termination at UGA258. In agreement with the enzymatic assay, the full-length luciferase protein was detected when 40 nM bacterially expressed SBP2-CT was added to the reaction. A comparable signal was obtained with 0.4 nM of SBP2-CT translation product. Taken together, the data suggest that the specific activity of the SBP2-CT translation product is approximately 50 to 100-fold higher than the recombinant protein.

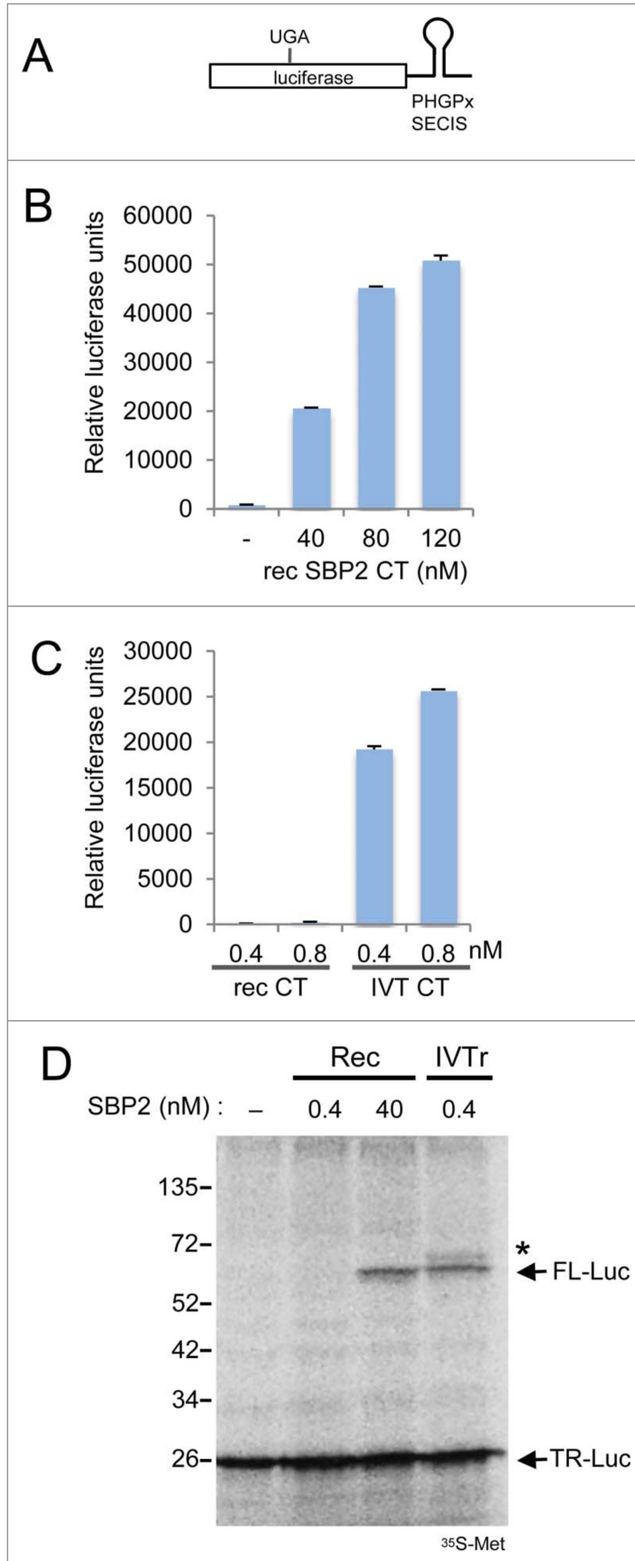

Figure 1. In vitro translated SBP2-CT is more active than recombinant protein. (**A**) Schematic diagram of the luc/UGA/PHGPx reporter construct. (**B**) The reporter mRNA was in vitro translated in the presence of increasing amounts of purified recombinant SBP2-CT as indicated. Translation products were analyzed for luciferase activity. The error bars represent one standard deviation. (**C**) The luc/UGA/PHGPx reporter RNA was in vitro translated in the presence of either purified recombinant SBP2-CT or in vitro translated SBP2-CT, as indicated. Translation products were analyzed for luciferase activity. The error bars represent one standard deviation. (**D**) The luc/UGA/PHGPx reporter was in vitro translated in the presence of either purified recombinant SBP2-CT or in vitro translated SBP2-CT, in the presence of ^{35}S-methionine. Two microliters of the translation products were analyzed by SDS-PAGE. TR-Luc, truncated luciferase; FL-luc, full-length luciferase; *, radio-labeled in vitro translated SBP2-CT.

In vitro translated SBP2-CT discriminates among SECIS elements in a competitive UGA recoding assay

In the standard UGA recoding assay described above and in,[33] the RRL is primed with a single type of SECIS-containing RNA. We previously developed a competitive assay, which more closely mimics the cellular environment where multiple selenoprotein mRNAs compete for the limiting UGA recoding machinery.[26] In this assay, the luciferase reporter RNA containing the Dio1 SECIS element (luc/UGA/Dio1) is translated in RRL in the presence of in vitro translated SBP2-CT and different SECIS RNAs, which act as decoys. Previously, the activity of SBP2-CT was

completely inhibited by 0.5 pmoles of SECIS RNA, which was the lowest amount that we tested. We have now modified the conditions by decreasing the amount of SECIS competitor RNAs (0.025 to 0.4 pmoles) to be within the linear range for the decoy effect. As shown in **Figure 2**, the PHGPx SECIS was a better competitor than the Deiodinase 2 (Dio2) and Glutathione Peroxidase 1 (GPx1) SECIS RNAs. Luciferase activity was inhibited by more than 80% when the translation assays were performed in the presence of 0.2 pmoles of the PHGPx SECIS whereas a comparable reduction required 0.4 pmoles of the Dio2 SECIS. The GPx1 SECIS was a much less effective decoy since 0.4 pmoles caused only a 60% reduction in activity. This effect was specific for UGA recoding, as the same SECIS RNAs did not inhibit translation of a control luciferase RNA (data not shown). Thus, the SBP2-CT translation product can discriminate among SECIS elements in vitro.

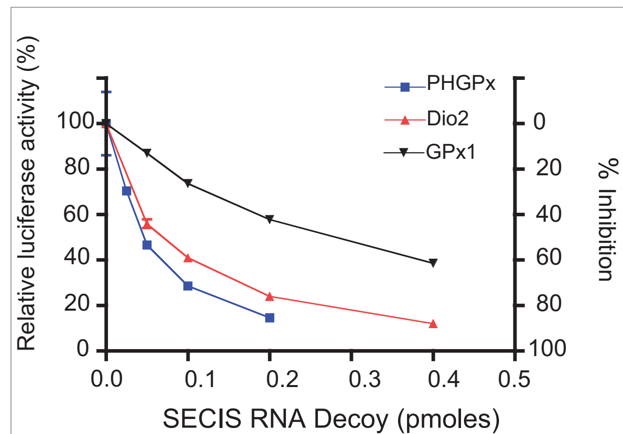

Figure 2. SBP2-CT is selective for different SECIS elements. The UGA recoding assays were performed in RRL using luc/UGA/Dio1 reporter in the presence of in vitro translated SBP2-CT, with PHGPx, Dio2 and GPx1 SECIS elements added as competitor RNAs, as indicated. Translation products were analyzed for luciferase activity. The error bars represent one standard deviation.

Purified SBP2-RBD is specific and selective in the absence of additional factors

Previously, we defined the RNA-binding domain of rat SBP2 as encompassing amino acids 517-777 (SBP2-RBD).[26] The in vitro translated SBP2-RBD was capable of discriminating between SECIS elements in an RNA electrophoretic mobility shift assay (REMSA). However, we could not exclude the possibility that other proteins in the RRL were facilitating the SECIS binding activity (specificity) and aiding in the differentiation among SECIS elements (selectivity). Indeed, it has been proposed that the SBP2: SECIS interaction may be stabilized by other factors present in RRL.[35] We decided to test whether bacterially expressed recombinant SBP2-RBD was capable of specifically and selectively interacting with SECIS elements in the same manner as the in vitro translated RBD. The SBP2-RBD was expressed as a His-tagged protein in bacteria, purified by nickel affinity chromatography, and analyzed for SECIS-binding activity by REMSA.

In our previous study with SBP2-RBD, we showed that the in vitro translated protein was specific for SECIS elements and did not interact with a mutant SECIS element. However, we could not determine an accurate K_D since the translation product was not pure as it contained other proteins from the RRL. In order to determine whether amino acids 517-777 are sufficient to mediate high affinity SECIS-binding, we tested the dose-dependent binding activity of recombinant SBP2-RBD to the ^{32}P-labeled PHGPx SECIS in a REMSA (**Fig. 3A**). Based on quantification of the signals corresponding to the free and bound probe, the protein binds with an apparent K_D of 17.2 +/− 2.1 nM (**Fig. 3B**).

In order to assess the specificity of binding, we performed REMSA competition experiments in which either wildtype or mutant PHGPx SECIS RNAs were added to the reaction in excess. Binding of purified SBP2-RBD to the ^{32}P-labeled probe was efficiently competed by the cold wildtype SECIS whereas a mutant SECIS that has a mutation in the SECIS core did not compete even at 160-fold molar excess (**Fig. 3C**). Thus the SBP2-RBD:SECIS interaction is specific, even in the absence of additional cellular factors.

In order to analyze the ability of the RBD to discriminate between SECIS elements, we tested the relative ability of the SECIS elements from Dio2 and GPx1 to compete for SBP2-RBD binding. As shown in **Figure 3C**, the recombinant SBP2-RBD also showed selective SECIS-binding activity in the competition experiments. A 5-fold molar excess of the PHGPx SECIS reduced binding by 50% whereas the SECIS elements of Dio2 and GPx1 required 40-fold and 320-fold molar excess, respectively, for a comparable reduction. Thus the purified SBP2-RBD has a 60-fold higher affinity for the PHGPx SECIS than the GPx1 SECIS, which is comparable to what we observed with the *in vitro* translated SBP2-RBD.[26] Given the good agreement of these results, the ability to discriminate between SECIS elements is a property intrinsic to the RNA-binding domain and is not significantly influenced by other factors in the RRL.

Conserved cysteine residues in SBP2 are not individually required for RNA-binding

Generation of multiple recombinant proteins can be very time-consuming and technically challenging depending on the protein in question. The above studies demonstrate the in vitro translated SBP2-RBD is specific and selective and thus, represents a versatile system in which to study relationships between protein structure and function. The ease of generating multiple mutant proteins in the cell free translation system encouraged us to investigate the importance of cysteine residues in the RNA-binding domain of SBP2. The SECIS-binding activity of SBP2-CT was previously shown to be sensitive to reducing conditions.[40] In the complete absence of DTT, little or no binding activity was observed. Pretreatment with oxidizing reagents diamide or N-ethylmaleimide also eliminated binding activity.[40] These observations suggest that free cysteines influence the RNA-binding activity of SBP2. Therefore, we decided to examine the contribution of cysteine residues in the RNA-binding domain of SBP2 to its SECIS-binding activity.

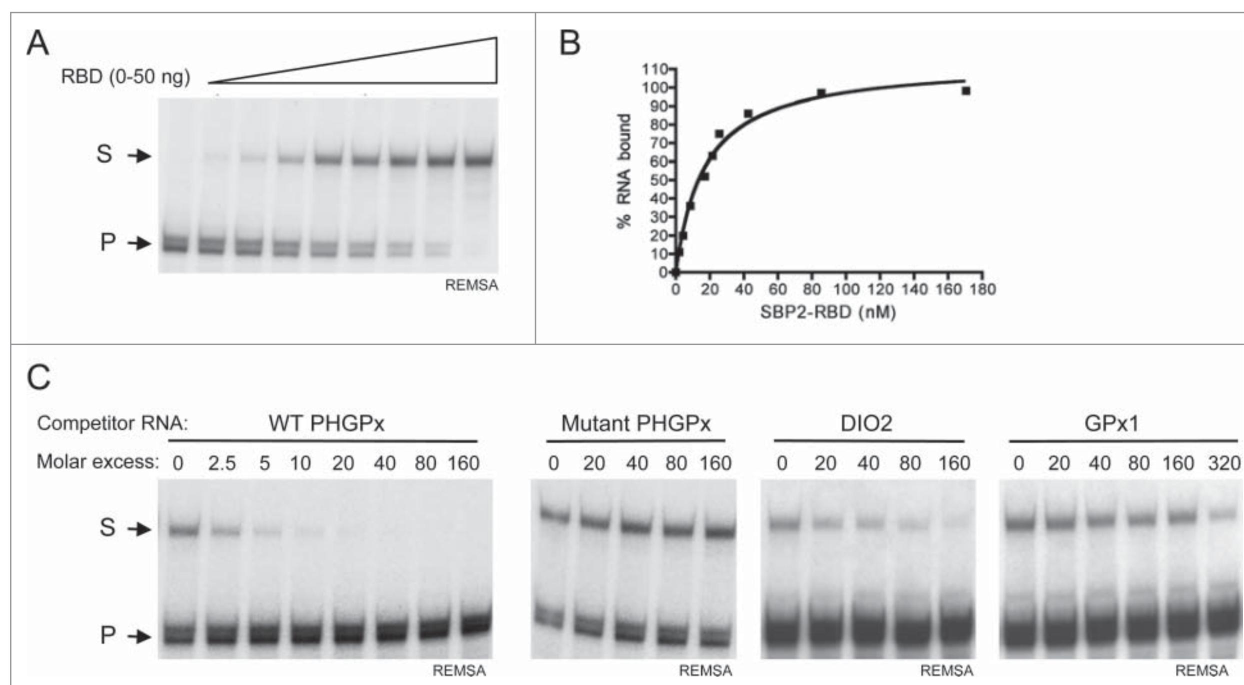

Figure 3. RBD is selective and binds the SECIS element with high affinity. (**A**) REMSA analysis was performed using [^{32}P]-labeled PHGPx SECIS RNA and increasing amounts of purified, recombinant SBP2-RBD protein. The RNA and protein were allowed to bind to equilibrium and then complex formation was analyzed by non-denaturing gel electrophoresis. (**B**) Quantification of binding data from REMSA in panel A. (**C**) Competition REMSAs using [^{32}P]-labeled PHGPx SECIS RNA as the probe and increasing amounts of PHGPx, Dio2 and GPx1 SECIS RNAs as competitors as indicated. P, unbound probe; S, shift due to functional SECIS:SBP2 interaction.

Using the rat SBP2-RBD as a guide, we assembled a set of over 70 vertebrate SBP2 sequences and a smaller set of 12 metazoan sequences (supplemental **Table 2**). A sequence alignment was performed on the vertebrate and metazoan sets independently to identify conserved cysteines. In the vertebrate lineage, the number of cysteine residues in the RNA-binding domain of these sequences ranges from 5 to 11, with a mode of 7. The sequences from the primate lineage have only 5 cysteines, C626, C637, C684, C691 and C712, (numbering refers to the rat sequence). These five cysteines appear to constitute the core cysteine signature of the domain. Multiple sequence alignment across this distal portion of the RNA-binding domain (rat aa 612-756) demonstrates that these cysteines are conserved across all vertebrate species. The exception is C712 in the fish lineage, which has a conserved cysteine in the -1 position relative to C712 (**Fig. 4A** and Supplemental **Fig. 1**).

The metazoan SBP2 proteins are very distinct from the vertebrate lineage, as they tend to be shorter (300–500aa), consisting primarily of the RNA-binding domain. Most invertebrate SBP2 sequences contain 6 to 8 cysteines across this region. However, only one is conserved across the set, which is positionally equivalent to C626. The cysteines at 684 and 711 (analogous to the fish) are also present in at least half of the set, as is a distal cysteine at 739, which is not present in the vertebrate lineage (Supplemental **Fig. 2**).

We focused on the 5 core vertebrate cysteines, in the context of rat SBP2-RBD, to examine their contribution to SECIS binding. A set of 5 SBP2-RBD constructs was created where a single cysteine was changed to encode serine. The mutant proteins were expressed using *in vitro* translation with ^{35}S-methionine (**Fig. 5A**) and equimolar amounts were subsequently tested for the ability to bind to the SECIS element of PHGPx using a REMSA. As shown in **Figure 5B**, the SECIS-binding activity was not perturbed by the mutation of any individual cysteine residue.

Multiple Cys mutations in SBP2-RBD impair SECIS-binding

Next, we investigated whether multiple cysteine mutations would affect the interaction of SBP2-RBD with the SECIS. The cysteine to serine mutations were made in combinations of 2, 3, 4, or 5, moving from left to right across the domain (**Fig. 6A**). Each mutant protein was produced by in vitro translation with ^{35}S methionine (**Fig. 6B**) and equimolar amounts were tested in a REMSA assay with the PHGPx SECIS (**Fig. 6C**). In this experiment, the amount of wildtype protein used results in a maximal shift, with very little free probe remaining. This facilitates the detection of any deficiency in the RNA-binding activity. A mild reduction in SECIS binding is already apparent in the 2X and 3X mutant proteins, as shown by the appearance of free probe. However, the 4X and 5X mutants were further impaired as they show both a decrease in the protein:RNA complex, and an increase in the amount of free probe. The impaired ability of the mutant 4X and 5X proteins to bind to the RNA also permits a lower-affinity interaction to occur between the SECIS probe and an endogenous protein in the RRL, indicated by the asterisk.

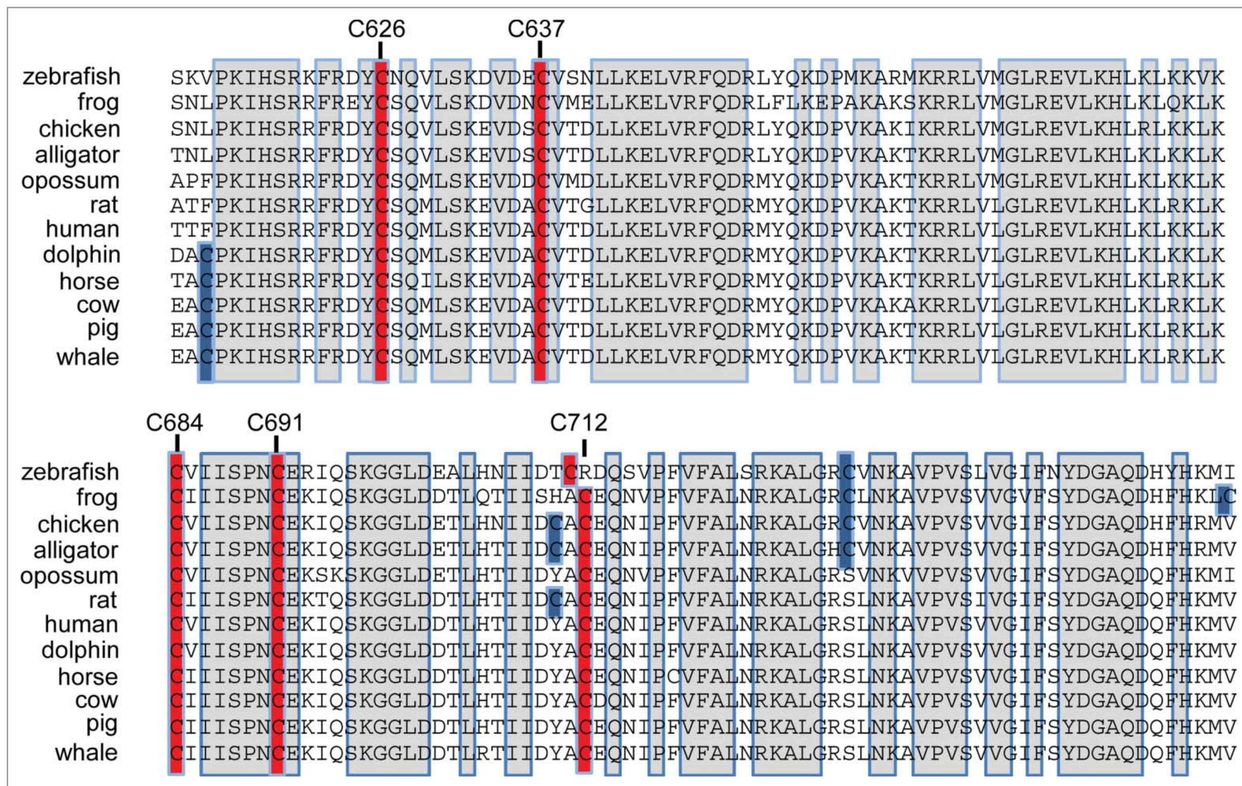

Figure 4. Vertebrate SBP2 RBD contains 5 conserved cysteines. A multiple sequence alignment of the second part of the RBD (612-756, rat numbering) including primate, rodent, ungulate, marsupial, reptile, avian, and fish and additional mammalian sequences. The red and blue highlighting indicates completely and partially conserved cysteine residues respectively. The gray boxes indicate regions of identity across the domain. The locations of the 5 conserved vertebrate cysteines are indicated and numbered with respect to the rat sequence.

These results show that while the RBD can compensate for the loss of an individual cysteine, the mutation of multiple cysteines reduces the RNA-binding activity of SBP2.

Localization is not affected by the mutation of the cysteines in the RBD

In addition to RNA-binding, this cysteine-containing region has been implicated in the subcellular localization of SBP2.[29] The oxidation state of SBP2 contributes to the subcellular localization of the protein in HEK293 cells, as hydrogen peroxide treatment caused SBP2 to translocate from the cytoplasm to the nucleus at steady state.[29] An observed decrease in selenoprotein synthesis was attributed to the altered localization of SBP2. However, any additional effects on its RNA-binding activity were not investigated. It was suggested that the cysteine residues in this region are directly involved in the regulation of SBP2 nuclear export.[29] SBP2 has been shown to shuttle between the cytoplasm and nucleus with putative nuclear localization signals being identified.[12,28,29] One previously identified nuclear export signal (NES) is centered around C637 and a model was suggested where the NES is masked under reducing conditions through the formation of disulfide bonds.

In order to test this model, we analyzed the localization of the individual cysteine mutants as well as a 4x compound mutant (C626S,C637S,C691S,C712S) in the context of SBP2-CT. We observed the steady-state subcellular localization of these mutants when transfected into McArdle 7777 rat hepatoma cells. As shown in **Figure 7A**, wildtype SBP2-CT and each of the individual cysteine mutants are found in the cytoplasm. We also treated our cells with Leptomycin B, which inhibits nuclear export to see whether these mutations impaired the nuclear accumulation of SBP2-CT. None of the mutations affected the accumulation of the protein in the nucleus upon Leptomycin B treatment (**Fig. 7B**), suggesting that their ability to shuttle between compartments is not significantly impaired. Finally, we also stressed the cells with hydrogen peroxide to see if the loss of the cysteine residues would affect the response to oxidative stress. However, as shown in **Figure 7C**, the SBP2-CT proteins were not translocated to the nucleus upon treatment with hydrogen peroxide. This difference from previous observations is likely due to cell type-specific differences, but may also be due to differences in the region of SBP2 included in the construct or the type of epitope tags added. We performed the same treatments on the 4x compound mutant (**Fig. 8**), and obtained similar results to the individual mutants. These results suggest that the RNA-binding activity is not a major determinant of subcellular localization, given that the loss of multiple cysteines impairs SECIS-binding but does not alter the localization characteristics. Future studies will be required to investigate the cell-type specificity and cellular mechanisms that control the localization of SBP2.

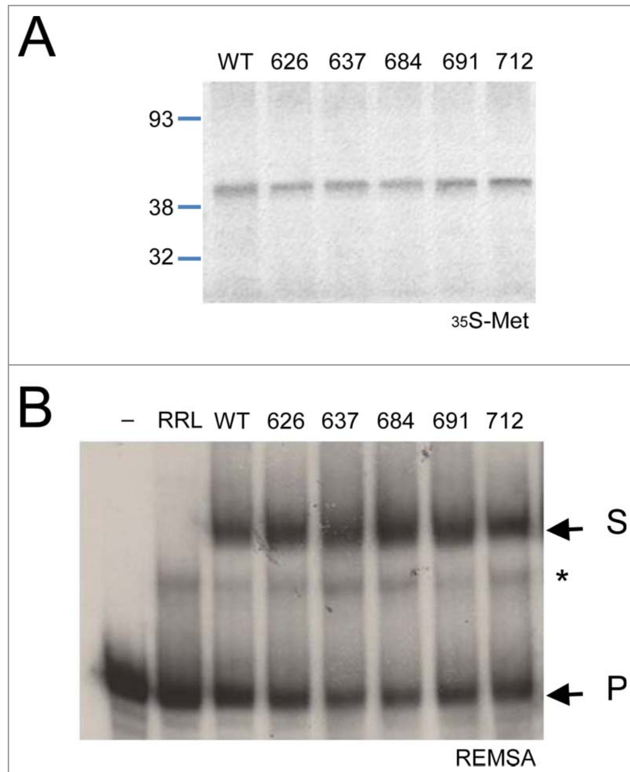

Figure 5. RNA-binding activity is not affected by the loss of an individual cysteine (**A**) SDS-PAGE analysis of in vitro translated SBP2-RBD and individual cysteine mutant proteins labeled with ^{35}S-methionine. (**B**) REMSAs were performed with equimolar amounts of the in vitro translated proteins incubated with PHGPx SECIS RNA as described in **Figure 3**. P, unbound probe; S, functional SECIS/SBP2 interaction; *, shift caused by interaction with an endogenous protein in the RRL.

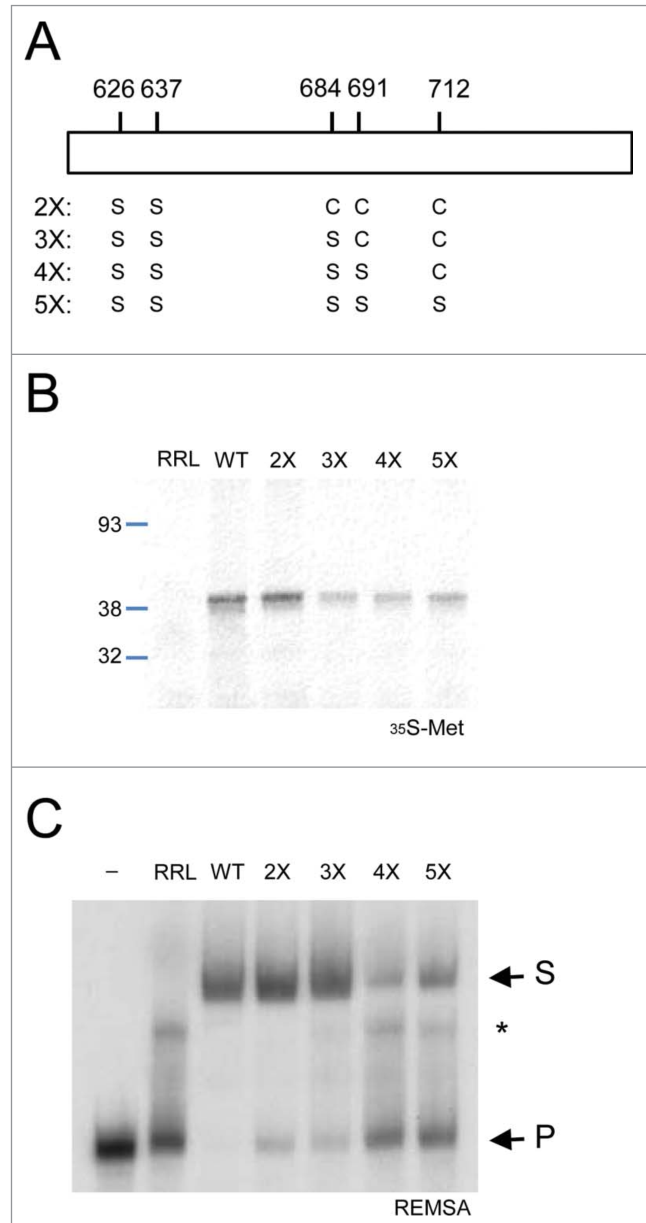

Figure 6. Multiple cysteine mutations impair the RNA-binding activity of SBP2. (**A**) Schematic representation of the multiple cysteine (C) to serine (S) mutations within the RBD from amino acid 612 to 777. All numbering refers to the rat sequence. (**B**) SDS-PAGE analysis of in vitro translated wildtype and mutant SBP2-RBD proteins labeled with ^{35}S-methionine. (**C**) REMSAs of wildtype and mutant translated proteins using PHGPx SECIS RNA as the probe as described in **Figure 3**. P, unbound probe; S, functional SECIS/SBP2 interaction; *, shift caused by interaction with an endogenous protein in the RRL.

Discussion

Traditional biochemical analysis of the relationships between the structure and function of a protein usually depends on the purification of the protein from a bacterial or cell culture model. Several technical pitfalls may need to be overcome including optimization of expression conditions, potential toxicity of the expressed protein to the host system, or retention of insoluble protein in cellular inclusion bodies. These problems are not present in a cell-free protein synthesis system such as RRL. There are several technical advantages including that the time required to progress from vector to expressed protein is much faster, and that many different proteins can be generated at the same time.

In this study, we directly compared the UGA recoding activity of SBP2-CT produced in a cell-free system, to protein that was purified from bacteria. The specific activity from the in vitro translated protein was 50–100x greater than the recombinant protein. This difference is not attributable to additional factors in the in vitro translation product compared to the bacterial protein, as the recoding assay is itself performed in RRL, and the same complement of additional proteins would be present. However, there are other properties of RRL that may contribute to the increased activity of in vitro translated SBP2. First, a known drawback of bacterially expressed recombinant protein is the lack of eukaryotic post-translational modifications. In contrast, a variety of protein modifications have been described in RRL with examples that include phosphorylation, acetylation, glycosylation, ubiquitination, and isoprenylation (reviewed in [41]) as well as sumoylation [42] and methylation.[43] The ability of RRL to

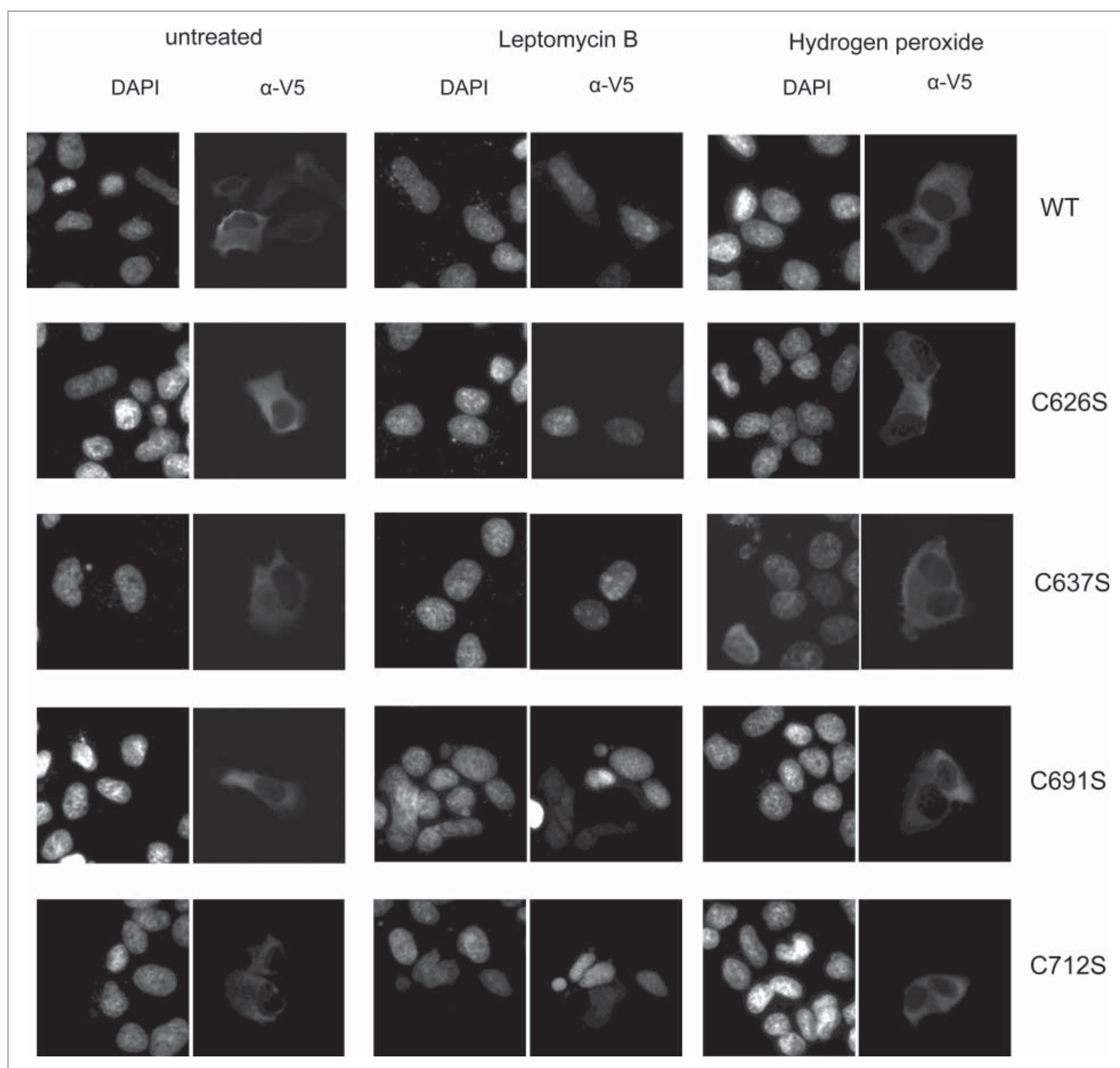

Figure 7. The subcellular localization of SBP2-CT is not affected by single cysteine mutations. (A) McArdle 7777 cells were examined for the localization of transiently transfected SBP2-CT, wildtype and single cysteine mutants, as described in Materials and Methods. Images were taken at 40x magnification. The SBP2-CT proteins were detected with an antibody directed against the V5 epitope tag, and the nuclei were detected with 4',6-diamidino-2-phenylindole (DAPI) stain. (**B**) Forty-8 hours post transfection, cells were treated with 2 ng/mL amount of Leptomycin B for 2 hours to prevent nuclear export, and the subcellular localization was determined as in A. (**C**) Forty-8 hours post transfection cells were treated with 1 mM hydrogen peroxide for 2 hours and the subcellular localization was detected as in A.

perform a wide range of post-translational modifications may positively contribute to the activity of SBP2-CT.

Another benefit of the RRL system is the presence of molecular chaperoning activities, which facilitates the proper folding of the expressed protein.[44] Initial in silico analysis of SBP2 amino acid sequences predicted that the protein is largely unfolded, and that the only structured region is within the RNA-binding domain (aa 611-770). Several biophysical approaches confirmed that much of the SBP2 protein is disordered.[45] Additionally, SBP2 has been shown to interact in a complex with the chaperone heat shock protein 90 (Hsp90) in cell culture. Inhibition of Hsp90 in cells also prevents the accumulation of SBP2, suggesting a role for Hsp90 in the biogenesis of SBP2.[46] RRL contains abundant amounts of Hsp90[44,47] and Hsp70,[44] which may facilitate the proper folding and expression of SBP2, thereby leading to a more active protein. Even in the absence of understanding the mechanism, it is clear that the in vitro translated SBP2-CT has greater activity and may provide a more sensitive platform for investigating the mechanism of selenocysteine insertion and for probing SBP2 function.

Our results also establish that amino acids 517-777 in rat SBP2 are sufficient to bind to the SECIS element with high

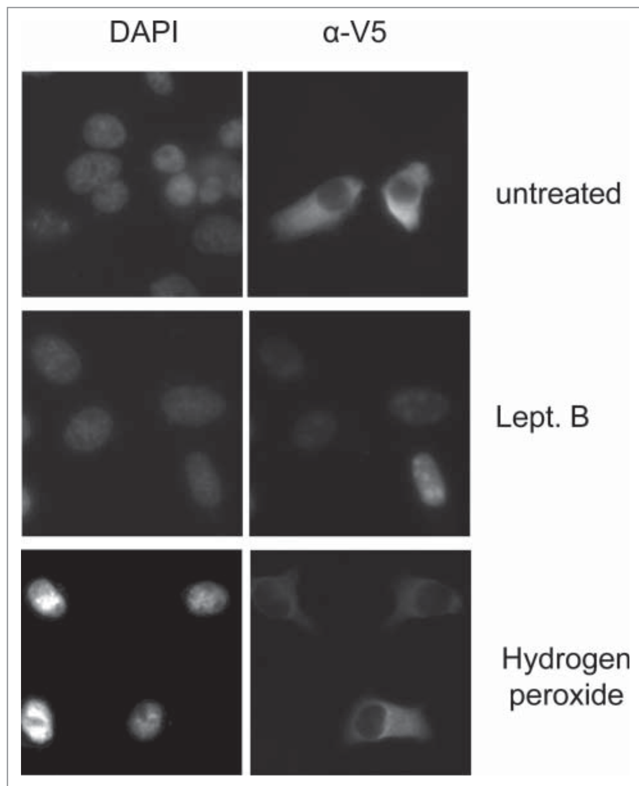

Figure 8. Nucleo-cytoplasmic shuttling is not perturbed when RNA-binding is impaired. McArdle 7777 cells were examined for the localization of transiently transfected SBP2-CT 4X(Cys to Ser) mutant. Images were taken at 40x magnification. The SBP2-CT 4x(Cys to Ser) mutant protein was detected with an antibody directed against the V5 epitope tag, and the nuclei were detected with 4′,6-diamidino-2-phenylindole (DAPI) stain. Leptomycin B and hydrogen peroxide treatments were performed as described in **Figure 7**.

affinity and specificity. Thus other sequences in SBP2 or factors in the cell are not required for this activity. A major challenge in the field is that the SBP2:SECIS structure has not been solved. Indeed, it has not been possible to obtain crystals of SBP2 alone or the SBP2:SECIS complex, presumably because SBP2 is an intrinsically disordered protein.[45] Even attempts with a truncated protein (starting at amino acid 525) have been unsuccessful. However, regions C-terminal to the RNA-binding domain are also expected to be disordered and may have interfered with attempts to crystallize the complex. Given that the RNA-binding domain alone is predicted to be structured, and is sufficient to mediate high affinity binding to the SECIS element, the ability to produce large amounts of highly purified bioactive SBP2-RBD should facilitate future structural studies on understanding how SBP2 discriminates between SECIS elements.

SBP2 is considered the master regulator of selenoprotein synthesis and the protein has been shown to exhibit different binding affinities for selenoprotein mRNAs *in vitro* and *in vivo.* Whether these differences correlate with the efficiency of selenocysteine incorporation has not been directly explored. Donovan and Copeland proposed that there is no connection between SBP2 binding affinity and UGA recoding efficiency *in vitro*.[35] This conclusion was based on comparing the results from 2 independent studies, which analyzed different SECIS elements for either SECIS binding in a REMSA [35] or UGA recoding activity in RRL.[36] However, each study analyzed SECIS elements of different sizes (minimal SECIS of 100 nt versus 140 nt), which influences both the RNA-binding and recoding activities. The stoichiometry between the protein and RNA is different in both systems as well, which may also complicate a direct comparison. However, a potential issue common to both studies it that these experiments were done in a noncompetitive environment in which only a single type of SECIS element was tested. In contrast, when performed under competitive conditions, the hierarchy that we observed for both SECIS-binding and UGA recoding efficiency (PHGPx > Dio2 > GPx1) parallels the physiological importance of these selenoproteins. The disruption of the PHGPx gene in mice is embryonic lethal[23] whereas Dio2 knockout mice have a modest phenotype.[48,49] Conversely, GPx1 knockout mice do not have an observable phenotype unless stressed.[50] Our data suggest that the ability of a SECIS element to inhibit UGA-recoding activity in RRL (**Fig. 2**) is directly related to the affinity of the SBP2:SECIS interaction (**Fig. 3**). In terms of the competitive cellular environment, Squires et al. [51] analyzed the relative expression levels of selenoprotein mRNAs *in vivo*. PHGPx mRNA is highly expressed and more abundant than GPx1 and Dio1 mRNAs in most mouse tissues. This data confirms that the molar ratios used in our decoy assays are well within physiologically relevant ranges, as they are even lower than what would be predicted to exist in a cell. Given that the PHGPx mRNA is abundant and that SBP2 has a high affinity for its SECIS element, one can envision that PHGPx may be preferentially expressed over other selenoproteins *in vivo*. While SBP2 binding affinity may dictate UGA recoding in RRL, other factors likely contribute to the efficiency of selenocysteine incorporation in vivo, including the ability of the SECIS element to bind L30, undergo kinking, and interact with regulatory proteins such as nucleolin and eIF4a3.

Many selenoproteins are involved in the redox homeostasis of cellular environments.[14] A decrease in SBP2 levels leads to increased detection of reactive oxygen species in the cell,[52] in keeping with its role in the production of selenoproteins, which have a role in anti-oxidant defense. The initial characterization of SBP2 also showed redox effects on its function as its SECIS-binding activity required the addition of DTT to the reaction.[40] Additional experimental support was provided by Papp et al.,[29] who demonstrated the presence of reversible disulfide bonds as well as a free thiol in the C-terminal part of SBP2 (aa584-854 of human SBP2). They also observed a change in subcellular localization in response to oxidative stress, although our results suggest that this is not uniformly true across all cell types. However, it appears that an elegant system exists where SBP2, the translational regulator of a class of anti-oxidant proteins, is susceptible to the oxidation state of the environment in multiple ways.

The conservation of a core signature of 5 cysteines across the vertebrate lineage suggests they play an important role in SBP2 function. In our study, in the context of SBP2-RBD, we show that individually disturbing the cysteine residues did not have an

effect on binding to the SECIS. However, multiple cysteine to serine mutations did interfere with RNA binding. Several individuals with heterozygous SBP2 mutations have been described in recent years, including a proband who received an allele from his mother where the third of the 5 core cysteines was mutated to arginine (C691R, in human nomenclature; corresponds to C684 in rat).[20] While his mother was not described as symptomatic, her selenoprotein expression levels were uniformly decreased, suggesting that this mutation is sufficient to perturb selenoprotein synthesis. In our study, this mutation did not affect the binding of SBP2-RBD to the PHGPx SECIS, but we cannot exclude the possibility that binding to other SECIS elements could be affected. The mutation could also impact another SBP2 function such as binding to the ribosome, or facilitating interactions with other proteins involved in selenoprotein synthesis.

The ease of producing multiple different mutant proteins in a cell free system makes it a feasible method for rapidly interrogating the various regions of interest of SBP2. This can be driven both by in silico analysis of the protein sequence to understand the relationship between conservation and function, as well as the continued discovery of complex patient mutations. A cell free system presents the opportunity to compare the activities of each of the mutations alone or in combination. This approach will accelerate studies aimed at understanding the functions and regulation of SBP2, which may lead to improved approaches for treating the patients with genetic SBP2 deficiency in the future.

Materials and Methods

Reagents

The constructs for the in vitro translation of SBP2-CT (aa 399-846) and SBP2-RBD (517-777) are in the pcDNA3.1V5/His construct (Life Technologies), as previously described.[25] For experiments using in vitro translated SBP2-CT in luciferase assays, the protein also contains the previously published M535A mutation to prevent the production of a truncated protein due to internal initiation.[26] The construct for bacterial expression of SBP2-CT has also been described elsewhere.[34] The SBP2-RBD region (aa 517-777) was cloned into pET200 Directional TOPO (Life Technologies) as a HIS_6- and Xpress-tagged protein for bacterial expression, with purification conditions as previously described for SBP2-CT.[34] The cysteine to serine mutations at positions C626, C637, C684, C691 and C712 were generated using Dpn1-directed mutagenesis using the primers listed in Supplementary **Table 1**. Briefly, primers containing the mutation of interest were used to amplify the parent plasmid. The resulting product was incubated with DpnI, which digests the template DNA. The remaining mutagenized DNA was transformed into Top10 cells and plated onto selective medium, and colonies were isolated and analyzed. All of the mutations were confirmed by DNA sequencing. Sequential rounds of mutagenesis were used to obtain the multiple mutants. The luciferase reporter constructs luc/UGA^{258}/PhGPx and luc/UGA^{258}/Dio1 have been previously described.[26,33] The phospholipid hydroperoxide glutathione peroxidase (PHGPx), glutathione peroxidase 1 (GPx1), and deiodinase 2 (Dio2) SECIS probe sequences were also previously described.[26,53]

DNA sequences

The sequences used in the multiple alignment of SBP2-RBD were obtained primarily from GenBank and Ensembl databases. Additional metazoan sequences were obtained from the supplemental data of reference.[30] A complete list of the organisms and their accession numbers is found in Supplementary **Table 2**. The multiple alignment was performed with **MU**ltiple **S**equence **C**omparison by **L**og- **E**xpectation (MUSCLE, http://www.edi.ac.uk/Tools/msa/muscle).

In vitro transcription

Plasmid DNAs were linearized and used as templates for in vitro transcription using T7 RNA polymerase as per manufacturer's guidelines (Ribomax T7; Promega). The transcription reactions were treated with DNAse I for 20 minutes and then phenol:chloroform extracted. The aqueous phase was passed through a Micro Bio-Spin P30 column according to manufacturer's instructions (BioRad). Radiolabeled PHGPx SECIS probe was synthesized using 1 mM GTP, 1 mM ATP, 1 mM CTP, 0.05 mM UTP and 25 μCi of ^{32}P-labeled UTP for 3 hours at 37°C. Probes were DNase I treated and cleaned as above, heated to 95°C for 2 minutes and slow cooled to room temperature.

In vitro translation

All in vitro translation reactions were performed in 70% rabbit reticulocyte lysate (Promega) as per manufacturer's suggestion. In order to generate SBP2-CT, reactions were assembled in a volume of 100 μl, and the reaction was programmed with 2 μg of mRNA. In order to label the SBP2-CT translation product, an amino acid mixture minus methionine and [^{35}S]-methionine were used. The translation reaction was incubated at 30°C for 15–30 minutes. For the labeled reactions, a 2-μl aliquot of each translation reaction was resolved on an SDS-PAGE gel and quantitated by PhosphorImager analysis. The concentrations were determined by comparison with known standards. Calculations were performed using a concentration of 5 μM cold methionine in the RRL lysate as indicated by the manufacturer (Promega). We also compared the SBP2 translation products synthesized in the presence of cold methionine or 35S-methionine and analyzed the products by SDS-PAGE and Western blotting. As shown in Supplementary **Figure 3**, there was no statistically significant difference in the amount of protein produced under the 2 conditions.

In vitro selenocysteine insertion assay

In vitro translation reactions were assembled for a total volume of 25 μl, including complete amino acid mixture and 100 ng of luc/UGA^{258}mRNA in the presence or absence of SBP2-CT. The reactions were incubated at 37°C for 30-60 minutes as indicated. Each reaction was tested in triplicate by adding 2.5 μl of the translation mixture to 50 μl of luciferase substrate (Promega) and reading the sample for 10 s in a

PerkinElmer Victor.[3] To examine the effect of multiple SECIS elements as decoys, increasing amounts of SECIS RNA was added to the translation reaction as indicated.

RNA electrophoretic mobility shift assays (REMSA)

For direct binding studies with SBP2-RBD, the PHGPx SECIS probe was synthesized as described above. Ten femtomoles of radiolabeled probe was incubated with increasing amounts of bacterially expressed SBP2-RBD. The reaction conditions were as previously described.[54] For the competition reactions, REMSA were performed with 10 ng of purified bacterial SBP2-RBD and increasing concentrations of unlabeled SECIS RNA were added in excess, as indicated. Complexes were resolved on 6% non-denaturing polyacrylamide gels in Tris-Glycine-EDTA buffer. The gels were dried, and the appearance of complexes was analyzed on a PhosphorImager. ImageQuant software was used to quantify the percent of probe that was bound by the protein. For analyzing the binding activity of the cysteine to serine mutants, 2.5 ng of in vitro translated proteins were analyzed for binding to the PHGPx SECIS as previously described.[26]

Cell culture, transfection and treatments

McArdle 7777 rat hepatoma cells were obtained from ATCC. All cells were cultured in a monolayer in Dulbecco's Modified Eagle's Media (DMEM) with 4.5 g/L glucose and 10% FBS in 5% CO_2 at 37°C. The transfection reactions were performed on cells seeded on coverslips at 2.5×10^5 cells in 6-well plates, using 500 ng of plasmid DNA per reaction and ExGen transfection reagent (Fermentas) according to manufacturer's instructions. Cells were analyzed 48 hours post-transfection. For the Leptomycin B and hydrogen peroxide experiments, cells were pre-incubated with cycloheximide at 10 μg/mL to inhibit new protein synthesis. The cycloheximide treatement was maintained upon the addition of 2 ng/mL Leptomycin B or 1 mM hydrogen peroxide and cells were further incubated for 2 hours prior to analysis.

Immunofluoresence

Cells grown on coverslips were washed with phosphate buffered saline (PBS) and fixed 4% paraformaldehyde for 30 minutes. After washing with PBS, cells were permeabilized with 0.2% Triton X-100 in PBS for 5 minutes at room temperature with gentle mixing. Cells were washed twice with PBS and then incubated with 3% bovine serum albumin (BSA)/0.1% Tween in PBS for 60 minutes. The cells were then incubated with an anti-V5 antibody (Life Technologies) at 1:200 dilution in 3%BSA/0.1%Tween for one hour at room temperature. The samples were washed twice in PBS followed by incubation with the anti-mouse Alexa Fluor 488 at 1:200 for one hour (Life Technologies). Cells were washed twice and mounted in ProLong Antifade mounting buffer with DAPI (Life Technologies) and left to cure overnight. Images were captured at 40x magnification on a Leica DM5500B upright microscope (Leica Microsystems, GmbH) using ImagePro Plus software (MediaCybernetics).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Kaushal N, Gandhi UH, Nelson SM, Narayan V, Prabhe KS. Selenium and Inflammation. In: Selenium: Its Molecular Biology and Role in Human Health. D Hatfield, MJ Berry, G Vadim, eds. New York: Springer, 2012:443-56.
2. Handy D, Loscalzo J. Selenoproteins in cardiovasculat redox pathology. In: Selenium: Its Molecular Biology and Role in Human Health. H DL, B MJ, G V., eds. New York: Springer, 2012:249-59.
3. Turanov A, Malinouski M, Gladyshev V. Selenium and Male Reproduction. In: Selenium: Its Molecular Biology and Role in Human Health. D Hatfield, MJ Berry, V Gladyshev, eds. New York: Springer, 2012:409-17.
4. Moghadaszadeh B, Petit N, Jaillard C, Brockington M, Quijano Roy S, Merlini L, Romero N, Estournet B, Desguerre I, Chaigne D, et al. Mutations in SEPN1 cause congenital muscular dystrophy with spinal rigidity and restrictive respiratory syndrome. Nat Genet 2001; 29:17-8; PMID:11528383; http://dx.doi.org/10.1038/ng713
5. Kryukov GV, Castellano S, Novoselov SV, Lobanov AV, Zehtab O, Guigo R, Gladyshev VN. Characterization of mammalian selenoproteomes. Science 2003; 300:1439-43; PMID:12775843; http://dx.doi.org/10.1126/science.1083516
6. Sunde R. Selenoproteins: Hierarchy, Requirements, and Biomarkers. In: Selenium: Its Molecular Biology and Role in Human Health. D Hatfield, MJ Berry, V Gladyshev, eds. New York: Springer, 2012:137-52.
7. Berry MJ, Banu L, Chen YY, Mandel SJ, Kieffer JD, Harney JW, Larsen PR. Recognition of UGA as a selenocysteine codon in type I deiodinase requires sequences in the 3' untranslated region. Nature 1991; 353:273-6; PMID:1832744; http://dx.doi.org/10.1038/353273a0
8. Tujebajeva RM, Copeland PR, Xu XM, Carlson BA, Harney JW, Driscoll DM, Hatfield DL, Berry MJ. Decoding apparatus for eukaryotic selenocysteine insertion. EMBO Rep 2000; 1:158-63; PMID:11265756; http://dx.doi.org/10.1093/embo-reports/kvd033
9. Fagegaltier D, Hubert N, Yamada K, Mizutani T, Carbon P, Krol A. Characterization of mSelB, a novel mammalian elongation factor for selenoprotein translation. EMBO J 2000; 19:4796-805; PMID:10970870; http://dx.doi.org/10.1093/emboj/19.17.4796
10. Lee BJ, Worland PJ, Davis JN, Stadtman TC, Hatfield DL. Identification of a selenocysteyl-tRNA(Ser) in mammalian cells that recognizes the nonsense codon, UGA. J Biol Chem 1989; 264:9724-7; PMID:2498338
11. Carlson BA, Martin-Romero FJ, Kumaraswamy E, Moustafa ME, Zhi H, Hatfield D, et al. Mammalian selenocysteine tRNA. In: Selenium: Its Molecular Biology and Role in Human Health. DL Hatfield, ed. Norwell, Massachusetts: Kluwer Academic Publishers, 2001:23-32.
12. Copeland PR, Fletcher JE, Carlson BA, Hatfield DL, Driscoll DM. A novel RNA binding protein, SBP2, is required for the translation of mammalian selenoprotein mRNAs. EMBO J 2000; 19:306-14; PMID:10637234; http://dx.doi.org/10.1093/emboj/19.2.306
13. Fletcher JE, Copeland PR, Driscoll DM, Krol A. The selenocysteine incorporation machinery: interactions between the SECIS RNA and the SECIS-binding protein SBP2. RNA 2001; 7:1442-53; PMID:11680849
14. Labunskyy VM, Hatfield DL, Gladyshev VN. Selenoproteins: molecular pathways and physiological roles. Physiol Rev 2014; 94:739-77; PMID:24987004; http://dx.doi.org/10.1152/physrev.00039.2013
15. Seeher S, Mahdi Y, Schweizer U. Post-transcriptional control of selenoprotein biosynthesis. Curr Protein Pept Sci 2012; 13:337-46; PMID:22708491; http://dx.doi.org/10.2174/138920312801619448
16. Allamand V, Richard P, Lescure A, Ledeuil C, Desjardin D, Petit N, Gartioux C, Ferreiro A, Krol A, Pellegrini N, et al. A single homozygous point mutation in a 3'untranslated region motif of selenoprotein N mRNA causes SEPN1-related myopathy. EMBO Rep 2006; 7:450-4; PMID:16498447
17. Dumitrescu AM, Liao XH, Abdullah MS, Lado-Abeal J, Majed FA, Moeller LC, Boran G, Schomburg L, Weiss RE, Refetoff S. Mutations in SECISBP2 result in abnormal thyroid hormone metabolism. Nat Genet 2005; 37:1247-52; PMID:16228000; http://dx.doi.org/10.1038/ng1654
18. Di Cosmo C, McLellan N, Liao XH, Khanna KK, Weiss RE, Papp L, Refetoff S. Clinical and molecular characterization of a novel selenocysteine insertion sequence-binding protein 2 (SBP2) gene mutation (R128X). J Clin Endocrinol Metab 2009; 94:4003-9; PMID:19602558; http://dx.doi.org/10.1210/jc.2009-0686
19. Azevedo MF, Barra GB, Naves LA, Ribeiro Velasco LF, Godoy Garcia Castro P, de Castro LC, Amato AA, Miniard A, Driscoll D, Schomburg L, et al. Selenoprotein-related disease in a young girl caused by nonsense

mutations in the SBP2 gene. J Clin Endocrinol Metab 2010; 95:4066-71; PMID:20501692; http://dx.doi.org/10.1210/jc.2009-2611
20. Schoenmakers E, Agostini M, Mitchell C, Schoenmakers N, Papp L, Rajanayagam O, Padidela R, Ceron-Gutierrez L, Doffinger R, Prevosto C, et al. Mutations in the selenocysteine insertion sequence-binding protein 2 gene lead to a multisystem selenoprotein deficiency disorder in humans. J Clin Invest 2010; 120:4220-35; PMID:21084748; http://dx.doi.org/10.1172/JCI43653
21. Hamajima T, Mushimoto Y, Kobayashi H, Saito Y, Onigata K. Novel compound heterozygous mutations in the SBP2 gene: characteristic clinical manifestations and the implications of GH and triiodothyronine in longitudinal bone growth and maturation. Eur J Endocrinol 2012; 166:757-64; PMID:22247018; http://dx.doi.org/10.1530/EJE-11-0812
22. Seeher S, Atassi T, Mahdi Y, Carlson BA, Braun D, Wirth EK, Klein MO, Reix N, Miniard AC, Schomburg L, et al. Secisbp2 is essential for embryonic development and enhances selenoprotein expression. Antioxid Redox Signal 2014; 21:835-49; PMID:24274065; http://dx.doi.org/10.1089/ars.2013.5358
23. Imai H, Hirao F, Sakamoto T, Sekine K, Mizukura Y, Saito M, Kitamoto T, Hayasaka M, Hanaoka K, Nakagawa Y. Early embryonic lethality caused by targeted disruption of the mouse PHGPx gene. Biochem Biophys Res Commun 2003; 305:278-86; PMID:12745070; http://dx.doi.org/10.1016/S0006-291X(03)00734-4
24. Matsui M, Oshima M, Oshima H, Takaku K, Maruyama T, Yodoi J, Taketo MM. Early embryonic lethality caused by targeted disruption of the mouse thioredoxin gene. Dev Biol 1996; 178:179-85; PMID:8812119; http://dx.doi.org/10.1006/dbio.1996.0208
25. Copeland PR, Stepanik VA, Driscoll DM. Insight into mammalian selenocysteine insertion: domain structure and ribosome binding properties of Sec insertion sequence binding protein 2. Mol Cell Biol 2001; 21:1491-8; PMID:11238886; http://dx.doi.org/10.1128/MCB.21.5.1491-1498.2001
26. Bubenik JL, Driscoll DM. Altered RNA binding activity underlies abnormal thyroid hormone metabolism linked to a mutation in selenocysteine insertion sequence-binding protein 2. J Biol Chem 2007; 282:34653-62; PMID:17901054; http://dx.doi.org/10.1074/jbc.M707059200
27. Takeuchi A, Schmitt D, Chapple C, Babaylova E, Karpova G, Guigo R, Krol A, Allmang C. A short motif in Drosophila SECIS Binding Protein 2 provides differential binding affinity to SECIS RNA hairpins. Nucleic Acids Res 2009; 37:2126-41; PMID:19223320; http://dx.doi.org/10.1093/nar/gkp078
28. de Jesus LA, Hoffmann PR, Michaud T, Forry EP, Small-Howard A, Stillwell RJ, Morozova N, Harney JW, Berry MJ. Nuclear assembly of UGA decoding complexes on selenoprotein mRNAs: a mechanism for eluding nonsense-mediated decay? Mol Cell Biol 2006; 26:1795-805; PMID:16478999; http://dx.doi.org/10.1128/MCB.26.5.1795-1805.2006
29. Papp LV, Lu J, Striebel F, Kennedy D, Holmgren A, Khanna KK. The redox state of SECIS binding protein 2 controls its localization and selenocysteine incorporation function. Mol Cell Biol 2006; 26:4895-910; PMID:16782878; http://dx.doi.org/10.1128/MCB.02284-05
30. Donovan J, Copeland PR. Evolutionary history of selenocysteine incorporation from the perspective of SECIS binding proteins. BMC Evol Biol 2009; 9:229; PMID:19744324; http://dx.doi.org/10.1186/1471-2148-9-229
31. Low SC, Grundner-Culemann E, Harney JW, Berry MJ. SECIS-SBP2 interactions dictate selenocysteine incorporation efficiency and selenoprotein hierarchy. EMBO J 2000; 19:6882-90; PMID:11118223; http://dx.doi.org/10.1093/emboj/19.24.6882
32. Caban K, Kinzy SA, Copeland PR. The L7Ae RNA binding motif is a multifunctional domain required for the ribosome-dependent Sec incorporation activity of Sec insertion sequence binding protein 2. Mol Cell Biol 2007; 27:6350-60; PMID:17636016; http://dx.doi.org/10.1128/MCB.00632-07
33. Mehta A, Rebsch CM, Kinzy SA, Fletcher JE, Copeland PR. Efficiency of mammalian selenocysteine incorporation. J Biol Chem 2004; 279:37852-9; PMID:15229221; http://dx.doi.org/10.1074/jbc.M404639200
34. Chavatte L, Brown BA, Driscoll DM. Ribosomal protein L30 is a component of the UGA-selenocysteine recoding machinery in eukaryotes. Nat Struct Mol Biol 2005; 12:408-16; PMID:15821744; http://dx.doi.org/10.1038/nsmb922
35. Donovan J, Copeland PR. Selenocysteine insertion sequence binding protein 2L is implicated as a novel post-transcriptional regulator of selenoprotein expression. PLoS One 2012; 7:e35581; PMID:22530054; http://dx.doi.org/10.1371/journal.pone.0035581
36. Latreche L, Jean-Jean O, Driscoll DM, Chavatte L. Novel structural determinants in human SECIS elements modulate the translational recoding of UGA as selenocysteine. Nucleic Acids Res 2009; 37:5868-80; PMID:19651878; http://dx.doi.org/10.1093/nar/gkp635
37. Howard MT, Moyle MW, Aggarwal G, Carlson BA, Anderson CB. A recoding element that stimulates decoding of UGA codons by Sec tRNA[SerSec. RNA 2007; 13:912-20; PMID:17456565; http://dx.doi.org/10.1261/rna.473907
38. Donovan J, Caban K, Ranaweera R, Gonzalez-Flores JN, Copeland PR. A novel protein domain induces high affinity selenocysteine insertion sequence binding and elongation factor recruitment. J Biol Chem 2008; 283:35129-39; PMID:18948268; http://dx.doi.org/10.1074/jbc.M806008200
39. Donovan J, Copeland PR. The efficiency of selenocysteine incorporation is regulated by translation initiation factors. J Mol Biol 2010; 400:659-64; PMID:20488192; http://dx.doi.org/10.1016/j.jmb.2010.05.026
40. Copeland PR, Driscoll DM. Purification, redox sensitivity, and RNA binding properties of SECIS-binding protein 2, a protein involved in selenoprotein biosynthesis. J Biol Chem 1999; 274:25447-54; PMID:10464275; http://dx.doi.org/10.1074/jbc.274.36.25447
41. Promega. Post-Translational Modifications. In Vitro Protein Expression Guide.
42. Chosed R, Mukherjee S, Lois LM, Orth K. Evolution of a signalling system that incorporates both redundancy and diversity: Arabidopsis SUMOylation. Biochem J 2006; 398:521-9; PMID:16740136; http://dx.doi.org/10.1042/BJ20060426
43. Denman RB. Protein methyltransferase activities in commercial in vitro translation systems. J Biochem 2008; 144:223-33; PMID:18463115; http://dx.doi.org/10.1093/jb/mvn061
44. Schumacher RJ, Hurst R, Sullivan WP, McMahon NJ, Toft DO, Matts RL. ATP-dependent chaperoning activity of reticulocyte lysate. J Biol Chem 1994; 269:9493-9; PMID:8144534
45. Olieric V, Wolff P, Takeuchi A, Bec G, Birck C, Vitorino M, Kieffer B, Beniaminov A, Cavigiolio G, Theil E, et al. SECIS-binding protein 2, a key player in selenoprotein synthesis, is an intrinsically disordered protein. Biochimie 2009; 91:1003-9; PMID:19467292; http://dx.doi.org/10.1016/j.biochi.2009.05.004
46. Boulon S, Marmier-Gourrier N, Pradet-Balade B, Wurth L, Verheggen C, Jady BE, Rothé B, Pescia C, Robert MC, Kiss T, et al. The Hsp90 chaperone controls the biogenesis of L7Ae RNPs through conserved machinery. J Cell Biol 2008; 180:579-95; PMID:18268104; http://dx.doi.org/10.1083/jcb.200708110
47. Matts RL, Hurst R. Evidence for the association of the heme-regulated eIF-2 alpha kinase with the 90-kDa heat shock protein in rabbit reticulocyte lysate in situ. J Biol Chem 1989; 264:15542-7; PMID:2768277
48. Schneider MJ, Fiering SN, Pallud SE, Parlow AF, St Germain DL, Galton VA. Targeted disruption of the type 2 selenodeiodinase gene (DIO2) results in a phenotype of pituitary resistance to T4. Mol Endocrinol 2001; 15:2137-48; PMID:11731615; http://dx.doi.org/10.1210/mend.15.12.0740
49. Ng L, Goodyear RJ, Woods CA, Schneider MJ, Diamond E, Richardson GP, Kelley MW, Germain DL, Galton VA, Forrest D. Hearing loss and retarded cochlear development in mice lacking type 2 iodothyronine deiodinase. Proc Natl Acad Sci U S A 2004; 101:3474-9; PMID:14993610; http://dx.doi.org/10.1073/pnas.0307402101
50. Cheng WH, Ho YS, Ross DA, Valentine BA, Combs GF, Lei XG. Cellular glutathione peroxidase knockout mice express normal levels of selenium-dependent plasma and phospholipid hydroperoxide glutathione peroxidases in various tissues. J Nutr 1997; 127:1445-50; PMID:9237936
51. Squires JE, Stoytchev I, Forry EP, Berry MJ. SBP2 binding affinity is a major determinant in differential selenoprotein mRNA translation and sensitivity to nonsense-mediated decay. Mol Cell Biol 2007; 27:7848-55; PMID:17846120; http://dx.doi.org/10.1128/MCB.
00793-07
52. Papp LV, Lu J, Bolderson E, Boucher D, Singh R, Holmgren A, Khanna KK. SECIS-binding protein 2 promotes cell survival by protecting against oxidative stress. Antioxid Redox Signal 2010; 12:797-808; PMID:19803747; http://dx.doi.org/10.1089/ars.2009.2913
53. Lesoon A, Mehta A, Singh R, Chisolm GM, Driscoll DM. An RNA-binding protein recognizes a mammalian selenocysteine insertion sequence element required for cotranslational incorporation of selenocysteine. Mol Cell Biol 1997; 17:1977-85; PMID:9121445
54. Miniard AC, Middleton LM, Budiman ME, Gerber CA, Driscoll DM. Nucleolin binds to a subset of selenoprotein mRNAs and regulates their expression. Nucleic Acids Res 2010; 38:4807-20; PMID:20385601; http://dx.doi.org/10.1093/nar/gkq247