Published in final edited form as:

Biochem J. 2014 September 15; 462(3): 555–565. doi:10.1042/BJ20140076.

Selenoprotein S is Involved in Maintenance and Transport of Multiprotein Complexes

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Abstract

Selenoprotein S (SelS) is a selenocysteine-containing protein with roles in endoplasmic reticulum (ER) function and inflammation. It has been implicated in ER-associated protein degradation (ERAD), and clinical studies revealed an association of its promoter polymorphism with cytokine levels and human diseases. However, the pathways and interacting proteins that could shed light on pathogenesis of SelS-associated diseases have not been studied systematically. We performed a large-scale affinity isolation of human SelS and its mutant forms and analyzed proteins that interact with them. All previously known SelS targets and nearly two hundred additional proteins were identified, which were remarkably enriched for various multiprotein complexes. Subsequent chemical cross-linking experiments identified the specific interacting sites in SelS and its several targets. Most of these interactions involved coiled coil domains. The data suggest that SelS participates in intracellular membrane transport and maintenance of protein complexes by anchoring them to the ER membrane.

Keywords

Selenoprotein S; selenocysteine; protein-protein interactions; multi-protein complexes; coiled coil domain

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INTRODUCTION

Selenoproteins are a diverse group of proteins that contain the 21st genetically encoded amino acid, selenocysteine (Sec, U), encoded by UGA codon [1]. Twenty five selenoprotein genes have been identified in the human genome [2], and at least three of them are essential [3]. Selenoproteins have different structural motifs, localize to various cellular compartments and are involved in diverse biological processes [4]. More than half of the human selenoproteins have thioredoxin-like fold (glutathione peroxidases, thyroid hormone deiodinases, Selenoproteins H, M, P, T, V, W, and Sep15) and may serve as enzymes that catalyze thiol-dependent reactions and participate in cellular redox homeostasis. Other human selenoproteins with established redox functions include thioredoxin reductases and methionine sulfoxide reductase B1. However, the precise functions of many other selenoproteins remain poorly characterized, even though many physiological effects associated with dysregulation of these proteins were found. The difficulties in elucidating their functions at the molecular level include the lack of data on interacting proteins and unknown types of their catalytic activity, which prevent assigning these selenoproteins to specific metabolic pathways/processes.

One selenoprotein with a poorly characterized function is Selenoprotein S (SelS, also known as SEPS1, VIMP, Tanis, and SELENOS). Previous studies have reported on the role of SelS in retrotranslocation of proteins from the ER to the cytosol [5–7], maintenance of ER homeostasis [8,9], and inflammatory response [10,11]. Several reports have also shown an association between SelS dysregulation and metabolic disorders [12–17]. In addition, SelS was proposed to serve as a thioredoxin-dependent reductase *in vitro* [18–20].

In this study, we investigated SelS by assessing its protein-protein interactions. Affinity isolation of SelS-bound proteins followed by mass spectrometry analyses identified nearly two hundred proteins which were specifically pulled down through the interaction with SelS. Surprisingly, many SelS targets were members of well-characterized cellular multi-protein complexes. Chemical cross-linking experiments confirmed the interaction of SelS with the components of different multi-protein complexes and revealed a role of the SelS conserved coiled coil domain in this interaction. These data indicate that the function of SelS is not only linked to its role in maintenance of ER homeostasis, ER-associated protein degradation (ERAD) and inflammation, but is also associated with maintenance and intracellular membrane-based transport of protein complexes.

EXPERIMENTAL

Antibodies and reagents

Mouse monoclonal antibodies against KDEL were from Abcam. Rabbit polyclonal affinity isolated antibodies against Derlin 1, HA-tag, SelS, SelK, and β -actin, as well as anti-HA agarose and dimethylformamide were from Sigma. Anti-p97 mouse affinity isolated antibodies were from BioLegend, and antibodies against EPRS from Abcam and against AIMP2, USP5 and NCKAP1 from Proteintech. Highly cross-adsorbed Alexa Fluor-488 goat anti-rabbit, Alexa Fluor-647 goat anti-mouse antibodies, and DAPI Fluoro-Pure grade were from Invitrogen. High purity, water soluble digitonin was from Calbiochem/EMD

Bioscience. AcTEV protease was from Invitrogen. Unless otherwise stated, all remaining reagents were from Sigma.

DNA constructs

cDNA for human SelS (IMAGE ID 2967406) was from Thermo Scientific. To overexpress the full-length Sec-containing SelS (189 amino acids) in mammalian cells, the ORF of SelS was cloned into XbaI/SalI restriction sites of pCI-HHT-Toxo-SECIS vector [21,22], yielding HHT-SelS (Figure 1A). This vector is a useful tool for overexpression and purification of ER-resident selenoproteins. It contains an H-2K^b-HA-TEV tag [21] that is used for efficient ER translocation of proteins (H-2K^b signal sequence from mouse MHC class I heavy chain) as well as for purification purposes (HA and TEV protease target sequences). This vector also has a *Toxoplasma gondii* Selenoprotein T SECIS element that drives the highly efficient Sec insertion in selenoproteins [22]. HHT-tag was cloned into EcoRI/XbaI restriction sites of the multiple cloning site of pCI-neo (Promega), and the *T. gondii* SelT SECIS element was cloned downstream of the multiple cloning site of this vector. Insertion of the SelS ORF between the HHT-tag (5'-end) and the SECIS element allows efficient expression of the HHT-tagged, full-length SelS. To overexpress H-2K^b-HA-TEV-tagged SelS lacking the coiled coil domain, a restriction site Eco47III was inserted in the SelS sequence instead of the coiled coil domain (amino acids 52-122) by subcloning, generating HHT-SelS-CC (Figure 1A). SelS lacking the coil coiled domain was also cloned into the XbaI/SalI restriction sites of pCI-HHT-Toxo-SECIS vector. To overexpress the cytosolic tail of SelS (amino acids 52-189), the corresponding sequence of SelS was cloned into the XbaI/SalI restriction sites of pCI-HT-Toxo-SECIS vector [21], giving HT-SelS-CT (Figure 1A). The pCI-HT-Toxo-SECIS vector contains HA-TEV tag (instead of H-2K^b-HA-TEV-tag) cloned in the EcoRI/XbaI restriction sites of the same initial pCI-Toxo-SECIS vector. QuikChange site-directed mutagenesis kit (Agilent) was used to generate SC (Cys174Ser, Sec188Cys), CC (Sec188Cys), SS (Cys174Ser, Sec188Ser) and C (truncated form, Sec188Stop) mutations in the HHT-SelS CU construct.

Cell culture, transfection and immunofluorescence microscopy

Human HEK 293T and HeLa cells (ATCC) were grown in high glucose DMEM supplemented with 10% calf serum (HEK 293T) or 10% fetal bovine serum (HeLa) and antibiotic/antimycotic solution (all from Invitrogen) at 37 °C in 5% CO₂. Transient transfection of HeLa cells was carried out using FuGENE HD (Roche Applied Science) according to the manufacturer's recommendations. HEK 293T cells were transfected by calcium phosphate co-precipitation. Immunofluorescence microscopy was performed as described previously [21]. HeLa cells were used for transfection with the SelS constructs and followed by immunostaining with anti-KDEL (ER marker), anti-SelS or anti-HA tag antibodies and DAPI (nuclear staining).

Anti-HA affinity purification and mass spectrometry analyses

5 x 10⁸ HEK 293T cells transfected with HHT-SelS CU, HHT-SelS CC, HHT-SelS C, HHT-SelS-CC and HT-SelS-CT constructs were collected in PBS buffer 48–61 h after transfection. Untransfected HEK 293T cells were used as control. The cells were lysed in ice-cold TNM buffer (1.8 % digitonin, 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM

MgCl₂) containing complete protease inhibitor tablets and 2.5 mM N-ethylmaleimide for 40 min. Affinity purification of HA-TEV-tagged SelS proteins was performed as described [23]. Samples for SDS PAGE, Western blotting and liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) analyses were prepared and analyzed as described [21]. For small-scale experiments, HEK 293T cells in 10 cm plates were transfected with HHT-SelS SC, HHT-SelS CC, HHT-SelS SS and HHT-SelS C constructs, cells were lysed in RIPA buffer, and SelS was immunoprecipitated on HA-agarose according to the manufacturer's protocol.

Anti-SelS affinity purification

Untransfected HEK 293 cells at 90 % confluency were washed twice with ice-cold PBS, scrapped and pelleted. Harvested cells were solubilized in TNM buffer containing protease inhibitors and 2.5 mM NEM for 40 min in end-to-end rotator at 4 °C. After clearing cell extracts by centrifugation, total protein was measured. The lysates were pre-cleared of materials that bind to Dynabeads ProteinG (Invitrogen) for 30 min at room temperature. For Western blotting, pre-cleared lysates were incubated with rabbit antibodies against SelS at 4 °C overnight with continuous rotation, followed by two hour incubation in the presence of Dynabeads ProteinG magnetic beads. Magnetic beads were then collected, washed five times with mild washing buffer (PBS with 0.01 % Tween-20), heated in protein sample buffer, and subjected to SDS-PAGE analyses. To prepare samples for MS/MS analyses, anti-SelS antibodies were cross-linked to Dynabeads ProteinG using BS3 according to manufacturer's instructions. Pre-cleared cell lysates were incubated with immobilized anti-SelS antibodies at 4 °C overnight with continuous rotation. Magnetic beads were collected, washed with PBS, and proteins eluted with glycine at pH 2.8. The eluted proteins were neutralized, precipitated with trichloroacetic acid, washed with cold acetone, and air-dried.

Chemical cross-linking, mass spectrometry and xQuest analysis

Affinity-purified SelS fractions were treated with a chemical cross-link as described [24,25]. An equimolar mixture of isotopically light (d0) and heavy (d12) labeled disuccinimidyl suberate (DSS, Creative Molecules) was dissolved in dimethylformamide at a concentration of 50 mM. The cross-linking reaction was performed by the addition of DSS (200 µM) to SelS-bound protein fractions eluted from HA-agarose by TEV protease. For cross-linking experiments, Tris-HCl was substituted with HEPES in TNM and TNE (0.2 % digitonin, 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 5 mM MgCl₂) buffers. The reaction mixture was incubated with mixing at 300 rpm for 30 min at 37 °C. Cross-linking was quenched by the addition of ammonium bicarbonate to a final concentration of 50 mM for 10 min at 37 °C. Efficiency of cross-linking was monitored by Western blotting of the SelS-bound protein mixtures before and after cross-linking with anti-SelS antibodies. Mass spectrometric analysis of the non-cross-linked protein complexes was used to generate a database for the *xQuest* search of cross-link spectra. High resolution MS/MS cross-linking analyses were done on the Q Exactive (Thermo). Xcalibur .raw files were converted into the open mzXML format by msconvert [26]. The *xQuest* databases established from the respective non-cross-linked affinity-purifications included the 188 validated SelS protein targets. The *xQuest* search was utilized with default parameters and *xQuest* score >20.

Bioinformatics and structural analysis

Functional protein-protein interactions were analyzed with STRING [27]. Coiled-coil domains were predicted with COILS [28]. Docking was conducted with Hex 6.12 [29], employing DARS (Decoys As the Reference State) potentials, with OPLS minimization as a post-processing procedure. As a constrain, the ligand was set to bind within 20 Å from the linker site (i.e., experimentally derived constrain, from cross link analyses); this represents a mild constrain, allowing docking a great degree of freedom. Only top scoring candidates, as ranked by Hex 6.2, were considered.

RESULTS

Strategy for unbiased identification of SelS targets

A previous yeast two-hybrid analysis identified serum amyloid A1 β (SAA1 β) as a SelS binding protein [16]. Several proteins involved in ERAD, including Derlin 1 [5], Derlin 2 [6], p97 ATPase [6,7], Hsp [30], Hrd [30], ataxin 3 [31] and SelK [21] were also found to interact with SelS based on co-immunoprecipitation experiments. In addition, a search for proteins interacting with an S-tagged SelS expressed in mammalian cells identified UBXD8, UBE4A, UBXD6, and KLHDC2, and confirmed Derlin 1, Derlin 2, p97 ATPase and SelK as SelS-interacting proteins [32]. Yet, besides an obvious link to ERAD, these interactions did not clarify SelS function. To carry out an unbiased screen for SelS-interacting proteins, we performed large-scale immunopurification of various SelS forms using an approach previously applied for ER-resident SEL1L [23] and SelK [21] proteins. We supplied SelS with an N-terminal HA-TEV tag (Figure 1A), preceded by H-2K^b, which efficiently directed the expressed SelS forms to the ER [21,23]. We then prepared constructs coding for H-2K^b-HA-TEV-tagged full-length SelS (HHT-SelS CU), Sec-to-Cys SelS mutant (HHT-SelS CC), and truncated Sec-to-Stop SelS mutant (HHT-SelS C) (Figure 1A). In addition, constructs coding for shortened forms of SelS (a form lacking the coiled coil domain, HHT-SelS-CC, and a form containing only the cytosolic tail of SelS (amino acids 52-189), HT-SelS-CT) were prepared (Figure 1A) for dissecting SelS function. All these SelS forms were then expressed in HEK 293T cells. The H-2K^b signal peptide was removed by endogenous proteases [33], wherein the expressed mature SelS forms contained only the N-terminal HA-TEV tag. The transfected cells were lysed with a mild detergent digitonin, and SelS forms (together with their interacting proteins) were enriched on anti-HA agarose beads and eluted by treatment with TEV protease (Figure 1B). An advantage of this approach is that the SelS-bound proteins were eluted with a protease, which limited elution of proteins nonspecifically bound to the beads. This resulted in high purity of SelS-interacting proteins. We noted that SelS specifically pulled down a large number of proteins. This was evident from comparison of affinity preparations of SelS versus SelK and various controls. The eluted fractions were subjected to SDS PAGE (Figure 2A). The proteins bound to various SelS forms were then identified by LC-MS/MS (Figure 2B). Depending on sample preparation, up to 600 proteins were identified in samples. By subtracting controls and applying stringent filters (Figure 2C), we narrowed down this list to approximately 200 proteins in the case of full-length SelS (Supplementary Table S1). Interaction of different SelS forms with p97 ATPase, SelK and Derlin 1 were also confirmed by Western blotting (Figure 2D).

Cellular localization of tagged SelS forms

Native SelS lacks ER targeting and retention signals and is targeted to the ER membrane due to the presence of a transmembrane domain in the N-terminal region [5]. Previous overexpression of myc-tagged SelS in COS cells altered ER morphology, resulting in long filaments [5,6], and accumulation of SelS in the Golgi-related perinuclear speckles was also reported [34]. To examine localization of HA-TEV-tagged SelS forms used in the target search, we performed immunofluorescence microscopy of HeLa cells transfected with HHT-SelS CU, HHT-SelS-CC and HT-SelS-CT constructs. The cells were stained either with anti-SelS or anti-HA-tag antibodies (Figure 3). HA-TEV-tagged SelS (Figure 3A) and HA-TEV-tagged SelS lacking the coiled coil domain (Figure 3B) co-localized with the ER-marker KDEL showing a characteristic ER staining pattern. When the transmembrane domain was deleted, the cytosolic tail of HA-TEV-tagged SelS was mainly localized in the nucleus and cytosol (Figure 3C). Overexpression of ER-anchored SelS forms altered ER morphology in some cells, resulting in long filaments (Figures 3D and 3E), as reported previously [5]. These observations were consistent with the expected ER localization of SelS forms.

Testing the role of Sec in SelS protein-protein interactions

In functionally characterized selenoproteins, Sec occurs in the active site and is directly involved in redox catalysis [4]. Recently, it was shown that two residues, Cys174 and Sec188, are responsible for the reductase [18–20] and peroxidase [19] enzymatic activities of SelS *in vitro*. Residue Sec188 was reported as the peroxidatic residue (the residue that is first oxidized by substrate into selenenic acid (Se-OH)) and Cys174 was proposed to serve as the resolving residue (the residue, which attacks the selenenic acid to form selenenylsulfide bond). It was shown that thioredoxin reduces the selenenylsulfide bond *in vitro* [18–20].

We attempted to identify proteins that could bind to different forms of SelS in a redox manner. These experiments were assisted by stabilizing possible inter-protein disulfide or selenenylsulfide bonds involving SelS and its target proteins, thereby the samples were treated with 2.5 mM N-ethylmaleimide in lysis buffer. Besides SelS itself, we tested several of its mutant forms that lacked possible catalytic or resolving Cys or Sec residues, which was expected to promote interprotein disulfide/selenenylsulfide. First, we performed large-scale affinity purification of the Sec188Cys mutant of HA-TEV-tagged SelS (CC), Sec188Stop mutant HA-TEV-tagged SelS (C, truncated form) and full-length HA-TEV-tagged SelS (CU) from HEK 293T cells, eluted the SelS forms and proteins that bind to them with TEV protease and subjected the SelS samples to LC-MS/MS. However, this approach did not identify proteins unique to SelS forms differing in Sec or predicted resolving Cys (data not shown). We further generated additional mutations in the Cys174/Sec188 redox pair of the HA-TEV-tagged full-length SelS. Four HA-TEV-tagged SelS forms were expressed in HEK 293T cells: SC (Cys174Ser, Sec188Cys), CC (Sec188Cys), SS (Cys174Ser, Sec188Ser) and C (truncated form, Sec188Stop). HA-TEV-tagged SelS proteins were purified on HA-agarose and eluted from beads by SDS PAGE sample buffer. Co-immunopurification of SelK and p97 ATPase with different SelS forms was then demonstrated by Western blotting (Figure 4A). There was no significant difference among

SelS forms in binding SelK and p97, suggesting that this binding is not controlled by the SelS redox state and that it does not involve the redox chemistry of Sec and Cys. However, as shown previously [18,19] we found that Cys174 and Sec188 (here replaced with Cys) could form an intermolecular selenenylsulfide under non-reducing conditions as evidenced by the formation of a band corresponding to the SelS dimer (Figure 4A). These observations excluded redox-dependent and Sec-dependent stable binding between SelS and p97 ATPase or SelK. More generally, our study did not detect Sec-dependent interactions involving SelS and protein complexes.

SelS co-purifies with multi-protein complexes

The set of 188 proteins identified as SelS targets following stringent CompPASS analysis (Figure 2B and Supplementary Table S1) included previously identified SelS interactors (SAA1 β , p97, SelK, Derlin 1 and Derlin 2) as well as other proteins known to be associated with ERAD (Figure 4B). In fact, seven out of eight previously identified SelS interactors involved in ERAD were found in our SelS target list (Figure 4C). The ERAD complex was not, however, the only system that was evident in the target list. The identified proteins were remarkably enriched for multiprotein complexes, including oligosaccharyltransferase (OST), multisynthetase (tRNA synthesis), coatomer (coats membrane-bound transport vesicles), exocyst (involved in vesicle trafficking), anaphase-promoting (cyclosome), nucleopore, proteasome cap, ERAD and other complexes (Figure 4D). Each of these complexes was represented by multiple proteins in the SelS target list. On the other hand, many proteins commonly found in proteomic experiments, such as ribosomal proteins, peroxiredoxins and other abundant proteins were not observed. It should be noted that the stringent criteria applied in order to narrow down SelS targets likely led to the removal of some true positive SelS interactors (e.g., if these proteins are commonly found in proteomics experiments). Gene ontology analysis of SelS targets showed a significant enrichment of functions associated with ER, Golgi and nuclear protein transport, retrotranslocation, unfolded protein response (UPR) and ubiquitin-dependent proteasomal degradation (Supplementary Table S2).

Identification of peptides directly interacting with SelS by chemical cross-linking

To determine how SelS interacts with such a great variety of proteins, fractions containing SelS targets were subjected to chemical cross-linking with an isotopically labeled homobifunctional crosslinker disuccinimidyl suberate. Efficiency of cross-linking was monitored by Western blotting of SelS in samples subjected or not to this treatment. High molecular weight protein complexes containing SelS and a known SelS target p97 ATPase were observed following crosslinking (Figure 5A). The cross-linked proteins were digested by trypsin and subjected to LC-MS/MS, and the spectra were analyzed with xQuest [24] using a database of 188 SelS targets. As expected, the majority of cross-links were mono and intrapeptide cross-links involving SelS, and the second most represented protein was p97. In addition, 14 SelS-containing interprotein crosslinks were identified by xQuest with an ID-score >20 (Table 1). As a control, the same SelS preparation, but without disuccinimidyl suberate treatment was analyzed. Only a limited number of low score spectra and no cross-links matching SelS targets were detected, indicating specificity of the xQuest analysis. To verify the detected SelS crosslinks, several proteins representing various

complexes (EPRS, AIMP2, USP5 and NCKAP1) were analyzed by Western blotting in initial, flow-through and HA-agarose bound fractions with antibodies specific for these proteins. All these proteins were enriched in SelS pull-down samples (USP5 was enriched weakly), whereas actin (control) was not (Figure 5B–F).

Interactions involving endogenous SelS

We further tested whether interactions involving overexpressed SelS could be detected with endogenous SelS. Immunoprecipitation experiments were carried out with anti-SelS antibodies in HEK 293 cells (Figure 5G). SelS could be quantitatively precipitated, whereas its interaction targets were partially enriched. The strongest signals were observed for p97 and Derlin 1, but other proteins were also detected, whereas actin (control) was not. It is possible, however, that the interactions between SelS and multiprotein complexes are somewhat enhanced by SelS overexpression. To further validate association of SelS with protein complexes identified in the SelS-overexpressing experiments, endogenous SelS was immunoprecipitated from HEK 293 cells, followed by identification of interacting proteins by LC-MS/MS. STRING analysis of top-scoring hits revealed several SelS-interacting protein complexes, such as multisynthase, proteasome cap and coatomer complexes (Supplementary Figure S1). Thus, endogenous SelS also engages in interaction with multiprotein complexes.

Structural analysis of SelS binding proteins

Almost all verified SelS targets, as well as SelS itself, had at least one coiled coil domain (Table 1). Moreover, in 5 out of 14 proteins for which interaction sites within proteins were detected (UBXN4, EPRS, ANAPC5, p97 ATPase, LRRC59), the crosslinks corresponded to the coiled coil domains or were immediately adjacent (in sequence) to them. The crosslinked sites within SelS were also found exclusively within its coiled coil domain, suggesting that SelS interacts with target proteins through this domain.

To get further insights into how SelS interacts with its targets, we subjected SelS and some of its targets to computational structural analysis (Figure 6, top scoring poses are shown). Among the 14 proteins mentioned above, 4 had sufficient structural information for docking simulations (known structure, model covering the full-length protein): UBP5, NCKP1, CYB5R3, and p97 ATPase, including three (UBP5, NCKP1, p97 ATPase) with coiled coil domains. We chose a representative of these coiled coil proteins (p97 ATPase) and CYB5R3 (as a protein without the coiled coil domain) for detailed docking simulations with SelS. These analyses suggested high affinity of the interacting regions, i.e., the top scoring SelS/target models pointed directly toward the regions connected by the crosslinker. The structural analyses also provided further clues on the binding modes. Specifically, the binding between SelS and p97 (Figure 6A) appeared to involve the interaction between parts of their coiled coil domains (GLEVDKRELQELVQY in p97 ATPase, and ALAAARLKMQEELN in SelS). CYB5R3 bound SelS with a different mode due to the lack of the coiled coil (Figure 6B): here, the sequence RQEALAAARLKM of SelS made contact with KDPDDHTV and NKHSAR in the target protein. A considerable similarity between poses in Figures 6A and 6B points to the role of the sub-sequence ALAAARLKM (located within the coiled coil domain) of SelS, which was directly involved in the contact with both

proteins. Overall, the N-terminal and middle parts of the coiled coil domain of SelS were most prone to interaction with its partners. It also appears that the SelS coiled coil domain can engage partners without coiled coil regions, perhaps relying on other features (e.g., basic residues in SelS interacting with acidic residues in the targets). Since SelS is a membrane protein, and the complexes are cytosolic, the interactions involving SelS cytosolic tail (Figure 6) could be pivotal in anchoring these complexes to the ER membrane.

DISCUSSION

SelS is a 189 amino acid protein with a single transmembrane domain in the N-terminal region. It has a short ER luminal segment (27 amino acids), a transmembrane domain (24 amino acids) and a longer (132 amino acids) cytosolic tail containing Sec in the C-terminal penultimate position [2]. While the specific function of SelS is not known, an earlier experiment involving co-precipitation of a GST-fused fragment of SelS (amino acids 48-187) with p97 ATPase suggested a functional link between these proteins [5]. SelS localizes to the ER and plasma membranes [2,5], and its cytosolic tail contains a coiled coil domain (amino acids 52-122) that may be responsible for binding other proteins and/or for SelS dimerization [5]. The C-terminal region of SelS does not have a pronounced secondary structure and was hypothesized to bind an unknown negatively charged target and/or serve as an oxidoreductase [18,20].

At a more physiological level, SelS expression is markedly increased by fasting (in the liver of diabetic animals) and is down-regulated (in liver, adipose tissue, and skeletal muscle) in fed state of diabetic animals [15]. Consistent with animal studies, SelS is dysregulated in diabetes patients [16,17]. A yeast two-hybrid screen revealed an interaction of SelS with serum amyloid A1 β (SAA1 β), an acute-phase inflammatory response protein [16]. An additional evidence for the role of SelS in inflammation came from a study [14] that associated a SelS promoter polymorphism, 105G $\rightarrow$ A, with higher levels of proinflammatory cytokines, IL-6, IL-1 β and TNF- α , in plasma of patients with chronic inflammation. Moreover, knockdown of SelS in RAW264.7 macrophages increased the release of IL-6 and TNF- α [13], whereas treatment of HepG2 cells with IL-1 β and TNF- α up-regulated SelS expression [14]. Based on these data, a regulatory loop was proposed wherein cytokines stimulate SelS expression, which in turn decreases cytokine production [14].

It was shown that SelS serves as a component of the ERAD system, a pathway that targets misfolded or unfolded proteins and transports them from the ER to the cytosol, followed by ubiquitin-proteasome degradation [5,35]. In this pathway, SelS was proposed to mediate the interaction between cytosolic p97 and integral ER-membrane proteins, such as Derlins [5,6]. On the other hand, it was shown that the interaction between p97 ATPase and Derlin 1 was not affected when SelS levels were reduced by shRNA [7]. In addition, an integral ER-membrane protein UBXD2 (Erasin) was suggested to mediate Derlin/p97 interactions [36]. Thus, the proposed function of SelS (i.e., binding between p97 ATPase and Derlins, anchoring cytosolic p97 ATPase to the ER membrane for efficient ERAD processing) may be dispensable and additional SelS partners need to be identified to elucidate SelS function in retrotranslocation or in others processes. In this regard, we previously identified SelK as a SelS interacting partner and an ERAD component [21]. The two selenoproteins share

domain organization and interacting partners (e.g., Derlins), but the specific functions are not known for either protein.

To clarify SelS function, we carried out large-scale affinity isolation of SelS from HEK 293T cells. The experiments were done under mild conditions involving digitonin-treated lysates, ensuring preservation of membrane-bound protein-protein interactions. We observed a dramatic increase in eluted proteins when SelS was used as bait (in contrast to other proteins as well as SelK [21]), and this enrichment occurred regardless of the presence of residues (Cys174/Sec188) that are involved in the predicted redox function and peroxidase activity of SelS [18–20]. Our strategy did not reveal proteins that interact with Cys174 and Sec188 residues. However, we identified numerous proteins that interact through the SelS coiled coil domain.

An unexpected finding from our study is that SelS specifically associates with multi-protein complexes anchoring them to the ER membrane. This is evidenced by unbiased identification of interaction partners of SelS and determining sites within these proteins involved in the interaction. Our findings implicate SelS in diverse cellular processes, in which it participates by supporting maintenance and transport of multiprotein complexes. Indeed, several tRNA synthetases were detected that take part in the multi-synthetase complex, several proteins of the proteasome cap (but not the proteasome itself), several oligosaccharyltransferase protein components as well as components of coatomer, exocyst, nucleopore, cyclosome and other multi-protein complexes. At the same time, many abundant cytosolic proteins were not detected at all. We also did not detect components of very large structures, such as ribosomes. These observations suggest that our protein preparations were of high quality and that the identified proteins are true SelS targets.

Why would so many proteins interact with SelS? One common theme among these protein complexes is their role in membrane-based intracellular transport. It is also possible that other components are involved in many of these interactions. One such protein may be p97 ATPase, a known SelS target, which had the most total spectral counts in the SelS preparations. Alternatively, SelS may be involved in storing multi-protein complexes. Assembly, processing and reconstitution of such complexes is an energy-expensive process, but very little is known about how multi-protein complexes are formed, disassembled and replaced. In any case, it is very likely that SelS does not interact directly with all proteins identified in our study. It interacts with specific components of various multi-protein complexes, whereas other proteins in the affinity preparations appear because they are components of these complexes.

To get further insights into the SelS/target protein interactions, we carried out cross-linking experiments and identified specific interacting sites within SelS and its targets. Fourteen such proteins were found, and most of them had predicted coiled coil regions. Moreover, in five of these proteins as well as in SelS itself, the interaction sites were within the coiled coil regions or immediately adjacent to them. These data, along with the fact that coiled coil regions are frequently used in protein-protein interactions, suggest that SelS interacts with components of multiprotein complexes through its coiled coil domain.

We carried out molecular modeling simulations of SelS interactions with selected targets (one with coiled coil domains, p97 ATPase, and one without, CYB5R3) to clarify these interactions. The results were well consistent with the experimental information (i.e., the best docking poses pointed directly to the cross-link regions), and showed that SelS binds its targets through its coiled coil domain (particularly within the ALAAARLKM subsequence). In the case of p97 ATPase, our simulations confirmed the active role of coiled coil interactions: the predicted binding mode is consistent with a direct involvement of SelS coiled domain in anchoring its targets to the ER membrane. But the data also suggest that the coiled coil of SelS domain may also engage in other interactions.

The findings of our study lead to new questions. Why is it important to transiently anchor multiprotein complexes to the ER membrane? Are other proteins involved in maintenance of these complexes and what are they? Does SelS binding support transport, storage, assembly, disassembly or some other process in multi-protein complex functions? How does SelS overexpression or knockdown affect the functions of the protein complexes? Does SelS function as an oxidoreductase *in vivo* and what are its targets? All functionally characterized selenoproteins are oxidoreductases with Sec being the catalytic redox-active residue, and the Cys174/Sec188 pair of SelS was proposed to be a redox motif [18,20]. However, SelS does not have a globular structure and its sequence has low conservation, so by itself it unlikely can function as an enzyme. An attractive possibility is that SelS is a cofactor for another protein and that Sec is involved in repair or protein modifications that require a redox function. It is also known that dietary selenium may elicit sex-specific effects and be associated in a sex-specific way with supplementation effects or disease risks. In this regard, it has been shown that SelS expression and regulation are sexually dimorphic [37]. Whether SelS binding to multiprotein complexes is altered by sex is unknown. Further studies should address all these questions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

FUNDING

This work was supported by the National Institute of Health (NIH) [grant number GM061603] to VNG.

Abbreviations

SelS	selenoprotein S
SelK	Selenoprotein K
ER	endoplasmic reticulum
ERAD	ER-associated degradation
HEK 293	human embryonic kidney cells

RPN	ribophorin
HA	hemagglutinin
TEV	Tobacco Etch Virus
IP	immunoprecipitation
DSS	disuccinimidyl suberate

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Figure 1. Design of the study to identify SelS targets

A) Schematic representations of SelS fusion proteins. Various forms of HHT-tagged SelS were cloned into pCI-ToxoSECIS vector resulting in HHT-SelS CU, HHT-SelS CC and HHT-SelS C constructs. SelS constructs without the coiled-coil domain (HHT-SelS-CC) or containing only the cytosolic part of SelS without H2-K^b (HT-SelS-CT) were prepared as described in the *Experimental section*. An asterisk shows the site for Eco47III restriction enzyme introduced in the SelS sequence.

B) Schematic representation of the SelS target search. HeLa cells were transfected [1] with various SelS constructs, subjected to fluorescent microscopy analysis [2.1]. HEK 293T cells were transfected [1] with the same constructs and used for large-scale affinity purification of SelS and its binding proteins on anti-HA-agarose followed by elution with TEV protease [2.2, 3, 4]. The eluted proteins were subjected to SDS-PAGE [5], and prepared [6,7] for LC-MS/MS [7] or cross-linking [8]. Mass-spectrometry data were filtered and analyzed [9] by Sequest and CompPASS to generate the SelS target list used for cross-linking analysis by xQuest. Crosslinked proteins were verified by Western blotting [10].

Figure 2. Analysis of SelS-binding proteins

A) Silver staining of SDS-PAGE performed on SelS samples eluted with TEV protease (corresponding to step 5 in Figure 1B). HEK 293T cells were left untransfected (lane 1) or transfected with the constructs coding for HHT-SelS CU (lane 2), HHT-SelS- Δ CC (lane 3) and HT-SelS-CT (lane 4).

B) SDS-PAGE analysis of SelS target preparations. SelS samples and control (SelS CU, SelS C, HEK untransfected) were fractionated by SDS-PAGE, and proteins were stained with silver staining. Positions of TEV protease and SelS are indicated by arrows on *right* and molecular weights in kDa are shown on *left*.

C) Flow chart illustrating analysis of mass-spec data for identification of SelS-interacting proteins.

D) Western blotting of SelS samples shown in Figure 2A with the corresponding antibodies (SelS - *top panel*, p97, SelK and Derlin 1 - *bottom panels*). Positions of SelS forms are indicated by arrows on *right* and molecular weights in kDa are shown on *left*.

Figure 3. Cellular localization of SelS

A) HeLa cells were transfected with the construct coding for the ER-targeted H2-K^b-HA-TEV-SelS.

B) HeLa cells were transfected with the construct coding for the ER-targeted H2K^b-HA-TEV-SelS that lacked the coiled-coil domain.

C) HeLa cells were transfected with the construct coding for the ER-targeted H2K-HA-TEV-SelS that lacked the transmembrane domain (TM).

D) HeLa cells were transfected with the construct coding for the ER-targeted H2K-HA-TEV-SelS.

E) HeLa cells were transfected with the construct coding for the ER-targeted H2K-HA-TEV-SelS that lacked the coiled-coil domain. Transfected cells were stained with SelS or HA antibodies (green) and KDEL antibodies (red) (*left* and *middle* panels). Nuclei were stained with DAPI (blue) and shown in the merged images (*right* panels).

Figure 4. Isolation of SelS-interacting protein complexes

A) HEK 293T cells were transfected with pCI-ToxoSECIS (vector) or with various HA-tagged SelS forms (see descriptions in the text). The SelS interacting proteins were isolated on HA-agarose and specific binding of SelS targets was assessed by Western blotting under non-reducing conditions with SelS (*upper* panel), SelK (*middle* panel) or p97 (*lower* panel) antibodies. Position of SelS dimers is shown with *asterisk*.

B) Known proteins involved in ERAD were visualized by STRING and the identified SelS targets indicated by red circles. Proteins with cross-links to SelS are highlighted with a black circle.

C) Previously identified SelS targets were visualized by STRING, and the SelS targets found in the current study are highlighted with red circles.

D) SelS interacts with diverse multiprotein complexes. Protein complexes were visualized by STRING. SelS targets are indicated by red circles. Proteins with cross-links to SelS are highlighted with black circles. *OST* – oligosaccharyltransferase complex, *MS* – multisynthetase complex, *APC* – anaphase-promoting complex, *NP* – nuclear pore complex.

Figure 5. Analysis of SelS-interacting proteins identified by cross-linking

A) HEK 293 cells were transfected with HA-tagged SelS (HHT-SelS CC), and SelS targets isolated on HA-agarose and treated with a cross-linker. Efficiency of cross-linking was analyzed with SelS (*left* panel) and p97 (*middle* panel) antibodies. Coomassie Brilliant Blue (CBB) stained membrane is shown on *right*. Molecular weight markers in kDa are shown on *left*.

B–E) SelS samples (initial - *input*, flow-through – *flow*, final – *IP:HA*) prior to crosslinking were analyzed by Western blotting with (B) EPRS, (C) AIMP2, (D) USP5 and (E) NCKAP1 antibodies. Actin served as a control.

F) Coomassie Blue stained membrane (*upper* panel) and Western blotting with SelS antibodies (*lower* panel) are shown on *right*.

G) SelS was immunoprecipitated and the samples analyzed by Western blotting with indicated antibodies. Arrows (on *right* of each blot) indicate the expected migration of proteins (indicated on *left* of each blot). Input – initial sample. IP:control – sample treated as IP:SelS except that no SelS antibodies were added during the procedure. Flow - sample after treatment with SelS antibodies. IP:SelS – proteins immunoprecipitated with SelS antibodies.

Figure 6. Binding modes of SelS

Two representative proteins have been selected among SelS targets for docking calculations: one containing a coiled coil domain (p97 ATPase, PDB structure: 3CF3; panel A) and one without it (CYB5R3/NADH-cytochrome b5 reductase 3, PDB structure: 1UMK; panel B). In both panels, SelS is shown in yellow, and the docking partner (p97, CYB5R3) in light grey (secondary structure only shown, for clarity). Contact regions of binding partners are presented as molecular surfaces; linked regions (from cross-link experiments, SRVAILKANL in p97, and RNKHSA in CYB5R3) are shown in bright red. Finally (panel A only), dark red colored sections of both SelS and p97 highlight interactions directly involving coiled coil domains in both proteins.

Table 1

SelS targets identified following cross-linking

Protein ^a	ID Score ^b	UniProt ^c	Length (a.a.) ^d	CC domain ^e	IP/WB ^f
<i>SelS</i>	-	<i>Q9BQE4</i>	<i>189</i>	+++	Yes
EPRS	38.13	P07814	1512	++	Yes
USP5	36.99	P45974	858	++	Yes
AIMP2	33.3	Q13155	320	+	Yes
UBXN4	30.57	Q92575	508	+++	
NCAPD2	29.55	Q15021	1401	++	
NB5R3	26.52	P00387	301		
UBR4	26.44	Q5T4S7	5183	+	
LRC59	25.47	Q96AG4	307	++	
ANAPC5	24.67	Q9UJX4	755	++	
PRKDC	23.75	P78527	4128	+	
EXOC4	22.94	Q96A65	974	++	
HECTD1	22.26	Q9ULT8	2610	++	
GBF1	21.81	Q92538	1859		
NCKP1	20.59	Q9Y2A7	1128	++	Yes

^aProteins identified by crosslink with SelS.

^b₁Quest score.

^cUniProt ID number.

^dLength of polypeptide in amino acids.

^ePresence of the coiled coil (CC) domain in protein sequence.

^fIP/WB, immunoprecipitation/Western blotting evidence of interaction.