



HHS Public Access

Author manuscript

Biol Trace Elem Res. Author manuscript; available in PMC 2020 November 01.

Published in final edited form as:

Biol Trace Elem Res. 2019 November ; 192(1): 60–68. doi:10.1007/s12011-019-01774-8.

Molecular mechanisms by which selenoprotein K regulates immunity and cancer

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Abstract

Many of the 25 members of the selenoprotein family function as enzymes that utilize their selenocysteine (Sec) residues to catalyze redox-based reactions. However, some selenoproteins likely do not exert enzymatic activity by themselves, and selenoprotein K (SELENOK) is one such selenoprotein family member that uses its Sec residue in an alternative manner. SELENOK is an endoplasmic reticulum (ER) transmembrane protein that has been shown to be important for ER stress and for calcium-dependent signaling. Molecular mechanisms for the latter have recently been elucidated using knockout mice and genetically manipulated cell lines. These studies have shown that SELENOK interacts with an enzyme in the ER membrane, DHHC6 (letters represent the amino acids aspartic acid, histidine, histidine, and cysteine in the catalytic domain), and the SELENOK/DHHC6 complex catalyzes the transfer of acyl groups such as palmitate to cysteine residues in target proteins, i.e. palmitoylation. One protein palmitoylated by SELENOK/DHHC6 is the calcium channel protein, the inositol 1,4,5-trisphosphate receptor (IP3R), which is acylated as a means for stabilizing the tetrameric calcium channel in the ER membrane. Factors that lower SELENOK levels or function impair IP3R driven calcium flux. This role for SELENOK is important for the activation and proliferation of immune cells, and recently a critical role for SELENOK in promoting calcium flux for the progression of melanoma has been demonstrated. This review provides a summary of these findings and their implications in terms of designing new therapeutic interventions that target SELENOK for treating cancers like melanoma.

Keywords

palmitoylation; calcium; selenium; selenoprotein; inositol 1,4,5-trisphosphate receptor; cancer therapy

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Introduction

Selenium (Se) is an essential dietary trace mineral that is important for various aspects of human health [1]. The biological effects of Se are mainly exerted through its incorporation into selenoproteins as the 21st amino acid, selenocysteine (Sec) [2]. The 25 members of the selenoprotein family exhibit a wide variety of functions including the control of reactive oxygen species and cellular redox tone, regulating thyroid hormone metabolism, facilitating sperm maturation/protection, and promoting optimal immunity [3]. Many selenoproteins exhibit a common thioredoxin-like fold within their catalytic domains comprised of a conserved cysteine (Cys)-X-X-Sec (CXXU) or Cys-X-X-Ser [4,5]. Many of these selenoenzymes carry out well defined catalytic activities (e.g. glutathione peroxidases, thioredoxin reductases, iodothyronine deiodinase, methionine sulfoxide reductases), while and other CXXU-containing selenoproteins have been proposed to serve as oxidoreductase enzymes [2]. Selenoprotein K (SELENOK) does not contain a CXXU motif nor does it appear to catalyze redox reactions like other established selenoenzymes. In fact, the intrinsically disordered nature of SELENOK [6] suggests its function is dependent on the partner proteins with which it complexes. The role of intrinsically disordered domains in proteins serving as docking platforms for signaling molecules or binding proteins has been established for other nonenzymatic proteins [7]. Indeed, much of what has been learned about SELENOK function has been gained by examining its binding partners [8–10]. This review will summarize what has been discovered regarding SELENOK function and possible therapeutic approaches that may center on this selenoprotein.

Tissue distribution, localization, Se-dependence of SELENOK

Dietary [Se] regulates the expression of selenoproteins at the levels of mRNA and protein. Under conditions of low Se status, the translation of selenoproteins stalls at the Sec-encoding UGA codon and both the mRNA and truncated protein get degraded through nonsense-mediated decay and destruction via C-end degrons, respectively [11,12]. Moreover, dietary [Se] impacts expression levels of different selenoproteins to different degrees [13]. This concept is part of what is referred to as ‘the hierarchy of selenoproteins’ and the ranking of least to most sensitive Se sensitive selenoproteins in this hierarchy depends on the tissue involved and varies between cell lines [14,13]. Differences between selenoproteins in sensitivity to limiting [Se] was first demonstrated by experiments comparing of the activities of glutathione peroxidases 1 and 4 (GPX1 and GPX4) in rat liver, kidney, and heart [15]. Under conditions of Se deficiency, GPX1 activity fell sharply while GPX4 activity was better maintained, and tissues showed varied differences between these two GPXs. It must be noted that, in addition to the tissue involved, sex also influences the selenoprotein hierarchy [16]. Our data in mice suggests that SELENOK levels are similar between male and females [17]. Also, there is high sequence homology between mouse and human SELENOK at the protein level. SELENOK amino acid sequences from human and mouse share 91% identity [18], which supports functional investigations using mouse models in combination with human cell lines.

While many selenoproteins exhibit some degree of tissue specificity, SELENOK mRNA and protein are widely expressed throughout mouse tissues, although particularly high levels

have been found in lymphoid tissues [19,17]. These results are consistent with findings from the Human Protein Atlas, where SELENOK protein was shown to be widely distributed but highest in the gastrointestinal tract that contains many lymph nodes, the immune system, and lung that is rich in mucosal lymphoid tissues [20]. Northern blot data from one report suggested high levels of expression in the heart [21], but this has not been supported by subsequent studies. SELENOK has been shown to exhibit altered levels of expression in response to changes in Se status in humans and mice [22,17], but it is not known exactly where SELENOK lies within the selenoprotein hierarchy. In some tissues like mouse colon and spleen, SELENOK was not found to be Se sensitive [23,24]. One study using human lymphocytes isolated from human donors before and after Se supplementation showed that SELENOK mRNA was among three selenoprotein transcripts upregulated after supplementation [25]. SELENOK has not been found to be upregulated in cancers like melanoma, despite an apparent dependence on SELENOK function for growth and metastasis [26]. The sensitivity of SELENOK levels to bioavailable Se very likely depends on tissue and cell type, with tissues such as the brain, muscle and testes receiving 'priority' for bioavailable Se to express SELENOK under Se deficiency at a cost to other tissues such as those comprising the immune system [13]. The 'bioactivity' of SELENOK has not yet been compared between tissues or under different [Se] conditions. Overexpression of GFP-tagged SELENOK in HeLa cells showed localization in the endoplasmic reticulum (ER) [21]. Subsequently, immunofluorescent staining of primary human monocytes confirmed colocalization of SELENOK with the ER marker KDEL and cell fractionation data also showed ER localization [17].

The role of SELENOK in regulating ER stress and Calcium Flux

The ER is a crucial organelle in which protein-folding takes place as well as maturation of secreted and membrane proteins. In many cases the correct folding and processing of proteins in the ER may not effectively occur and the errant proteins must undergo ER-associated degradation (ERAD) so that ER stress does not build up [27]. ER homeostasis is tightly regulated and may involve one or more of the ER-resident selenoproteins: type 2 iodothyronine deiodinase and selenoproteins F, K, M, N, S, and T [28]. SELENOK has been shown to participate in ERAD by binding in an ERAD complex with other proteins such as Derlin-1 [10]. Importantly, the gene encoding SELENOK contains a functional ER stress response element and SELENOK was found to be upregulated by treatment of cells that cause misfolded protein accumulation in the ER [5]. Along with another selenoprotein, SELENOS, SELENOK participates in ERAD by recruiting cytosolic valosin-containing protein (VCP/p97) to promote translocation of misfolded proteins from the ER lumen [8]. It remains unclear how the Sec residue may or may not participate in retrograde translocation of misfolded proteins and in SELENOK knockout mice there are no apparent signs of ER stress [29,17]. The latter finding suggests some redundancy or compensation, perhaps involving SELENOS, may occur in the absence of SELENOK expression to mitigate ER stress.

A major finding with the SELENOK knockout mice was that a deficiency in this selenoprotein led to an impaired flux of calcium from the ER in activated immune cells. In particular, ex vivo experiments in our laboratory suggested that SELENOK was crucial for

promoting store-operated calcium entry (SOCE) in T cells, neutrophils, and macrophages stimulated with chemokines and other reagents that trigger G-coupled protein receptor signaling in these immune cells [17]. The impaired SOCE in SELENOK knockout immune cells resulted in 50% decreased levels of activation as determined by downstream activation markers, and lowered immunity in knockout mice led to higher levels of death during West Nile virus infection compared to controls [29,17]. The molecular mechanism by which SELENOK regulates SOCE was not clear in these initial studies, but subsequent investigations revealed a specific role for SELENOK in the posttranslational modification (i.e. palmitoylation) of the calcium channel protein, the inositol 1,4,5-trisphosphate receptor (IP3R) [30]. SELENOK was found to be crucial for stable expression of IP3R in the ER membrane and thereby a critical regulator of SOCE through this calcium channel protein. The details of these findings are described in more detail below.

The role of SELENOK in promoting protein palmitoylation

Our investigation into the role that SELENOK plays in the immune system during atherosclerosis revealed that SELENOK was required for lipid raft localization of the oxidized low density lipoprotein receptor, CD36, on macrophages [31]. Using mouse knockout models and bone-marrow transplants, SELENOK-deficient macrophages were found to have decreased CD36 palmitoylation, which occurs at the ER membrane and is crucial for stabilizing CD36 expression and its clustered expression in the plasma membrane [32]. S-palmitoylation of cysteine residues is a post-translational modification occurring on integral and peripheral membrane proteins that contribute to membrane association [33]. Our laboratory subsequently found that palmitoylation of other proteins required SELENOK including Arf-GAP with Src homology 3 (SH3) domain, ankyrin repeat, and plekstrin homology domain-containing protein 2 (ASAP2) and the IP3R [34,9]. There are very likely other proteins that required SELENOK for this posttranslational modification. Protein palmitoylation involves the addition of the 16-carbon fatty acid, palmitate, to cysteine residues on target substrate proteins through a thioester bond [35]. Since SELENOK itself is not an enzyme, it was apparent to us that it must bind to a partner enzyme to promote palmitoylation. A major clue to this puzzle was the fact that SELENOK is the only selenoprotein with an SH3 binding domain and that one member of the enzyme family involved in palmitoylation reactions contained an SH3 domain. Indeed, SELENOK interacts with the acyltransferase, DHHC6 (letters represent the amino acids aspartic acid, histidine, histidine, and cysteine in the catalytic domain), in the ER membrane through SH3/SH3 binding domain interactions and this is how SELENOK participates in palmitoylation reactions [9].

The specific mechanism by which SELENOK exerts its effects on DHHC6 to increase its catalytic efficiency required new tools to be built so that the enzymatic reaction of acyl transfer could be examined in more detail. The palmitoylation reactions catalyzed by DHHC enzymes have been shown to proceed via a two-step ping-pong mechanism [36]. In the first autopalmitoylation step, a Cys residue within the DHHC catalytic domain reacts with palmitoyl-CoA to form a palmitoyl-PAT intermediate. In the second step, the palmitoyl group is transferred from the PAT to a Cys residue on the target protein. The first step is rapid, whereas the second is much slower. Thus, after the first step, the unstable thioester

bond between Cys on the PAT and the palmitic acid may hydrolyze before transfer of the palmitic acid to a target protein, termed the “futile cycle” [37]. Our laboratory was able to synthesize soluble versions of DHHC6 and shown that they bind recombinant SELENOK in a complex that exhibited acyl transferase activity. The data from these experiments showed that SELENOK increases the catalytic efficiency of palmitoylation by stabilizing the acyl-DHHC6 intermediate and protecting it from hydrolysis, thereby preventing the futile cycle [38]. This binding of a PAT enzyme with a cofactor to promote intermediate stabilization was previously found in another protein pair in yeast. In particular, the yeast ethylene-responsive factor-2 (ERF2) is an ortholog for mammalian DHHC9 and binds to yeast ERF4 in a manner that stabilizes the acyl-ERF2 intermediate and increases its catalytic efficiency [39]. ERF4 is not a selenoprotein (yeast do not express selenoproteins), so the manner in which the two cofactors increase catalytic efficiency must be different. As discussed below in more detail, the Sec residue in SELENOK plays a critical role in promoting palmitoylation.

The Sec residue in SELENOK is responsible for its bioactivity

SELENOK is a relatively small protein (94 amino acids) that is localized to the ER membrane (Fig. 1). This single spanning transmembrane has a short ER luminal domain and a longer cytosolic domain containing the Sec residue. The topology of this transmembrane protein was first described for *Drosophila* SELENOK (G-rich protein) showing the N-terminus in the lumen with no N-terminal signal peptide [40]. The Sec residue is located near the C-terminus of SELENOK (amino acid 92), which is a feature found in approximately one-third of the selenoproteins [41]. While the Sec residue has been shown to exhibit some antioxidant activity in vitro [42,21], a role for regulating redox reactions in vivo has not been established. As described above, we generated SELENOK knockout mice and found impaired SOCE in T cells, neutrophils, and macrophages stimulated with chemokines and other reagents that trigger G-coupled protein receptor signaling in these immune cells [17]. Importantly, when these immune cells were stimulated with thapsigargin, a drug that induces SOCE in a manner that bypasses the IP3R, the wild-type and knockout cells did not differ. This implicated the IP3R as a molecule somehow involved in SELENOK-dependent SOCE.

The exact mechanism by which SELENOK regulated SOCE was not initially clear, although the requirement of the Sec residue in this process became evident when we subsequently found that a calpain protease (calpain-2) that cleaved the C-terminal end of SELENOK (residues 81–94, including Sec₉₂) away from the remaining protein abolished the SOCE promoting activity of SELENOK [43]. Interestingly, resting macrophages actively cleave SELENOK via calpain-2 to maintain low levels of calcium flux, and upon activation the endogenous inhibitor of calpains, calpastatin, is upregulated to increase levels of full-length SELENOK and endow the cells with full potential of calcium flux via IP3R. We subsequently used CRISPR/Cas9 to generate cell lines lacking the 81–94 amino acid portion to confirm that this region is the functional domain of SELENOK [26]. Cells lacking the functional domain of SELENOK were defective in generating calcium flux through the IP3R in a manner similar to those with active calpain-2 cleavage of SELENOK. In this sense, dietary Se along with the calpain-2/calpastatin system are both regulators of SELENOK

function. In fact, the endogenous inhibitor of calpain-2, calpastatin, protects SELENOK from cleavage and thereby serves as a positive regulator of SELENOK function [43,44]. Dietary Se levels regulate the levels of Sec incorporation into SELENOK while calpain-2 regulates cleavage of the functional domain. To date, no other selenoproteins have been described as being proteolytically modulated by calpain enzymes, although investigations into this issue have not been extensive.

The definitive experiments showing the requirement of the Sec residue at position 92 of SELENOK for its function were recently published as described above. In particular, recombinant versions of SELENOK containing either Sec₉₂, Cys₉₂, or Ala₉₂ complexed to a soluble version of DHHC6 were used in PAT reactions to test the bioactivity of each. Our results showed that only Sec₉₂ improved the catalytic efficiency of DHHC6, while Cys₉₂ or Ala₉₂ were equally insufficient to allow SELENOK to increase the catalytic efficiency of DHHC6. Selenoester bonds have been shown to have high reactivity in acyl transfer reactions [45]. This is consistent with the Sec residue in SELENOK being particularly suited to combine with DHHC6 for catalyzing protein palmitoylation as an important post-translational modification, and the fact that our studies showed that Sec cannot be replaced with Cys for protecting the thioester bond in the acyl-DHHC6 intermediate from hydrolysis. The role SELENOK plays in palmitoylation reactions and how it is regulated are illustrated in Figure 2.

SELENOK in cancer

The exact role of SELENOK in carcinogenesis or tumor progression has not been well studied. The effects of SELENOK loss-of-function or gain-of-function in different tumor cell lines has been studied to a limited extent. For example, reducing SELENOK in human choriocarcinoma cells increased the proliferative, migratory, and invasive capacity of these cells while over-expressing SELENOK had the opposing effect [46]. Similarly, overexpression of SELENOK in human gastric cancer BGC-823 cells inhibited cell adhesion and migration [47]. Studies in other cancer cells have shown that reduced SELENOK levels reduced proliferation and migration [26]. Micro RNAs may also play a role in regulating SELENOK levels in certain cancers [48], and this may depend on the type of cancer or may even vary between individual cancer cells within tumors. Given that SELENOK complexes with other protein partners to exert its functions, manipulating the levels of SELENOK may perturb the stoichiometry of the members within complexes. Thus, it may be that the increases or decreases in SELENOK that alter the optimum level within complexes can disrupt functions required by different cancer cells at different stages, or different cell lines immortalized at different stages. Investigation into the mutational status of SELENOK in various cancers using The Cancer Genome Atlas Program (TCGA) revealed 37 somatic mutations in *SELENOK* from 39 cancer associated cases [49]. Most mutations occurred within either the 5' or 3' UTR. However, several mutations were observed in the protein-coding region of *SELENOK*, resulting in 1 frameshift mutation, 9 missense mutations, and 1 mutation that occurred within a splice donor site of *SELENOK*. Additionally, 853 individual cases were affected by 819 copy number variation events in *SELENOK* whereby most resulted in loss of function. On the other hand, patients involved in a study of germ cell neoplasms demonstrated mostly an increase in *SELENOK* copy number.

When different cell-types transform into malignant tumor cells, calcium homeostasis and calcium-dependent signaling may be altered in a process referred to as calcium remodeling [50]. SOCE and the downstream calcium-dependent signaling cascades are exploited by cancer cells to increase their capacity to proliferate, invade and migrate. Because SOCE is important for both primary tumor progression and metastasis to other tissues, molecules regulating SOCE may serve as important targets for developing anti-cancer therapeutics. In fact, cancer cells may develop a stringent dependence on highly efficient SOCE along with the signaling pathways and gene expression programs shaped by calcium remodeling [51,52]. The importance of calcium flux in the regulation of melanoma proliferation and cell migration was previously suggested by the knockdown of stromal-interacting molecule 1 (STIM1) or Orai1 in human melanoma cell lines as well the use of a pharmacological inhibitor of SOCE [53]. Similar effects were shown for breast tumor cells and glioblastoma cells [54,55]. Our findings using cell-based assays and animal models of melanoma indicated that SELENOK was required for progression of melanoma [26]. In particular, our in vitro and in vivo findings suggest that melanoma requires SELENOK expression for the IP3R dependent maintenance of stemness, tumor growth and metastatic potential. This suggests that SELENOK may represent a new potential therapeutic target for treating melanoma and possibly other cancers.

Designing and testing drugs that inhibit SELENOK function

Although published data suggest that targeting SELENOK with inhibitors may be an effective approach to treating cancers that rely on SOCE for disease progression, this approach presents several challenges. Our unpublished work has established that anti-SELENOK antibodies targeting the C-terminal portion of SELENOK can interfere with the palmitoylation reaction catalyzed by the SELENOK/DHHC6 complex. Thus, antibody-based inhibitors (i.e. immunotherapeutics) may represent an effective form of therapy to treat melanoma or other cancers. However, SELENOK is an intracellular protein and this renders standard immunotherapeutic approaches that rely on antibody-based treatment unrealistic since targets for these drugs are conventionally extra-cellular proteins. Reassessment of intracellular proteins as immunotherapeutic targets like SELENOK may need to be prioritized since intracellular molecules constitute nearly half of the human proteome and represent an untapped reservoir of potential therapeutic targets [56]. Even though antibodies may exist that recognize an intracellular protein of interest discovered through mechanistic studies, the promising new field of immunotherapy is not a viable option due to the hurdles of delivering large antibodies (Ab ~150 kDa) or other antibody fragments (Fab ~50 kDa, scFv ~25 kDa) into cells to inhibit these proteins. In addition, the multiple chains comprising Ab reagents also make recombinant production of biologics very challenging. One possible way to overcome these barriers involves a novel strategy that combines two emerging technologies: single domain antibodies (sdAbs, a.k.a. nanobodies) from camelids [57,58] and cell penetrating peptides (CPPs) [59–61]. In theory, these small versions of Abs exhibit the high specificity of conventional Abs required for effective therapeutics. In fact, studies have demonstrated the use of humanized sdAb fused to CPP to effectively inhibit their intracellular targets. For example, CPP-sdAb directed against different hepatitis C virus proteins inhibited viral replication within cells [62–64]. Our

laboratory is currently exploring this approach for the development of intracellular immunotherapeutics to inhibit SELENOK, and this approach may yield a novel set of inhibitory reagents.

Synthesis of small molecules that bind to vital regions of target proteins is a more conventional approach for developing inhibitors of intracellular proteins like SELENOK. However, this approach also presents challenges, such as a need for insight on the structure of the target protein. Gaining structural information on SELENOK for designing small molecules to inhibit its function is limited by the fact that the exposed, cytosolic portion of SELENOK is intrinsically disordered [42]. Intrinsically disordered proteins have long been challenging for the field of inhibitor development, but some new technologies are emerging to overcome the lack of structural detail. Small molecules that bind to intrinsically disordered proteins have been successfully identified through screens utilizing surface plasmon resonance, nuclear magnetic resonance (NMR) spectroscopy, and fluorescence methods [65]. Identifying small molecule inhibitors specific for blocking SELENOK-dependent palmitoylation would provide a very useful research tool and, perhaps, a lead compound for therapy development in the context of cancers such as melanoma. In addition, techniques have been developed for the synthesis of recombinant SELENOK [66] and our laboratory has shown that this recombinant SELENOK bound to soluble DHHC6 is functional in cell-free palmitoylation enzyme assays [38]. Thus, screens that successfully identify small molecules with high affinity binding to SELENOK would be able to use downstream in vitro assays to confirm inhibitory activity.

A third possible approach for targeting SELENOK for functional inhibition could take advantage of the SH3 binding domain in its cytosolic region. Our previous work demonstrated that the SH3 binding domain in SELENOK binds to the SH3 domain of DHHC6 [38,9], and this interaction is required for function of the SELENOK/DHHC6 complex. SH3/SH3 binding domains are an abundant mechanism used within cells for promoting protein-protein interactions [67]. Although a multitude of SH3/SH3 binding domain interactions have been identified, there may be ways to include 'context' or to modify residues adjacent to the motifs to provide specificity to inhibitor design [68,69]. The most successful efforts to increase SH3 ligand specificity have focused primarily on replacing sequences flanking the PxxP motif with natural or non-natural moieties [70,71]. Since the SH3 binding domain is well defined in SELENOK, this approach may prove successful for peptide inhibitors designed to disrupt the interaction between two binding domains of SELENOK and DHHC6. This is another area of research that we have focused our efforts, with the goal of developing the smallest peptide or peptide mimetic that can specifically and effectively interrupt interaction between SELENOK and DHHC6

Concluding remarks

Much progress has been made in understanding the role that SELENOK plays in the immune system and in cancer cells. Insight into the molecular mechanisms by which this selenoprotein regulates calcium-dependent signaling was provided with the discovery that SELENOK interacts with the DHHC6 enzyme to promote protein palmitoylation. A major target of SELENOK/DHHC6 palmitoylation is the calcium channel protein, the IP3R.

SELENOK deficiency results in impaired calcium flux in immune cells that leads to ~50% maximal activation of immune cells including T cells, macrophages, and neutrophils. However, SELENOK deficiency appears to have a more dramatic effect on cancer cells that rely on calcium-dependent signaling to grow and metastasize. Thus, therapeutics that inhibit SELENOK will likely impair immunity, but this may not be as dramatic as the static effect they may exert on tumors. Furthermore, combining cancer-static SELENOK inhibitors with cancer-toxic chemotherapeutics may prove to be an effective strategy for treating cancers that are particular dependent on calcium signaling. It is important to note that the anti-cancer effects of SELENOK have thus far only been investigated in a mouse melanoma model and a human melanoma cell line. Much more needs to be determined in terms of the broader range of cancer types for which SELENOK inhibitors may be effective. Also, effects that inhibiting SELENOK have on ER stress or other physiological processes needs to be considered. The potential of SELENOK as a therapeutic target is only beginning to be studied and challenges remain regarding its ‘drugability’, but basic science investigations have been revealing and SELENOK is now much less of an enigmatic member of the selenoprotein family.

Acknowledgements

This research was supported by NIAID/NIH grant R01AI089999.

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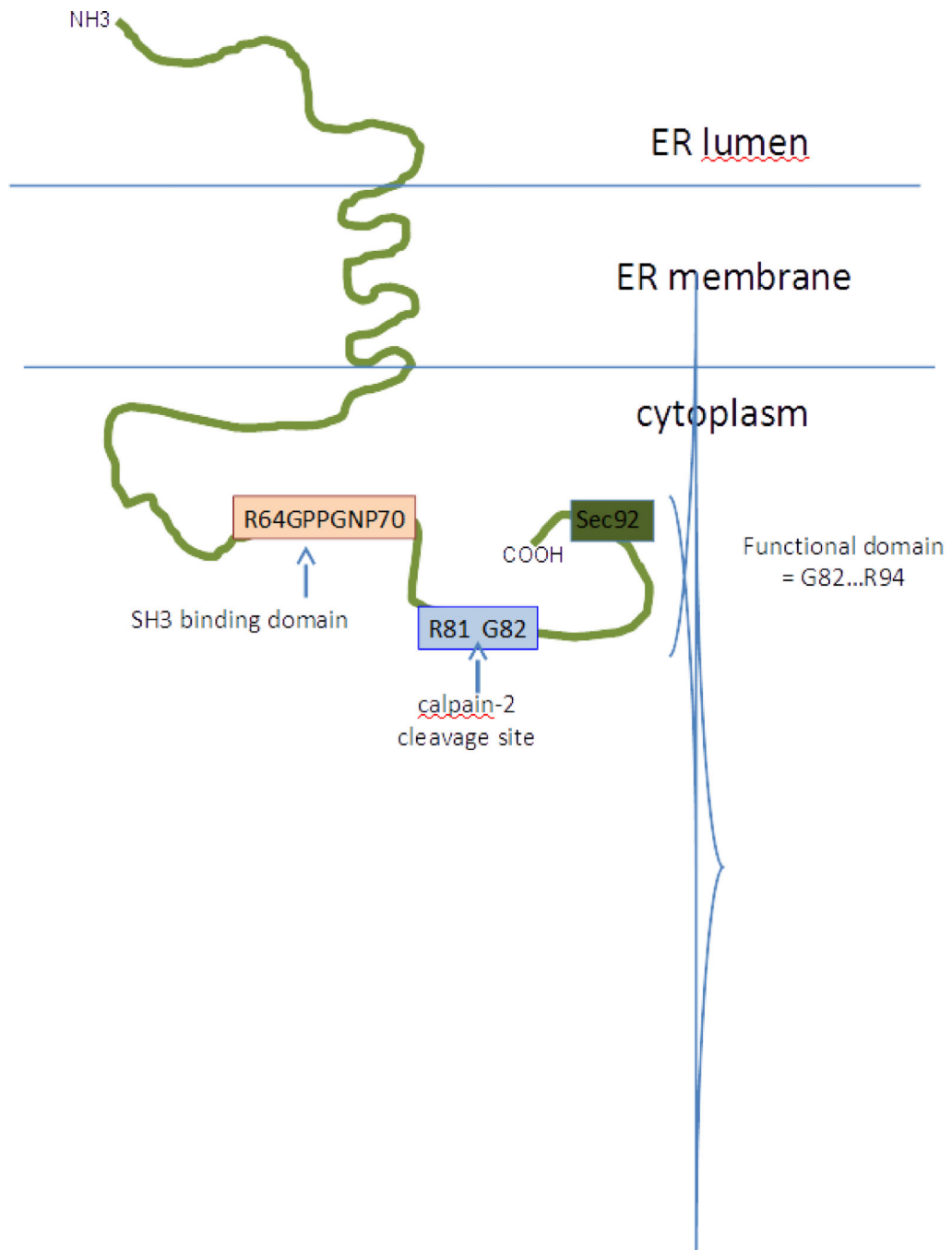
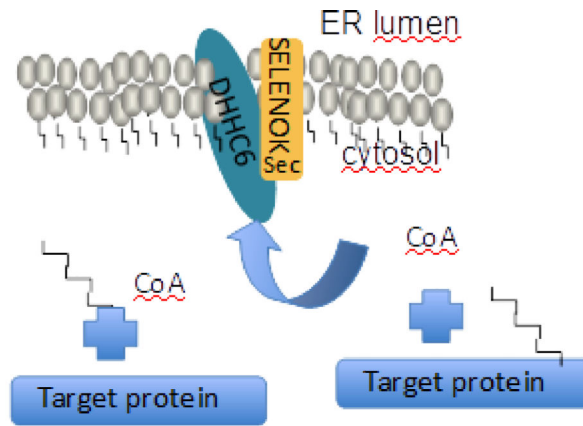


Figure 1. The topology and functional domains of SELENOK. SELENOK is a transmembrane protein in the ER membrane with its functional domains localized to the cytoplasm. It is regulated by calpain-2 proteolysis, which cleaves the functional domain from the remaining protein.



Palmitoylation reaction impeded by:

- low [Se] that leads to low [SELENOK]
- genetic factors that lead to low [SELENOK]
- calpain-2 cleavage of SELENOK that releases functional domain
- Defects in SH3 binding domain in SELENOK in binding to SH3 domain in DHHC6
- therapeutic inhibitors (?)

Figure 2.

The protein acyl transferase reaction catalyzed by the SELENOK/DHHC6 complex at the ER membrane. Acyl groups such as palmitate are transferred from palmitoyl-coenzyme A (CoA) to target proteins like the IP3R. These reactions are regulated by different factors as shown.