Published in final edited form as:

Biosci Rep. ; 29(5): 329–338. doi:10.1042/BSR20080146.

Selenium status highly-regulates selenoprotein mRNA levels for only a subset of the selenoproteins in the selenoproteome

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Synopsis

Gpx (glutathione peroxidase)-1 enzyme activity and mRNA levels decrease dramatically in selenium (Se) deficiency, whereas other selenoproteins are less affected by Se deficiency. This hierarchy of Se regulation is not understood, but the position of the UGA selenocysteine codon is thought to play a major role in making selenoprotein mRNAs susceptible to nonsense-mediated decay. Thus in the present paper we studied the complete selenoproteome in the mouse to uncover additional selenoprotein mRNAs that are highly-regulated by Se status. Mice were fed Se-deficient, Se-marginal, and Se-adequate diets (0, 0.05 and 0.2 µg Se/g, respectively) for 35 days, and selenoprotein mRNA levels in liver and kidney were determined using microarray analysis and quantitative real-time PCR analysis. Se-deficient mice had liver Se concentrations and liver Gpx1 and thioredoxin reductase activities that were 4, 3 and 3%, respectively, of the levels in Se-adequate mice, indicating that the mice were Se-deficient. mRNAs for Selh (selenoprotein H) and Sepw1 (selenoprotein W) as well as Gpx1 were decreased by Se deficiency to <40% of Se-adequate levels. Five and two additional mRNAs were moderately down-regulated in Se-deficient liver and kidney, respectively. Importantly, nine selenoprotein mRNAs in liver and fifteen selenoprotein mRNAs in kidney were not significantly regulated by Se deficiency, clearly demonstrating that Se regulation of selenoprotein mRNAs is not a general phenomenon. The similarity of the response to Se deficiency suggests that there is one underlying mechanism responsible. Importantly, the position of the UGA codon did not predict susceptibility to Se regulation, clearly indicating that additional features are involved in causing selenoprotein mRNAs to be sensitive to Se status.

Keywords

glutathione peroxidase-1 (Gpx1); mouse; nonsense-mediated decay (NMD); selenium deficiency; selenoprotein W (Sepw1); selenoprotein H (Selh)

INTRODUCTION

Gpx (glutathione peroxidase)-1 was the first-identified biochemical role for the essential nutrient, Se (selenium) [1], and this activity is used to assess the risk of Se-dependent disease and to determine Se status. This discovery was followed rapidly by the identification of Se-dependent enzymes in bacteria [2] and then by the identification of Sec (selenocysteine) as the Se cofactor present and incorporated into the peptide backbone of these selenoproteins [3,4]. These discoveries were followed grudgingly over the next 30

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years by the identification of additional selenoproteins in higher animals as well as bacteria, including plasma Sepp1 (selenoprotein P) containing ten selenocysteines, all three mammalian deiodinases, all three mammalian Txnrd (thioredoxin reductases), and three additional Gpxs with unique tissue distribution or substrate utilization [5,6]. Characterization of this growing superfamily of selenoproteins revealed that Sec is encoded by a UGA codon [7], that a unique 3'-UTR (untranslated region) stem-loop structure called a SECIS (Sec insertion sequence) element is required in the mRNA to reinterpret the UGA codon for Sec rather than as a termination of translation codon [8,9], and that a series of unique and novel gene products are required to synthesize an activated form of Se and then use serine attached to a unique tRNA to synthesize Sec [10,11]. Se in the form of Sec is incorporated co-translationally via this machinery into the growing polypeptide chain at the position specified by the UGA codon. Previously, a computational method based on these elements was developed to identify in-frame UGA-containing selenoproteins [12] in sequenced genomes. Kryukov et al. [12] thus elegantly found that the human selenoproteome consists of 25 selenoproteins and that the rodent selenoproteome consists of 24 selenoproteins. This is the first example where the complete proteome specific for a cofactor has been identified.

Gpx1 activity decreases dramatically under conditions of Se deficiency, and increases during Se repletion, thus making Gpx1 a useful biomarker for Se status [13]. Other selenoproteins, such as Gpx4 (phospholipid hydroperoxide Gpx), only decrease in activity to approx. 40–50% of maximal activity in Se-deficient animals [14]. A more complete understanding of this regulation emerged when it was found that Gpx1 mRNA also decreases dramatically under conditions of Se deficiency [15], whereas Gpx4 mRNA levels do not decrease [14,16]. Other selenoprotein levels, such as Txnrd, decrease to nearly the same extent as Gpx1, but Txnrd mRNA levels are only modestly decreased [17]. This hierarchy of Se regulation of the various selenoproteins and their mRNAs is not currently understood.

The degradation of mRNA levels in Se deficiency appears to be due to NMD (nonsense-mediated decay) [18,19]. The decrease in Gpx1 mRNA levels is not due to transcriptional regulation, but rather due to NMD of Gpx1 mRNA when Se is lacking. mRNA levels for several other well-characterized selenoproteins, however, are not susceptible to NMD. The underlying mechanism appears to require the UGA codon to be positioned >55 nt upstream of an intron splice junction [20–22], but this condition is also true for Gpx4 mRNA, which decreases in Se-deficient cultured cells but not in intact Se-deficient animals, whereas Gpx1 mRNA is decreased by nearly an order of magnitude [14,23,24]. A number of hypotheses have been proposed to explain the hierarchy of the susceptibility of selenoprotein mRNAs to degradation [8,18,19,25–28], but this issue is complicated because Se deficiency has a much more profound impact in intact animals than in cultured cells [18,23]. Thus we conducted the present study of the complete selenoproteome in mice fed differing levels of dietary Se, with the hypothesis that we would uncover additional selenoprotein mRNAs that are highly regulated by Se status and so might help to better understand the underlying mechanism of Se regulation of mRNA stability.

To evaluate the complete selenoproteome for Se regulation of mRNA levels, selenoprotein mRNA levels in total RNA from liver and kidney of Se-deficient, Se-marginal, and Se-adequate mice were determined using microarray analysis and quantitative real time polymerase chain reaction qRT-PCR (quantitative real-time PCR) analysis. We found two selenoprotein mRNAs in addition to Gpx1 mRNA that were highly down-regulated in Se-deficient tissue, and six additional mRNAs that were moderately down-regulated. We also found, however, that the majority of selenoprotein mRNAs were not decreased significantly

by Se status. Furthermore, the sensitivity of these selenoprotein mRNAs to Se regulation is not predicted by current models for the underlying mechanism.

MATERIALS AND METHODS

Reagents

Molecular biology reagents were purchased from Promega (Madison, WI, U.S.A.), Invitrogen (Carlsbad, CA, U.S.A.) or Sigma (St. Louis, MO, U.S.A.). All other chemicals were of molecular biology or reagent grade.

Animal husbandry

Male mouse pups from Se-adequate dams in our wild-type colony (predominantly C57 Black background) were weaned 18 days after birth and housed individually in hanging-wire cages for 35 days. The basal Se-deficient diet was a rodent torula-yeast diet containing 0.005 μg Se/g diet and supplemented with 100 mg/kg of all-rac- α -tocopherol acetate to ensure prevention of liver necrosis, and supplemented with 0.4% L-methionine to ensure adequate growth, as described previously [16]. Mice were fed the basal diet supplemented with 0, 0.05, or 0.2 μg Se/g as Na_2SeO_3 to provide Se-deficient, Se-marginal or Se-adequate diets [29], and had free access to feed and water. The care and treatment protocol was approved by the University of Wisconsin institutional animal care and use committee.

Tissue analyses

Mice were anaesthetized with ether, blood was drawn by cardiac puncture using heparinized syringes, and were then killed by exsanguination under anaesthesia. Livers were perfused in situ with ice-cold 0.15 M potassium chloride, and all tissues were removed and quick-frozen in liquid nitrogen for later enzyme and RNA analysis. Blood was centrifuged [1500 g for 15 min at 4°C, Eppendorf 5415R centrifuge, F-45-24-11 rotor (Brinkmann, Westbury, NY, U.S.A.) to separate the plasma from red cells, and the red cells were reconstituted to the original volume using PBS [76 mM NaCl and 50 mM sodium phosphate (pH 7.4)]. Liver, kidney and diet Se concentrations were determined by neutron-activation analysis [30].

Enzyme assays

To prepare tissue supernatant, frozen tissues were homogenized in nine volumes of sucrose buffer [20 mM Tris/HCl (pH 7.4), 0.25 M sucrose, 1 mM EDTA and 0.1% peroxide-free Triton X-100] and centrifuged [10,000 g for 15 min at 4°C, model J2-21M, JA-21 rotor (Beckman Instruments, Palo Alto, CA, U.S.A.)]. Gpx1 activity in liver, kidney and red blood cells (designated as Gpx1) and Gpx3 activity in plasma [designated as Gpx3 (plasma Gpx)] were measured by the coupled assay procedure [31] using 120 μM H_2O_2 . Gpx4 activity was measured by the coupled assay procedure [14] using 78 μM phosphatidylcholine hydroperoxide, the specific substrate. For both assays, 1 unit is the amount of enzyme that will oxidize 1 μmole of GSH per min under these conditions. Txnrd was measured using the gold-inhibition assay with DTNB [5,5'-dithiobis-(2-nitrobenzoic acid)] as a substrate [32]. The protein concentration of each sample was determined by the method of Lowry et al. [33].

RNA isolation and processing

Total RNA from mouse liver and kidney (50–100 mg of tissue, $n=3$ per diet group) was isolated using the guanidinium isothiocyanate method using TRIzol reagent (Invitrogen), following the manufacturer's protocol. mRNA was purified using an oligo(dT)-linked Oligotex resin (Qiagen, Valencia, CA, U.S.A.), and then used to synthesize double-stranded cDNA [34]. The cDNA was purified using phenol/chloroform extraction [35], and was used

for *in vitro* transcription of biotin-labeled cRNA using the Enzo BioArray HighYield RNA Transcript Labeling Kit (Enzo Life Sciences, Farmingdale, NY, U.S.A.). Finally, purified cRNA was fragmented at 94°C for 35 min in a buffer containing 200 mM Tris/acetate (pH 8.0), 500 mM potassium acetate and 150 mM magnesium acetate. The integrity and size of total RNA, cRNA and fragmented cRNA for each sample was assessed by formaldehyde-agarose-gel electrophoresis. RNA or DNA obtained at each step was quantified using an ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, U.S.A.).

Microarray analysis

Each fragmented cRNA sample (10 µg) was hybridized to one Affymetrix Mouse Genome 430 2.0 array following the manufacturer's protocols (Affymetrix, Santa Clara, CA, U.S.A.). After hybridization, the arrays were washed and stained with streptavidin phycoerythrin (Invitrogen) in a GC450 Fluidics Station (Affymetrix) and scanned using a GC3000 7G scanner (Affymetrix). Hybridization, washing and scanning of the arrays were performed by the Gene Expression Center at University of Wisconsin-Madison. Expression data were generated from raw fluorescence values using Microarray Suite 5.0 (MAS 5.0) (Affymetrix) within the GCOS (Gene Chip Operating Software) version 1.4 (Affymetrix). The GCOS-generated detection calls and scaled expression values for the probe sets, targeting each of the 24 mouse selenoprotein genes, were obtained from each single array analysis. If multiple probe sets were found to target the same gene, the probe set giving the highest expression in Se-adequate samples was used for analysis. In addition, the expression of eight non-selenoprotein genes was determined, including three house-keeping genes [Gapdh (glyceraldehyde-3-phosphate dehydrogenase), Actb (β-actin) and Rps14 (ribosomal S14)], and including five orthologs of selenoprotein genes that encode cysteine-containing rather than selenocysteine-containing proteins (Mrps14 (mitochondrial Rps14), Gpx7, Msra (methionine sulfoxide reductase), Msrb2, and Msrb3]. Selenoprotein mRNA levels were normalized to the expression of all of the probe sets within each array by the GCOS software, the expression ratios for each gene from each of the nine arrays per tissue were calculated relative to the average Se-adequate expression (set to 100) and visualized using Treeview software [36].

qRT-PCR

The relative mRNA abundance was also determined by quantitative qRT-PCR. RNA (1 µg) was reverse transcribed to cDNA using the RETROscript kit (AM1710, Ambion, Austin, TX, U.S.A.), following the manufacturer's instructions. Gene-specific primers were designed to span a splice junction and amplify an approx. 150 base-pair segment (see Table 1). The final real time reaction (25 µl final volume) contained 10 ng of reverse-transcribed RNA, 0.2 mM gene-specific forward and reverse primers, and 1x SybrGreen PCR Master Mix (#4309155, Applied Biosystems, Foster City, CA, U.S.A.). Reactions were performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems), with initial stages of 50°C for 2 min and 95°C for 10 min, followed by 50 cycles consisting of 95°C for 15 s and 60°C for 2 min. A dissociation curve was run for each plate to confirm the production of a single product. The amplification efficiency for each gene was determined using the DART program [37]. The relative abundance of each mRNA was calculated using the method of Pfaffl [38], accounting for gene-specific efficiencies, normalized to Actb levels, and set relative to the mean of the Se-adequate (0.2 µg Se/g diet) animals.

Statistics

Data are presented as means ± SEM (n = 3/diet group). Results were analyzed by ANOVA using a fixed model testing the main effect of diet. F-tests and least-squares means were calculated using the mixed procedures of SAS (SAS, Cary, NC, U.S.A.). When the main

effect of diet was significant ($P<0.05$), the means were separated by the least significant difference test.

RESULTS

Physiological markers

The weanling mice weighed 8.4 g at the start of the study, and after 35 days the final masses were 28.1 ± 1.2 , 27.5 ± 0.9 and 28.7 ± 2.3 ($P=0.82$) for mice fed 0, 0.05 and 0.2 $\mu\text{g Se/g}$ diet, respectively. The average gain was 0.57 g/day. There was no effect of dietary Se level on body mass throughout the study (see Supplementary Figure S1 at <http://www.bioscirep.org/bsr/029/bsr0290329add.htm>). Similarly, there was no effect of dietary Se on liver and kidney masses, expressed as total organ mass or as a percentage of body mass (results not shown). There were no overt signs of Se deficiency in these mice at any point in the study.

Se analysis indicated that tissue Se concentrations in mice fed the Se-deficient diet decreased to 0.5 and 2.3 nmol/g tissue in liver and kidney, respectively (Figure 1A). Liver Se was 4% of the level in mice fed an Se-adequate diet containing 0.2 $\mu\text{g Se/g}$ diet, clearly indicating that the mice were Se deficient. Kidney Se in Se-deficient mice was only 17% of Se-adequate levels. Liver and kidney Se levels in mice fed the 0.05 $\mu\text{g Se/g}$ diet were 49% and 64%, respectively, of the levels in Se-adequate mice, showing that these Se-marginal mice were intermediate in Se status.

Biochemical markers

Plasma Gpx3 activity was decreased in Se-deficient mice to 13% of Se-adequate levels (Figure 1B), with plasma Gpx3 activity in Se-marginal mice 26% of the level in Se-adequate mice. Gpx1 activity in Se-deficient red blood cells (Figure 1B) only decreased to 37% of Se-adequate levels, as expected due to the long half-life of the red blood cell in rodents [39]; Se-marginal red blood cell Gpx1 activity was 57% of Se-adequate levels.

In Se-deficient liver, liver Gpx1 activity was 3.4% of Se-adequate levels, and Se-marginal liver only had 40% of the Se-adequate Gpx1 activity (Figure 1C). The Se-deficient kidney appeared to be less Se deficient, with kidney Gpx1 activity at 17% of Se-adequate levels. In Se-deficient liver, Txnrd activity was 2.8% of Se-adequate levels, whereas Se-marginal liver had 68% of Se-adequate Txnrd activity (Figure 1D). Kidney Txnrd and Gpx4 activities were not determined due to lack of sufficient material. Thus, based on these biochemical markers, mice fed the Se-deficient diet were clearly Se-deficient; mice fed the Se-marginal diet had less than half of the level of Se-adequate enzyme activity for liver and kidney Gpx1, for liver Txnrd and for plasma Gpx3, indicating that these mice were marginal in Se status.

Gpx4 activity in Se-deficient liver, in contrast to Gpx1, Gpx3 and Txnrd activities, was only 34% of Se-adequate levels (Figure 1D). Se-marginal liver had 79% of Se-adequate Gpx4 activity.

Microarray studies

Studies with the Affymetrix Mouse Genome 430 2.0 array were then conducted to determine the Se regulation of all 24 selenoprotein mRNAs in the complete selenoproteome in the mouse. Microarray analysis identified 17 liver selenoprotein mRNAs as 'present' (Figure 2), including nine that were significantly down-regulated in Se-deficient liver [Gpx1, Selh (selenoprotein H), Sepw1 (selenoprotein W), Txnrd1, Txnrd2, Selk (selenoprotein K), Dio1 (deiodinases, iodothyronine, type I), Selt (selenoprotein T) and Sep15 (15 kDa selenoprotein)]. In kidney, 20 selenoprotein mRNAs were identified as 'present', including

eight that were significantly down-regulated in Se-deficient kidney [Gpx1, Selh, Sepw1, Selm (selenoprotein M), Txnrd1, Dio1, Sep15 and Sels (selenoprotein S)]. In addition, eight non-selenoprotein mRNAs (Gapdh, Actb, Rps14, Mrps14, Gpx7, Msra, Mrsb2 and Mrsb3) were also evaluated by microarray analysis and found not to be significantly affected by Se status.

Normalized fluorescence of the selenoprotein mRNAs in the microarray analysis suggested that Sepp1, Gpx1, Gpx4 and Sepx1 (also known as Mrsb1) (highest to lowest) were the selenoprotein transcripts with the apparent highest abundance in Se-adequate liver (see Supplementary Figure S2 at <http://www.bioscirep.org/bsr/029/bsr0290329add.htm>). These estimates match previous analyses that found that Sepp1, Gpx1, and Gpx4 transcript levels were comparable to Gapdh levels in Se-adequate liver [16,23]. In Se-adequate kidney, Gpx3, Sepp1, Gpx1 and Gpx4 (highest to lowest) were the selenoprotein transcripts with the apparent highest abundance (see Supplementary Figure S3 at <http://www.bioscirep.org/bsr/029/bsr0290329add.htm>).

qRT-PCR studies

To confirm that levels of these mRNAs are regulated by Se status, qRT-PCR analyses were conducted for all selenoprotein mRNAs identified by microarray analysis as being Se-regulated in either tissue, plus Sepp1, Gpx4, and Sepx1 in both tissues, and Gpx3, Selo (selenoprotein O), and Txnrd3 in kidney. The resulting expression profiles, determined by both microarray analysis and qRT-PCR, are shown in Figures 3 and 4. In general, there was very good agreement between microarray analysis and qRT-PCR analysis for the regulation of mRNA levels by Se status. Using qRT-PCR, transcript levels of three housekeeping genes, Gapdh, Actb and Rps14, were also found not to be significantly affected by Se status (results not shown).

Analysis by qRT-PCR showed that Gpx1 mRNA levels in Se-deficient liver were decreased to 26% of Se-adequate levels (Figure 3A). Unlike liver Se and liver Gpx1 activity, however, liver Gpx1 mRNA levels in Se-marginal liver were not significantly different from Se-adequate Gpx1 mRNA levels. In kidney, a similar pattern was observed, with Se-deficient and Se-marginal Gpx1 mRNA levels decreased to 27% and 79% of Se-adequate levels respectively (Figure 4A). Gpx4 mRNA levels were not significantly affected by the level of dietary Se in either liver or kidney (Figures 3G and 4F). Thus the present study with mice shows differential regulation of mRNA levels as well as selenoenzyme activities.

In the present study, Sepw1 and Selh mRNA levels in Se-deficient liver were decreased to 26 and 33% of levels in Se-adequate liver respectively, and thus the transcript levels of these genes, along with Gpx1, are highly regulated by Se status. In addition, mRNAs for Txnrd1 and Txnrd2 were moderately and significantly decreased in Se-deficient liver to 45–50% of Se-adequate levels, and mRNAs for Sepp1, Selk and Selm were significantly decreased to 60–70% of Se-adequate levels. In all, eight selenoprotein mRNAs were significantly decreased by Se deficiency in mouse liver to <70% of Se-adequate levels. The mRNA levels in Se-marginal liver were not significantly different from Se-adequate levels for most Se-regulated mRNAs. For the Se-regulated genes, the response curves appeared to be hyperbolic, with the largest increase in mRNA level occurring in the transition from Se-deficient to Se-marginal states; mRNA levels in Se-marginal liver were not significantly different from Se-adequate levels for most Se-regulated mRNAs. Lastly, nine of seventeen selenoprotein mRNAs present in liver, including Gpx4 and Sepx1 mRNAs (Figures 3G and 3H), were not significantly regulated by Se status.

In kidney, Gpx1, Sepw1 and Selh mRNAs were also highly regulated by Se status, decreasing in Se-deficient kidney to 27, 32 and 37% of Se-adequate levels respectively

(Figures 4A–4C) as assessed by qRT-PCR. These values are again very similar to the values determined by microarray analysis. In Se-deficient kidney, *Selm* was 64% of Se-adequate levels by qRT-PCR (Figure 4E), and 47% of Se-adequate levels by microarray analysis. Kidney Se-deficient *Gpx3* mRNA level was 64% of Se-adequate levels by qRT-PCR (Figure 4D), but not significantly different by microarray analysis. Thus five selenoprotein mRNAs were significantly decreased by Se deficiency in mouse kidney. Again the response curves for the regulated kidney selenoprotein mRNAs appeared to be hyperbolic, as stated for liver. Lastly, fifteen of the twenty selenoprotein mRNAs present in kidney (Figure 4), including *Gpx4* mRNA (Figure 4F), were not significantly regulated by Se status.

DISCUSSION

The impact of Se deficiency in the present study was very similar to previous reports studying pups from Se-adequate dams [39,40]. There was no significant effect on growth or the apparent health of these mice supplemented with normal levels of vitamin E and the sulfur amino acids. Feeding of an Se-deficient diet for 35 days, however, dramatically decreased liver Se, and liver *Gpx1* and *Txnrd* activity to 4, 3, and 3% of Se-adequate levels respectively. These decreases are very similar to levels reported previously [40]. This clearly shows that these young rapidly growing mice were biochemically Se deficient without overt alterations in their overall physiology.

Liver and kidney mRNA levels for *Gpx1* mRNA were also decreased to 26 and 27% of Se-adequate levels respectively. These decreases are not as large as observed in rats [23], most probably due to the differences between these two rodent models in growth and thus in the dilution of initial Se stores. Just as in rats, *Gpx4* activity was only moderately affected by Se deficiency, and *Gpx4* mRNA was unchanged by Se deficiency in these mice, illustrating that these mRNAs for *Gpx1* and *Gpx4* are differentially regulated by Se status in mice. Thus this mouse model allowed us to detect alterations in mRNA levels for the complete selenoproteome due to changes in Se status, including the six newly discovered but uncharacterized rodent selenoproteins [12].

In both liver and kidney, *Sepw1* and *Selh* mRNA levels assessed by microarray analysis were highly regulated by Se status, as was *Gpx1* mRNA, with all three mRNAs decreasing in Se deficient mice to <40% of Se-adequate levels. This regulation was confirmed by qRT-PCR analysis for *Gpx1*, *Sepw1* and *Selh* mRNAs. The present study is the first report in which *Selh* mRNA is regulated directly by Se status. *Sepw1* mRNA has been reported to be decreased by Se deficiency in mouse muscle [41], but other researchers were not able to detect *Sepw1* mRNA in liver [42]. In addition, *Txnrd1*, *Txnrd2*, *Sepp1*, *Selk* and *Selm* mRNAs were moderately down-regulated in Se-deficient liver, and *Selm* and *Gpx3* mRNAs were moderately down-regulated in Se-deficient kidney. Importantly, nine selenoprotein mRNAs in liver and fifteen selenoprotein mRNAs in kidney, including *Gpx4* mRNA in both tissues, were not significantly regulated by Se deficiency, clearly demonstrating that Se regulation of selenoprotein mRNAs is not a general phenomenon for all selenoproteins.

The mechanism underlying the Se regulation of the selenoprotein mRNA level is not understood. For *Gpx1* and *Gpx4* mRNA, at least, it is clear that this regulation is not due to transcriptional regulation or mRNA processing and export. Previous studies with a recombinant chimera of *Gpx1* indicate that the decrease in *Gpx1* mRNA in Se deficiency is due to NMD [18,19]. Positioning of an in-frame UGA codon sufficiently upstream of a splice junction in β -globin along with a SECIS element in the 3'UTR will confer Se regulation onto β -globin mRNA [18]. Susceptibility to NMD appears to require a UGA codon placed at least 55 nt up-stream of the exon/intron splice junction [20–22]. This explains the high regulation of *Gpx1* mRNA (UGA codon at 105 nt upstream), but it does

not explain why Gpx4 mRNA (UGA codon at 105 nt upstream) is not susceptible to Se regulation *in vivo* [14,23]. This is further complicated by previous studies which show that transiently expressed Gpx4 mRNA or endogenous Gpx4 mRNA *in cultured cells* is susceptible to NMD [24], indicating that additional factors are likely to be involved.

The present study was conducted in part to survey the full selenoproteome for additional selenoprotein mRNAs which are susceptible to Se regulation, presumably by NMD, and to relate this susceptibility to the position of its cognate UGA codon relative to the downstream splice junction (Table 2). Highly regulated Gpx1 mRNA has its UGA codon located 105 nt from the splice junction. Two additional highly regulated mRNAs were identified: Selh fits the >55 nt rule, with its UGA codon 136 nt upstream of the splice junction, but the UGA codon for Sepw1 only lies 15 nt upstream. For the moderately regulated mRNAs identified in this study, the UGA codon positions for Txnrd1 and Txnrd2 fit the >55 nt rule, but the UGA codon positions for Selk, Selm, and Gpx3 and the first UGA codon for Sepp1 are 5, 21, 22, and 26 nt upstream of the splice-junction respectively. And several of the selenoprotein mRNAs found to have low Se regulation in the present study, including Gpx4 and Dio1, have UGA codons positioned much greater than 55 nt upstream of the splice junction. The present study thus provides a number of new examples showing that the >55 nt rule does not explain the susceptibility to degradation.

There are several alternative hypotheses that may be involved in Se regulation of selenoprotein mRNA level. Some studies using chimaeric constructs expressed in cultured cells suggest that regions in both the coding region and in the 3'UTR are involved in making selenoprotein mRNAs susceptible to decay in Se-deficient cells [25]. The extent of Se regulation in cultured cells, however, is much smaller than in the intact animal [18] making these studies difficult to interpret. NMD in mammals appears to be a consequence of conditions in the pioneering round of translation [22], and so competition for a limited supply of Se for translation may switch the destination of an mRNA from translation to NMD [23]. The UGA codon position itself relative to the start codon or to the SECIS element, or the local UGA context, can all affect translational efficiency and thus might affect mRNA sensitivity to NMD [43]. Early studies with chimaeric mRNA in cultured cells or oocytes found that SECIS elements differ in their ability to facilitate Sec incorporation in translation [8], and thus varied translational efficiency due to differences in SECIS elements might confer differential sensitivity to NMD [8,44]. Differential affinities of SECIS elements for isoforms of the Sec-tRNA also influence translational efficiency and are accompanied by changes in mRNA levels, and so may influence mRNA stability [26]; these shifts in relative levels of Sec-tRNA isoforms, however, are modest in Se-deficient mice [45], making it unlikely that this alone regulates selenoprotein mRNA stability in intact animals. Similarly, differing affinities of SBPs (SECIS-binding proteins), such as SBP2, for the SECIS element have also been proposed to explain the hierarchy of sensitivity to NMD [27,28]; these studies in cultured cells may explain the differential effects of translation of selenoprotein mRNAs, but studies with chimaeric constructs have shown that SECIS elements from selenoprotein mRNAs not susceptible to NMD are as sufficient in conferring NMD sensitivity as those from NMD-susceptible mRNAs, thus indicating that Se regulation of NMD involves more than the SECIS element and SBPs [18]. Lastly, the presence of a putative second SECIS stem-loop immediately downstream of the UGA codon in the coding region in some selenoprotein mRNAs is thought to influence translational efficiency [46] but only Sepn1 (selenoprotein N1) mRNA, which we did not find to be present in mouse liver or kidney, has been studied. The two new mRNAs shown in the present study to be highly regulated *in vivo* may prove useful to better understand the mechanism underlying this regulation.

One overall striking feature of the Se response curves in the present study (Figures 3 and 4) is that a marginal level of dietary Se (0.05 µg Se/g diet) raises selenoprotein mRNA levels to Se-adequate levels; the dramatic impact on selenoprotein mRNA stability of these Se-regulated mRNAs occurs at or <0.05 µg Se/g diet. In rats fed intermediate levels of dietary Se to better define Se regulation, the response curves for Gpx1, Selh and Sepw1 mRNAs are hyperbolic, all overlap, and reach plateau responses at or near 0.05 µg Se/g diet [47]. This strongly suggests that there is one underlying mechanism in play in Se regulation of selenoprotein mRNA levels, but further studies will be required to unravel this mechanism.

In summary, microarray and qRT-PCR analysis of mRNA levels for the complete selenoprotein proteome found two additional selenoprotein mRNAs, in addition to Gpx1, that were highly regulated by Se status. Several additional selenoprotein mRNAs were moderately regulated by Se status, but the majority of selenoprotein mRNAs were not down-regulated by Se deficiency in mouse liver and kidney. This clearly indicates that Se regulation of mRNA level is not a general phenomenon of selenoprotein mRNAs. The similarity of the Se response curves for those mRNAs sensitive to Se status, however, suggests that there is one underlying mechanism in play. Lastly, the position of the Sec codon, UGA, in the Se-regulated mRNAs does not predict at all the susceptibility to Se regulation, clearly indicating that additional features are involved in making selenoprotein mRNA levels sensitive to Se status. For these selenoproteins with Se-regulated mRNA levels, it will also be important to determine if the protein levels of the selenoproteins are also regulated by Se status.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

FUNDING

This work was supported in part by the National Institutes of Health [grant numbers DK74184, T32-DK07665 (training grant supporting K.M.B.)], and by the University of Wisconsin Agricultural Experiment Station [grant number WIS04909].

Abbreviations used

Actb	β-actin
Dio1	deiodinase, iodothyronine, type I
Gapdh	glyceraldehydes-3-phosphate dehydrogenase
GCOS	GeneChio Operating Software version 1.4
Gpx	glutathione peroxidase
Gpx3	plasma Gpx
Gpx4	phospholipid hydroperoxide glutathione peroxidase
Msr	methionine sulfoxide reductase
NMD	nonsense-mediated decay
qRT-PCR	quantitative real-time polymerase chain reaction
Rps14	ribosomal protein S14
Mrps14	mitochondrial Rps14

Sec	selenocysteine
SECIS	Sec insertion sequence
SBP	SECIS binding protein
Selh	selenoprotein H
Selk	selenoprotein K
Selm	selenoprotein M
Sep15	15 kDa selenoprotein
Sepp1	selenoprotein P
Sepw1	selenoprotein W
Sepx1	Msrb1
Txnrd	thioredoxin reductase
UTR	untranslated region

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Figure 1. Effect of Se status on tissue Se concentration and on tissue selenoenzyme activity

At weaning, mice were fed diets containing 0, 0.05, or 0.2 μg Se/g to provide Se-deficient, Se-marginal or Se-adequate diets, and killed 35 days later. Values are means $\pm$ S.E.M. (n=3) and, for a given analysis, values not sharing a common letter (uppercases or lowercase) are significantly different ($P < 0.05$). (A) Tissue Se concentration. Kidney, $\bullet$; liver, $\circ$. (B) Gpx3 ($\bullet$) and red blood cell Gpx1 ($\circ$) activities. (C) Tissue Gpx1 activities. Kidney, $\bullet$; liver, $\circ$. (D) Liver Gpx4 ($\circ$) and Txnrd ($\bullet$) activities.

Figure 2. Visualization of effect of Se status on selenoprotein mRNA expression as assessed by microarray analysis

Expression ratios for each gene from each of the nine arrays per tissue were calculated relative to average Se-adequate expression and set to 100, as described in the Materials and methods section. Expression levels were visualized using Treeview software [36], as shown in the colour scale. (A) Liver and (B) kidney mRNA from mice fed diets containing 0, 0.05, or 0.2 µg Se/g for 35 days were subjected to microarray analysis for all 24 selenoproteins in the mouse genome, and for the expression of eight non-selenoprotein genes, including three house-keeping genes (Gapdh, Actb, and Rps14), and including five orthologues of selenoprotein genes that encode cysteine-containing rather than Sec-containing proteins (Mrps14, Gpx7, Msra, Msrb2 and Msrb3). Shown are the seventeen liver selenoprotein mRNAs and twenty kidney selenoprotein mRNAs called as 'present' by the GCOS software. Seli, selenoprotein I; Selo, selenoprotein O; Sels, selenoprotein S; Selt selenoprotein T; Sephs2, selenophosphate synthetase 2.

Figure 3. Effect of Se status on liver selenoprotein mRNA expression

Liver mRNA from mice fed diets containing 0, 0.05, or 0.2 µg Se/g for 35 days was subjected to microarray analysis (○) and to qRT-PCR analysis (●), as described in the Materials and methods section. For microarray analysis, relative expression (%) was calculated relative to the mean of the Se-adequate (0.2 µg Se/g) abundance (set to 100) after the GCOS software had normalized selenoprotein mRNA levels to the expression of all probe sets within each array. For qRT-PCR analysis, relative mRNA abundance was calculated using the method of Pfaffl [38] to account for gene-specific efficiencies, normalized to Actb levels in each sample, and set relative to the mean of the Se-adequate (0.2 µg Se/g diet) abundance. Values are means ± S.E.M. (n=3) and, for a given analysis, values not sharing a common letter are significantly different ($P < 0.05$). Shown are mRNA expression levels for (A) Gpx1, (B) Selh, (C) Sepw1, (D) Txnrd1, (E) Txnrd2, (F) Selk, (G) Gpx4 and (H) Sepx1.

Figure 4. Effect of Se status on kidney selenoprotein mRNA expression

Kidney mRNA levels in mice fed diets containing 0, 0.05 or 0.2 µg Se/g. Values are described in detail in the legend to Figure 3. Shown are mRNA expression levels for (A) Gpx1, (B) Selh, (C) Sepw1, (D) Gpx3, (E) Selm and (F) Gpx4.

Table 1

qRT-PCR Primers for mouse selenoproteins used in the present study

Gene	Forward Primer	Reverse Primer
ActB	5'-AGCCATGTACGTAGCCATCC-3'	5'-CTCTCAGCTGTGGTGGTGAA-3'
Dio1	5'-GGAACCATAGGCATTGGAAA-3'	5'-AGTGCCAGAGAGCCAGATTTC-3'
Gapdh	5'-GTGTTCTACCCCCAATGTG-3'	5'-AGGAGACAACCTGGTCCTCA-3'
Gpx1	5'-GGTTCGAGCCCCAATTTTACA-3'	5'-CCCACCAGGAACCTCTCAAA-3'
Gpx3	5'-GATGTGAACGGGGAGAAAGA-3'	5'-CCCACCAGGAACCTCTCAAA-3'
Gpx4	5'-CTCCATGCACGAATTCTCAG-3'	5'-ACGTCAGTTTTGCCTCATTG-3'
Rps14	5'-TGTCTGCCACATCTTTGCAT-3'	5'-GAGGACTCATCTCGGTCAGC-3'
Selh	5'-GCGAGATTGAACTTTGCATC-3'	5'-TTGTCCACCGTCTCCATAGG-3'
Selk	5'-GCTGGTGGATGAGGAAGGTA-3'	5'-CTCATTATCTGTGGGGACA-3'
Selm	5'-TTTGTACCGAGGACATTCA-3'	5'-TGTACCAGCGCATTGATCTC-3'
Selo	5'-TGCACAGAAAGCCATTGAAG-3'	5'-GGAAGATTGCTCCTCAGTGC-3'
Sels	5'-GCCTTACGCACACTTTTACA-3'	5'-GTGGCCTAATGGCAATGTCT-3'
Selt	5'-TGTGGCAACAGAAAGGGATT-3'	5'-CAGGTGGCATCAACATCAAG-3'
Sep15	5'-CTCACCAGTGAAACGCTTTG-3'	5'-TCAAAGAGCACACAGCAAGG-3'
Sepp1	5'-GCAATTGCTTGACAGTGTGC-3'	5'-TTCATGGGCTGATTTTGTCA-3'
Sepw1	5'-CCCAAGTACCTCCAGCTCAA-3'	5'-GCCATCACCTCTCTTCTTGG-3'
Sepp1	5'-ACAGTTGTTGCCCCATTAGC-3'	5'-GGAGTGGGTCTCAGCTTCAG-3'
Txnrd1	5'-TTCGACCTGATCATCATTGG-3'	5'-CCACATTCACACAGTTCCT-3'
Txnrd2	5'-CCCTAGAGTGTGCTGGCTTC-3'	5'-AAGCATGATCCTCCCAAGTG-3'
Txnrd3	5'-CCTTTCCCAGTTGCTAGTGC-3'	5'-GTGCTACACTCTGGGCAACA-3'

Selo, selenoprotein O; Sels, selenoprotein S; Selt selenoprotein T;

Table 2

Effect of UGA Codon Position on Se Regulation

Se gene	UGA Exon (nth of n exons)	Distance (nt)	Liver Se Regulation	Kidney Se Regulation
Gpx1	1st of 2	105	High	High
Selh	2nd of 4	136	High	High
Sepw1	2nd of 6	15	High	High
Selk	4th of 5	5	Moderate	Low
Selm	2nd of 5	21	Moderate	Moderate
Txnrd1	15th of 15	1685	Moderate	Low
Txnrd2	17th of 18	83	Moderate	Low
Sepp1	2nd of 5	26	Moderate	Low
Gpx3	2nd of 5	22	A	Moderate
Dio1	2nd of 4	103	Low	Low
Selt	2nd of 6	101	Low	Low
Gpx4	3rd of 7	105	Low	Low
Sepx1	3rd of 5	34	Low	Low
Sep15	3rd of 5	28	Low	Low
H47/Sels	6th of 6	559	Low	Low
Selo	9th of 9	363	nd	Low
Txnrd3	16th of 16	738	A	Low
Sephs1	1st of 1	1852	nd	nd
Seli	10th of 10	5464	nd	nd
Dio2	2nd of 2	5271	A	nd

The susceptibility to Se regulation of selenoprotein mRNA levels in Se0deficient mouse tissue (liver and kidney) as assessed by qRT-PCR in Se-deficient versus Se-adequate mouse samples (high = 20–45% of Se-adequate; moderate = 45–70% of Se-adequate; Low = >70% of Se-adequate). The exon location of the UGA codon in each selenoprotein gene [data from National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/>)], and the distance in nucleotides from the UGA (A) codon and the 3' exon/intron splice junction are indicated. A, absent as determined by Affymetrix GCOS software analysis of microarray data; nd, not determined by qRT-PCR analysis, present but not Se regulated in microarray analysis; Dio2, deiodinase, iodothyronine, type II; Seli, selenoprotein I; Selo, selenoprotein O; Sels, selenoprotein S; Selt, selenoprotein T; Sephs2, selenophosphate synthetase 2.