Selenium regulation of transcript abundance and translational efficiency of glutathione peroxidase-1 and -4 in rat liver

Sherri WEISS SACHDEV[1] and Roger A. SUNDE[2]

Department of Biochemistry, 217 Gwynn Hall, University of Missouri, Columbia, MO 65211, U.S.A., and Department of Nutritional Sciences, 217 Gwynn Hall, University of Missouri, Columbia, MO 65211, U.S.A.

Glutathione peroxidase (GPX)1 mRNA in rat liver falls dramatically during Se deficiency to levels that are approx. 10% of Se-adequate levels. This regulation is mediated by mRNA stability, and is hypothesized to involve nonsense-mediated mRNA decay. mRNA levels for GPX4 and other selenoproteins are much less regulated by Se status. To evaluate the relative contribution of mRNA abundance versus translational efficiency to overall regulation of GPX1 expression, we quantified GPX1, GPX4 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts per cell in rat liver. Surprisingly, we found that GPX1 transcripts in Se deficiency are moderately abundant and similar in abundance to GAPDH and other selenoprotein mRNAs; Se supplementation increases GPX1 mRNA so that it is 30-fold higher than GAPDH mRNA. Translational efficiency of GPX1 mRNA is half of that of GPX4. Translational efficiency of GPX1 mRNA increases approx. 20-fold with Se supplementation and appears to switch GPX1 mRNA from nonsense-mediated degradation to translation. This regulatory switch can explain why GPX1 expression is an excellent parameter for assessment of Se status.

Key words: gene expression, mRNA, nonsense-mediated decay, selenoproteins, switch.

INTRODUCTION

Glutathione peroxidase (GPX)1 (also known as classical GPX or GSH:H_2O_2 oxidoreductase; EC 1.11.1.9) and GPX4 (also known as phospholipid hydroperoxide GPX; EC 1.11.1.12) are two members of the GPX family that contain Se in the form of selenocysteine (Sec) [1–3]. GPX1 activity falls dramatically in Se deficiency, and this regulation makes it an excellent parameter for the assessment of Se status [4]. In all selenoproteins, the Sec is encoded by UGA. In eukaryotic selenoproteins, an in-frame UGA codon and a conserved 3′-untranslated region (UTR) stem-loop called the Sec insertion sequence (SECIS) are the two necessary and sufficient *cis*-acting elements that must be present in mRNA for selenoprotein translation (reviewed in [5]). Sec for translation is formed from inorganic Se and serine [6], while the serine is esterified to a unique $tRNA^{Sec}$. The SECIS element recruits the Sec-$tRNA^{Sec}$, and the Sec is incorporated co-translationally at the position specified by the UGA codon in selenoproteins, such as GPX1, GPX4, selenoprotein P (Sel P) and thioredoxin reductase (TRR). Thus Se availability can limit the translation of all selenoproteins. In addition, GPX1 expression is uniquely regulated at the level of mRNA stability [7–10], whereas mRNA levels for GPX4 and other selenoproteins remain relatively unchanged by Se status [10–12].

The transcription rate of GPX1 is unaffected by Se status [8,13]. Recent studies suggest that GPX1 mRNA is degraded by a nonsense-mediated mRNA decay mechanism when Sec is not available for translation [14,15]. Nonsense mutations are known to destabilize many different mRNA species, particularly when located upstream from an intron (reviewed in [16,17]). Selenium regulation of GPX1 mRNA levels requires the Sec UGA codon, and requires that this Sec codon be located upstream from the intron [14,15], whereas for Sec translation, the Sec codon can be either upstream or downstream from the intron [18]. Thus the mechanism for Sec translation can be distinguished at least partially from the mechanism for GPX1 mRNA stability, but the efficiency of translation in Se-deficient compared with Se-adequate cells may determine whether transcripts are shunted toward translation or toward mRNA degradation. To determine the translational efficiency of different selenoproteins, however, absolute mRNA abundance must be measured.

We previously studied the features of GPX1 mRNA that specifically target this transcript for decay, and found that the GPX1 3′-UTR can convey significant Se regulation to β-globin mRNA when a UGA nonsense mutation is engineered upstream from the first β-globin intron [14]. This shows that sequences upstream from the GPX1 3′-UTR do not contain any specific elements required for Se regulation of mRNA level other than the UGA codon and the intron. Interestingly, the GPX4 3′-UTR in chimaeric constructs can fully substitute for the GPX1 3′-UTR both for Sec insertion and in Se regulation of GPX1 mRNA levels [14], indicating that the GPX1 3′-UTR alone cannot fully explain the unique Se regulation of GPX1 mRNA. Studies like these with recombinant mRNAs in cultured cells, however, are limited because GPX1 mRNA in Se-deficient cells only decreases to 50% of Se-adequate levels, whereas GPX1 mRNA levels fall to 10% of Se-adequate levels in Se-deficient rat liver. Thus the full effects of Se regulation on mRNA abundance and translational efficiency will not be apparent in cultured cells, and so we conducted the present study in intact rats.

The purpose of the present study was to use the well-regulated rat liver model and to quantify the effect of Se status on mRNA abundance and on translational efficiency, with the objective of discerning the respective roles of mRNA abundance and translational efficiency in the regulation of GPX1 expression. To date, no one has directly compared transcript abundance and the

Abbreviations used: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPX, glutathione peroxidase; Sec, selenocysteine; SECIS, Sec insertion sequence; Sel P, selenoprotein P; TRR, thioredoxin reductase; UTR, untranslated region.

[1] Present address: GSF-National Research Center for Environment and Health, Institute of Mammalian Genetics, 85764 Neuherberg, Germany

[2] To whom correspondence should be addressed (e-mail SundeR@missouri.edu).

resulting translational efficiency of different selenoprotein mRNAs to see if these measures match with the known features of nonsense-mediated mRNA decay. The tacit assumption has been that GPX1 mRNA levels are decreased dramatically in Se deficiency relative to other mRNA species, including other selenoprotein mRNAs. We determined the number of GPX1, GPX4, Sel P and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts per liver cell and we used SDS/PAGE analysis to quantify ^{75}Se incorporation into GPX1, GPX4 and TRR. Surprisingly, we found that GPX1 transcripts in Se deficiency are equally abundant when compared with other mRNA species, including other selenoprotein mRNAs; Se supplementation further stimulates a 4–7-fold increase in GPX1 mRNA so that GPX1 mRNA is 33–37-fold higher than GAPDH mRNA levels. Although GPX1 mRNA is highly abundant, we found that the translational efficiency of GPX1 transcripts is half, or less, of that of GPX4. These findings support the model that Sec translational efficiency is a major factor in determining the susceptibility of GPX1 mRNA to nonsense-mediated decay.

EXPERIMENTAL

Animals

Care and treatment of experimental animals was approved by the Animal Care and Use Committee at the University of Missouri. Male and female weanling rats (Holtzman, Madison, WI, U.S.A.) were fed a torula yeast-based Se-deficient diet (0.008 μg of Se/g of diet) [9] supplemented with 0 or 0.2 μg of Se/g of diet (as Na_2SeO_3) for 30 days. Food and deionized water were provided *ad libitum*. Each rat was injected (by intraperitoneal injection) with 50 μCi of ^{75}Se (as Na_2SeO_3) (2030 μCi/μg of Se; University of Missouri Research Reactor). After 24 h, the rats were anaesthetized with ether, the liver was perfused with ice-cold 0.15 M KCl, and liver samples were frozen immediately in liquid nitrogen or at -20 °C for RNA isolation and enzyme analysis respectively.

RNA isolation and analysis

The rat GPX1 and GPX4 cDNA clones isolated previously in our laboratory [4,19] and the rat Sel P cDNA [20] (provided by Dr Ray Burk and Dr Kris Hill, Vanderbilt Medical Center, Nashville, TN, U.S.A.) were subcloned into the pBluescript II (SK-) vector (Stratagene, La Jolla, CA, U.S.A.). The rat GAPDH cDNA fragment was purchased within the pTRI vector (Ambion, Austin, TX, U.S.A.). *In vitro* transcription of antisense RNA probes was performed essentially according to the manufacturer's protocol (Promega, Madison, WI, U.S.A.). The specific radioactivity of each probe was modified to balance the intensity of the signals for the different mRNA species within the same gel lane. The GPX4 and GAPDH probes were synthesized to the highest specific radioactivity using 50 μCi of [^{32}P]UTP (3000 Ci/mmol; NEN, Boston, MA, U.S.A.) per reaction. The GPX1 and Sel P probes were synthesized to a lower specific radioactivity, using 20 and 10 μCi of [^{32}P]UTP respectively, per reaction. The amount of unlabelled UTP in each reaction was as follows: 20 μl transcription reactions for the GPX1, GPX4, GAPDH and Sel P probes contained 6, 2, 4 and 5 μl of 100 μM unlabelled UTP respectively. Full-length probes were purified by electrophoresis through a 6 % (w/v) polyacrylamide gel, eluted in 2 M ammonium acetate plus 1 % (w/v) SDS, and ethanol-precipitated. The GAPDH probe protects 316 nt, the GPX1 probe protects 547 nt (bases 411–957 [21]), the GPX4 probe protects 445 nt (bases 209–653 [22]), and the Sel P probe protects 266 nt (bases 987–1252 [20]).

The *in vitro*-synthesized sense-strand transcripts were made by transcribing the full-length cDNAs for GPX1, GPX4 and Sel P. The template for the GAPDH sense transcript, however, was not full-length. Therefore in all of the subsequent calculations for GAPDH, we corrected for the difference in size between the 316 nt GAPDH fragment and the full-length rat GAPDH cDNA (1233 bases [23]).

Total RNA was isolated from rat liver homogenate by centrifugation on 5.7 M CsCl as described previously [7]. RNA samples (10 μg of total RNA/sample) from Se-deficient and Se-adequate male and female rat livers, and 0.01, 0.05, 0.1, 0.5, 1, 5, 10 or 50 ng of each sense transcript were hybridized overnight at 45 °C with antisense RNA probes for GPX1, GPX4, GAPDH and Sel P mRNA species. The hybridization reactions were then treated with RNase (40 μg/ml RNase A and 2 μg/ml RNase T1) for 45 min at 30 °C. After RNase inactivation, the protected-probe fragments were ethanol-precipitated and the samples were analysed on a 6 % (w/v) non-denaturing polyacrylamide gel. For each liver RNA sample, the GPX1, GPX4 and Sel P mRNA signals were normalized to the GAPDH mRNA signal. All samples were analysed in duplicate.

Enzyme assays

To prepare liver-cell supernatant, samples of liver tissue were homogenized in 9 vol. 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 (10000 ***g*** for 15 min at 4 °C; model J2-21M; JA-21 rotor; Beckman Instruments, Palo Alto, CA, U.S.A.). GPX1 activity was measured by the coupled assay procedure [24], using 120 μM H_2O_2. GPX4 activity was measured by the coupled assay procedure [10], using 78 μM phosphatidylcholine hydroperoxide. For both GPX1 and GPX4 assays, 1 enzyme unit is the amount of enzyme that will oxidize 1 μmol of GSH/min under these conditions. The protein concentration of each sample was determined by the method of Lowry et al. [25].

^{75}Se-labelled GPX1 and GPX4 protein standards

To obtain authentic ^{75}Se-labelled GPX1 and GPX4 proteins, which could be used as standards, an adult male chow-fed rat was injected intravenously with 200 μCi of ^{75}Se and killed 3 days later as described above for the other experimental animals. The liver was perfused with ice-cold 0.15 M KCl, and liver cytosol was prepared from a 3 g portion of the fresh tissue and separated by Sephadex G-150 chromatography as described by Sunde and Evenson [6]. Fractions (5 ml) were collected and assayed for ^{75}Se (Packard Auto-Gamma 5650; Packard Instruments, Downers Grove, IL, U.S.A.), protein (A_{280}; Uvicon 940; Kontron Instruments, Zurich, Switzerland), and GPX1 and GPX4 activity as described above. Fractions that contained the GPX1 and GPX4 activity peaks were pooled separately, concentrated and desalted by ultrafiltration (YM10 > 10000 MW membrane; Amicon Corporation, Danvers, MA, U.S.A.) in 50 mM sodium phosphate, 0.5 mM GSH and 0.25 mM EDTA, pH 6.85. Prepared this way, GPX4 only contains the 19 kDa ^{75}Se-labelled GPX4; the approximately 90 kDa GPX1 peak contains principally the 23 kDa ^{75}Se-labelled GPX1 subunit, but also the 57 kDa ^{75}Se-labelled TRR subunit [26].

SDS/PAGE analysis

Liver samples from the male and female weanling rats (1500 μg of protein) were analysed for ^{75}Se-labelled proteins as previously

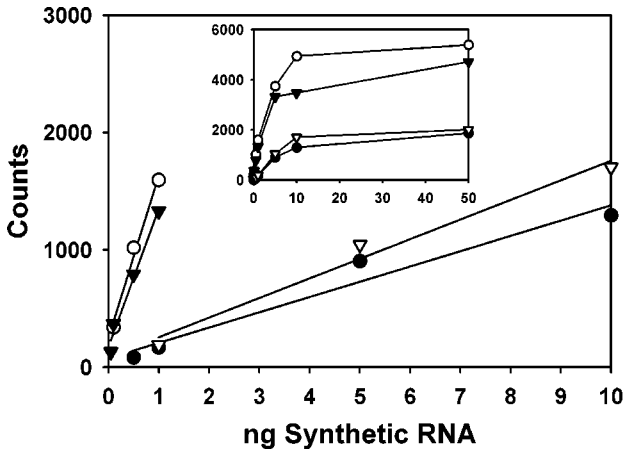

Figure 1 Standard concentration curves generated with *in vitro*-synthesized sense-strand RNA

Individual probes for GPX1 (●), GPX4 (○), GAPDH (▼) and Sel P (▽) were incubated with increasing amounts of sense transcript plus 10 μg of yeast tRNA, then subjected to RNase digestion. The protected probe fragments were quantified by direct imaging of the RNase protection assay gel. Linear regression (first order) is shown for the region of each standard curve which includes the working concentrations of the specific transcripts in liver RNA samples. The working concentrations of mRNA in the 10 μg total RNA samples were: GPX4, 0.4–0.6 ng; GAPDH, 0.3–0.4 ng; Sel P, 5.7–10.5 ng; and GPX1, 0.8–9.4 ng. The inset shows the full standard curves for each probe, including the non-linear regions.

described [26]. In addition, a sample of non-radioactive liver supernatant (1500 μg of protein) spiked with the partially purified ^{75}Se-labelled GPX1 and/or GPX4 was also analysed simultaneously so that the migration position of the GPX1 and GPX4 proteins in liver supernatant samples could be identified precisely. Standard molecular mass markers (Wide Range Color Markers; Sigma, St Louis, MO, U.S.A.) were also included in each gel. Each gel lane was sliced horizontally into 2 mm slices, which were counted individually for ^{75}Se. All counts were corrected for decay to the day of injection.

RESULTS

Absolute quantification of the abundance of selenoprotein transcripts in rat liver

The purpose of these experiments was to directly compare the transcript abundance and translation efficiencies of GPX1 and GPX4 in Se-deficient and Se-adequate rat liver. To quantify the number of selenoprotein transcripts in rat liver, we generated standard curves (Figure 1) for each mRNA species using known amounts of *in vitro*-synthesized RNA. As expected, with increasing amounts of target RNA, the system became saturated (Figure 1, inset) and no longer accurately reflected transcript abundance. The same probes calibrated by the standard curves were used for RNase protection assays of the rat liver RNA samples (Figure 2). The signal intensities resulting from probe protection by liver mRNA were quantified using linear-range standard curves, which included working concentrations of each mRNA species (Figures 1 and 2).

The resulting abundance of each mRNA species was first determined as the mass proportion of each mRNA in total RNA (Figure 3A). On the basis of mass, Sel P and Se-adequate GPX1 mRNA were the most abundant of the four mRNA species studied, making up 6×10^{-4} to 1×10^{-3} of the mass of the original

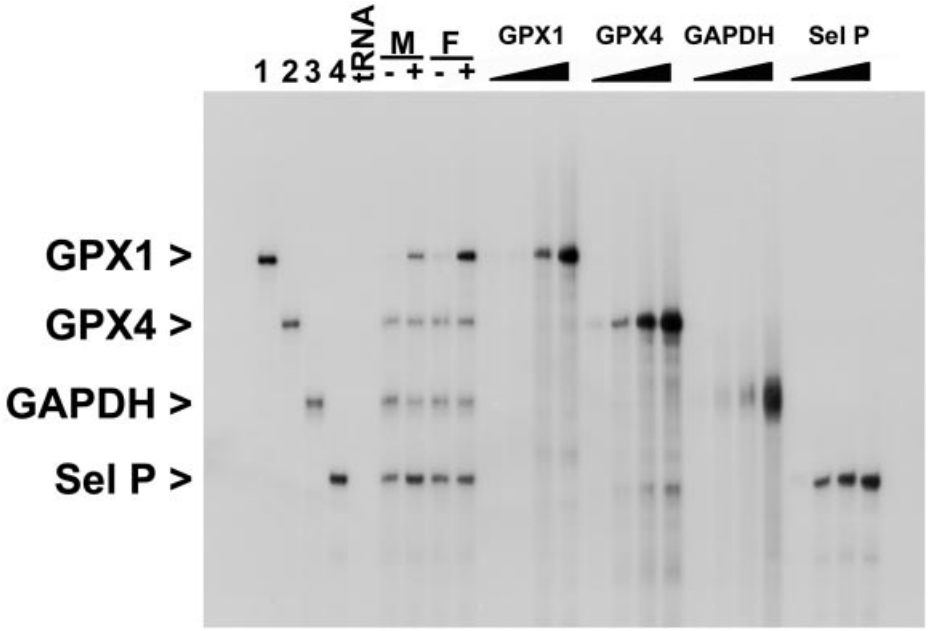

Figure 2 RNase protection assay autoradiogram for rat liver RNA with *in vitro*-synthesized RNA standards

Samples of total RNA (10 μg) from Se-adequate female rat liver protect a single specific mRNA fragment when incubated with the individual probes for GPX1, GPX4, GAPDH and Sel P (lanes 1–4). Yeast tRNA (10 μg) did not protect any probe fragments when incubated with all four of the probes (lane tRNA). Total RNA (10 μg) from Se-deficient (−) and Se-adequate (+) male (M) and female (F) rat liver was incubated with all four probes simultaneously. A subset of the *in vitro*-synthesized RNA standard curves (Figure 1), which encompasses the working concentration of each transcript in rat liver, is shown in the gel lanes marked by wedges. Concentrations of the standards shown in this Figure are: GPX1, 0.5, 1, 5 and 50 ng; GPX4, 0.1, 0.5, 1 and 5 ng; GAPDH, 0.1, 0.5, 1 and 5 ng; and Sel P, 1, 5, 10 and 50 ng.

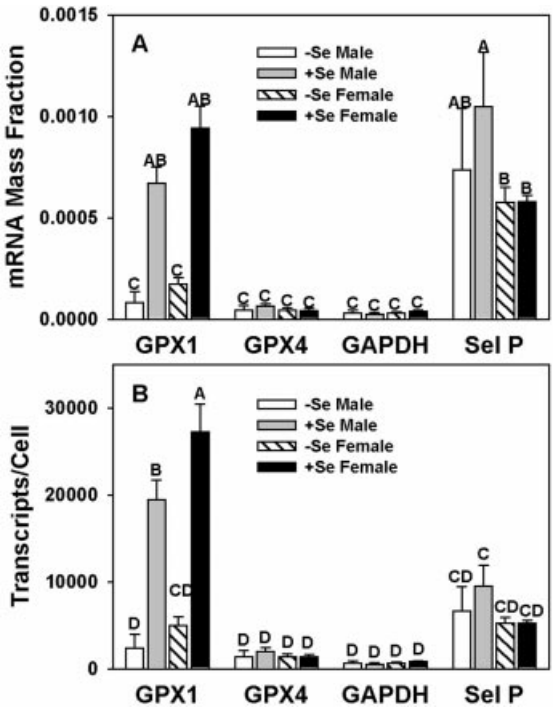

Figure 3 Quantification of mRNA abundance by mass (A) and by transcript number (B)

(**A**) The mass of each mRNA species within 10 μg of total RNA was determined by RNase protection assay analysis using the standard curves shown in Figure 1. (**B**) Transcript number was determined as: (mass of mRNA/mass of total RNA) $\times$ Avogadro's constant $\times$ molecular mass of transcript $\times$ (2.1×10^{-2} ng of total RNA/rat liver cell) = molecules of mRNA/cell. Bars indicate the mean ($\pm$S.E.M., $n = 3$) GPX1, GPX4, GAPDH and Sel P mRNA abundance in Se-deficient ($-$Se) and Se-adequate ($+$Se) male and female rat liver. Within a graph, bars sharing a common letter are not significantly different ($P < 0.01$) by analysis of variance.

total RNA sample from rat liver. Moderately abundant mRNAs, such as β-actin, are reported to contribute up to 3×10^{-4} of the total RNA mass [27].

To better understand translational efficiency, the data were converted from fractional transcript mass into number of transcripts/cell (Figure 3B). This was calculated using the molecular mass of each transcript and the amount of total RNA per liver cell (2.1×10^{-2} ng of total RNA/rat liver cell) reported by Lee and Costlow [28]. When expressed as transcripts/cell, Se-adequate GPX1 mRNA was by far the most abundant of the four mRNA species studied. In the present study, GPX1 expression was highest in the female rats, consistent with past studies in which GPX1 expression in female rats was reported to be 1.7–3-fold greater than the level observed in male rats [9,29,30]. In this sense, female rats are a natural overexpression model for studies investigating GPX1 expression and regulation. GPX1 transcript abundance is significantly affected both by dietary Se status and by sex of the animal, whereas transcript abundance of GPX4, GAPDH and Sel P is not affected by dietary Se or by gender. The abundance of GPX1 mRNA in Se-adequate male and female rat liver was 33–37-fold higher than GAPDH mRNA, which is considered to be a moderately abundant transcript [31]. This demonstrates that, in spite of the dramatic destabilization of GPX1 mRNA in Se-deficiency, the abundance of GPX1 transcripts in Se-deficient rat liver does not fall below the abundance of other selenoprotein transcripts in either Se-deficient or Se-adequate rat liver (Figure 3B).

Table 1 Effect of dietary Se on rat liver GPX1 and GPX4 enzyme activity

GPX1 and GPX4 activities (enzyme units/g of protein) were measured in Se-deficient ($-$Se) and Se-adequate ($+$Se) male and female rat liver supernatants. Values represent the means $\pm$ S.E.M. ($n = 3$). Within the GPX1 column or the GPX4 column, values sharing a common symbol are not significantly different ($P < 0.05$) by analysis of variance.

	Enzyme activity (enzyme units/g of protein)			
	GPX1		GPX4	
Gender	$-$Se	$+$Se	$-$Se	$+$Se
Male	16 ± 3‡	504 ± 43†	0.63 ± 0.23†	3.27 ± 0.15*
Female	31 ± 4‡	1213 ± 92*	0.70 ± 0.15†	3.00 ± 0.42*

GPX1 and GPX4 enzyme activities

In this experiment, both GPX1 and GPX4 activities in liver supernatant were significantly reduced in the Se-deficient animals (Table 1), with GPX1 and GPX4 activities falling to 3 and 25% respectively, of Se-adequate levels. In other similar experiments where rat liver cytosol, rather than supernatant, was measured, GPX1 activity in Se-deficient cytosol decreased to less than 1% of Se-adequate levels [9,12]. The increased GPX1 expression in female rats compared with male rats was more pronounced for GPX1 activity levels (Table 1) than for GPX1 mRNA levels (Figure 3).

Quantification of GPX1 and GPX4 polypeptide synthesis

GPX1 and GPX4 activities reflect the steady-state levels of the two proteins. To estimate the synthesis rates for GPX1 and GPX4 proteins, ^{75}Se incorporation into GPX1 and into GPX4 over a 24 h period was determined by SDS/PAGE analysis and ^{75}Se counting of the Se-labelled protein bands. GPX1, GPX4 and TRR each have one Se per subunit, so ^{75}Se incorporation directly indicates the number of subunit polypeptides synthesized. To ensure that the GPX1 and GPX4 Se peaks were correctly identified, authentic ^{75}Se-labelled GPX1 and GPX4 were used to spike non-radioactive liver supernatant samples (Figures 4A and 4B). The native GPX1 protein is made up of four 23 kDa subunits [1], and a second selenoprotein, TRR, composed of two 57 kDa subunits [32], migrates with the Sephadex G-150 GPX1 peak. Thus when non-radioactive liver supernatant was spiked with this fraction, SDS/PAGE analysis detected both the ^{75}Se-labelled 23 kDa GPX1 subunit, as well as the ^{75}Se-labelled 57 kDa TRR. When non-radioactive liver supernatant was spiked with both authentic GPX1 and GPX4, a third ^{75}Se-labelled band corresponding to the 19 kDa GPX4 subunit [2] was clearly detected. These control experiments affirmed the migration positions of the GPX1 and GPX4 subunits, and showed that the SDS/PAGE procedure could sufficiently separate and resolve the two ^{75}Se-labelled bands for quantification purposes. Incorporation of ^{75}Se into Sel P (80 kDa) was not detected in the present study because Sel P is a secreted plasma selenoprotein [20]. Separation of unfractionated ^{75}Se-labelled liver supernatant by SDS/PAGE resulted in a profile of ^{75}Se-labelled bands identical with that of the GPX1- plus GPX4-spiked liver supernatant (Figures 4C–4F).

GPX1 synthesis is higher than the other selenoproteins in Se-adequate rats

In Se-adequate rats, GPX1 subunits are the predominant ^{75}Se-labelled selenoprotein in both male and female rats 24 h after

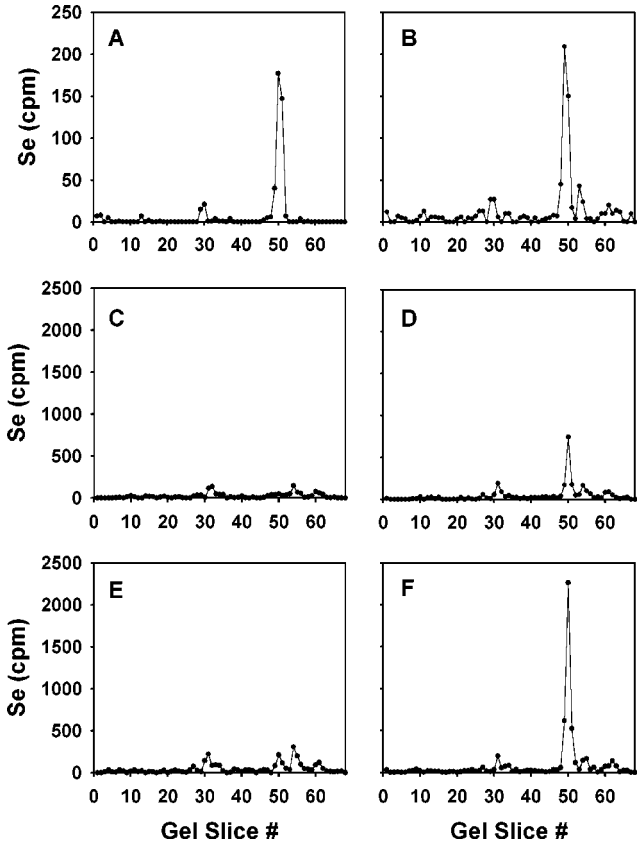

Figure 4 Analysis of ^{75}Se-labelled proteins in rat liver by SDS/PAGE

Rat liver supernatant (1500 μg of protein) was electrophoresed using 3 mm gels [14 cm × 16 cm; 0.1% SDS/7.5–20% (w/v) acrylamide gradient, pH 8.8; 4.5% (w/v) acrylamide stacking gel, pH 6.8]. ^{75}Se incorporation into selenoproteins was determined by counting 2 mm slices of each gel lane. (**A**) Non-radioactive liver supernatant spiked with partially purified ^{75}Se-labelled GPX1. (**B**) Partially purified ^{75}Se-labelled GPX1 plus partially purified ^{75}Se-labelled GPX4. (**C**) ^{75}Se-labelled rat liver supernatant from a Se-deficient male rat. (**D**) Se-adequate male rat ^{75}Se-labelled liver supernatant. (**E**) Se-deficient female rat ^{75}Se-labelled liver supernatant. (**F**) Se-adequate female rat ^{75}Se-labelled liver supernatant.

^{75}Se injection (Figures 4D and 4F, and Figure 5). This ^{75}Se incorporation into GPX1 in female rats was 9-fold higher than ^{75}Se incorporation into GPX4 and TRR. In male Se-adequate rats, ^{75}Se incorporation into GPX1 was 3-fold higher than incorporation into GPX4 and TRR. In Se-deficient rats, in contrast, ^{75}Se incorporation into GPX1, GPX4 and TRR was similar, and was also virtually the same as the level of ^{75}Se incorporation into TRR and GPX4 in Se-adequate rats (Figure 5). These apparently similar rates of ^{75}Se incorporation in Se-deficient and Se-adequate rat liver do not reflect total Se incorporation rates, because of dilution of ^{75}Se in Se-adequate liver. Based on previous experiments, the specific radioactivity of Se in Se-deficient rat liver 24 h after ^{75}Se injection is approximately 15 times that in Se-adequate liver in both male and female rats [33,34]. Adjusting the ^{75}Se incorporation values shown in Figure 5 to total Se incorporation values (by multiplying the Se-adequate values by this factor of 15; results not shown) only further amplifies the increased synthesis of GPX1 in Se-adequate liver relative to Se-deficient liver. The resulting calculated increase in total Se incorporation into GPX1 with Se supplementation (60-fold and 150-fold in male and female rats respectively) was of the same order of magnitude as differences in measured enzyme activity in rat liver cytosol [9,10,12].

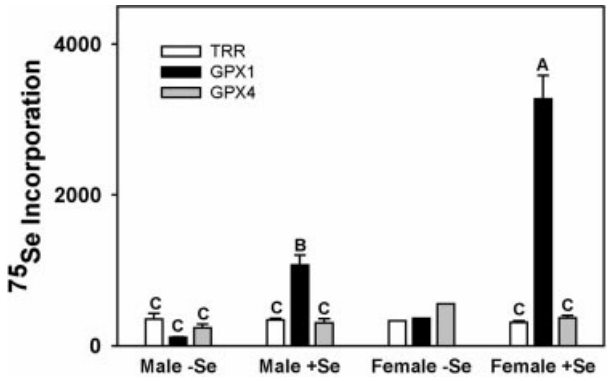

Figure 5 Quantification of 24 h ^{75}Se incorporation into rat liver selenoproteins

^{75}Se incorporation into TRR, GPX1 and GPX4 protein bands was determined by SDS/PAGE as shown in Figure 4. Bars indicate the mean ^{75}Se c.p.m. (±S.E.M., $n = 3$; $n = 2$ for Se-deficient females) in each protein in Se-deficient (−Se) and Se-adequate (+Se) male and female rat liver. Bars sharing a common letter are not significantly different ($P < 0.05$) by analysis of variance. In this Figure, ^{75}Se incorporation into Se-adequate selenoproteins was not adjusted for differences in ^{75}Se specific radioactivity.

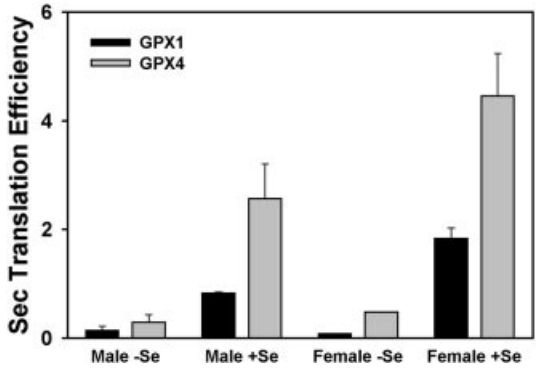

Figure 6 Assessment of relative translation efficiency for GPX1 and GPX4 transcripts

Within each individual rat, the relative Se incorporation into GPX1 and into GPX4 was divided by the number of transcripts for GPX1 and GPX4 (see Figure 3B). Bars indicate the mean Sec translation efficiencies (arbitrary units) for GPX1 and GPX4 transcripts (±S.E.M., $n = 3$; $n = 2$ for Se-deficient females) in each protein in Se-deficient (−Se) and Se-adequate (+Se) male and female rat liver. For Se-adequate samples, the ^{75}Se incorporation was multiplied by a factor of 15 to adjust for differences in specific radioactivity (see the text) so that total Sec translational efficiency could be compared with that in Se-deficient animals.

GPX1 translational efficiency is half of GPX4 translation efficiency

To determine the relative contribution of mRNA abundance versus Sec translational efficiency on overall regulation of GPX1 expression, Sec translational efficiency was calculated as the ratio of Se incorporation to transcript abundance (Se-labelled subunits/mRNA transcripts) for both GPX1 and GPX4 (Figure 6). In Se-adequate males and females, translational efficiency of GPX1 synthesis was 50%, or less, than that of GPX4, demonstrating that more successful Se incorporation events occur per GPX4 transcript than per GPX1 transcript. In Se-deficiency, GPX1 translational efficiency was also 50%, or less, of that of GPX4 in both male and female rats (Figure 6). These comparisons of translational efficiency are direct comparisons within the same gender and Se status, and thus are not affected by differences in

Se specific radioactivity. In Figure 6, the plotted translational efficiencies were corrected for Se isotope dilution (see above) to show the relative Sec translational efficiency in Se-adequate versus Se-deficient liver. In Se-deficiency, the relative Sec translational efficiencies for both GPX1 and GPX4 decreased to 4–6 % and 6–10 % respectively, of Se-adequate translational efficiencies, showing the impact of impaired Sec availability on selenoprotein expression in Se-deficient cells. Thus in liver cells in intact rats, Se supplementation increases the translational efficiency of GPX1 mRNA approx. 20-fold and increases the translational efficiency of GPX4 mRNA approx. 12-fold, relative to the translational efficiency in Se-deficient cells.

DISCUSSION

In past experiments, we and others used standard Northern-blot and RNase protection assay analyses to determine the effect of cellular Se status on selenoprotein mRNA levels. These standard methods, however, do not allow direct comparison of abundance of two or more mRNA species, because different probes can have different specific activities and can hybridize to different proportions of the target transcript. In the present study we implemented an assay for absolute quantification of mRNA in which the probes were calibrated using known concentrations of *in vitro*-transcribed RNA. This new approach permits direct comparison of GPX1 mRNA abundance with that of other transcripts, such as GPX4, which are not regulated by Se status.

Our previous Northern-blot experiments indicated that GPX1 mRNA levels decrease to visually undetectable amounts in Se-deficient rat liver relative to Se-adequate rat liver. In the current study, using the more sensitive RNase protection assay method, we found that steady-state GPX1 mRNA in Se-deficient liver was at least as abundant as GAPDH mRNA and thus can be considered a moderately abundant transcript rather than a rare transcript. Selenium status had no effect on transcript abundance of GAPDH, as well as GPX4 and Sel P, but Se supplementation increased GPX1 mRNA 4–7-fold (Figure 3). Importantly, these measures have revised our model for Se regulation of GPX1 mRNA; the present study now makes it clear that GPX1 mRNA levels in Se-adequate conditions are uniquely up-regulated when compared with the other selenoprotein mRNAs (Figure 7).

Using these measures of mRNA abundance, we were able to directly calculate translational efficiencies of GPX1 and GPX4 using ^{75}Se incorporation into these selenoproteins. Under both Se-deficient and Se-adequate conditions, the efficiency of GPX1 translation was half, or less, of that of GPX4. In addition to changes in mRNA abundance, relative Sec translational efficiency increased 17–24-fold and 10–15-fold for GPX1 and GPX4 respectively (Figure 6), under Se-adequate conditions compared with Se-deficient conditions. The combined increase in translational efficiency and in GPX1 mRNA abundance explains the greater than 100-fold increase in measured GPX1 activity in Se-adequate compared with Se-deficient liver, and explains why elevated GPX1 expression in Se-adequate rat liver accounts for 50 and 75 % of rat liver Se in males and females respectively [9]. For GPX4, Se regulation only utilizes changes in translational efficiency, thus explaining the rather modest changes in rat liver GPX4 activity in Se-adequate compared with Se-deficient liver [10].

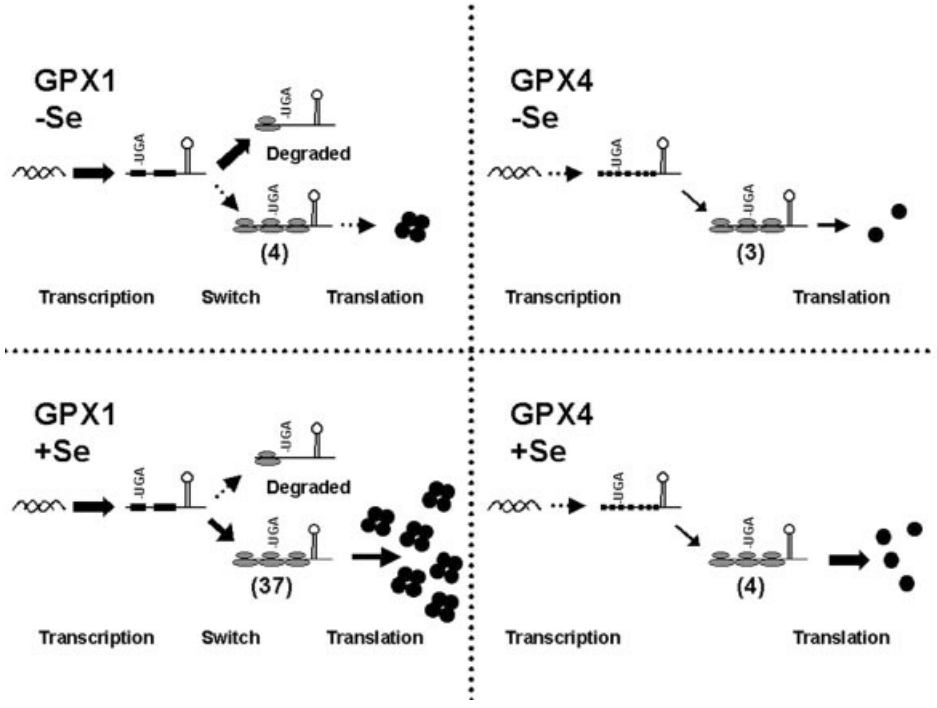

Figure 7 Model for the differential Se-regulation of GPX1 and GPX4 expression

The level of GPX1 (left-hand panels) and GPX4 (right-hand panels) expression in Se-deficient (−Se; top panels) and Se-adequate (+Se; bottom panels) rat liver is determined by transcription rate, by a Se-dependent switch that directs mRNA towards nonsense-mediated decay versus translation, and by translational efficiency. The switch is not shown for GPX4 because GPX4 mRNA is not susceptible to nonsense-mediated decay. Relative flux at each step is indicated by the magnitude of the arrow. Absolute levels of mRNA (untranslated plus translated) relative to GAPDH transcript levels in +Se male rats (see Figure 3B, 1 ≈ 500 GAPDH transcripts per cell) are shown in parentheses. Resulting steady-state levels of GPX1 and GPX4 enzyme are illustrated by ❀ and ● respectively, in −Se and +Se liver.

So, what does this tell us about the mechanism of Se regulation of GPX1 mRNA abundance? It has become clear that Se regulation of GPX1 mRNA requires a SECIS element and a Sec codon followed by a downstream intron [14,15]. The requirement for intron sequences downstream from the UGA codon strongly suggests that GPX1 mRNA is destabilized by a nonsense-mediated mRNA decay mechanism. This mechanism is similar to the destabilization caused by nonsense mutations in a number of other mRNA species [16,17]. The GPX1 SECIS element can convey Se responsiveness to β-globin mRNA containing a UGA nonsense mutation [14]. Furthermore, a Sec → Cys mutation will stabilize GPX1 mRNA [14,15]. Collectively, this suggests that Sec translation can abrogate nonsense-mediated mRNA decay. The present experiments were undertaken to better understand the Se-specific regulation of GPX1 mRNA in the context of more general regulatory mechanisms for mRNA stability.

A co-translational mRNA surveillance system is known to recognize transcripts containing a premature termination codon and to target these transcripts for degradation [35]. Part of this recognition process involves a scanning mechanism that searches for a splice junction in sequences located downstream from the nonsense codon. The positions of introns within heteronuclear RNA are suggested to be marked by ribonucleoproteins that become associated with the mRNA during transcription and splicing. This marking mechanism shunts aberrant transcripts to degradation during the first round of translation [36]. Sec insertion at a UGA codon offers the ability to convert this general nonsense-mediated decay process into an Se-dependent switch between translation and degradation (Figure 7). The rule is that an exon junction located at least 50–55 nt downstream from a termination codon defines the transcript as aberrant [17]. Notably, in the mouse GPX1 gene, an intron is located 105 nt downstream from the Sec codon [37]. In mouse GPX4, by contrast, the intron is located 45 nt downstream from the Sec codon [38]. This GPX4 genomic structure does not satisfy the nonsense codon-splice junction rule, predicting that GPX4 mRNA would not be subject to nonsense-mediated decay (Figure 7), thus accounting for the observed minimal effect of Se status on steady-state GPX4 mRNA levels (Figure 3).

Our studies presented in this paper have compared the translational efficiencies of GPX1 and GPX4 in an intact animal system, and are the first to quantify absolute selenoprotein transcript abundance. In recombinant cultured cell systems, mammalian selenoprotein mRNAs are translated with very low efficiency when compared with Sec → Cys mutants [5,18,39–44]. In prokaryotes, the ratio of selenoprotein mRNA, the Se-specific Sel B translation factor and selenocysteyl-tRNA has been shown to be critical for optimal translation of the Sec codon [45,46]. Translation of a β-galactosidase reporter is considerably faster when it is located before the bacterial formate dehydrogenase Sec codon, and translation slows considerably when placed after the Sec codon, suggesting that assembly of the Sec translation complex is also rate-limiting for bacterial selenoprotein synthesis [47]. Similar assembly constraints, along with differences in SECIS affinity, local codon context and UGA position [5,48,49], all appear to contribute to the reduced efficiency of selenoprotein synthesis in higher animals, and may contribute to the difficulty in overexpressing selenoproteins in cultured cells.

The present studies affirm that GPX1 mRNA is the most highly abundant selenoprotein mRNA in rat liver under Se-adequate conditions. More interestingly, these studies show that even under Se-deficient conditions, GPX1 mRNA is at least as abundant as other moderately abundant transcripts in spite of its instability. Steady-state levels of mRNA reflect the dynamic interaction between synthesis and degradation. This suggests that GPX1 transcription is elevated relative to the transcription of other selenoprotein mRNAs (Figure 7), but, paired with accelerated degradation, this results in moderate constitutive expression at steady-state in Se-deficiency (Figure 7). The impact of this dynamic condition is that an increase in Se status will cause an increase in Sec translational efficiency of both efficiently-translated GPX4 and less efficiently-translated GPX1 (Figure 7). For GPX1, this will also cause the marked switch from mRNA degradation to GPX1 translation, resulting in increased GPX1 mRNA abundance. The product of these effects is the increase in GPX1 synthesis so that GPX1 can account for up to 75% of total liver Se. We have suggested that GPX1 serves as a biological Se buffer or Se store; our hypothesis is that this ability of GPX1 to dramatically respond to changes in Se status is an important aspect of GPX1 function [3,4]. Evolution may have taken advantage of co-translational Sec insertion combined with nonsense-mediated decay to provide a mechanism for control of intracellular storage of Se. This regulatory switch appears to have the capacity to respond to changes in Se status almost as readily as the iron-mediated de-repression of ferritin synthesis [50], and perhaps at the same order of magnitude as zinc-dependent up-regulation of metallothionein transcription [51]. The control of GPX1 expression provided by such a regulatory switch – a Se 'thermostat' or selenostat – can explain why GPX1 expression is the fundamental parameter of choice for assessment of Se status and setting of Se requirements.

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