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Selenium regulation of thioredoxin reductase activity and mRNA levels in rat liver☆

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Abstract

Mammalian thioredoxin reductase (TRR; $NADPH_2$:oxidized thioredoxin oxidoreductase, E.C. 1.6.4.5) is a new member of the family of selenocysteine-containing proteins. TRR activity in Se-deficient rat liver is reported to decrease to 4.5 to 15% of the activity in Se-adequate rat liver, similar to the fall in Se-dependent glutathione peroxidase-1 activity. Both glutathione peroxidase-1 enzyme activity and mRNA levels decrease dramatically in Se deficiency, whereas glutathione peroxidase-4 activity only decreases to 40% of Se-adequate levels and mRNA level is little affected by Se deficiency. The purpose of these experiments is to study the effect of Se status on TRR mRNA levels and enzyme activity in our well-characterized rat model, and to compare this regulation directly to the regulation of other Se-dependent proteins in male weanling rats fed Se-deficient diets or supplemented with dietary Se for 28 days. In two experiments, TRR activity in Se-deficient liver decreased to 15% of Se-adequate activity as compared to 2% and 40% of Se-adequate levels for GPX1 and GPX4, respectively. Using ribonuclease protection analysis, we found that TRR mRNA levels in Se-deficient rat liver decreased to 70% of Se-adequate levels. This decrease in TRR mRNA was similar to the GPX4 mRNA decrease in Se-deficient liver in these experiments, whereas GPX1 mRNA levels decreased to 23% of Se-adequate levels. This study clearly shows that TRR represents a third pattern of Se regulation with dramatic down-regulation of enzyme activity in Se deficiency but with only a modest decrease in mRNA level. The conservation of TRR mRNA in Se deficiency suggests that this is a valued enzyme; the loss of TRR activity in Se deficiency may be the cause of some signs of Se deficiency.

Keywords: Thioredoxin; Requirements; Glutathione peroxidase-1; Glutathione peroxidase-4; Selenoprotein P; Ribonuclease protection analysis

1. Introduction

Thioredoxin reductase (TRR; $NADPH_2$:oxidized thioredoxin oxidoreductase, E.C. 1.6.4.5) is a member of the Type-I pyridine nucleotide-disulfide oxidoreductase enzyme family [1]. Like the other members of this family, TRR catalytically transfers two electrons from NADPH, via FAD and an N-terminal disulfide active-site, to the substrate [2]. Unlike many other members of this family, mammalian TRR contains a penultimate selenocysteine (Sec) residue at its carboxyl terminus [3–6]. In addition to mammals, TRR has also been identified as the sole selenoprotein in *C. elegans* [7,8]. Careful investigations to determine the role of the penultimate Sec residue in TRR during catalysis indicate that the Se atom transfers electrons to oxidized thioredoxin (Trx) [2,9–11]. Reduced Trx subsequently serves as an important source of reducing equivalents for ribonucleotide reductase and other proteins and enzymes containing redox-active disulfide groups [12], and TRR has recently been shown to reduce dehydroascorbate and ascorbate radical, providing a new antioxidant role for selenium [13,14]. TRR activity is likely to be an important aspect of selenium's role in mammalian biology.

Presently 18 known selenoproteins have been identified in mammals [15,16], including three isoforms of TRR (TRR1, TRR2, and TRR3) that all have Sec as the penultimate amino acid [5,12]. Classic selenium-dependent glutathione peroxidase-1 (GPX1, glutathione: H_2O_2 oxidoreductase, E.C. 1.11.1.9) activity in rat liver decreases

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dramatically in Se deficiency to 1% of Se-adequate levels [17,18]. In contrast, phospholipid hydroperoxide glutathione peroxidase (GPX4, E.C. 1.11.1.12) only decreases to 40% of Se adequate levels in the same animals [19]. Most interestingly, GPX1 mRNA levels are also regulated by Se status, falling to <10% of Se adequate levels in Se-deficient tissue, whereas GPX4 mRNA levels are little affected. Se regulation of GPX1 mRNA occurs at the level of mRNA stability and is controlled by nonsense mediated decay [20, 21]. Se-dependent thyroxine deiodinase-1 and selenoprotein P protein levels are reported to fall to approximately 10% of Se-adequate levels, with only modest decreases in mRNA levels [22,23]. TRR activity in rat liver is reported to decrease to as little as 4.5% of Se adequate levels in rats fed a Se-deficient diet for 14 weeks [24], suggesting that the mechanism responsible for substantial down regulation of TRR activity in Se deficiency may also include altered TRR mRNA stability.

The purpose of these experiments is to study the effect of Se status on TRR mRNA levels and enzyme activity in our well-characterized rat model, and to compare this regulation directly to the regulation of other Se-dependent parameters. We found that TRR mRNA levels in Se-deficient rat liver decreased to 70% of Se-adequate levels whereas TRR activity fell to 15% of Se-adequate levels. This indicates that Se regulation of TRR expression is a third pattern of selenoprotein regulation in addition to the patterns characteristic of GPX1 and GPX4.

2. Methods and materials

2.1. Animals and diets

These studies were conducted using rapidly-growing weanling rats fed torula-yeast diets [18]. Male weanling rats, 21 days old, were obtained from Holtzman (Madison, WI USA) and housed individually in hanging wire mesh cages. Initial body weights ranged from 50 to 80 g. The care and treatment protocol of the experimental animals was approved by the Institutional Animal Care and Use Committee at the University of Missouri. The diet was a 30% torula-yeast diet [25] supplemented with 4 g/kg D, L-methionine (U.S. Biochemical, Cleveland, OH USA) and 100 mg/kg all-rac-α-tocopheryl acetate to allow adequate growth and to prevent liver necrosis, respectively. In experiment 1, rats were assigned randomly to one of several dietary treatment groups and fed the basal diet (0.008 μg Se/g) or supplemented with 0.2 μg Se/g diet as Na_2SeO_3 (Sigma Chemical Co., St. Louis, MO), or in experiment 2, supplemented with graded levels of dietary Se (0.02 to 0.3 μg Se/g diet). Rats had free access to food and deionized water. Body weights were recorded weekly during the 28 day experiments. At sacrifice, rats were anesthetized with ether, and whole blood was obtained by cardiac puncture using heparinized syringes and assayed the same day. Livers were perfused in situ with ice-cold 150 mM KCl to flush out erythrocytes, and then frozen for later analysis. All named chemicals, unless otherwise indicated, were purchased from Sigma Chemical (St. Louis, MO USA).

2.2. Enzyme activity assays

For GPX activity measurements, whole blood was centrifuged (1400 $\times$ g, 15 min, GPR tabletop centrifuge, GH-3.7 rotor, Beckman instruments, Palo Alto, CA) to separate plasma from erythrocytes. Red blood cells were reconstituted to the original volume using buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3). Approximately 0.5 g of each large left liver lobe was homogenized (Polytron homogenizer, Model PT 10/35, Brinkman, Westbury, NY) in 9 vol of 0.25 M sucrose, pH 7.4, containing 20 mM Tris-HCl, 0.1% Triton X-100 [19]. Homogenates were centrifuged (15,000 $\times$ g, 20 min, Model J2-21M, rotor JA-21, Beckman Instruments). GPX activity was determined for red blood cells, plasma and liver supernatants by the coupled assay procedure [26] using 12 mM H_2O_2 so that only Se-dependent GPX activity was measured. GPX4 activity was measured using 78 nmoles phosphatidyl choline hydroperoxide as described previously [19]. For each assay, an enzyme unit (EU) is defined as the amount of enzyme that oxidizes one micromole GSH per minute under the specified conditions [19,26]. Protein concentration was determined by the Lowry method [27].

TRR activity was assayed according to the method of Holmgren and Björnstedt [1] as described by Hill et al. [24] with slight modification. Briefly, 0.5 g of liver was homogenized in 9 vol of buffered saline (described above). Homogenates were centrifuged (13,000 $\times$ g, Model JM-21, rotor, JA-21, Beckman Instruments) and supernatants containing 10 mg protein were dialyzed against 100 volumes buffered saline for 16 hours at 4°C. Dialysates were heated at 55°C for 10 minutes and microcentrifuged at 13,000 g $\times$ 30 minutes. The TRR final assay mixture contained 5 μM thioredoxin (from Spirulina sp., Sigma T-3658, adjusted for purity), 0.39 mg insulin, 0.82 mM NADPH in a final volume of 180 μL of 85 mM HEPES with 3.4 mM EDTA. The mixture was preincubated at 37°C for 5 minutes, and the reaction started by the addition of heat-denatured supernatant (100 μg protein, warmed to 37°C). After 10.0 minutes, the reaction was stopped by the addition of 750 μL of ice-cold 6.0 M of guanidine-HCL, containing 0.4 mg/ml DTNB (5,5′-dithiobis(2-nitrobenzoic acid)). For each sample, a duplicate reaction with H_2O in place of thioredoxin served as a blank. The absorbance at 412 nm of the resulting reduced chromophore (ε = 13,600 M^{-1} cm^{-1}) was measured. Thioredoxin-dependent NADPH reduction of insulin was determined by subtracting the 412 nm absorbance of duplicate reactions without thioredoxin. One enzyme unit (EU) of TRR activity is that amount of enzyme that reduces 1 micromole of thioredoxin per minute under these condi-

tions; 1 EU is equivalent to 0.06838 A_{412} units [1,24] after correction for final sample volume (0.93 × 1/13.6).

2.3. RNA isolation and ribonuclease protection analysis

Total RNA was isolated from 0.3 g portions of liver by homogenization in guanidine isothiocyanate buffer followed by centrifugation (20 hr) on 5.7 M CsCl as described previously [28]. The RNA pellet was dissolved in diethyl pyrocarbonate-treated water and quantitated spectrophotometrically by A_{260} (ε = 25 mL · mg^{-1} · cm^{-1}).

Ribonuclease protection analysis was conducted as described previously by Weiss and Sunde [20,29]. DNA probe templates were subcloned into the pBluescript II (SK-) vector (Stratagene, La Jolla, CA), which contains the T3 and T7 promoters. In vitro transcription of antisense RNA probes was performed essentially according to the manufacture's protocol (Promega, Madison, WI). The TRR probe used in this study was amplified from the TRR est prepared by Lee [30] in pBluescript vector using PCR primers corresponding to the T7 promoter and rat TRR cDNA (bp 1396–1415 of rat TRR [6], accession no. H34190). The TRR probe was synthesized to high specific activity using 80 μCi [α-^{32}P]UTP (3,000 Ci/mmol, 10 mCi/mL) (NEN, Boston, MA) per reaction. The resulting probe protects 798 nucleotides (1396–2193) of TRR. Probes for GPX1 (bases 411–957 [31]), GPX4 (bases 209–653 [32]), GAPDH (Ambion, Austin, TX USA) and Sel P (bases 987–1252 [33]) were synthesized using 2.5 to 7.5 μCi [α-32P]UTP from above mentioned stock to protect 547, 445, 316, and 266 nucleotides, respectively. Probes were purified by electrophoresis in a 6% polyacrylamide gel, eluted in 2 M ammonium acetate containing 1% SDS, and ethanol-precipitated.

For ribonuclease protection analysis of TRR, 20 μg of total liver RNA were hybridized overnight at 45°C with a balanced mixture of the single-strand antisense RNA probes described above. Yeast tRNA served as a negative control. Each probe was also hybridized individually at twice the concentration used in the balanced mixture with RNA from a Se-adequate (0.2 μg Se/g diet) liver to verify that the probe was in excess. Hybridization reactions were treated with RNase (40 μg/mL RNase A, 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 analyzed on a 6% polyacrylamide gel. RNA protected from ribonuclease was visualized by autoradiography. Protected probe fragments in each sample were quantitated by direct imaging of the gel (Instant-Imager, Packard Instrument Company, Meriden, CT USA). The signals for TRR, GPX1, GPX4 and Sel P were normalized to the signal for GAPDH mRNA.

2.4. Statistical analysis

Data are presented as means ± SEM. In experiment 1, data analysis comparing two treatments was conducted using the unpaired student's t test ($p < 0.05$). In experiment 2, results for each dietary treatment were subjected to one-way analysis of variance (ANOVA), and differences between means were assessed by Duncan's multiple range analysis [34] ($p < 0.05$). The plateau breakpoint for each Se response curve, defined as the intersection of a line tangent to the point of steepest slope and the plateau, was calculated as described by Weiss et al. [18,35] using polynomial regression analysis (Sigma Plot, Jandel Scientific, Corte Madera, CA) to estimate the minimum dietary Se necessary to obtain plateau response.

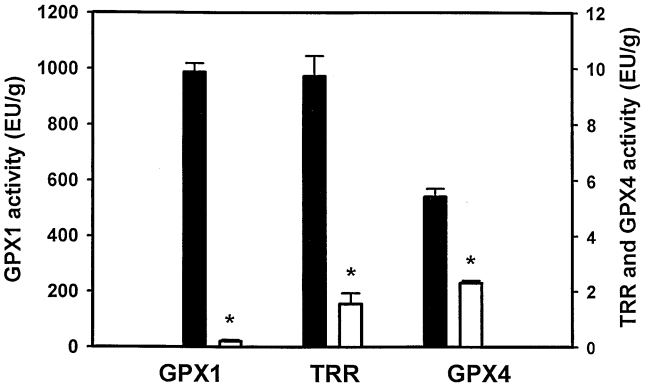

Fig. 1. Selenoenzyme activity in Se-adequate and Se-deficient rat liver. Glutathione peroxidase-1 (GPX1), glutathione peroxidase-4 (GPX4), and thioredoxin reductase (TRR) enzyme activities were assayed in rats fed either Se-adequate (filled bars, 0.2 μg Se/g diet as Na_2SeO_3) or Se-deficient (open bars, 0.008 μg Se/g diet) diets for 28 d. Values are means ± SEM (n = 10 rats per group). For each parameter, significant differences ($p < 0.05$) are designated by an asterisk (*).

3. Results

3.1. Experiment 1

To determine the effect of Se status on TRR activity and mRNA levels in Se-deficient and Se-adequate rat liver, ten weanling rats were assigned randomly to one of two dietary treatment groups and fed the basal diet or supplemented with 0.2 μg Se/g diet for 28 days. Rats fed the Se-deficient diet had the same growth rate (7.26 g/d) as rats fed the Se-adequate diet (7.22 g/d). Red blood cell and plasma GPX activities in the Se-deficient rats were 33% and 7.7% of Se adequate levels (277.3 ± 9.7 and 104.1 ± 1.6 EU/g protein, respectively), indicating that the rats were selenium deficient. In liver, Se-deficient rats had 2% of Se-adequate GPX1 activity (Fig. 1). These samples were homogenized in buffer containing Triton X-100 to permit concomitant assay of GPX4 activity, and thus the apparent GPX1 activity in Se-deficient liver supernatants was low but not at the undetectable levels observed in liver cytosol prepared with just sucrose buffer [18]. In contrast to GPX1 activity, GPX4 activity in Se-deficient liver supernatant was 43% of Se-

adequate levels (Fig. 1). Thus these two selenoperoxidase activities illustrate two distinct patterns of Se-dependent regulation, despite sharing 40% amino acid sequence identity [36].

TRR activities in Se-deficient rat liver were 16% of Se adequate levels (Fig. 1), similar to the decreases reported by Hill and colleagues [37]. To determine the effect of Se status on TRR mRNA, we used ribonuclease protection analysis to determine mRNA levels for GPX1, GPX4, selenoprotein P (Sel P), and TRR in Se-deficient and Se-adequate rats (Fig. 2a). Visually, there was little effect of Se status on SelP and GPX4 mRNA levels, but Se status had a dramatic effect on GPX1 mRNA levels as reported previously [18,19]. There was no effect of Se status on GAPDH mRNA levels, similar to other control mRNAs such as 18S rRNA [19,35]. Quantitation by direct imaging (Fig. 2b) showed that GPX1 mRNA levels decreased to 20% of Se-adequate levels. In contrast, GPX4 mRNA levels in Se-deficient liver decreased nonsignificantly to 81% of Se-adequate levels, and SelP mRNA levels in this experiment were not reduced significantly by Se deficiency. Ribonuclease protection analysis showed that TRR mRNA in Se-deficient rat liver decreased significantly to 65% of Se-adequate levels. These results indicate that TRR mRNA levels decreased to a greater extent in Se deficiency than did GPX4 and Sel P mRNA levels, but did not decrease as dramatically as GPX1 mRNA levels.

3.2. Experiment 2

A second experiment was conducted to compare the patterns of Se regulation of TRR enzyme activity and mRNA level to those of GPX1 and GPX4. Male weanling rats were fed the basal diet or supplemented with graded levels of dietary Se (0.02, 0.05, 0.075, 0.1, 0.2, and 0.3 μg Se/g diet) for 28 days. Initial body weights for the rats in this experiment ranged from 52 to 78 g. Growth was not affected by dietary Se level (8.0 $\pm$ 0.1 g/d) and final body weights ranged from 274 $\pm$ 9 to 296 $\pm$ 4 g for rats supplemented with 0.3 and 0.05 μg Se/g diet, respectively. RBC GPX1 activity in Se-deficient rats was 93 $\pm$ 4 EU/g protein as compared to 328 $\pm$ 31 EU/g protein for rats fed 0.2 μg Se/g diet. The plateau inflection point occurred at 0.11 μg Se/g diet as in earlier experiments [18,35]. In this experiment RBC GPX1 activities in rats fed 0.3 μg Se/g were not significantly higher than in rats fed 0.2 μg Se/g diet (data not shown). Plasma GPX activity in Se-deficient rats was 7.6% of the activity in Se-adequate rats (0.2 μg Se/g diet) (Fig. 3). In this study plasma GPX activity increased linearly with increasing dietary Se and reached a plateau between 0.075 and 0.1 μg Se/g diet. The plateau breakpoint for plasma GPX activity was 0.08 μg Se/g diet, calculated as described by Weiss et al. [35]. These response curves for RBC and plasma GPX activities are similar to those reported previously [18,35], indicating that Se regulation in these animals is the same as we have seen previously.

3.3. Liver selenoenzyme activity

Liver GPX1 activity levels were 584 $\pm$ 24 EU/g in rats treated with 0.2 μg Se/g diet (Fig. 4). Rats fed the basal diet had 3% of the activity in the Se-adequate group. GPX1 activity increased sigmoidally with increasing dietary Se, with a plateau breakpoint at 0.1 μg Se/g diet. In contrast, GPX4 enzyme activity in rats fed the basal diet was 53% of plateau levels of GPX4 activity in Se-adequate rats (Fig. 4), with a plateau breakpoint at 0.05 μg Se/g diet. In this experiment, liver TRR activity showed a slightly sigmoidal response curve with increasing dietary Se. Se-deficient TRR activity levels were 14% of levels in rats fed 0.2 μg Se/g diet (Fig. 4). Importantly, a clearly defined breakpoint in TRR activity occurred at 0.07 μg Se/g, showing that this level of dietary Se was sufficient to saturate rat liver TRR activity. These response curves clearly show different Se regulation patterns for these three selenoenzyme activities.

3.4. Liver selenoenzyme mRNA levels

Ribonuclease protection analysis was used to quantitate selenoprotein mRNA levels simultaneously for GPX1, GPX4, TRR, and SelP (Fig. 5A). The dramatic effect of Se deficiency on GPX1 mRNA was again observed in this study. There was no effect of dietary Se treatment on GAPDH mRNA levels. Protected probe fragments were quantitated by direct imaging to quantitate relative mRNA levels for each of the selenoproteins, and these levels were normalized to GAPDH mRNA levels to control for loading differences (Fig. 5B–E). In this study, rats fed the unsupplemented diet had GPX1 mRNA levels that were 23% of the GPX1 mRNA levels in rats supplemented with 0.2 μg Se/g diet. The plateau breakpoint for GPX1 mRNA was 0.06 μg Se/g diet. Rat liver GPX4 mRNA levels were only moderately responsive to changing Se status. In this experiment, Se-deficient GPX4 mRNA levels decreased significantly to 83% of plateau values, and reached plateau levels by 0.04 μg Se/g diet. There was no effect of Se status on SelP mRNA in this experiment.

TRR mRNA levels in Se-deficient rat liver in experiment 2 were 70% of TRR mRNA levels in Se-adequate rats (Fig. 5). Similar to GPX4, the breakpoint in the Se response curve for TRR mRNA occurred at 0.05 μg Se/g diet showing that this level of dietary Se was sufficient to support maximal levels of TRR mRNA. In summary, there was no effect of Se deficiency on Sel P mRNA levels, there was modest effect of Se-deficiency on TRR and GPX4 mRNA levels, and there was a dramatic effect of Se-deficiency on GPX1 mRNA levels.

4. Discussion

In two experiments, we have studied effect of Se status on TRR enzyme activities and mRNA levels, and compared

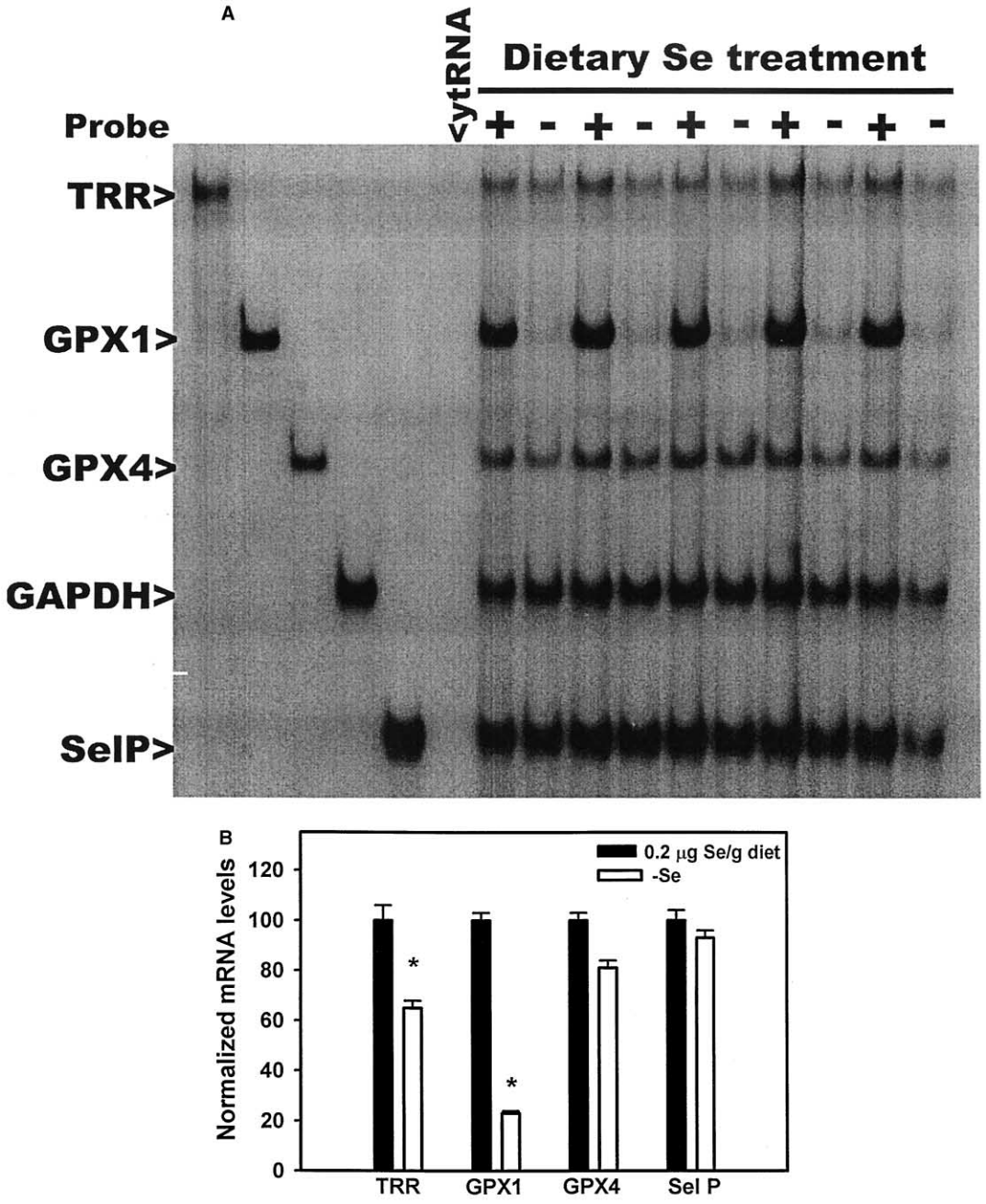

Fig. 2. Ribonuclease protection analysis (A) and quantitation (B) of selenoprotein mRNA in Se-adequate and Se-deficient rat liver. Rats were fed either Se-adequate or Se-deficient diets for 28 d. Total RNA (20 μg) was analyzed for thioredoxin reductase (TRR), glutathione peroxidase-1 (GPX1), glutathione peroxidase-4 (GPX4), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and selenoprotein P (SelP) mRNA. A Se-adequate RNA sample was hybridized individually with 2×-concentration of a single probe (lanes 1–5), or alternating Se-adequate (+) and Se-deficient (−) RNA samples (lanes 7–16) were hybridized with [^{32}P]UTP-labeled probe mixture, digested with RNase, and protected fragments analyzed on 6% polyacrylamide gels and visualized by autoradiography. Yeast tRNA (lane 6, ytRNA) was analyzed with the probe mixture as a negative control. Protected [^{32}P]UTP labeled fragments were quantitated by direct imaging of the gel and normalized to GAPDH mRNA levels in the same sample. Normalized TRR, GPX1, GPX4, and SelP mRNA in Se-adequate or Se-deficient rat liver are shown relative to Se-adequate levels. Values are means ± SEM (n = 10 rats per group). For each parameter, significant differences ($p < 0.05$) are designated by an asterisk (*).

TRR regulation to the regulation of other selenoproteins in rat liver. In both experiments there was no effect of Se-deficiency on growth whereas plasma GPX and liver GPX1 activities decreased to 8% and 2–3% of Se-adequate levels, respectively, indicating that these animals were biochemically Se-deficient but otherwise little affected by feeding a Se-deficient diet for four weeks.

Liver GPX1 activity falls exponentially and dramatically in rats fed Se-deficient diets [18,19,28,35,38–41]. In our experiments liver GPX1 activity decreased to near zero

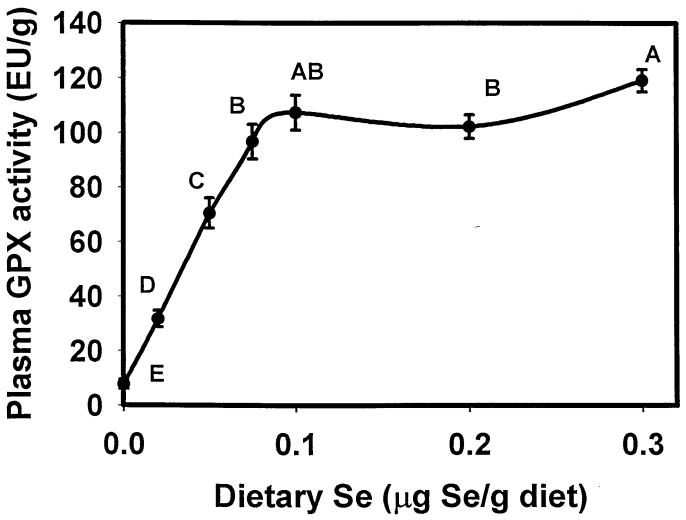

Fig. 3. Plasma glutathione peroxidase (GPX3) activity in rats fed graded levels of dietary Se. Male weanling rats were fed the basal diet (0.007 μg Se/g diet) or supplemented with 0.02, 0.05, 0.075, 0.1, 0.2, or 0.3 μg Se/g diet as Na_2SeO_3 for 28 days. Values are means ± SEM (n = 4 rats per group). Means not sharing a common letter are significantly different ($p < 0.05$).

levels, similar to previous results using liver homogenized with buffers containing detergent so that full GPX4 activity could be measured in the same supernatants [19]. Plasma GPX activity in these Se-deficient rats, as in other studies [18,35], also decreased to <10%. Se-deficient liver GPX4 activity in these two experiments decreased to 40–50% of Se-adequate levels, similar to previous results [19,42]. Hill and colleagues [24,37] showed that rat liver TRR activity decreases to 4.5–10% using either the insulin-based assay or the gold thioglucose inhibition assay [37]. In the present experiments using the insulin-based assay, we found that TRR activity decreased to 15% of Se-adequate activity in rat liver. Collectively, these studies show that TRR activity, like GPX1 and plasma GPX, is one of the Se-dependent enzyme activities that decreases substantially in Se-deficient rat liver.

The focus of these studies was to determine the impact of Se-deficiency on mRNA levels for TRR. Using ribonuclease protection analysis and a probe specific for rat TRR1, we found that TRR mRNA levels decreased to 70% of levels found in Se-adequate rat liver. In the same analysis, GPX1 mRNA decreased to 20% of Se-adequate levels. This decrease is similar to falls in GPX1 mRNA reported using ribonuclease protection analysis [20], but is not as low as sometimes observed in studies using Northern blot analysis [19]. Se-deficient GPX4 mRNA levels in these experiments fell to 81–83% of Se-adequate levels; this decrease was only statistically significant in one of the two experiments, whereas similar decreases in previous studies using Northern blot analysis were not significant [19]. We were also able to quantitate SelP mRNA levels in the same analysis; as we reported previously [18], there was no significant effect of Se status on SelP mRNA expression in this rat model. In other studies where rats were fed Se-deficient diets for 4.5 weeks, Sel P mRNA was found to decrease to 67% of Se-adequate levels [23]. The present study indicates, in contrast to the effect of Se-deficiency on enzyme activity of TRR, mRNA levels for TRR1, like GPX4 and SelP, are only modestly affected by Se-deficiency.

We have been using plateau breakpoint analysis to determine Se requirements for various selenium-dependent parameters [18,35]. A plateau-breakpoint is the minimum dietary Se required for the plateau response, as determined graphically by a line tangent to the steepest slope and the line through the plateau region of a Se response curve. The plateau breakpoint for GPX1 activity is always near 0.1 μg Se/g diet, whereas, the plateau breakpoint for GPX4 is near 0.05 μg Se/g diet. Similar responses were observed in these experiments (Fig. 4). Analysis of the response of liver TRR enzyme activity to graded levels of dietary Se shows that the plateau breakpoint occurs at 0.07 μg Se/g diet indicating that the minimum dietary Se requirement for maximal TRR activity in rat liver is 0.07 μg Se/g diet. The plateau breakpoint for GPX1 mRNA in these experiments is 0.07 μg Se/g diet, similar to previous studies [18,19,35]. GPX4 mRNA had a plateau breakpoint at 0.04 μg Se/g diet, whereas we found no significant effect of Se status on GPX4 mRNA in our previous studies [19]. The lack of significant effect of dietary Se on SelP mRNA levels in this study suggests that the minimal Se requirement for SelP mRNA expression in this model is less than 0.007 μg Se/g diet (Se in the basal diet). Finally, we found that the minimal Se requirement for TRR mRNA expression was 0.05 μg Se/g diet.

To more carefully explore this differential regulation of selenoenzyme expression, we plotted individual levels of GPX1, GPX4, and SelP mRNA levels normalized not to GAPDH, but rather to TRR mRNA level in the same sample (Fig. 6). This analysis clearly shows differential regulation of GPX1 relative to TRR mRNA. GPX1 mRNA still falls to 25% of the levels in Se-adequate animals, showing the

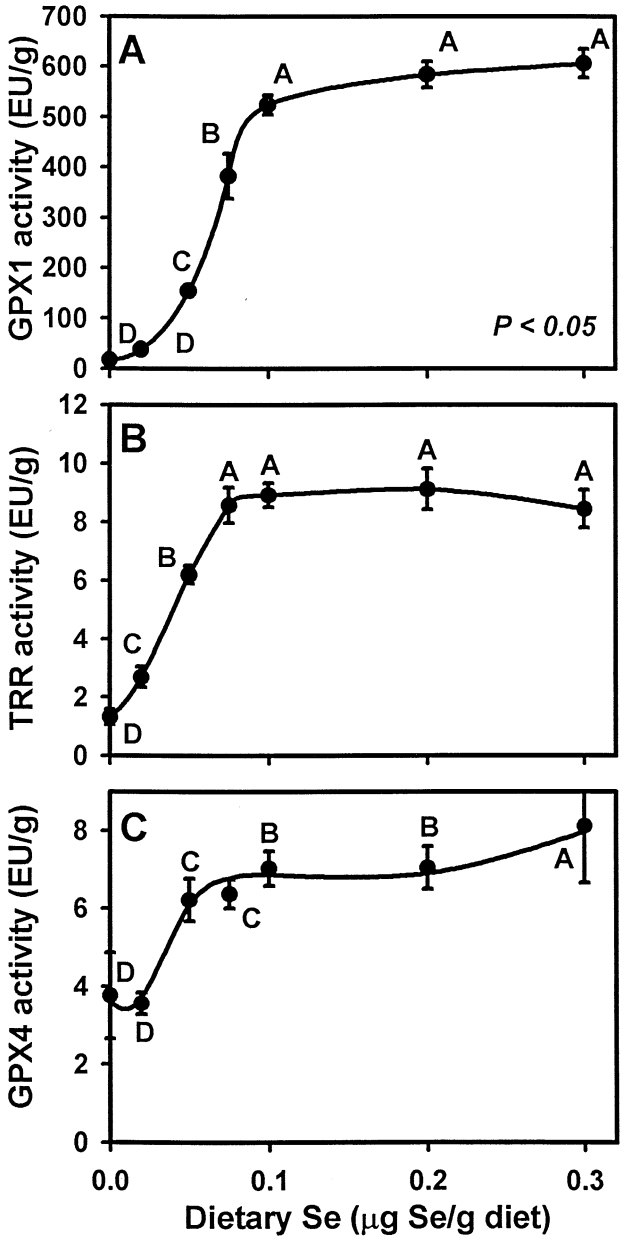

Fig. 4. Selenoenzyme activity in rats fed graded levels of dietary Se. Glutathione peroxidase-1 (GPX1) (A), thioredoxin reductase (TRR) (B), and glutathione peroxidase-4 (GPX4) (C) enzyme activities were assayed in rats fed the basal diet (0.007 μg Se/g diet) or supplemented with indicated levels of dietary Se for 28 days. Values are means ± SEM (n = 4 rats per group). Means not sharing a common letter are significantly different ($p < 0.05$).

specific effect of Se-deficiency on GPX1. Recent research indicates that this specific regulation of GPX1 mRNA is mediated by mRNA stability. When Se is limiting, the UGA in GPX1 is interpreted as a stop or nonsense codon instead of a Sec codon; this specifically targets GPX1 mRNA for degradation by a process called nonsense-mediated decay [20,21,29]. Premature termination of translation at a nonsense codon is thought to cause the ribosome to fall off the mRNA before the ribosome strips downstream mRNA of marker proteins left from the intron splicing process, which leaves these markers on untranslated mRNA as a signal to target this mRNA for decay. Only GPX1 mRNA appears to have the UGA codon located sufficiently upstream (>50 nucleotides) from an exon/intron junction to be susceptible to nonsense-mediated decay [43]. The relative stability of GPX4, SelP, and TRR mRNAs in Se-deficiency suggests that these are not susceptible to nonsense-mediated decay. Normalizing GPX4 mRNA to TRR mRNA completely eliminated any effect of dietary Se, indicating that whatever mechanism responsible for the modest decreases is acting uniformly on both mRNAs. This also suggests that the AU-rich regions identified in TRR [44] are not responsible for the Se-regulation of TRR mRNA. Interestingly, normalized SelP mRNA actually increases in Se-deficiency relative to TRR, further indicating that the decrease in TRR mRNA in Se-deficiency is modest but real. Increased nonspecific degradation of inactive messages in Se-deficient cells may account for the modest changes in GPX4 and TRR mRNAs [41].

The differences in dietary Se required for maximal levels of selenoenzyme activity or mRNA clearly demonstrate that there is differential regulation of selenoprotein expression. While differences have been consistently reported for selenoenzyme activity and mRNA [19,35], this study shows clear differences in Se regulation of different selenoprotein mRNA species in the same analysis of an individual sample, by use of ribonuclease protection analysis. These analyses show a hierarchy of minimal Se requirements necessary for maximal mRNA expression, and our study here adds TRR to these hierarchies. Fig. 6 indicates that the hierarchy of dietary Se requirements for mRNA is (lowest to highest) SelP < GPX4, TRR < GPX1. For enzyme activity, GPX4 and GPX1 assays were done on the same supernatants and showed clear differences in Se regulation, and the liver TRR and plasma GPX3 analysis was done on samples from the same animals. To date, the only other report of a graded response of TRR to increasing Se concentration is in cultured cells where TRR activity increased before GPX1 activity [45]. The hierarchy of dietary Se requirements for enzyme activity is (lowest to highest) GPX4 < TRR, plasma GPX3 < GPX1 [41].

Previously, we thought that there were two clear patterns of Se regulation. One, typified by GPX1, had dramatic down-regulation of both enzyme activity and mRNA levels in Se-deficiency. The other pattern, typified by GPX4, showed decreases in Se-deficiency to approximately half of the activity seen in Se-adequate animals, but with little or no change in mRNA expression. This study clearly shows that TRR represents a third pattern with dramatic down-regulation of enzyme activity in Se-deficiency but with only modest decrease in mRNA level.

This experiment has characterized the effect of Se-deficiency on TRR activity and mRNA levels. TRR stands out as a selenoenzyme that decreases significantly in Se-deficiency without a dramatic fall in mRNA. This represents a

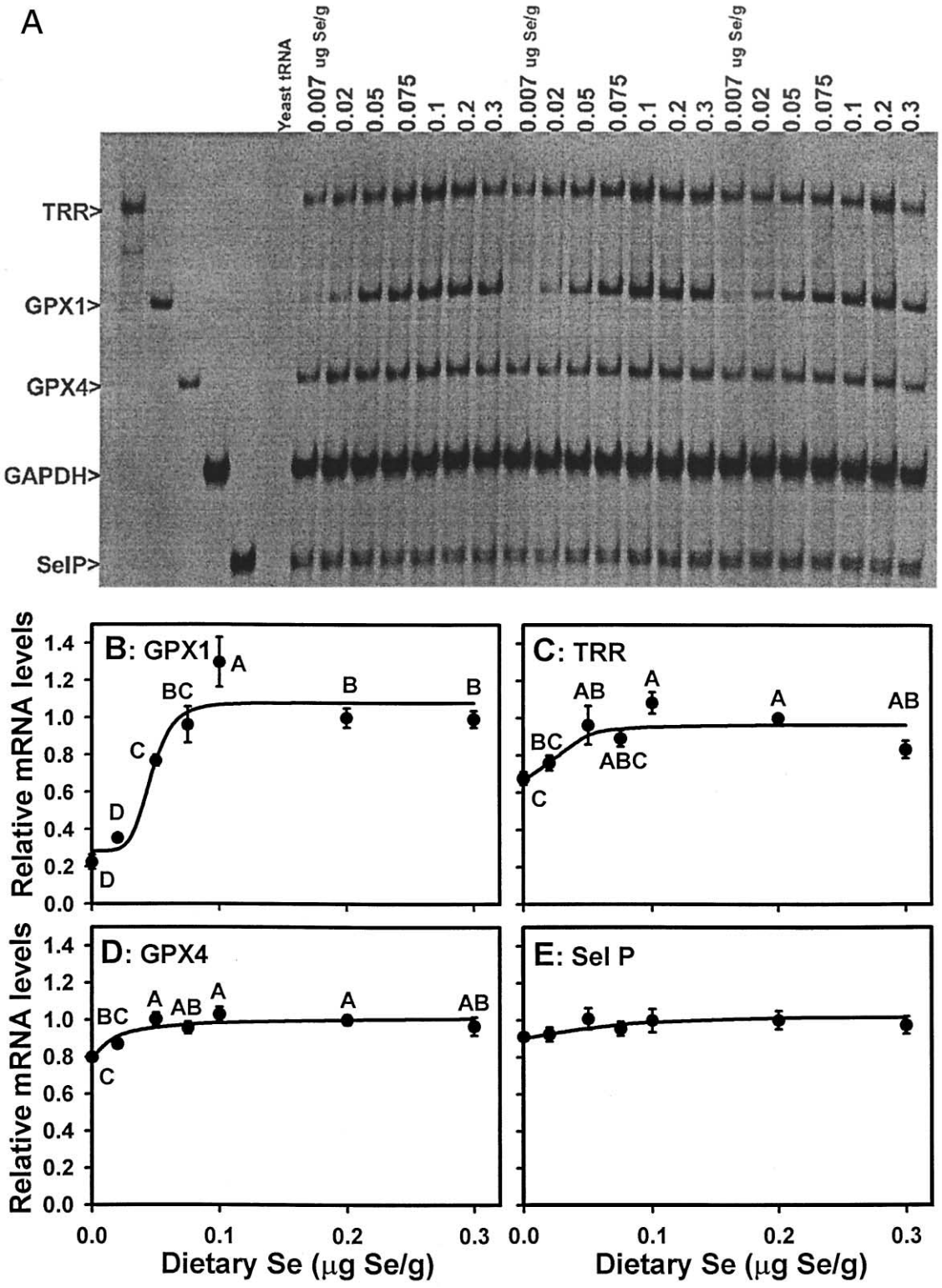

Fig. 5. Ribonuclease protection analysis (A) and quantitation (B–E) of selenoprotein mRNA in rats fed graded levels dietary Se. Male weanling rats were fed the basal diet (0.007 μg Se/g diet) or supplemented with 0.02, 0.05, 0.075, 0.1, 0.2, or 0.3 μg Se/g diet as Na_2SeO_3 for 28 days. Total RNA (20 μg) was analyzed for thioredoxin reductase (TRR), glutathione peroxidase-1 (GPX1), glutathione peroxidase-4 (GPX4), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and selenoprotein P (SelP) mRNA. A Se-adequate RNA sample was hybridized individually with 2×-concentration of a single probe (lanes 1–5). RNA samples from rats fed the indicated levels of dietary Se were hybridized with the [^{32}P]UTP-labeled probe mixture, digested with RNase and protected fragments analyzed on 6% polyacrylamide gels and visualized by autoradiography. Yeast tRNA (lane 6) was analyzed with the probe mixture as a negative control. Protected [^{32}P]UTP-labeled fragments were quantitated by direct imaging of the gel and normalized to GAPDH mRNA levels in the same sample. Normalized GPX1 (B), TRR (C), GPX4 (D), and SelP (E) mRNA are plotted relative to Se-adequate levels (0.2 μg Se/g diet). Values are means ± SEM (n = 4 rats per group). Means not sharing a common letter are significantly different ($p < 0.05$).

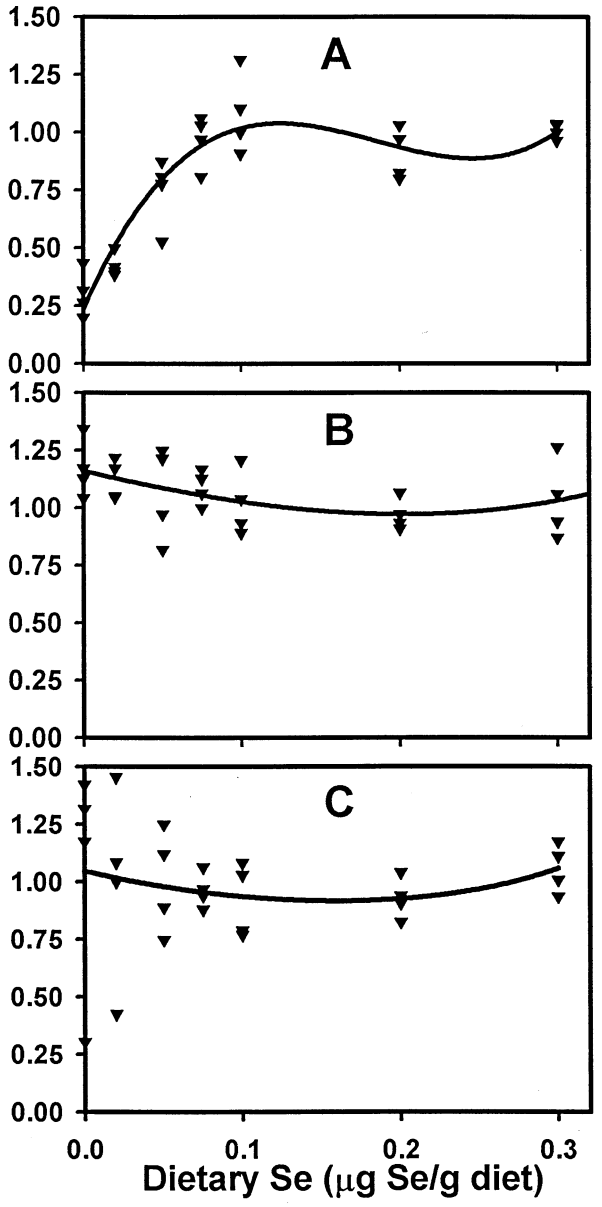

Fig. 6. Differential expression of GPX1 (A), GPX4 (B), and SelP (C) mRNA levels, plotted relative to TRR mRNA levels. For each individual sample, protected [^{32}P]UTP-labeled fragments were quantitated by direct imaging of the gel and normalized to TRR mRNA levels in the same sample, for each individual sample. Points are individual values (n = 4 per dietary treatment group). Lines through the points are best-fit lines based on polynomial least squares analysis (Sigma Plot, Jandel Scientific, Corte Madera, CA).

third pattern of Se regulation in addition to GPX1, with large changes in both activity and mRNA, and in addition to GPX4, with modest changes in activity with little or no change in mRNA. The conservation of TRR mRNA in Se-deficiency suggests that TRR is a valued enzyme and that the loss of TRR enzyme activity in Se-deficiency may be the cause of some signs of Se-deficiency. Absence of thioredoxin in the thioredoxin-knockout mouse is embryonic lethal [46], which makes the TRR-knockout mouse an interesting future question. Further studies are needed to understand the role of Se-dependent TRR in mammals.

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