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Bradley A. Carlson

*NCI, National Institutes of Health, Bethesda, Maryland*

Sergey V. Novoselov

*University of Nebraska-Lincoln*

Easwari Kumaraswamy

*NCI, National Institutes of Health, Bethesda, Maryland*

Byeong Jae Lee

*Seoul National University, Seoul 151-742, South Korea*

Miriam R. Anver

*Pathology/Histotechnology Laboratory, Science Applications International Corporation-Frederick, Inc., National Cancer Institute, Frederick, Maryland*

*See next page for additional authors*

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## Authors

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# Specific Excision of the Selenocysteine tRNA<sup>[Ser]Sec</sup> (*Trsp*) Gene in Mouse Liver Demonstrates an Essential Role of Selenoproteins in Liver Function\*

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Bradley A. Carlson‡, Sergey V. Novoselov§, Easwari Kumaraswamy‡¶, Byeong Jae Lee||, Miriam R. Anver\*\*, Vadim N. Gladyshev§, and Dolph L. Hatfield‡ §§

From the ‡Section on the Molecular Biology of Selenium, Basic Research Laboratory, Center for Cancer Research, NCI, National Institutes of Health, Bethesda, Maryland, 20892, §Department of Biochemistry, University of Nebraska, Lincoln, Nebraska 68588, ||Laboratory of Molecular Genetics, School of Biological Sciences, Seoul National University, Seoul 151-742, South Korea, and \*\*Pathology/Histotechnology Laboratory, Science Applications International Corporation-Frederick, Inc., National Cancer Institute, Frederick, Maryland 21702

Selenium is essential in mammalian embryonic development. However, in adults, selenoprotein levels in several organs including liver can be substantially reduced by selenium deficiency without any apparent change in phenotype. To address the role of selenoproteins in liver function, mice homozygous for a floxed allele encoding the selenocysteine (Sec) tRNA<sup>[Ser]Sec</sup> gene were crossed with transgenic mice carrying the *Cre* recombinase under the control of the albumin promoter that expresses the recombinase specifically in liver. Recombination was nearly complete in mice 3 weeks of age, whereas liver selenoprotein synthesis was virtually absent, which correlated with the loss of Sec tRNA<sup>[Ser]Sec</sup> and activities of major selenoproteins. Total liver selenium was dramatically decreased, whereas levels of low molecular weight selenocompounds were little affected. Plasma selenoprotein P levels were reduced by about 75%, suggesting that selenoprotein P is primarily exported from the liver. Glutathione S-transferase levels were elevated in the selenoprotein-deficient liver, suggesting a compensatory activation of this detoxification program. Mice appeared normal until about 24 h before death. Most animals died between 1 and 3 months of age. Death appeared to be due to severe hepatocellular degeneration and necrosis with concomitant necrosis of peritoneal and retroperitoneal fat. These studies revealed an essential role of selenoproteins in liver function.

Selenium is an essential micronutrient in the diet of higher vertebrates, including humans and other mammals. Numerous health benefits have been attributed to this element. For ex-

ample, evidence suggests that selenium has cancer chemopreventive properties, inhibits viral expression, and delays the progression of AIDS in patients who are positive for the human immunodeficiency virus (HIV<sup>+</sup>) (reviewed in Ref. 1). Furthermore, it appears to reduce the risk of heart disease and other cardiovascular and muscle disorders, to slow the aging process, and to have roles in mammalian development, male reproduction, and immune function (1).

Selenoproteins are most certainly responsible for many of the health benefits of selenium. Humans encode 25 selenoproteins in their genome and mice 24, but the functions of only about half of these proteins are now known (2). Determining the identity and functions of selenoproteins is essential to understanding the role of selenium in human health. Selenium makes its way into protein as the amino acid selenocysteine (Sec) (reviewed in Ref. 3). Sec has its own code word, UGA, and its own tRNA designation, Sec tRNA<sup>[Ser]Sec</sup>. Sec is biosynthesized on its tRNA after the tRNA is aminoacylated initially with serine by seryl-tRNA synthetase. The presence of a stem-loop structure, designated a Sec insertion sequence element, in selenoprotein mRNAs dictates a UGA codon within an open reading frame to function as Sec and not as stop (4). In addition, much of the Sec protein insertion machinery is unique to this amino acid, and in mammals, there is a specific Sec insertion sequence-binding protein, SBP2 (5), and a specific elongation factor, EFsec (6, 7), required for Sec insertion into protein.

Selenoproteins are the only known class of proteins for which expression is determined by the presence of a single tRNA. Thus, manipulating the expression of Sec tRNA<sup>[Ser]Sec</sup> can perturb the expression of selenoproteins, which in turn provides an important tool in elucidating the biological functions of the various members of this class of proteins and their potential roles in promoting better health. One approach to elucidating the cellular roles of selenoproteins is to knock out the corresponding gene. Indeed, several laboratories have targeted specific selenoproteins for removal from the mouse genome, including glutathione peroxidases 1, 2, and 4 (GPx1 (8), GPx2 (9), GPx4 (10)), thyroid hormone deiodinase 2 (DIO2) (11), and selenoprotein P (SelP)<sup>1</sup> (12, 13). These studies have provided insights into the roles of these selenoproteins in development and cellular metabolism.

In a different approach, and to provide alternative models for

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¶ Current address: The Wistar Institute, 3601 Spruce St., Philadelphia, PA 19104.

§§ To whom correspondence should be addressed: NCI, National Institutes of Health, Bldg. 37, Rm. 6016, 9000 Rockville Pike, Bethesda, MD 20892. Tel.: 301-496-2797; Fax: 301-435-4957; E-mail: hatfield@dc37a.nci.nih.gov.

<sup>1</sup> The abbreviations used are: SelP, selenoprotein P; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; GPx, glutathione peroxidase; RPC-5, reverse phase chromatographic column-5.

examining the roles of selenium in health, we initially generated a transgenic mouse carrying a mutant Sec tRNA<sup>[Ser]Sec</sup> transgene wherein the expressed tRNA lacked a highly modified base, N<sup>6</sup>-isopentenyladenosine (i<sup>6</sup>A), at position 37 (14). The i<sup>6</sup>A-tRNA<sup>[Ser]Sec</sup>-deficient mice manifested a reduction in selenoproteins that occurred in a protein- and tissue-specific manner. More recently, we generated a conditional knockout of the Sec tRNA<sup>[Ser]Sec</sup> gene encoding flanking *loxP* sites whereby the gene is receptive to removal by the *Cre* recombinase, which may be under the control of promoters targeted for specific organs or tissues (15). Mice that are homozygous for this floxed allele, designated *Trsp*<sup>f/f</sup>, were crossed to transgenic mice carrying the *Cre* recombinase under the control of two promoters targeting mammary epithelium. Neither *Cre* recombinant resulted in complete removal of *Trsp*. However, one of the promoters, *MMTV-Cre*, removed about 80% of the *Trsp*, which resulted in an altered selenoprotein expression in mammary epithelium (15) similar to that observed in mouse liver with the mutant transgene (14). However, no apparent phenotypic changes due to selenoprotein deficiency were detected. In the present study, *Trsp* was selectively removed from liver by mating floxed mice with transgenic mice carrying the *Alb-Cre* transgene (16, 17). Characterization of these mice provided important insights into selenoprotein synthesis and transport and identified an essential role of selenoproteins in liver function.

#### EXPERIMENTAL PROCEDURES

**Materials**—<sup>75</sup>Selenium (specific activity 1000 Ci/mmol) was obtained from the Research Reactor Facility, University of Missouri, Columbia, MO, and all other reagents were commercial products of the highest grade available. Floxed *Trsp* (designated *Trsp*<sup>f</sup>), strain C57B6, mice have been described (15), and heterozygous albumin *Cre* (designated *Alb-Cre*<sup>+/-</sup>), strain C57B6, transgenic mice (16, 17) were purchased from Jackson Laboratories. Antibodies to SeLP were kindly provided by Drs. K. E. Hill and R. F. Burk (Vanderbilt University) and by Drs. U. Schweizer and L. Schomberg (Charité Universitätsmedizin, Berlin, Germany) and those to TR1 were used as described previously (14). The care of animals was in accordance with the National Institutes of Health institutional guidelines under the expert direction of D. L. Sly (SAIC, NCI, National Institutes of Health).

**Identification of *Trsp*, *Trsp*<sup>f/f</sup>, and *Alb-Cre*<sup>+/-</sup> and Selective Removal of *Trsp* in Liver**—Mice carrying homozygous floxed *Trsp* (*Trsp*<sup>f/f</sup>) were identified by PCR analysis of tail DNA as described previously (15), and the *Alb-Cre* transgene was identified by PCR analysis of tail DNA with primers 5'-ACCTGAAGATGTTCGCGAT-TATCT-3' and 5'-ACCGTCAGTACGTGAGATATCTT-3', which resulted in a 370-bp fragment (16). Heterozygous floxed *Trsp*-heterozygous albumin *Cre* (*Trsp*<sup>f/+</sup>·*Alb-Cre*<sup>+/-</sup>) mice were generated by mating *Trsp*<sup>f/f</sup> mice with *Alb-Cre*<sup>+/-</sup> mice, selecting for the appropriate offspring, and then mating the resulting mice heterozygous for both genes to obtain homozygous floxed *Trsp*-heterozygous albumin *Cre* (*Trsp*<sup>f/f</sup>·*Alb-Cre*<sup>+/-</sup>), *Trsp*<sup>f/+</sup>·*Alb-Cre*<sup>+/-</sup>, and *Trsp*<sup>f/f</sup> offspring for Sec tRNA<sup>[Ser]Sec</sup>, selenoprotein, blood, and pathological analyses.

**Isolation, Fractionation, and Identification of Specific tRNA Isoforms**—Total tRNA was isolated from tissues (18) and fractionated by RPC-5 chromatography (19) or by polyacrylamide gel electrophoresis as described (14). Sec tRNA<sup>[Ser]Sec</sup> and serine tRNA<sup>Ser</sup><sub>1</sub> were identified by northern blotting and quantitated as given (14).

**Labeling of Selenoproteins and GPx1 and TR1 Assays**—Mice with genotypes *Trsp*<sup>f/f</sup>·*Alb-Cre*<sup>+/-</sup>, *Trsp*<sup>f/+</sup>·*Alb-Cre*<sup>+/-</sup>, and *Trsp*<sup>f/f</sup> were labeled with <sup>75</sup>Se, tissues and organs excised, proteins extracted and electrophoresed, gels stained with Coomassie Blue, and proteins transferred to nylon membranes. The resulting transblots were exposed to a PhosphorImager as described (14, 15, 20).

GPx1 activity was assayed directly, and TR1 activity was assayed after enrichment on ADP-Sepharose as described (14). A major protein differentially expressed between knockout and control livers was identified by N-terminal Edman degradation at the University of Nebraska-Lincoln proteomics core facility.

**Pathology Evaluation**—Mice were sacrificed using CO<sub>2</sub> inhalation. Necropsy examination was performed on a subset of mice that were sacrificed or that died spontaneously. The total numbers of mice exam-

ined were as follows: *Trsp*<sup>f/f</sup>, scheduled sacrifice, 7 males and 0 females; *Trsp*<sup>f/f</sup>·*Alb-Cre*<sup>+/-</sup>, scheduled sacrifice, 4 males and 2 females; and *Trsp*<sup>f/f</sup>·*Alb-Cre*<sup>+/-</sup>, spontaneous death/clinically ill, 3 males and 6 females. A comprehensive set of organs and tissues was collected and fixed in 10% buffered neutral formalin. Tissues were paraffin-embedded, sectioned at 5 μm, and stained with hematoxylin and eosin. The TUNEL assay (Apoptag, Serologicals Corp.) was performed on sections of liver from all mice. Prussian blue stain for iron was performed on samples of necrotic fat.

**Blood and Selenium Analyses**—Blood samples were taken from mice prior to necropsy by cardiac puncture. The serum was obtained by centrifugation and used for determining blood chemistries (run by the Pathology/Histotechnology Laboratory) using standard techniques. The analytes tested were urea nitrogen, total protein, albumin, aspartate transaminase, alanine transaminase, alkaline phosphatase, γ-glutamyl transpeptidase, and total bilirubin.

To determine the levels of selenium in the form of low molecular weight selenocompounds or selenoproteins, 300 mg of liver were homogenized in 5 ml of a lysis buffer (14, 15, 20), and proteins were precipitated in trichloroacetic acid and collected as described previously (21). Selenium levels in the extracts, pellets, and trichloroacetic acid supernatants and in all other tissues and organs were determined by the Oscar E. Olsen Biochemistry Laboratories at South Dakota State University.

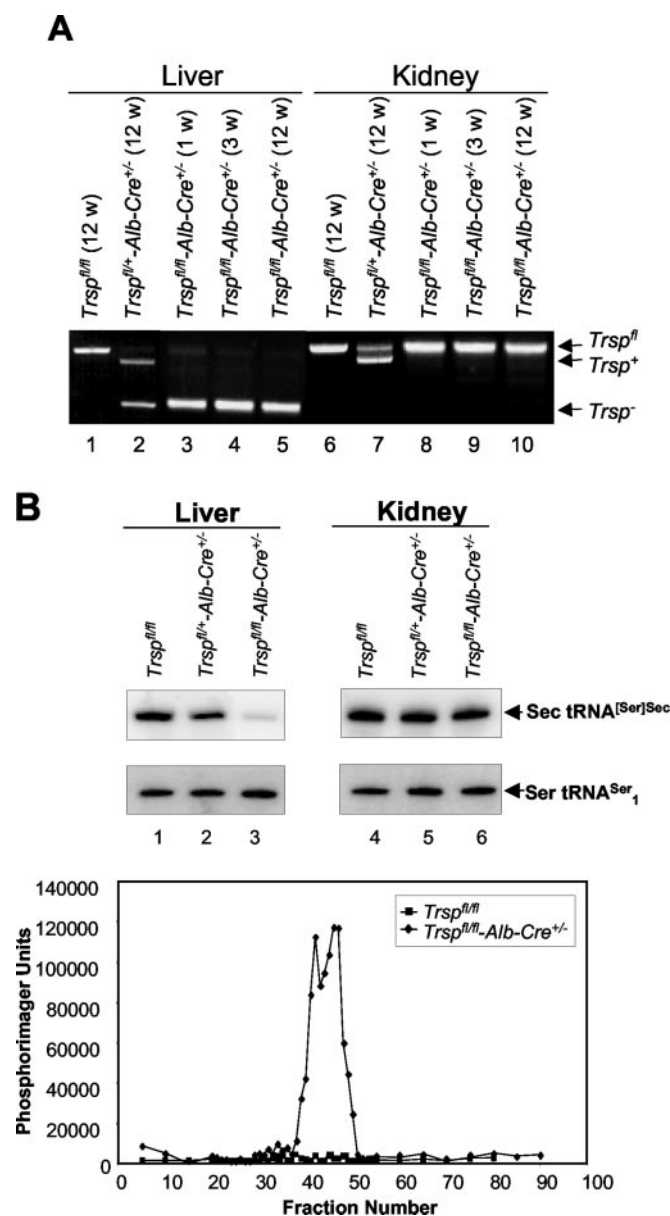
#### RESULTS

**Specific Removal of *Trsp* from Liver—*Alb-Cre*** has been reported to be highly specific to liver and virtually 100% efficient in hepatocytes when crossed with floxed alleles (16). Its complete expression in liver occurred after an initial time lag of only a few weeks (17). We, therefore, examined the status of *Trsp* in liver of mice with genotypes *Trsp*<sup>f/f</sup>, *Trsp*<sup>f/+</sup>·*Alb-Cre*<sup>+/-</sup>, and *Trsp*<sup>f/f</sup>·*Alb-Cre*<sup>+/-</sup> at 1, 3, and 12 weeks of age using the kidney as a control organ. As shown in Fig. 1A, between 80 and 90% of the gene was removed from the liver of mice about 1 week old (lane 3) and virtually completely removed in older mice (lanes 4 and 5). *Trsp*<sup>f/f</sup> was unaffected in the kidney of *Trsp*<sup>f/f</sup>·*Alb-Cre*<sup>+/-</sup> mice (lanes 8–10). The wild type gene (lacking the floxed allele, designated *Trsp*<sup>+</sup>) was observed in heterozygous mice encoding *Trsp*<sup>f</sup>, *Trsp*<sup>+</sup>, and *Alb-Cre*<sup>+/-</sup> where *Trsp*<sup>f</sup> was removed in liver (Fig. 1A, lane 2) but retained in kidney (lane 7). The small amounts of *Trsp*<sup>f</sup> observed in *Trsp*<sup>f/+</sup>·*Alb-Cre*<sup>+/-</sup> or *Trsp*<sup>f/f</sup>·*Alb-Cre*<sup>+/-</sup> mice are likely because of the presence of cell forms other than hepatocytes (see "Discussion").

Total tRNA was isolated from perfused livers and from kidneys of *Trsp*<sup>f/f</sup>, *Trsp*<sup>f/+</sup>·*Alb-Cre*<sup>+/-</sup>, and *Trsp*<sup>f/f</sup>·*Alb-Cre*<sup>+/-</sup> mice. Sec tRNA<sup>[Ser]Sec</sup> levels were examined by Northern analysis and by RPC-5 chromatography (Fig. 1B). Relative to the amount of serine tRNA<sub>1</sub>, more than 90% of the Sec tRNA<sup>[Ser]Sec</sup> had been removed from livers lacking the corresponding gene (Fig. 1B, lane 3), whereas in the kidneys of these mice, the Sec tRNA<sup>[Ser]Sec</sup> population was unaffected (lane 6), as shown by Northern blot analysis in the upper portion of Fig. 1B. Interestingly, the Sec tRNA<sup>[Ser]Sec</sup> level was reduced to about 70% of the wild type amount in the liver of *Trsp*<sup>f/+</sup>·*Alb-Cre*<sup>+/-</sup> mice (see legend to Fig. 1). This reduction in Sec tRNA<sup>[Ser]Sec</sup> expression was similar to that observed in mice heterozygous for the standard tRNA<sup>[Ser]Sec</sup> knockout (15). Virtually all of the Sec tRNA<sup>[Ser]Sec</sup> appeared to be absent in the liver of mice lacking *Trsp* in this tissue as compared with *Trsp*<sup>f/f</sup> mice following fractionation of total tRNA from corresponding organs by RPC-5 chromatography, dot blotting on nylon membranes, and hybridization (see graph in lower portion of Fig. 1B).

**Selenoprotein Expression**—The expression of selenoproteins in liver, kidney, plasma, brain and testes of *Trsp*<sup>f/f</sup>, *Trsp*<sup>f/+</sup>·*Alb-Cre*<sup>+/-</sup>, and *Trsp*<sup>f/f</sup>·*Alb-Cre*<sup>+/-</sup> mice was assessed by <sup>75</sup>Se labeling of the corresponding mice and by examining the resulting labeled proteins from these tissues following gel electrophoresis. Coomassie Blue-stained gels of total proteins from livers of these





**FIG. 1. Selective removal of *Trsp* and Sec tRNA<sup>[Ser]Sec</sup> from liver.** A, *Trsp*. DNA was isolated from liver and kidney, and products were generated by PCR with the primers given under "Experimental Procedures" using DNA from *Trsp*<sup>fl/fl</sup>, *Trsp*<sup>fl/+</sup>-*Alb-Cre*<sup>+/-</sup>, and *Trsp*<sup>fl/+</sup>-*Alb-Cre*<sup>+/-</sup> mice. PCR of *Trsp*<sup>fl</sup> yielded a 1.1-kb fragment, *Trsp*<sup>+</sup> a 900-bp fragment, and *Trsp*<sup>-</sup> a 450-bp fragment. B, Sec tRNA<sup>[Ser]Sec</sup>. Total tRNA was extracted from liver of *Trsp*<sup>fl/fl</sup>, *Trsp*<sup>fl/+</sup>-*Alb-Cre*<sup>+/-</sup>, and *Trsp*<sup>fl/+</sup>-*Alb-Cre*<sup>+/-</sup> mice and either electrophoresed on a polyacrylamide gel (upper panel) or chromatographed on a RPC-5 column (lower panel). Following transblotting of the tRNA from gels onto nylon membranes, membranes were hybridized with <sup>32</sup>P-oligonucleotide probe for either Sec tRNA<sup>[Ser]Sec</sup> or Ser tRNA<sup>Ser1</sup> as given under "Experimental Procedures" (upper panel). PhosphorImager analysis showed that the Sec tRNA<sup>[Ser]Sec</sup> level was 72.5% in liver of *Trsp*<sup>fl/+</sup>-*Alb-Cre*<sup>+/-</sup> mice relative to that observed in *Trsp*<sup>fl/fl</sup> mice, whereas the level was 6.1% in *Trsp*<sup>fl/+</sup>-*Alb-Cre*<sup>+/-</sup> mice relative to that observed in *Trsp*<sup>fl/fl</sup> mice. Following RPC-5 chromatography, fractions were dot-blotted onto membranes and hybridized with <sup>32</sup>P-oligonucleotide probe for Sec tRNA<sup>[Ser]Sec</sup> as given under "Experimental Procedures" (lower panel).

mice appeared similar (Fig. 2, lower panels) with the exception of an enriched band in the extract of *Trsp*<sup>fl/+</sup>-*Alb-Cre*<sup>+/-</sup> mice (designated by an arrow). We extracted this 25-kDa band from gels and sequenced the first 10 residues, PMILGYWNVR. This sequence is identical to that of mouse glutathione S-transferase,

demonstrating that the expression of this enzyme is elevated in the liver of selenoprotein-deficient mice.

The selenoprotein population was dramatically different in the livers of the three lines of mice. The liver in the *Trsp*<sup>fl/fl</sup>-*Alb-Cre*<sup>+/-</sup> strain lacked virtually any selenoprotein expression. The three major bands observed on the gels of *Trsp*<sup>fl/fl</sup> and *Trsp*<sup>fl/+</sup>-*Alb-Cre*<sup>+/-</sup> mice were TR1 (57 kDa) and GPx1 (22 kDa), and an uncharacterized selenoprotein at 52 kDa (14, 15, 20). To verify that the band at 57 kDa was TR1, crude extracts of liver were enriched on ADP-Sepharose columns, which are known to serve as an affinity matrix for this enzyme (14). The small panel attached to the right of the liver panel shows the Western blot of the corresponding extracts enriched for TR1 from the three mouse lines, demonstrating that the labeled band at 57 kDa was indeed TR1. Interestingly, protein from the three mouse lines, including that from *Trsp*<sup>fl/fl</sup>-*Alb-Cre*<sup>+/-</sup>, appeared to be present in virtually the same amounts. These data suggested that the TR1 polypeptide was efficiently made in the liver knockout mouse, but the open reading frame terminated at the penultimate UGA codon, and thus did not contain Sec (see below). In contrast to the <sup>75</sup>Se labeling pattern observed in these three mouse lines in liver, labeling of the corresponding selenoprotein population in kidney was virtually identical. The four major selenoprotein bands in kidney were likely TR1 (57 kDa), an uncharacterized 52-kDa selenoprotein, GPx1 (22 kDa), and GPx 4 (20 kDa) (14, 15, 20).

GPx1 and TR1, which are major selenoproteins expressed in the liver, often exhibit changes in expression that are opposite to each other (14, 15, 27). To confirm the decreased expression of these two selenoproteins in *Trsp* knockout liver, we assayed GPx1 and TR1 activities in mouse lines carrying the *Trsp*<sup>fl/fl</sup> and *Trsp*<sup>fl/+</sup>-*Alb-Cre*<sup>+/-</sup> genotypes. Consistent with the <sup>75</sup>Se labeling assays, the liver of *Trsp*<sup>fl/+</sup>-*Alb-Cre*<sup>+/-</sup> mice had less than 5% of the GPx1 activity of that found in the floxed control mouse, whereas the activities in kidney were virtually identical in these two mouse lines (data not shown). TR1 activity was similarly reduced in the liver of the *Trsp*<sup>fl/+</sup>-*Alb-Cre*<sup>+/-</sup> mice (data not shown), but TR1 levels were virtually unchanged (see Fig. 2 and its legend), providing further evidence that the protein is terminated at the penultimate UGA Sec codon.

Two major selenoproteins, SelP and GPx3, have been described in plasma (Ref. 22 and references therein). SelP has been reported to be synthesized in the liver and transported to plasma (23, 24) and to be imported into the brain and testes (25), whereas the kidney-proximal tubules are the major source of plasma GPx3 (26). Brain has been reported to express SelP mRNA, but not testes, suggesting that SelP is also synthesized in brain but not testes (25). The SelP level was reduced about 75% in plasma in *Trsp*<sup>fl/+</sup>-*Alb-Cre*<sup>+/-</sup> mice as shown in Fig. 2, "Plasma" panel. To verify that the major labeled band observed in plasma was SelP, a membrane containing the <sup>75</sup>Se-labeled liver extracts from these three mouse lines was exposed to antibodies for SelP (Fig. 2, see small panel (insert) attached to the right of the Plasma panel). The Western blot shows that the first and second lanes (Fig. 2) with plasma of *Trsp*<sup>fl/fl</sup> and *Trsp*<sup>fl/+</sup>-*Alb-Cre*<sup>+/-</sup> mice yielded a positive signal with SelP antibodies, but that the lane with plasma from *Trsp*<sup>fl/+</sup>-*Alb-Cre*<sup>+/-</sup> mice did not respond to SelP antibodies. These results demonstrate that the major <sup>75</sup>Se-labeled band in plasma is SelP (see also Ref. 22). It should also be noted that SelP antibodies do not detect SelP in tissues.<sup>2</sup> In contrast, the Coomassie Blue-stained gels of total proteins from plasma of the three mouse lines appeared to be similar. The <sup>75</sup>Se-selenoprotein labeling pattern observed in brain was similar in the different

<sup>2</sup> K. E. Hill and R. F. Burk, personal communication.

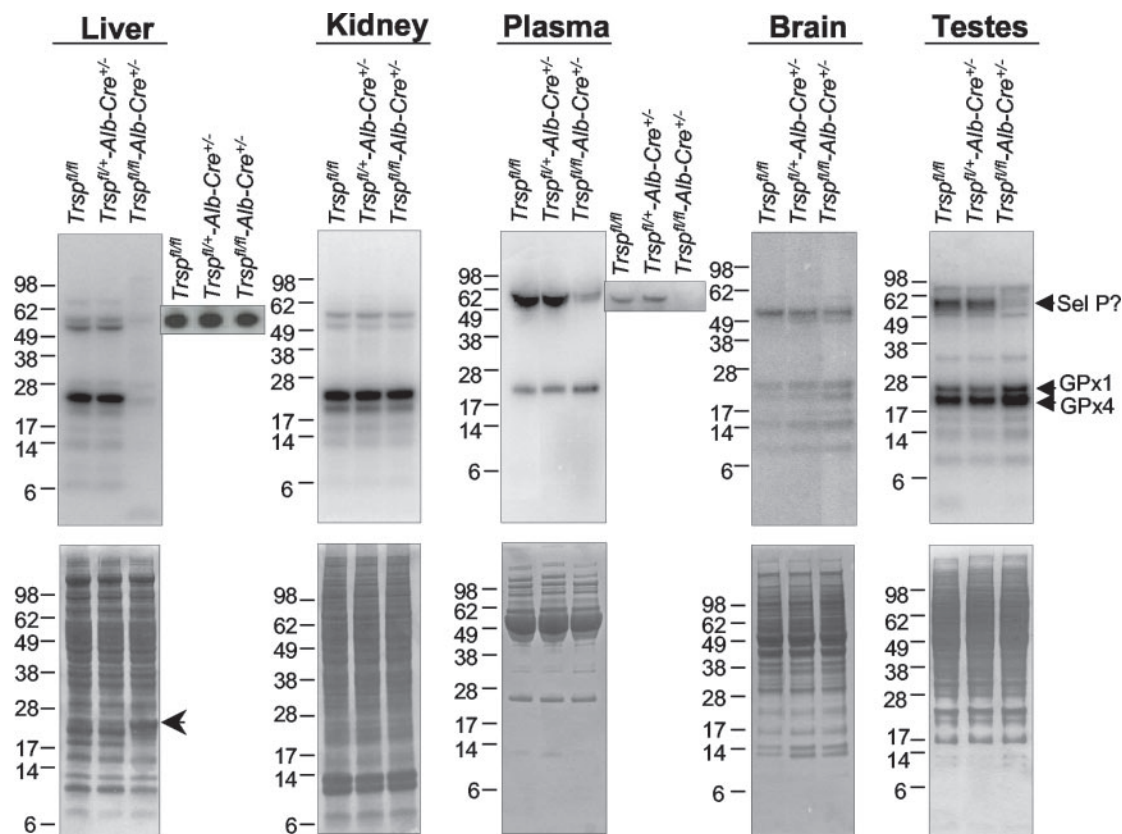


FIG. 2. **Protein and selenoprotein analysis in tissues of floxed *Trsp* and *Alb-Cre* mice.** Mice were labeled with  $^{75}\text{Se}$ , protein was extracted from liver and kidney or used directly from plasma and electrophoresed, the developed gels were stained with Coomassie Blue, and  $^{75}\text{Se}$ -labeled proteins were detected with a PhosphorImager as given under "Experimental Procedures."  $^{75}\text{Se}$ -labeled proteins from liver, kidney, plasma, brain, and testes of *Trsp*<sup>fl/fl</sup>, *Trsp*<sup>fl/+</sup>-*Alb-Cre*<sup>+/-</sup>, and *Trsp*<sup>fl/-</sup>-*Alb-Cre*<sup>+/-</sup> mice are shown in the upper panels, and Coomassie-stained proteins in the lower panels. Membranes containing labeled selenoproteins from liver extracts enriched for TR1 were exposed to antibodies specific to this selenoprotein (see "Experimental Procedures" and Ref. 14), and those from plasma were exposed to antibodies to SelP (see "Experimental Procedures") as shown in the smaller panel (insert) attached to the right of the liver panel (TR1) and the right of the plasma panel (SelP), respectively. TR1 was not detected by Western analysis in crude extracts or in the flow-through material from ADP-Sepharose columns (14).

genetic backgrounds of each animal examined, whereas in testes a band that migrates at the expected position of SelP was reduced. The labeling pattern of GPx3 in plasma suggests that its level may not be affected, providing further evidence that its presence in this tissue is the result of its synthesis in kidney (26).

**Selenoprotein mRNA Analysis**—Because the selective loss of *Trsp* in liver affects the expression of selenoproteins at the translation step, we examined the amounts of several selenoprotein mRNAs in mouse lines with *Trsp*<sup>fl/fl</sup>-*Alb-Cre*<sup>+/-</sup> and *Trsp*<sup>fl/+</sup>-*Alb-Cre*<sup>+/-</sup> genotypes. Of the selenoprotein mRNAs examined, the level of GPx1, and possibly that of SelP, appeared to be reduced in liver of *Trsp*<sup>fl/fl</sup>-*Alb-Cre*<sup>+/-</sup> mice, whereas those of GPx4, SPS2, D1, and TR1 appeared to be similar as compared with the corresponding levels in *Trsp*<sup>fl/+</sup>-*Alb-Cre*<sup>+/-</sup> mice (Fig. 3). The instability of GPx1 mRNA in liver not expressing this protein due to selenium deficiency generated by diet (28) or by genetic analysis (14) has been noted in other studies.

**Mortality and Pathology Findings**—Mice that were *Trsp*<sup>fl/fl</sup>-*Alb-Cre*<sup>+/-</sup> died at an early age ranging from 23 to 168 days. Highest mortality rates occurred in the 33 to 38 (a total of 7 males and 1 female) and 85 to 105 (a total of 5 males and 3 females) day range. Fourteen males and 12 females used in this study died peracutely. The mean life span was 60 and 73 days for liver *Trsp* knockout males and females, respectively, with an overall mean value of ~65 days for males and females.

A subset of mice were for pathological analysis. Findings were markedly different in *Trsp*<sup>fl/fl</sup>-*Alb-Cre*<sup>+/-</sup> mice, which

were found dead or were sacrificed when clinically ill compared with scheduled sacrifices of clinically normal mice. Spontaneous deaths and moribund mice had two major lesions with 100% incidence: hepatocellular degeneration/necrosis and necrosis of peritoneal/retroperitoneal fat. In contrast, neither of these phenotypes was present in clinically healthy *Trsp*<sup>fl/fl</sup>-*Alb-Cre*<sup>+/-</sup> mice. Many of the hepatocytes were swollen with an indistinctly vacuolated cytoplasm as shown in Fig. 4A. A number of these swollen cells had karyorrhectic nuclei. Other hepatocytes had undergone massive coagulative necrosis, and there was no apparent lobular pattern to this damage. Some of the necrotic hepatocytes were mineralized (dystrophic calcification). Livers in two females had foci of birefringent crystals (data not shown). Foci of subacute inflammation were present in damaged livers but were less extensive than expected with the large amount of cell death. The majority of hepatocytes that had karyorrhectic or more normal nuclei were positive for DNA laddering (apoptosis) with the TUNEL technique (Fig. 4B). Their nuclei did not have typical apoptotic morphology of shrinkage and pyknosis. The DNA laddering was confirmed by direct examination of the DNA (Fig. 4C), but none of the abnormal liver cells manifested enriched p53 levels as determined by direct assay (data not shown). Liver and fat lesions were absent in sacrificed *Trsp*<sup>fl/fl</sup> mice, which served as age-matched controls.

Necrosis of peritoneal and retroperitoneal fat occurred in the absence of pancreatic damage (Fig. 4D). The necrotic adipocytes had intact cell membranes but had undergone saponification. They had pale pink or blue cytoplasm and sometimes



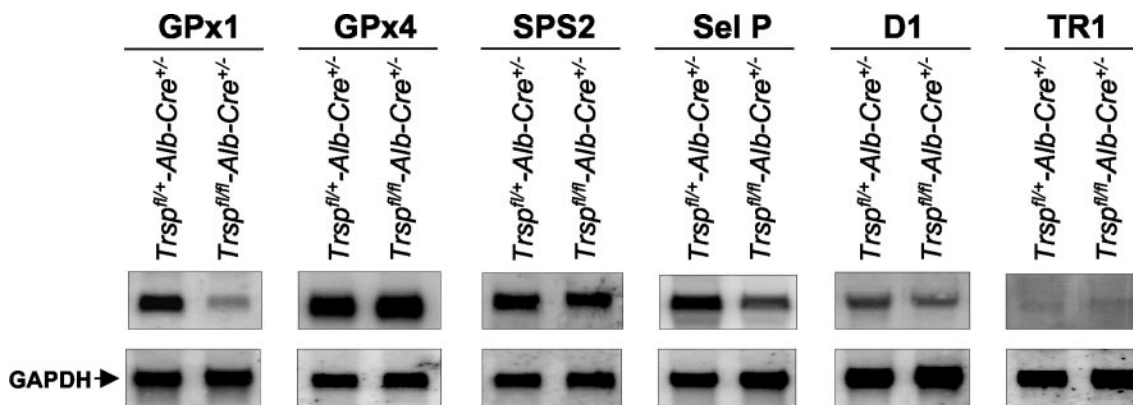


FIG. 3. Northern analysis of selenoprotein mRNAs. mRNA was extracted from liver of *Trsp*<sup>fl/fl</sup>-*Alb-Cre*<sup>+/-</sup> and *Trsp*<sup>fl/fl</sup>-*Alb-Cre*<sup>+/-</sup> mice, electrophoresed, and transblotted onto membranes. Membranes were hybridized with <sup>32</sup>P-labeled probes complementary to each mRNA shown, their levels quantitated with a PhosphorImager, and the filters stripped and rehybridized with glyceraldehyde-3-phosphate dehydrogenase probe (GAPDH) as a loading control (see "Experimental Procedures").

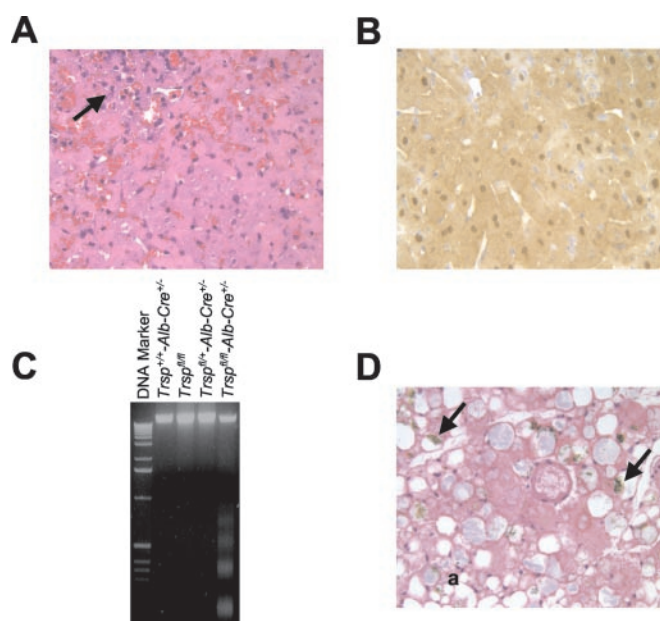


FIG. 4. Liver and fat necrosis and apoptosis in *Trsp*<sup>fl/fl</sup>-*Alb-Cre*<sup>+/-</sup> mice. A, hepatocellular degeneration and necrosis. Many hepatocytes are swollen with cytoplasmic vacuolation. Necrotic hepatocytes have condensed eosinophilic cytoplasm and indistinct or absent nuclei. There is a focus of accompanying subacute inflammation as indicated by the arrow. B, TUNEL assay. Many hepatocyte nuclei are dark brown, indicative of DNA laddering (apoptosis). Nuclei are of normal size or are swollen, which is not typical of apoptotic cell morphology. C, hepatocellular DNA was isolated from the different mouse lines and electrophoresed on an agarose gel as indicated in this panel. Laddering is present only in *Trsp*<sup>fl/fl</sup>-*Alb-Cre*<sup>+/-</sup> liver with the lesions seen in A and B. D, necrotic peritoneal fat. Adipocytes have cytoplasmic saponification (pink or blue cytoplasm) and spicular brown pigment as indicated by the arrows. There is no accompanying inflammatory reaction. A cluster of relatively unaffected adipocytes was observed as indicated by "a." Panels A, B, and D were magnified 40 $\times$ ; panels A and D were stained with hematoxylin and eosin.

brown pigmented spicules. This pigment was negative for iron. The cytoplasm of necrotic adipocytes was strongly birefringent under polarized light. There was no accompanying inflammatory reaction. The cause of death in the *Trsp*<sup>fl/fl</sup>-*Alb-Cre*<sup>+/-</sup> mice examined was attributed to liver failure and concomitant severe fat necrosis of abdominal fat.

**Selenium Levels and Blood Chemistries**—Selenium levels were determined in the liver, kidney, plasma, brain, and testes of *Trsp*<sup>fl/fl</sup>, *Trsp*<sup>fl/fl</sup>-*Alb-Cre*<sup>+/-</sup>, and *Trsp*<sup>fl/fl</sup>-*Alb-Cre*<sup>+/-</sup> mice. Substantially reduced levels of this element were found in liver

and plasma of *Trsp*<sup>fl/fl</sup>-*Alb-Cre*<sup>+/-</sup> mice (Fig. 5). Slightly lower selenium levels were observed in testes and brain of *Trsp*<sup>fl/fl</sup>-*Alb-Cre*<sup>+/-</sup> mice, compared with *Trsp*<sup>fl/fl</sup> mice, but these amounts did not appear to be significantly lower in these tissues of liver *Trsp* knockout mice (see "Discussion").

To examine whether the decreased selenium levels in the liver Sec tRNA<sup>[Ser]</sup>Sec liver knockout mice were due only to selenoprotein deficiency, we determined the relative amounts of selenoproteins and low molecular weight selenocompounds in liver of *Trsp*<sup>fl/fl</sup>, *Alb-Cre*<sup>+/-</sup>, *Trsp*<sup>fl/fl</sup>-*Alb-Cre*<sup>+/-</sup>, and *Trsp*<sup>fl/fl</sup>-*Alb-Cre*<sup>+/-</sup> mice. Extracts were prepared from all liver samples, and the selenium contents were assayed in the extracts as well as in the supernatants and precipitates following treatment with trichloroacetic acid (Table I). We found that the levels of the low molecular weight selenocompounds did not change significantly, whereas the differences in the protein and the trichloroacetic acid-precipitated fraction accounted for the difference in selenium amounts between the control and *Trsp*<sup>fl/fl</sup>-*Alb-Cre*<sup>+/-</sup> mice.

Blood chemistries (urea nitrogen, total protein, albumin, aspartate transaminase, alanine transaminase, alkaline phosphatase,  $\gamma$ -glutamyl transpeptidase, and total bilirubin) were performed on clinically normal mice (ages 49–76 days) of all three genotypes: *Trsp*<sup>fl/fl</sup> (total number of animals = 3), *Trsp*<sup>fl/fl</sup>-*Alb-Cre*<sup>+/-</sup> (3 animals), and *Trsp*<sup>fl/fl</sup>-*Alb-Cre*<sup>+/-</sup> (6 animals). Interestingly, there were no significant differences in the eight parameters tested among the different genotypes (data not shown).

## DISCUSSION

*Trsp* is virtually absent in the livers of 3–4-week-old mice when the floxed gene is targeted by *Alb-Cre*. *Alb-Cre* is expressed in hepatocytes (16, 17); about 80% of the liver is composed of this cell type. The remaining liver cells are biliary, endothelial, Pit, Kupffer, and fat-storing cells (29). Thus, the small amounts of Sec tRNA<sup>[Ser]</sup>Sec and selenoproteins observed in the liver of *Trsp*<sup>fl/fl</sup>-*Alb-Cre*<sup>+/-</sup> mice after 3 weeks are likely due to the presence of liver cells other than hepatocytes. Although blood chemistries, which are indicative of liver function, are normal in *Trsp*<sup>fl/fl</sup>-*Alb-Cre*<sup>+/-</sup> mice before the onset of illness, these mice do not have a normal life expectancy and die acutely within 3–24 weeks. The average life span of all mice lacking *Trsp* in their livers was ~65 days.

One characteristic of mice that fail to make selenoproteins in hepatocytes is that their liver is selenium-deficient. Reduced selenium levels have also been observed in the liver of GPx1 gene knockout mice suggesting that this selenoprotein provides a major source of selenium in this organ (8). We find that the

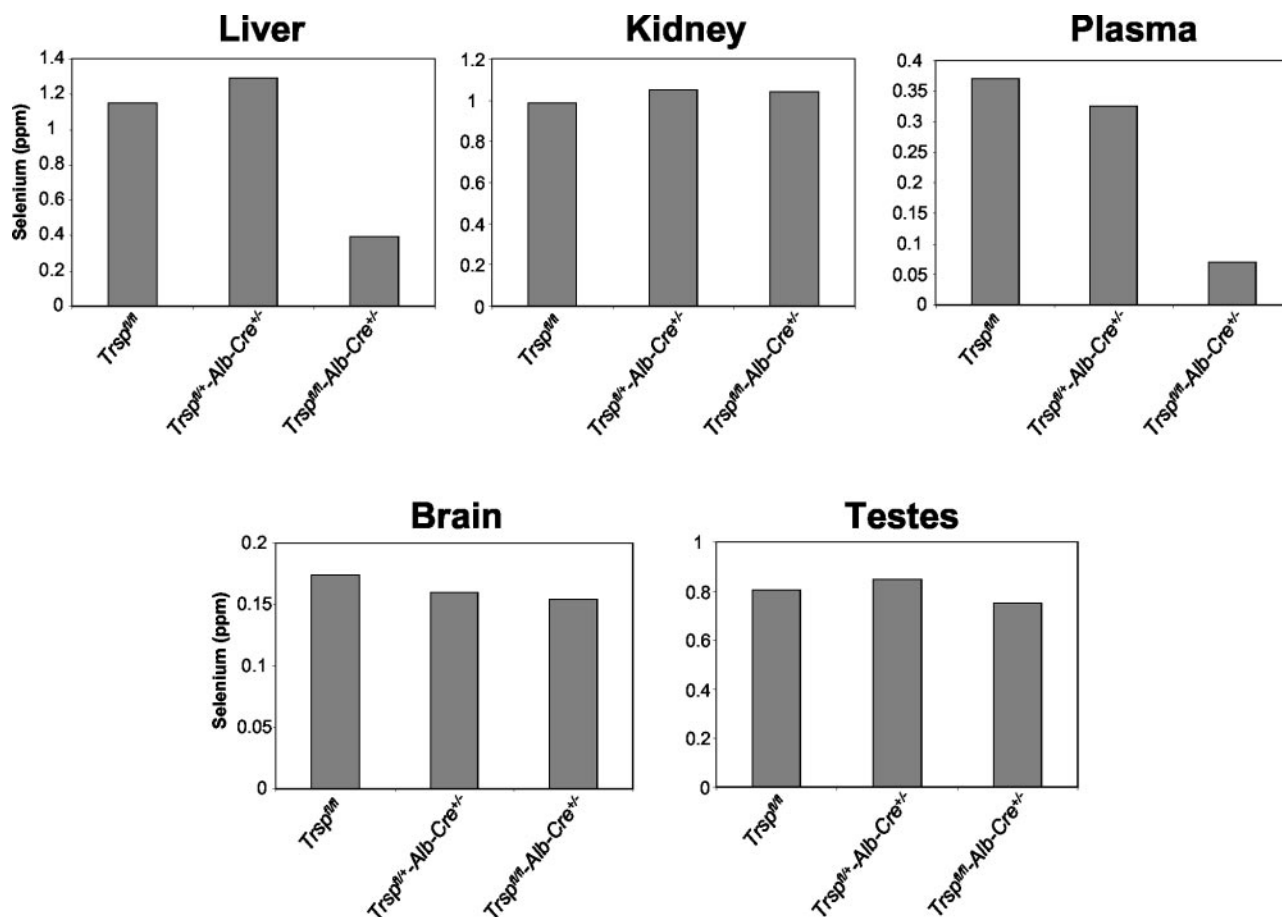


FIG. 5. **Selenium levels in floxed *Trsp* and *Alb-Cre* mice.** The amount of selenium was determined in duplicate in liver, kidney, plasma, brain, and testes, and the values shown represent the average of assays from two separate animals of the same genotype.

TABLE I

Selenium content in selenoproteins and low molecular weight selenocompounds in liver of *Trsp*<sup>fl/fl</sup>, *Alb-Cre*<sup>+/-</sup>, *Trsp*<sup>fl/+</sup>-*Alb-Cre*<sup>+/-</sup>, and *Trsp*<sup>fl/fl</sup>-*Alb-Cre*<sup>+/-</sup> mice

Liver extracts were prepared and protein and low molecular weight selenocompounds separated as described under "Experimental Procedures." Supernatant represents selenium present in the form of low molecular weight selenocompounds after removal of protein, and the trichloroacetic acid (TCA) fraction represents selenium present in the form of proteins.

Sample	Genotype	Selenium levels <sup>a</sup>
Total liver extract	<i>Trsp</i> <sup>fl/fl</sup>	ppm
	<i>Alb-Cre</i> <sup>+/-</sup>	1.14
	<i>Trsp</i> <sup>fl/+</sup> - <i>Alb-Cre</i> <sup>+/-</sup>	0.99
	<i>Trsp</i> <sup>fl/fl</sup> - <i>Alb-Cre</i> <sup>+/-</sup>	1.02
Supernatant	<i>Trsp</i> <sup>fl/fl</sup>	0.295
	<i>Alb-Cre</i> <sup>+/-</sup>	0.315
	<i>Trsp</i> <sup>fl/+</sup> - <i>Alb-Cre</i> <sup>+/-</sup>	0.315
	<i>Trsp</i> <sup>fl/fl</sup> - <i>Alb-Cre</i> <sup>+/-</sup>	0.250
TCA fraction	<i>Trsp</i> <sup>fl/fl</sup>	0.267
	<i>Alb-Cre</i> <sup>+/-</sup>	3.035
	<i>Trsp</i> <sup>fl/+</sup> - <i>Alb-Cre</i> <sup>+/-</sup>	2.690
	<i>Trsp</i> <sup>fl/fl</sup> - <i>Alb-Cre</i> <sup>+/-</sup>	2.415
		0.603

<sup>a</sup> The values represent  $\mu\text{g/g}$  liver used in preparing the total extract (Total liver extract),  $\mu\text{g/g}$  liver used in generating the supernatant (Supernatant), and  $\mu\text{g/g}$  protein found in the dried trichloroacetic acid pellet (TCA fraction). The data represent the average of assays from two animals performed in duplicate.

low molecular weight selenocompounds constitute about one-third of the selenium in liver, and this amount varied only slightly in selenoprotein-deficient mice. Therefore, most of the selenium in the liver is accounted for by selenium-containing

proteins, as specific excision of the Sec tRNA<sup>[Ser]Sec</sup> gene resulted in disrupted selenoprotein synthesis and a concomitant decrease in liver selenium levels but with little or no change in the low molecular weight selenocompounds.

Selenium levels appeared to be similar in brain as well as testes in mice that were either liver *Trsp*<sup>-</sup> or *Trsp*<sup>+</sup>. Selenium levels in the plasma of these mice, however, were reduced, suggesting that adequate amounts of this element in plasma are largely determined by SelP content, which supports the earlier observation of Burk *et al.* (22).

Our results confirm earlier reports that SelP is primarily synthesized in liver (23, 24). However, the finding that SelP is still present in plasma in the liver of *Trsp*<sup>-</sup> mice suggests that other organs contribute to the occurrence of SelP in the plasma, at least under conditions of disrupted SelP synthesis in the liver. Our finding that SelP levels are not decreased in brain of liver *Trsp*<sup>-</sup> mice, but they are decreased in testes, provides strong evidence that the former organ synthesizes its own SelP but the latter does not (see also 23,24). Enriching the diet with selenium in SelP knockout mice restores the neurologically defective phenotype observed in these animals to almost normal (12, 13), providing further evidence that the role of SelP, at least in part, is to transport selenium to various organs (see Ref. 30 for review). Interestingly, the finding of widespread necrosis of peritoneal and retroperitoneal fat in the absence of pancreatic damage in this study suggests that this circulating selenoprotein may also have a role in adipocyte homeostasis.

An interesting observation regarding the loss of selenoprotein expression and/or selenium deficiency in the liver of *Trsp*<sup>-</sup> mice is that the glutathione *S*-transferase levels were elevated, suggesting that this genotype may have up-regulated a detox-



ification program. Because many selenoproteins have roles as antioxidants (3), it would then seem that an interplay may exist between selenoproteins and other proteins involved in stress-related phenomena whereby they can compensate for each other.

Clinically ill mice used in this study had severe hepatocellular swelling and necrosis. DNA laddering was observed in the ill mice, suggesting that numerous cells were undergoing apoptosis. A TUNEL assay confirmed the presence of apoptotic cells. However, a direct assay of p53 showed that the levels of this protein were not elevated in liver cells of ill mice. Although enhanced expression of p53 often accompanies apoptosis, this is not true in all cases (*e.g.* see Ref. 31 and references therein), and apparently liver cells lacking selenoprotein expression undergo apoptosis without enhanced p53 expression.

An interesting feature of the removal of selenoprotein biosynthesis in hepatocytes is that liver requires their expression for proper function. In addition to demonstrating an essential role of selenoproteins in liver function, the present study has also provided new insights into the synthesis of SelP, its transport to other tissues, and a possible new function of this selenoprotein in fat metabolism.

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