ACCELERATED PUBLICATION

Gene disruption discloses role of selenoprotein P in selenium delivery to target tissues

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Selenoprotein P (SePP), the major selenoprotein in plasma, has been implicated in selenium transport, selenium detoxification or antioxidant defence. We generated SePP-knockout mice that were viable, but exhibited reduced growth and developed ataxia. Selenium content was elevated in liver, but low in plasma and other tissues, and selenoenzyme activities changed accordingly. Our data reveal that SePP plays a pivotal role in delivering hepatic selenium to target tissues.

Key words: glutathione peroxidase, selenium transport, targeted gene disruption, thioredoxin reductase.

INTRODUCTION

Selenium (Se) is an essential trace element that exerts its biological function as a catalytic entity in a variety of enzymes [1,2]. Typically it is present as a selenocysteine residue (SeCys) that is co-translationally inserted into the growing peptide chain at UGA codons within the open reading frame [3,4]. A specific stem–loop structure, the SeCys-insertion sequence (SECIS), characterizes those transcripts that encode selenoproteins. A complex machinery of SECIS-recognizing proteins, a specific elongation factor and a SeCys-loaded tRNA are required to decode the UGA codons as SeCys in mammals [5]. Many SeCys-containing proteins catalyse redox reactions, with SeCys acting as a component of the active site. In mammals, selenoproteins constitute the families of Se-dependent glutathione peroxidases (GPxs) [6], thioredoxin reductases (TrxRs) [7] and deiodinases [8]. These participate in peroxide degradation and antioxidant defence or control the intracellular redox potential and thyroid-hormone metabolism respectively. Labelling experiments with ^{75}Se in Se-deficient rats imply that mammals express up to 30–50 different selenoproteins, about 20 of which have already been identified, cloned and characterized, but the majority have not yet been functionally analysed (reviewed in [2]).

A still mysterious selenoprotein is selenoprotein P (SePP). Despite its biochemical identification two decades ago, the physiological function of SePP is still controversial. SePP is a plasma protein secreted by liver and accounts for more than 60 % of total plasma Se in rats. It contains up to ten SeCys residues, whose co-translational insertion is directed by two distinct SECIS elements within the 3′-untranslated region of the transcript [9]. This peculiar feature, together with its extracellular localization, prompted the idea that SePP might be little else than a transport or storage form of the trace element [10]. Fast delivery of Se from ^{75}Se-labelled SePP to the brain could indeed be demonstrated [11]. More recently, SePP was also proposed to protect against Se toxicity, to sequester Cd^{2+} and Hg^{2+} ions [12], to have an antioxidant function with thioredoxin as cofactor [13,14] or to serve as a barrier towards hydroperoxide- or peroxynitrite-mediated endothelial damage [15]. We therefore decided to analyse the physiological role of SePP directly by generating mice with a targeted inactivation of the *SEPP* gene.

MATERIALS AND METHODS

Materials

All chemicals were of analytical grade and were obtained from Sigma (München, Germany) or Merck (Darmstadt, Germany). [α-^{32}P]dCTP was from Hartmann Analytic (Braunschweig, Germany). Oligonucleotides were from MWG Biotech (Ebersberg, Germany), restriction endonucleases were from New England Biolabs (Frankfurt, Germany) and *Taq* polymerases were from Qiagen (Hilden, Germany).

Construction of a replacement vector

A targeting vector was constructed from plasmids pPNT and pHM2 and from a genomic Lambda clone of murine *SEPP* [16]. A 4.3 kb portion of 5′-sequence (including 0.6 kb upstream of the

Abbreviations used: cGPx, cellular glutathione peroxidase (GPx-1); ES, embryonic stem; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Nbs_2, 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent; 'DTNB'); PH-GPx, phospholipid hydroperoxide glutathione peroxidase (GPx-4); SePP, selenoprotein P; SECIS, selenocysteine (SeCys)-insertion sequence; *SEPP*, gene encoding SePP, *SEPP*KO, *SEPP* knock-out; TrxR, thioredoxin reductase.

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transcriptional start site, the non-coding exon 1, the complete 3.6 kb of intron 1 and 34 bp of exon 2, including the start ATG codon), were fused in-frame to a bacterial *lacZ* gene (Figure 1A). This construct was inserted 5′ to the neomycin phosphotransferase gene of pPNT. A 695 bp *Xba*I/*Bsp*I fragment 1.6 kb downstream of the *SEPP* poly(A) site was isolated and inserted 3′ to the neomycin phosphotransferase gene. The individual cloning steps were confirmed by DNA sequencing.

Gene targeting in embryonic stem (ES) cells

E14 ES cells were electroporated with the targeting vector after linearization at the unique *Not*I site. G418-resistant clones were screened by PCR. Candidate clones were verified by Southern hybridization. Two of the resulting chimaeric males were mated to C57BL/6 females and transmitted the targeted allele. Homozygous mutants were produced by cross-breeding of heterozygous mice. All mice were housed in a temperature-controlled (22 °C) animal room with a 12 h:12 h light/dark cycle with water and conventional lab chow (containing 0.24 p.p.m. Se; Altromin, Lage, Germany) *ad libitum*. After verification of the germline transmission of the mutant allele and of the proper homologous recombination, a PCR test was set up to genotype further progeny. Amplifications were performed using three primers: FlacZ (5′-CTGTAAGTCTGCAGAAATTGATG), FSePKO (5′-GTCTTAAGGAGAGAATGGATAG), and RSePKO (5′-GCTTCTACTCATCTCTACTTC), yielding products of 345 bp (wild-type allele) and/or 395 bp (knock-out allele). Animal experimentation was approved by the Regierung von Unterfranken (Government of Lower Franconia), Würzburg, Germany.

Northern- and Southern-blot analysis

Total RNA was extracted from the livers and brains of individual animals using peqGOLD TriFast reagent (peqLab Biotechnologie GmbH, Erlangen, Germany). Samples (20 μg/lane) were size-fractionated in a denaturing formaldehyde–agarose gel and transferred on to a nylon membrane (Nytran NY 12 N; Schleicher und Schüll, Dassel, Germany). Mouse tail DNA was digested by the indicated restriction endonucleases, size-fractionated, denatured and blotted. cDNA fragments for probe synthesis were amplified by PCR, isolated and labelled with [α-^{32}P]dCTP by random priming using Klenow polymerase (Gibco BRL, Eggenstein, Germany). Hybridizations and washings were carried out under high-stringency conditions as described in [17]. Signals were detected by exposure to X-ray films (X-Omat™; Kodak), and Northern-blot results were quantified using a phosphoimager (Cyclone Storage Phosphor Autoradiography System; Packard BioScience, Dreieich, Germany). The signals obtained for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were used to verify uniform loading and to calculate relative expression levels.

Sample collection and preparation of tissue homogenates

At the age of 5 weeks, mice were anaesthetized with diethyl ether, and blood was drawn from the heart for plasma collection. Liver, brain, kidneys and testis were removed, frozen in liquid nitrogen and stored at −80 °C until analysis. For enzyme activity assays and Se determinations, the tissues were powdered under liquid nitrogen using a dismembrator (Braun, Melsungen, Germany). Aliquots were homogenized on ice in approx. 5 vol. of 20 mM Hepes, pH 7.0, containing 250 mM sucrose and 1 mM EDTA using a glass/Teflon homogenizer. Homogenates were centrifuged at 10000 ***g*** for 20 min, supernatants were collected, and pellets were resuspended in homogenization buffer supplemented with 1 mM dithiothreitol. Protein concentrations were determined by a modified Bradford assay using IgG as a standard.

GPx assay

GPx activities were determined with the supernatants by a coupled test procedure [18] that monitors glutathione reductase-catalysed NADPH consumption at 340 nm as a measure of the rate of GSSG formation in the GPx reaction. The enzyme activity was measured at 20 °C in a buffer containing 20 mM potassium phosphate, pH 7.0, 0.6 mM EDTA, 0.15 mM NADPH, 4 units of glutathione reductase (Sigma, München, Germany), 2 mM GSH and 0.1 mM t-butylhydroperoxide and was expressed as nmol of NADPH consumed/min per mg of tissue protein. Non-specific NADPH oxidation was determined and subtracted by inhibiting Se-dependent GPx activity with 100 mM mercaptosuccinate before addition of the substrate.

TrxR assay

TrxR activities were assayed in supernatants by monitoring the NADPH-dependent reduction of 5,5′-dithiobis-(2-nitrobenzoic acid) (Nbs_2) determined as the increase in absorbance at 412 nm at 20 °C [31]. TrxR activities were assayed in 0.1 M potassium phosphate, pH 7.0, 10 mM EDTA, 2 mg/ml Nbs_2 and 0.2 mg/ml NADPH. The change of absorption at 412 nm was monitored, and TrxR activity is expressed as nmol of NADPH oxidized/min per mg of protein. The reliability of TrxR activities thus obtained was verified by a test system based on Trx-mediated insulin reduction [19].

Fluorimetric Se determination

The fluorimetric Se assay was essentially performed as described in [20]. Briefly, 50 μl of mouse plasma or 100 μl of crude tissue homogenate (10–500 μg of protein) were digested with 500 μl of $HNO_3/HClO_4$ (4:1, v/v) at 190 °C for 90 min in duplicate or triplicate. After cooling to room temperature, 500 μl of HCl was added and the open glass tubes were heated to 150 °C for 30 min. Then 2 ml of EDTA (2.5 mM) and 500 μl of diaminonaphthalene reagent (6.3 mM 2,3-diaminonaphthalene in 0.1 M HCl) was added at room temperature and the mixtures were left at 55 °C for 30 min. A 1 ml portion of cyclohexane was used to extract the piazselenol, and fluorescence was measured using a PerkinElmer fluorimeter (LS 50B; $\lambda_{excitation}$ 364 nm; $\lambda_{emission}$ 520 nm). Standard dilution curves of Na_2SeO_3 in water (0, 5, 10, 25, 50 and 100 μg of Se/litre), which were determined in parallel with every set of measurements, were linear and yielded a detection limit of 10 μg of Se/litre. A standard serum and a standard urine (Sero AS, Billingstad, Norway) were used to validate the method.

Statistical analysis

Student's *t* test was used to compare differences between the mean values obtained in *SEPP* knock-out (*SEPP*KO) and wild-type mice in each tissue analysed. Data are expressed as means ± S.E.M. Statistical significance was defined as $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***). To test the significance in multigroup comparisons, one-way ANOVA was used; differences at the 5 % level ($P < 0.05$) were assessed by Bonferroni's *post hoc* probability test.

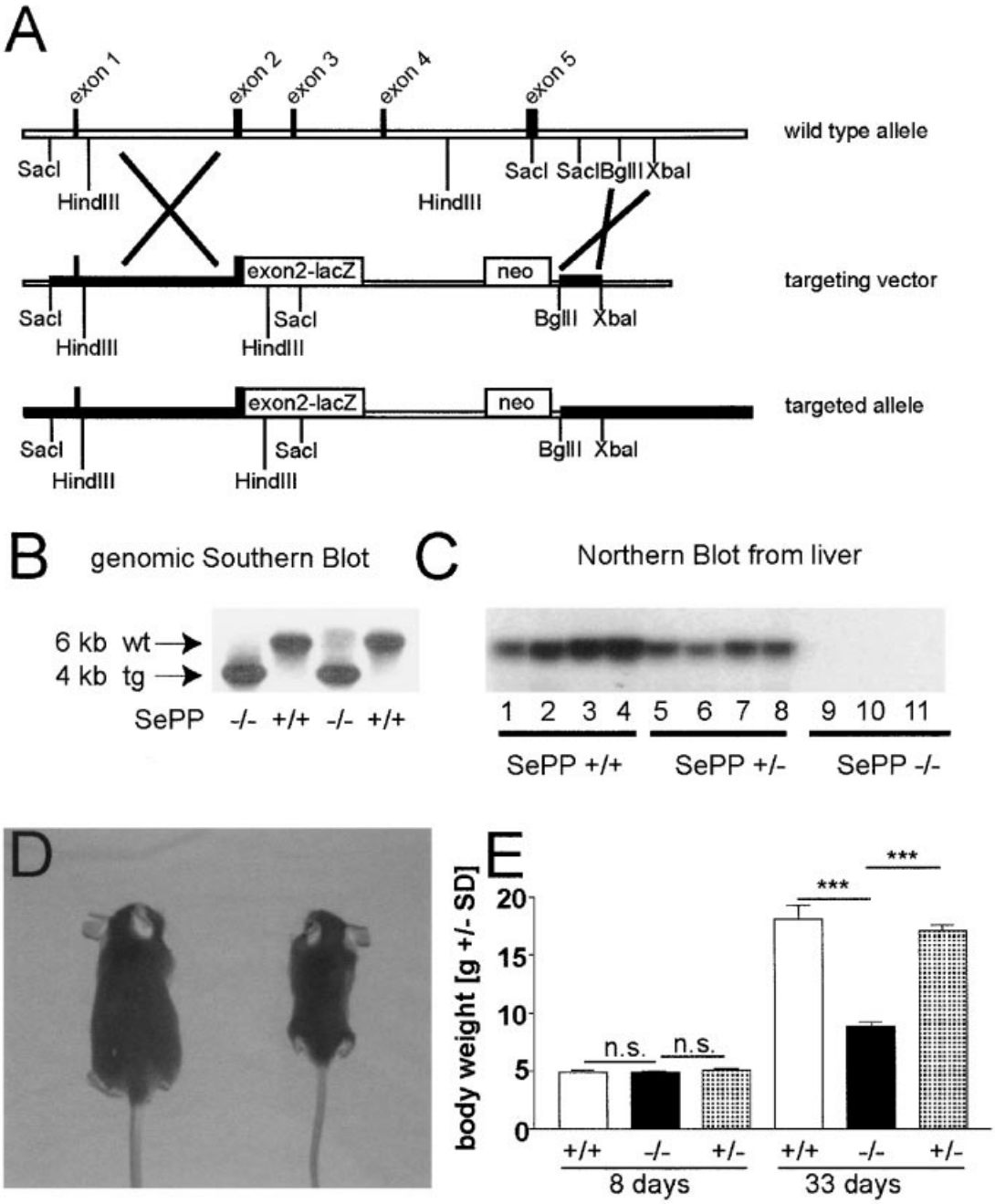

Figure 1 Generation of *SEPP*KO mice

(**A**) *SEPP* gene structure and targeting vector construction. Numbered boxes denote the five exons. The main part of exon 2 as well as exons 3–5 are replaced by vector sequences containing a PGK promoter-driven neomycin phosphotransferase gene ('neo'). Bold crosses (**X**, ***X***) represent homologous recombination. (**B**) Southern-blot analysis of genomic DNA isolated from tails of wild-type and homozygous *SEPP*KO mice after cleavage with enodnuclease *Hin*dIII. Arrows denote fragments obtained from wild-type (wt) and targeted alleles (tg) respectively. (**C**) Northern-blot analysis from livers of individual animals showing complete absence of *SEPP* mRNA in homozygous (−/−) *SEPP*KO mice. Note the significant decrease in mRNA in heterozygous (+/−) *SEPP*KO mice. (**D**) Photograph of a homozygous *SEPP*KO mouse (right) next to a wild-type littermate (left) at 5 weeks of age. (**E**) Body weights of homozygous *SEPP*KO mice are decreased in 5-week-old animals, but not 1 week after birth. +/+, $n = 8$; +/−, $n = 10$; −/−, $n = 7$; n.s. non-significant; $^{***}P < 0.001$ (ANOVA and Bonferroni's *post hoc* comparison).

RESULTS

Mice deficient in SEPP are viable and born without an obvious phenotype

In humans and mice [16], SePP is encoded by a single gene (*SEPP1*) that contains five exons. A targeting vector was constructed which retained the non-coding exon 1 and intron 1 of murine *SEPP* (Figure 1A). The open reading frame was replaced by vector sequences, and correct gene targeting was confirmed by Southern analysis (Figure 1B). The absence of *SEPP* transcripts in liver (Figure 1C) and brain (see Figure 4 below) was verified by Northern blotting. In heterozygous

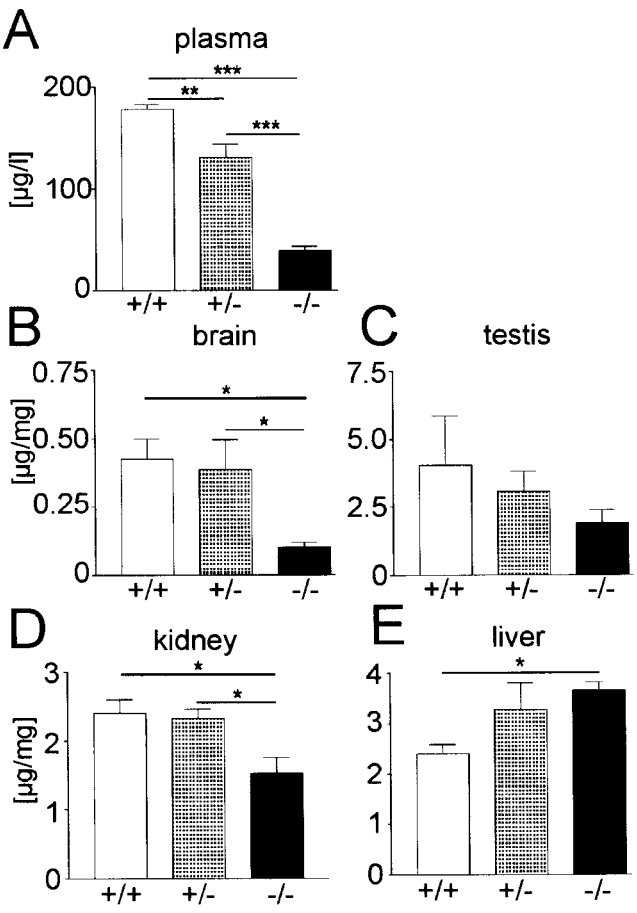

Figure 2 Se levels are altered in the organs of *SEPP*KO mice

(**A**) Plasma Se levels are gene-dose-dependently decreased in *SEPP*KO mice, confirming SePP as the most abundant selenoprotein in plasma. (**B**) Brain Se levels are severely decreased in homozygous *SEPP*KO mice, suggesting a role for SePP in Se accumulation in brain. Note the tenfold lower amount of Se in brain as compared with testis (**C**). (**D**) Se levels are significantly decreased in kidneys from homozygous *SEPP*KO mice. (**E**) *SEPP* deficiency leads to Se accumulation in liver. Se levels are expressed as μg of Se/litre of plasma (**A**) or μg of Se/mg of protein (**B**–**E**). $+/+$, $n = 8$; $+/-$, $n = 8$; $-/-$, $n = 7$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (ANOVA and Bonferroni's *post hoc* comparison).

animals the *SEPP* transcripts were decreased to $51 \pm 7\%$ and $53 \pm 8\%$ of the wild-type value respectively, indicating a simple gene-dose relationship. The transgene was back-crossed three times on to a C57BL/6 genetic background. Genotyping of the progeny showed Mendelian inheritance (1:2:1), with an average litter size of 10.6 and an unbiased ratio of the sexes. There were no indications of embryonic lethality in the *SEPP*KO mice. Male and female heterozygous and homozygous *SEPP*KO mice appeared normal at birth.

Development of phenotype in *SEPP*KO mice

In the first 2 weeks, wild-type and transgenic mice showed normal weight gain, growth and behaviour. However, by their third week of life, homozygous *SEPP*KO mice, but not their heterozygous littermates, developed signs of ataxia with a wide clumsy gait. To quantify the extent of the impediment, mice were tested on a RotaRod treadmill (Ugo Basile, Comerio, Italy). While heterozygous *SEPP*KO mice performed like wild-type mice and did not show impaired motor co-ordination or fatigue resistance, homozygous *SEPP*KO mice performed poorly (results not shown). Also, beginning at an age of 20 days, sporadic fatalities were observed and, by the end of the fifth week, nine of 39 homozygous *SEPP*KO mice had died spontaneously. When killed at postnatal day 33, homozygous *SEPP*KO mice presented with a markedly reduced body weight (Figures 1D and 1E) and shorter body and tail length.

Disturbed Se distribution in organs of *SEPP*KO mice

SePP was reported to account for more than 60% of plasma Se in rats [9]. Accordingly, plasma Se was found to be reduced in homozygous *SEPP*KO mice to 22% of the wild-type level. In heterozygotes, the decrease in plasma Se to about 60% of the control value is consistent with a gene-dose effect and excludes compensatory up-regulation of SePP synthesis from the remaining allele (Figure 2A). Attempts to correlate mouse SePP protein levels in plasma directly with Se levels in heterozygous animals have not been successful, as commercially available antibodies are directed towards the signal peptide, which is not present in circulating SePP [21]. Brain Se levels were strongly reduced in the homozygous *SEPP*KO mice, too, whereas heterozygous mice presented with an almost unchanged level (Figure 2B). Testis (Figure 2C) and kidney (Figure 2D) of homozygous *SEPP*KO mice showed moderate decreases in Se content (to 47 and 64% of wild-type levels respectively). Renal Se levels of heterozygous animals were unaltered. In contrast with these tissues, Se content was clearly elevated in liver in a gene-dose-dependent manner (to 1.5 times the control value in the homozygous *SEPP*KO mice; Figure 2E).

Activities of Se-dependent enzymes mirror altered Se content in tissues of *SEPP*KO mice

The impact of the disturbed Se distribution on the synthesis of selected selenoproteins was studied by functional tests. In principle the GPx and TrxR tests detect all members of the respective enzyme families, but nevertheless allow a realistic estimate of individual isoenzymes if their relative tissue-specific abundance is considered [22]. Cellular GPx (cGPx; GPx-1) is expressed ubiquitously and responds to Se-deprivation most readily, while phospholipid hydroperoxide GPx (PH-GPx; GPx-4) is most abundant in testis and is less responsive to Se deficiency [22,23]. Accordingly, the slightly decreased GPx activities in brain, kidney and testis of the heterozygous animals (Figure 3) likely reflect a decline in cGPx. In the homozygous *SEPP*KO mice the decrease of GPx activity is most prominent in kidneys (27%), where cGPx prevails, is intermediate in brain (33%), where both isoenzymes are expressed (see Figure 4 below), and is least pronounced in testis (42%), where PH-GPx is the abundant species. In contrast, GPx activities in liver, which primarily reflect cGPx levels, were clearly elevated (to 1.5 or 2.0 times control levels). The less Se-responsive TrxR activities were not significantly altered in liver and kidneys, but significantly decreased in brain and testis of homozygous *SEPP*KO mice (to 57% and 68% of control values respectively) (Table 1). In short, the variations of selenoenzyme activities, depending on their Se-responsivenes of the particular enzymes, mirror the disturbed Se distribution in the *SEPP*KO mice.

Selenoprotein transcripts in *SEPP*KO mice

Se deficiency may impair selenoprotein synthesis by both shortage of SeCys-loaded tRNA and degradation of the selenoprotein message [24]. In particular, the mRNA of cGPx is known to be

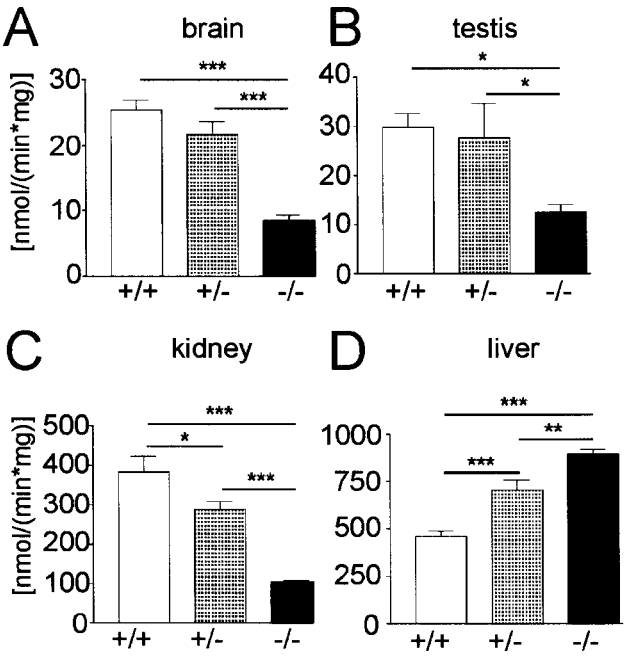

Figure 3 Changes in GPx activities in different tissues

GPx activites in brain (**A**) and testis (**B**) are drastically diminished in homozygous *SEPP*KO mice, but not in heterozygous littermates. GPx activities in kidneys (**C**) are significantly decreased in both homozygous and heterozygous *SEPP*KO mice in a gene-dose-dependent manner. In liver (**D**), markedly increased GPx activities parallel the elevated hepatic Se concentrations in *SEPP*KO mice. $+/+$, $n = 8$; $+/-$, $n = 8$; $-/-$, $n = 7$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (ANOVA and Bonferroni's *post hoc* comparison).

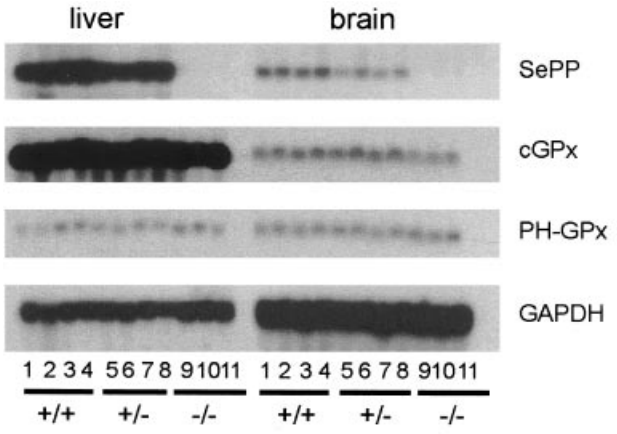

Figure 4 Gene expression of selenoproteins is altered in *SEPP*KO mice

Northern-blot analysis was performed with total RNA from liver and brain of homozygous *SEPP*KO mice, heterozygous *SEPP*KO mice or wild-type littermates. GAPDH RNA was used as loading control and for quantitative analysis. The amount of *SEPP* mRNA is about 50% in the preparations from heterozygous *SEPP*KO mice compared with wild-type controls. Absence of *SEPP* leads to a decrease of transcript levels for cGPx in brain, whereas only marginal effects are observed for PH-GPx levels in liver or brain.

more readily degraded in Se deficiency, whereas that of PH-GPx remains fairly stable or increases upon Se deprivation [22]. Accordingly, we found the cGPx transcripts decreased in brains of the homozygous *SEPP*KO mice [to $76 \pm 5\,\%$ of the wild-type level; $P < 0.01$ (ANOVA)], whereas transcript levels of PH-GPx were slightly increased [$127 \pm 6\,\%$ of the wild-type level; $P < 0.05$ (ANOVA)]. In liver, mRNA levels of cGPx from *SEPP*KO mice were decreased to $65 \pm 12\,\%$ of the wild-type level [$P < 0.05$ (ANOVA)] and PH-GPx levels were likewise slightly decreased ($82 \pm 6\,\%$), despite the significantly increased total GPx activities (Figure 4).

Table 1 Enzymic activity of TrxR

Statistical significance: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ (ANOVA and Bonferroni's *post hoc* comparison).

		Enzyme activity (fmol/min per mg) (mean ± S.E.M.)		
Organ	Mouse ...	+/+	+/−	−/−
Brain		5.08 ± 0.41	3.80 ± 0.27*	2.87 ± 0.23***
Liver		5.71 ± 0.37	6.29 ± 0.30	6.35 ± 0.44
Kidney		5.49 ± 0.36	5.00 ± 0.33	5.14 ± 0.65
Testis		4.55 ± 0.47	3.78 ± 0.23	3.07 ± 0.43*

DISCUSSION

Lack of any obvious phenotype of *SEPP*KO mice at birth, a normal number of offspring and an expected ratio of transgenic pups reveal that *SEPP* is evidently not essential for general embryonic development and likely not relevant to basic cellular functions either. Thus SePP cannot account for the vital importance of Se in mammals that became evident upon disruption of the SeCys-tRNA gene [25], which prevented the synthesis of all SeCys-containing proteins.

The appearance of a distinct phenotype during the third week after delivery followed by early fatalities nevertheless unmasked a biological role of SePP that is of pivotal importance to the adolescent or adult animal. The most obvious consequence of the SePP deficiency is a major disturbance of the Se distribution within the organism. A Se-deficiency status in tissues except liver, as demonstrated here for the *SEPP*KO mice, is also underscored by decreased GPx activities. In the liver, instead, synthesis of selenoproteins is within the normal range (TrxR) or markedly increased (GPx) in the absence of SePP. However, hepatic mRNA levels of cGPx and PH-GPx are decreased. This may tentatively be explained by the obvious Se overload that might reach the borderline of Se toxicity [26,27]. Hepatic accumulation of Se, associated with drastic Se decline in plasma, brain, testis and kidney in homozygous *SEPP*KO mice, implies that other tissues depend on the delivery of Se by SePP from the liver. In line with this view, the gross phenotype in part resembles Se-deficiency syndromes – retarded growth was reported for Se-deprived rats [28] and it is observed as 'unthriftiness' in Se-deficient sheep. Ataxia has so far not been reported to result from Se deficiency. The failure to detect this syndrome may be due to the difficulty to critically lower brain Se levels by dietary regimens alone. Even after 12–16 generations of continuously feeding a Se-deficient diet to rats, brain Se content decreased only to 90% of control [29]. The phenotype of the *SEPP*KO mice thus points to a novel role of Se in the central nervous system that has so far escaped attention.

The drastic disturbance of Se homoeostasis and selenoprotein expression in our transgenic animals renders it difficult, if not impossible, to draw any solid conclusions as to possible enzymic functions of SePP [13–15]. We are thus left with the minimum conclusion that at least one role of SePP is to incorporate Se reaching the liver from nutritional sources and to deliver it to sites of demand via the circulation. In other words, the *SEPP*KO

model revives the almost abandoned idea of Motsenbocker and Tappel [10] that SePP might be primarily a Se-transport protein. The biological significance of the SePP-dependent trafficking of Se appears conceivable in various respects. Dietary-derived Se-containing compounds that reach the liver via the portal vein are unstable and may be toxic [30] and therefore have to be instantly metabolized to SeCys-loaded tRNA, which is used for biosynthesis of selenoproteins. First of all, therefore, channelling of Se into SePP, which is the preferred metabolic path in the liver, represents a mechanism of detoxification and disposal. Subsequent release of SePP into the circulation provides Se in a comparatively inert, but functionally valuable, form, to distant tissues. The use of this transport form by peripheral tissues would likely require a receptor-mediated uptake, break-down of SePP and resynthesis of selenoproteins. The existence of SePP receptors has for long been inferred from binding studies [31], fast delivery of Se to the brain by means of injected SePP [11], and is also corroborated by highly different abilities of cell lines to utilize serum-bound Se, which essentially is SePP [32,33]. Receptor-dependent SePP utilization might thus offer a key to the understanding of the so-far-mysterious phenomenon of privileged Se supply to particular tissues such as testis, thyroid gland and brain [34].

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