Short Form Glutathione Peroxidase 4 Is the Essential Isoform Required for Survival and Somatic Mitochondrial Functions

Hanyu Liang‡, Si-Eun Yoo‡, Ren Na‡, Christi A. Walter‡¶, Arlan Richardson‡¶, and Qitao Ran‡¶††

From the ‡Sam and Ann Barshop Institute for Longevity and Aging Studies and Departments of ¶Cellular and Structural Biology, University of Texas Health Science Center, San Antonio, Texas 78229 and the ††South Texas Veterans Health Care System, San Antonio, Texas 78229

Glutathione peroxidase 4 (Gpx4) is an essential antioxidant enzyme having multiple functions. A long form Gpx4 protein and a short form Gpx4 protein, which are distinguishable by the presence or lack of a mitochondrial signal peptide at the N terminus, are generated from the Gpx4 gene. In this study, we generated transgenic mice using mutated GPx4 genes encoding either the long form Gpx4 (lGpx4 gene) or the short form Gpx4 (sGpx4 gene). Our results showed that transgenic mice with the sGpx4 gene had increased Gpx4 protein in all tissues and were protected against diquat-induced apoptosis in liver. Moreover, the sGpx4 gene was able to rescue the lethal phenotype of the mouse Gpx4-null mutation. In contrast, transgenic mice with the lGpx4 gene had increased Gpx4 protein only in the testes, and the lGPX4 gene failed to rescue the lethal phenotype of the mouse Gpx4-null mutation. In Gpx4-null mice rescued by the sGpx4 gene, the Gpx4 protein was present in mitochondria isolated from somatic tissues, and the subcellular distribution pattern of the Gpx4 protein in these mice was identical to that in wild-type mice. Interestingly, the male Gpx4-null mice rescued by the sGpx4 gene were infertile and exhibited sperm malformation. Together, our results demonstrated for the first time that the short form Gpx4 protein is present in somatic tissue mitochondria and is essential for survival and protection against apoptosis in mice, whereas the long form Gpx4 protein is important for male fertility.

Glutathione peroxidase 4 (Gpx4) is a major intracellular antioxidant that repairs lipid peroxidation (1). It is a member of the glutathione peroxidase (Gpx) superfamily. All Gpxs contain at the active site a selenocysteine, which participates in a two-electron reduction of peroxides to alcohols using glutathione as a reducing equivalent. Their common substrates are hydrogen peroxide and alkyl hydroperoxides (e.g. cumene hydroperoxide, tert-butylhydroperoxide, and hydroperoxy fatty acids). However, Gpx4 is unique in that it also reduces peroxides in complex lipids such as phosphatidylcholine and the hydroperoxide groups of thymine, lipoproteins, and cholesterol esters (2). More importantly, Gpx4 has the ability to reduce hydroperoxide-associated membranes. It is believed that its small size and large hydrophobic surface allow Gpx4 to react with complex lipids in membranes. Gpx4 is found in small amounts in the mitochondria, microsomes, cytosol, and nuclei of all tissues; however, it is highly expressed in testes (3). Gpx4 exhibits pleiotropic functions. First, Gpx4 is essential for survival; Gpx4-null mice die at midgestation (embryonic day 7.5), although the underlying mechanism of embryonic lethality is not clear (4). Second, Gpx4 has been shown to be important in repairing lipid peroxidation (5, 6). Third, Gpx4 is important in regulating mitochondrial apoptosis and protecting mitochondrial ATP generation under oxidative stress (7–9). Fourth, Gpx4 has been shown to control the activities of lipoxygenase and cyclooxygenase, thereby regulating eicosanoid production (10). Finally, Gpx4 is a major selenoprotein in testes and plays an important role in male fertility by affecting the maturation and function of sperm (11, 12).

Three forms of Gpx4 proteins are generated from the Gpx4 gene: a long form (lGpx4), a short form (sGpx4), and a nuclear form (nGpx4). The lGpx4 protein is synthesized from the first translation start codon. Because it has a mitochondrial signal at the N terminus, lGpx4 protein is believed to be targeted to mitochondria (13). Once inside mitochondria, the mitochondrial signal peptide of lGpx4 protein is cleaved. The sGpx4 protein is synthesized using the second translation start codon. Because of the lack of an N-terminal mitochondrial signal, the sGpx4 protein is believed to be the nonmitochondrial Gpx4 protein found in subcellular locations such as cytoplasm, nucleus, and microsome (13). However, because the mature lGpx4 protein and sGpx4 protein are identical, their distinct functions in vivo remain unclear. The nGpx4 protein is encoded from an alternative first exon called exon Ib and is expressed in somatic tissues; nGpx4 proteins are dispensable for both somatic functions and fertility (14).

In this study, we generated transgenic mice that overexpress either the sGpx4 protein or the lGpx4 protein using mutated human GPX4 genes. We show for the first time that sGpx4 protein is present in mitochondria as well as in other subcellular compartments of somatic tissues, and we show that the sGpx4 protein is essential for the protection against apoptosis...
and for the survival of mice. On the other hand, Igpx4 protein is primarily expressed in testis and is important for male fertility.

**EXPERIMENTAL PROCEDURES**

**Generation of Tg(sGPX4) Mice and Tg(lGPX4) Mice**—The sGPX4 and lGPX4 genes were generated by a site-directed mutagenesis technique from a phagemid P1 clone, which contains the human endogenous GPX4 gene (8). Briefly, the 12-kb fragment containing the GPX4 gene was removed from the P1 clone by digesting with ScaI and NsiI restriction enzymes and cloned into the pCR-2.1-TOPO vector to generate pCR-Wt-GPX4 construct. Point mutations were introduced to the GPX4 gene by synthesized PCR primers using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). To generate the GPX4 gene expressing only sGpx4 protein (sGPX4 gene), the first ATG start codon in exon Ia of the GPX4 gene was mutated to ATG, and the resultant construct was designated as pCR-sGPX4. To generate the GPX4 gene expressing only lGPx4 protein (lGPX4 gene), the second ATG start codon in exon Ia of the GPX4 gene was mutated to ATC, and the resultant construct was designated as pCR-lGPX4. The resulting construct was injected to fertilized oocytes derived from B6D2F1 mice to yield the GPX4 gene expressing only sGpx4 protein (sGPX4 gene), the second ATG start codon in exon Ia of the GPX4 gene was mutated to ATC, and the resultant construct was designated as pCR-lGPX4.

DNA from pCR-sGPX4 and pCR-lGPX4 were isolated and injected to fertilized oocytes derived from B6D2F1 mice to make transgenic mice overexpressing either sGpx4 protein or Igpx4 protein (Tg(sGPX4) and Tg(lGPX4) mice) at the University of Michigan Transgenic Animal Model Core. A PCR-based genotyping protocol was developed to genotyped these mice (T3, 5'-GGG CTA CAA CGT CAA ATT CGA TAT GTT CAG-3'; T4, 5'-AAG TTC ACC CAC TTC TTG AGC GTA GCA GAA AAG AAG-3'). We obtained multiple transgenic founder lines for Tg(sGPX4) mice and Tg(lGPX4) mice. The Tg(sGPX4) line and the Tg(lGPX4) line used in this study had similar levels of total GPx4 transcripts.

The Tg(sGPX4) and Tg(lGPX4) mice were crossed to C57/B6 mice twice and maintained as hemizygous mice. The mice were fed *ad libitum* a commercial diet and maintained under pathogen-free barrier conditions on a 12/12-h dark/light cycle. Male and female mice at 6–10 months of age were used for this study. All procedures for handling the mice in this study were reviewed and approved by the IACUCs (Institutional Animal Care and Use Committees) of the University of Texas Health Science Center at San Antonio and the South Texas Veterans Health Care System.

**Gpx4 Expression**—Total Gpx4 mRNA expressions (mRNA levels of both sGpx4 and lGpx4 transcripts) in various tissues were measured by quantitative real-time PCR. Briefly, total RNA was isolated from tissues using Tri Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s instructions. One µg of RNA was reverse transcribed to cDNA using a RETROscript kit (Ambion, Inc., Austin, TX) in 20 µl of total reaction volume. The synthesized cDNA was subsequently diluted with nuclease-free water. Real time PCR amplification mixtures (20 µl) contained an optimal amount of template cDNA, SYBR Green Master Mix (Applied Biosystems, Foster City, CA), and forward and reverse primers (forward, TGC ATC GTC ACC AAC GTG GC; reverse, CTT CAC CAC GCA GCC GTT CT). Reactions were run on an ABI 7500 real time PCR system (Applied Biosystems) with the standard PCR profile: 50 °C for 2 min, 95 °C for 10 min, and then 40 cycles of 95 °C for 15 s followed by 60 °C for 1 min. Dissociation curve analysis was performed after a completed PCR to evaluate the presence or absence of nonspecific amplifications. ∆Ct values were used to calculate the relative copy numbers of total Gpx4 transcripts.

Gpx4 protein levels in various tissues were determined by Western blotting as described previously (4). Briefly, tissues were homogenized in phosphate-buffered saline supplemented with 0.5% Triton X-100 and protease inhibitor. Protein concentrations were determined by the Bradford method. Equal amounts of protein were then separated by SDS-PAGE, transferred to nitrocellulose membranes, probed with a primary antibody to Gpx4 and the corresponding secondary antibody, and detected by ECL Western blotting (Amersham Biosciences). The intensities of the bands on the blots were quantified with ImageQuant 5.0 and normalized to loading control actin using anti-actin antibody (69100; MP Biomedicals, Inc., Aurora, OH).

To detect Gpx4 protein in subcellular fractions, tissues were homogenized in buffer 1 (250 mM mannitol, 75 mM sucrose, 500 µM EGTA, 100 µM EDTA, and 10 mM Hepes, pH 7.4) supplemented with the protease inhibitor mixture. The homogenates were centrifuged at 600 × g for 10 min at 4 °C to pellet nuclei. The resulting supernatant was then centrifuged at 10,000 × g for 10 min at 4 °C to obtain the mitochondria pellet. The supernatant was further centrifuged at 100,000 × g for 60 min at 4 °C to yield the cytosol. Gpx4 protein levels in each fraction were determined by Western blotting as described above.

To determine the levels of Gpx4 in submitochondrial fractions, liver mitochondria were further subfractioned as we described previously (15). Briefly, tissues were homogenized in ice-cold buffer A (0.25 m sucrose, 1 mM EDTA, 10 mM Hepes, pH 7.4). The homogenates were centrifuged at 600 × g for 10 min at 4 °C to pellet nuclei and unbroken cells. The resultant supernatant was then centrifuged at 3,000 × g for 10 min at 4 °C to obtain a crude heavy mitochondrial pellet. The crude heavy mitochondrial pellet was washed once and further purified by a discontinuous Nycodenz gradient (16). Briefly, the heavy mitochondrial pellet resuspended in 3.5 ml of 25% Nycodenz placed over 1.5 ml of 40%, 1.5 ml of 34%, and 2.5 ml of 30% Nycodenz and this was topped off with 2.5 ml of 23% and 0.5 ml of 20% Nycodenz. The gradient was centrifuged at 95,000 × g for 2 h at 4 °C in a Beckman SW40 Ti rotor. Mitochondria were collected from the 25% to 30% Nycodenz interface, washed, and resuspended in a small amount of buffer A. A sample was taken and designated as total mitochondrial. The remainder of mitochondria suspension was adjusted so that the final protein concentration was 100 µg/µl. Digitonin was slowly added to the mitochondria (1.5 mg of digitonin for every 10 mg of mitochondrial proteins). After incubation at 4 °C for 15 min with gentle rocking, the suspension was diluted with 3 volumes of buffer A and then centrifuged at 15,000 × g for 10 min at 5 °C. The supernatant was further centrifuged at 144,000 × g for 20 min at 5 °C, and the resultant pellet and supernatant were designated as mitochondrial outer membrane and intermembrane space, respectively. The pellet from the 15,000 × g centrifugation spin, containing mitoplasts, was resuspended in a small
In Vivo Functions of Gpx4 Isoforms

amount of buffer A supplemented with the protease inhibitor and subjected to three freeze-thaw cycles. The mitochondrial suspension was centrifuged at 144,000 × g for 50 min at 5 °C, and the resulting pellet and supernatant were designated as mitochondrial inner membrane and matrix, respectively. Protein concentrations for total mitochondria and each submitochondrial fraction were determined, and equal amounts of protein were loaded for the detection of Gpx4 by Western blotting. Antibodies to voltage-dependent anion channel, Smac/DIABLO, ATPase β subunit, and manganese superoxide dismutase were used to confirm the identities of mitochondrial outer membrane, intermembrane space, inner membrane, and matrix fractions, respectively.

Measurement of Apoptosis—Apoptotic nuclei in the liver were determined in situ by the presence of double-stranded DNA breaks observed in paraffin-embedded tissue sections using and in situ oligonucleotide ligation (ISOL) kit (Chemicon International) with oligonucleotide B according to the manufacturer’s instructions. Compared with the conventional terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling assay, the ISOL assay uses hairpin oligonucleotide probe to detect more specific DNA fragmentation caused by apoptosis, avoiding randomly damaged DNA (17). Slides were visualized under light microscopy, and the number of positive cells was determined in 10 random fields at 400 × magnification for each liver. Data are expressed as the mean of the ratio of the number of positive nuclei and the total number of nuclei in all 10 random fields.

Caspase-3 protein levels in liver tissues were determined by Western blotting using an anti-caspase-3 antibody as we had described previously (8). The intensities of bands corresponding to p20 of caspase-3 were quantified and used as indicators of caspase-3 activation.

Rescue Study—To determine whether sGpx4 and/or lGpx4 transgenes could rescue the lethal phenotype of Gpx4-null mutation, Tg(sGPX4) and Tg(lGPX4) mice were first crossed with mice heterozygous for the mouse Gpx4 gene (Gpx4+/−) to generate Gpx4+/− mice that carried either the sGpx4 or lGpx4 transgene, i.e. Tg(sGPX4)/Gpx4+/− mice and Tg(lGPX4)/Gpx4+/− mice. Tg(sGPX4)/Gpx4+/− or Tg(lGPX4)/Gpx4+/− mice were then bred with Gpx4−/− mice. The offspring were genotyped for the presence of sGPX4/lGPX4 transgene as well as the status of the endogenous mouse Gpx4 gene (i.e. Gpx4+/+, Gpx4+/−, Gpx4−/−). The successful rescue was indicated by the presence of live Gpx4−/− mice that carried the transgene.

Assessment of Fertility—To assess male fertility, individual males were mated continuously for 8 weeks periods with 2 wild-type (Wt) females. Individual females were mated with 1 Wt male for 8 weeks. All females were maintained for 4 weeks after the mating period to assess possible pregnancy. The number and size of litters at birth were recorded.

Sperm Analysis—Sperm were collected as described previously (18). Briefly, the epididymis was excised and placed in a prewarmed 35-mm Petri dish containing 1 ml of phosphate-buffered saline at 37 °C and then placed in a 37 °C incubator for 15 min. For the determination of sperm mobility, 1 drop of sperm suspension was placed on a prewarmed microscope slide, and a 22 × 22-mm coverslip was added. A minimum of 100 sperm/mouse were observed at ×400 magnification using a standard light microscope for motility and expressed as percentage of motility. Sperm were counted as motile with any type of movement. The sperm suspensions were filtered through 100-μm pore size mesh to remove tissue fragments. The sperm suspensions were diluted, and the sperm count was determined using a hemocytometer and expressed as ×10^6/ml. For the determination of abnormality, a drop of filtered sperm suspension was smeared onto a slide. The slide was air-dried, fixed in 1% acetic acid in 100% ethanol for 2 min, stained for 30 min in Tresolin (Statlab, Lewisville, TX), washed three times in 70% ethanol, and examined at ×400 magnification by counting and expressed as percentage of abnormality.

Statistical Analysis—All data are represented as the mean value of each group ± standard error (S.E.) of measurement, and Student’s t test was used for analysis. Statistical significance was assumed at p < 0.05.

RESULTS

Generation of Transgenic Mice Overexpressing Either sGpx4 Protein or lGpx4 Protein—We used a site-directed mutagenesis strategy to produce mutated Gpx4 genes that express either the sGpx4 protein or the lGpx4 protein from a human endogenous GPX4 gene that we have described previously (8). As shown in

FIGURE 1. Generation of Tg(sGPX4) and Tg(lGPX4) mice. A, Wt GPX4, the DNA sequence of exon 1 (capital letters) and intron 1 of human endogenous Gpx4 gene, with the two start codons responsible for the production of sGpx4 and lGpx4 proteins shown. B, the first start codon for synthesizing Igpx4 protein in Wt GPX4 was mutated from ATG to ATC. C, the second start codon for synthesizing sGpx4 protein in Wt GPX4 was mutated from ATG to ATC. B, total Gpx4 transcript levels in tissues from Tg(sGPX4) mice and Wt controls. C, total Gpx4 transcript levels in tissues from Tg(lGPX4) mice and Wt controls. Levels of Gpx4 transcripts were measured by quantitative real time PCR. All values are expressed as mean ± S.E. of data obtained from three or four mice. *, p < 0.05.
Fig. 1, the sGpx4 protein and lGpx4 protein arise from different translation start sites localized in exon Ia of the GPX4 gene. To generate the sGPX4 gene expressing only the sGpx4 protein, the first ATG start codon of the Wt GPX4 gene, which is responsible for synthesizing the lGpx4 protein, was mutated to ATC. To generate the lGPX4 gene expressing only the lGpx4 protein, the second ATG start codon, which is responsible for synthesizing the sGpx4 protein, was mutated to ATC. We performed restriction analysis and sequencing to confirm the presence of the introduced mutations in the sGPX4 gene and lGPX4 gene (data not shown). The sGPX4 gene and lGPX4 gene contain 5 kb of 5’ upstream sequence and 5 kb of 3’ downstream sequence, which include the cis-regulatory elements necessary for expression (8).

Transgenic mice carrying the sGPX4 gene (Tg(sGPX4) mice) or lGPX4 gene (Tg(lGPX4) mice) were generated by microinjecting the transgenes into fertilized oocytes. To characterize Gpx4 expression in the Tg(sGPX4) and Tg(lGPX4) mice, we first measured the total Gpx4 mRNA levels by quantitative real-time PCR. The PCR primers we used recognized all forms of human and mouse Gpx4 transcripts, including sGPX4 and lGPX4 transcripts. As shown in Fig. 1B, Tg(sGPX4) mice had a 3.8–7.5-fold increase in the total levels of Gpx4 transcripts in various tissues compared with Wt mice. Data in Fig. 1C show...
that Tg(lGPX4) mice had a 3.9–12-fold increase in the total levels of Gpx4 transcripts compared with Wt controls.

We also determined the levels of Gpx4 proteins in tissues of the transgenic mice. Gpx4 protein levels were elevated (a 1.7–2.5-fold increase) in tissue homogenates from Tg(sGPX4) mice compared with those from Wt mice (Fig. 2A). In contrast, aside from a 1.5-fold increase in Gpx4 protein in testes tissue, no significant increase in Gpx4 protein in somatic tissues, such as brain, kidney, and liver, was detected in Tg(lGPX4) mice (Fig. 2D). Pushpa-Rekha et al. (19) reported previously in rats the existence of two Gpx4 transcription initiation sites in vivo: an upper site in testes responsible for a long transcript that encodes the lGpx4 protein and a lower site predominantly in somatic tissues responsible for a short transcript that encodes the sGpx4 protein. Our data on Gpx4 protein levels in the two transgenic mice are consistent with this report. In addition, our results showed that long transcripts of Gpx4 in Wt mice were less than 1% of the total Gpx4 transcripts in somatic tissues but accounted for about 80% of the total Gpx4 in testes tissue (data not shown). These data also indicate that the short transcript is predominant in somatic tissues whereas the long transcript is predominant in testis tissues. Because our mutation strategy affects the transgene expression only at the translation level, the total Gpx4 transcripts from Tg(lGPX4) mice did not show a tissue-specific pattern, i.e. levels of Gpx4 transcripts were found to be increased in all tissues from the Tg(lGPX4) mice. The data in Fig. 2 also show the subcellular distribution of Gpx4 in tissues of the Tg(sGPX4) and Tg(lGPX4) mice. Gpx4 levels are increased in both the cytosolic and mitochondrial fractions of liver and testes from Tg(sGPX4) mice; however, the increase was greater for the cytosolic fraction than the mitochondrial fraction (Fig. 2, B and C). In contrast, Gpx4 levels were only increased in the mitochondrial fractions of testes from the Tg(lGPX4) mice (Fig. 2E).

Tg(sGPX4) Mice Were Protected against Oxidative Stress-induced Apoptosis—In cell cultures studies, it was shown that the overexpression of Igpx4 protein, but not sGpx4 protein, suppressed apoptosis (20). We showed previously that the overexpression of a Gpx4 gene with intact translation start sites in exon Ia for both sGpx4 and lGpx4 proteins protected against diquat-induced apoptosis in liver (8). To determine whether the overexpression of sGpx4 protein or lGpx4 protein is responsible for this protection, we compared diquat-induced apoptosis in Tg(sGPX4) mice and Tg(lGPX4) mice. We treated Tg(sGPX4) mice and Tg(lGPX4) mice with diquat, and liver apoptosis was determined by measuring apoptotic nuclei and caspase-3 activation. As shown in Fig. 3, A and B, Tg(sGPX4) mice had significantly reduced apoptotic nuclei and caspase-3 activation compared with Wt controls (Fig. 3C). In contrast, Tg(lGPX4) mice did not show any difference in the level of apoptotic nuclei and caspase-3 activation compared with Wt mice (Fig. 3D). Therefore, the overexpression of sGpx4 protein is able to protect against oxidative stress-induced apoptosis in vivo.

sGpx4 Protein Is Critical for Mouse Embryonic Development—Previously, we and others reported that Gpx4 homozygous null embryos (Gpx4−/−) die at embryonic day 7.5–8.5 (4, 21), and we further showed that the lethal phenotype of the Gpx4-null mutation could be rescued by the human GPX4 gene encoding both sGpx4 and lGpx4 proteins (8). To determine whether the lack of the sGpx4 protein or lGpx4 protein is responsible for the lethal phenotype in the Gpx4-null mutation, we measured the abilities of sGpx4 and lGpx4 transgenes to rescue the lethal phenotype of the Gpx4-null mutation. Using a breeding strategy described under “Experimental Procedures,” we determined whether we could obtain live mice null for endogenous Gpx4 but expressing the sGpx4 protein (Tg(sGPX4)/Gpx4−/− mice) or mice null for endogenous Gpx4 but expressing the lGpx4 protein (Tg(lGPX4)/Gpx4−/− mice), Fig. 4A shows the genotype results of one litter of 4-week-old offspring derived from Tg(sGPX4)/Gpx4−/− mice bred to Gpx4+/− mice, demonstrating that we were able to obtain live Tg(sGPX4)/Gpx4−/− mice. Fig. 4B shows a summary of the rescue study using the sGPX4 gene. Thirty-two Tg(sGPX4)/Gpx4−/− mice were obtained from a total of 189 offspring. The number of Tg(sGPX4)/Gpx4−/− mice we obtained was similar to what was predicted by Mendel’s law of segregation, indicating that the sGPX4 transgene is able to rescue the lethal phenotype of...
Gpx4-null mutation. We performed a similar rescue study using the Tg(lGPX4) mice. As indicated in the summary data of this experiment in Fig. 4C, we did not obtain any live Tg(lGPX4)/Gpx4<sup>−/−</sup> mice of a total of 195 offspring from the crossing of Tg(lGPX4)/Gpx4<sup>−/−</sup> mice with Gpx4<sup>−/−</sup> mice. Therefore, the lGPX4 transgene is unable to rescue the lethal phenotype of the Gpx4<sup>−/−</sup> mutation.

sGpx4 Protein Is Targeted to Mitochondria—The sGpx4 protein, which lacks the mitochondrial signaling peptide, is believed to be the nonmitochondrial form or cytosolic form of Gpx4 (13). However, whether sGpx4 protein can be targeted to mitochondria is unknown. Because the Tg(sGPX4)/Gpx4<sup>−/−</sup> mice are viable but null for lGpx4 protein, we were interested in knowing the subcellular distribution of the Gpx4 protein in those mice. We measured the total levels of Gpx4 as well as Gpx4 levels in cytosolic and mitochondrial fractions in liver and brain from Tg(sGPX4)/Gpx4<sup>−/−</sup> mice and compared the levels with those of Wt controls. Subcellular fractionation data in Fig. 5A show that the levels of Gpx4 protein in whole tissue homogenates and cytosolic fractions isolated from the liver and brain from the Tg(sGPX4)/Gpx4<sup>−/−</sup> mice were similar to what were observed in Wt mice, further confirming that the sGpx4 protein is the predominant isoform in somatic tissues. Interestingly, the Tg(sGPX4)/Gpx4<sup>−/−</sup> mice did not show a reduction of the Gpx4 protein in their mitochondrial fractions compared with Wt mice, even though they did not express the lGpx4 protein. Thus, these results indicate that the sGpx4 protein was able to be targeted to mitochondria.

We next asked whether the sGpx4 protein was targeted correctly to mitochondria. We obtained submitochondrial fractions from liver tissues of Tg(sGPX4)/Gpx4<sup>−/−</sup> mice and Wt mice and compared the distribution of Gpx4 protein in mitochondria from Tg(sGPX4)/Gpx4<sup>−/−</sup> mice and Wt mice. As shown in Fig. 5B, in the mitochondria from Wt mice, Gpx4 protein was found in both the inner and outer mito-
In Vivo Functions of Gpx4 Isoforms

| Genotype                  | Litter number | Litter size | Sperm count (10⁶/ml) | Sperm motility (%) |
|---------------------------|---------------|-------------|----------------------|--------------------|
| Wt                        | 2.33 ± 0.33   | 5.89 ± 0.31 | 10.13 ± 1.08         | 74.75 ± 3.07       |
| Tg(sGPX4)/Gpx4<sup>-/-</sup> | 0*           | 0*          | 6.70 ± 0.15*         | 27.67 ± 1.86*      |

FIGURE 6. Tg(sGPX4)/Gpx4<sup>-/-</sup> male mice are infertile and show defects in sperm. A, fertility assessment and sperm analysis were performed in Tg(sGPX4)/Gpx4<sup>-/-</sup> and Wt male mice as described under “Experimental Procedures.” B, morphology of sperm from Tg(sGPX4)/Gpx4<sup>-/-</sup> mice and Wt mice. Arrows indicate the Tg(sGPX4)/Gpx4<sup>-/-</sup> sperm that had unusual morphology, a kink between midpiece and principal piece. C, photograph of representative Western blot showing levels of Gpx4 protein in testis tissues and sperm from Wt mice and Tg(sGPX4)/Gpx4<sup>-/-</sup> mice.

In the mitochondria of Tg(sGPX4)/Gpx4<sup>-/-</sup> mice, Gpx4 was also enriched in inner membrane and outer membrane, and the distribution pattern of the Gpx4 protein in mitochondria from Tg(sGPX4)/Gpx4<sup>-/-</sup> mice was identical to that in Wt mice. Therefore, the sGpx4 protein can be targeted correctly to mitochondria.

**IGpx4 Protein Is Essential for Male Fertility**—Because Gpx4 has been shown to play an important role in fertility (12), we next asked whether the Tg(sGPX4)/Gpx4<sup>-/-</sup> mice, which were null for IGpx4 protein, showed any difference in fertility compared with Wt mice. The female Tg(sGPX4)/Gpx4<sup>-/-</sup> mice were fertile and were not different from Wt females in terms of litter size and the number of litters produced during the mating period (data not shown). However, after repetitively breeding Tg(sGPX4)/Gpx4<sup>-/-</sup> male mice with fertile female Wt mice, we were unable to obtain any offspring (Fig. 6A), indicating that the male Tg(sGPX4)/Gpx4<sup>-/-</sup> mice are infertile. The Tg(sGPX4)/Gpx4<sup>-/-</sup> males showed no gross abnormality in their testes and epididymus by histological examination (data not shown); however, they had reduced sperm count and decreased sperm motility (Fig. 6A). Microscopic comparison of sperm from the Tg(sGPX4)/Gpx4<sup>-/-</sup> mice and Wt mice indicated that the majority of the sperm from the Tg(sGPX4)/Gpx4<sup>-/-</sup> mice had a “kink morphology,” i.e. a bend between the principle piece and the midpiece (Fig. 6B). Because Gpx4 has been shown to be a major structural protein in the mitochondrial capsules at the midpiece (12), we measured Gpx4 levels in the testis and sperm of Wt mice and Tg(sGPX4)/Gpx4<sup>-/-</sup> mice. As shown in Fig. 6C, testes from Tg(sGPX4)/Gpx4<sup>-/-</sup> mice had reduced Gpx4 protein levels compared with testes from Wt mice, and we were unable to detect Gpx4 protein in sperm from the Tg(sGPX4)/Gpx4<sup>-/-</sup> mice. Thus, our data indicate that the lack of IGpx4 protein results in Gpx4 deficiency in sperm, leading to sperm malformation and infertility.

**DISCUSSION**

Gpx4 exists in three isoforms and has several important functions in vivo. However, the discrete functions for specific Gpx4 isoforms have not been investigated in depth. In this study, we generated transgenic mice using mutated GPX4 genes that encode either the sGpx4 protein or the IGpx4 protein to study the function, tissue distribution, and subcellular localization of these two isoforms. It should be noted that our mutation strategy affects the transgene expression only at the level of translation, i.e. the levels of total Gpx4 transcripts (both sGpx4 and IGpx4 transcripts) are expected to be elevated in both Tg(sGPX4) mice and Tg(lGPX4) mice. Indeed, our results indicate that both Tg(sGPX4) mice and Tg(lGPX4) mice had increased total Gpx4 transcripts. However, Tg(sGPX4) mice had increased levels of Gpx4 protein in tissues such as liver, brain, and kidney, whereas Tg(lGPX4) mice had an increased level of total Gpx4 protein only in testes. Previously, Pushpa-Rekha et al. (19) showed that two transcription initiation sites in rat Gpx4 gene are responsible for a short Gpx4 mRNA and a long Gpx4 mRNA, respectively. They further showed that the short transcript was found primarily in somatic tissues, whereas the long transcript was found primarily in testes. Our results from the Tg(sGPX4) mice and Tg(lGPX4) mice are consistent with those results, indicating that the sGpx4 protein is the predominant form in somatic tissues whereas the IGpx4 protein is expressed primarily in testes.

Gpx4 is an essential gene for survival of mouse because the null mutation of this gene results in early embryonic lethality (4, 21). Conrad et al. (14) reported that mice with a targeted deletion of nGpx4 protein were viable and fertile, indicating that nGpx4 protein is dispensable for survival. However, the roles of the sGpx4 protein and IGpx4 protein in development and survival were not clear. It has been shown that sGpx4 mRNA is present in embryonic and somatic tissues whereas IGpx4 mRNA is detectable only in testicular tissue (22). This suggests that IGpx4 protein may be dispensable for embryonic development. However, in another study, both sGpx4 and IGpx4 mRNA were found at high concentrations during the course of embryogenesis, and the knockdown of IGpx4 during in vitro embryogenesis strongly impaired hindbrain development and induced cerebral apoptosis (23), suggesting that IGpx4 plays a...
role in organogenesis and embryonic development. We showed previously that a human GPX4 gene expressing both sGpx4 and lGpx4 proteins could substitute the need of the mouse endogenous Gpx4 for development and survival (8). In this study, we further tested the ability of sGpx4 protein and lGpx4 protein to rescue the embryonic lethal phenotype of Gpx4-null mutation. We performed these rescue studies by crossing either Tg(sGPX4)/H11001 mice or Tg(lGPX4)/H11002 mice with Gpx4−/− mice. We found that the sGpx4 protein was able to rescue the lethal phenotype of Gpx4-null mutation because we obtained Gpx4−/− mice that carried the sGPX4 transgene (Tg(sGPX4)/Gpx4−/− mice) at the expected Mendelian ratio among the offspring mice derived from the breeding of Tg(sGPX4)/Gpx4−/− mice and Gpx4+/− mice. In contrast, no viable Gpx4−/− mice that carried the lGPX4 transgene (Tg(lGPX4)/Gpx4−/− mice) were found among a large number of offspring mice generated from the breeding of Tg(lGPX4)/Gpx4−/− mice and Gpx4+/− mice. This is the first direct evidence showing that the sGpx4 protein is essential for embryonic development whereas lGpx4 protein is dispensable.

Previous studies indicate that Gpx4 plays an import role in protecting mitochondria from oxidative damage and regulating mitochondrial apoptosis (8). Because the sGpx4 protein lacks the mitochondrial targeting signal peptide, it was assumed that the mitochondrial function of Gpx4 was due to the lGpx4 protein (24). However, our data indicate that Tg(sGPX4) mice were protected against diquat-induced apoptosis. In addition, Tg(sGPX4)/Gpx4−/− mice, which have no lGpx4 protein, had levels of Gpx4 protein in their mitochondria similar to the levels in Wt mice. Furthermore, the submitochondrial localization patterns of Gpx4 protein in Tg(sGPX4)/Gpx4−/− and Wt mice were identical. Therefore, our data indicate that the sGpx4 protein is targeted to mitochondria and that the mitochondrial function of Gpx4 in somatic tissues is due to the sGpx4 protein.

How does sGpx4 protein enter mitochondria without a mitochondrial leader sequence? It has been shown that Cu,Zn-superoxide dismutase, a cytosolic antioxidant enzyme, can cross the mitochondrial outer membrane and get trapped in the intermembrane space when metallated by the copper chaperone of superoxide dismutase in that space (16). Moreover, Gpx1 is present in the mitochondria even though it does not have an obvious mitochondrial leader sequence (25). Thus, it appears that there are other mechanisms in addition to the mitochondrial leader sequence that regulate protein import into mitochondria. Because sGpx4 protein exists in mitochondria and because Gpx4 plays an important role in mitochondrial function (7–9), we recommend that the Gpx4 nomenclature should be revised to sGpx4 and Gpx4. They are more appropriate than the current terms: cytosolic Gpx4 (or nonmitochondrial Gpx4) and mitochondrial Gpx4.

We also used the Tg(sGPX4)/Gpx4−/− mice to study the respective roles of Gpx4 isoforms in male fertility. We observed that male Tg(sGPX4)/Gpx4−/− mice, which are null for lGpx4 protein, were infertile and showed profound sperm defects such as reduced sperm motility and abnormal sperm morphology. It has been shown that in the midpiece of mature sperm, Gpx4 represents at least 50% of the capsule material that embeds the helix of mitochondria (12). We found that sperm from Tg(sGPX4)/Gpx4−/− mice had no detectable Gpx4 protein. Thus, our data suggest that the lack of Gpx4 protein in the sperm from Tg(sGPX4)/Gpx4−/− mice results in sperm structural abnormality and decreased motility, thereby contributing to infertility. Recently, Schneider et al. (26) reported that the homozygous lGpx4 knock-out mice are viable, but the male knock-out mice exhibit severe structural abnormalities in the midpiece of sperm and are infertile. Thus, both studies demonstrate independently using different methods the vital role of lGpx4 protein in male fertility.

In summary, our data show that in whole animals, sGpx4 protein is the predominant isoform found in somatic tissues. The sGpx4 protein is targeted to mitochondria and is essential for embryonic survival. In contrast, the lGpx4 protein is the major isoform expressed in tests. Although lGpx4 is not required for embryonic survival, it is required for normal sperm function and male fertility.

REFERENCES

1. Imai, H., and Nakagawa, Y. (2003) Free Radic. Biol. Med. 34, 145–169
2. Brigelius-Flohe, R. (1999) Free Radic. Biol. Med. 27, 951–965
3. Knopp, E. A., Arndt, T. L., Eng, K. L., Caldwell, M., LeBoeuf, R. C., Deeb, S. S., and O’Brien, K. D. (1999) Mammm. Genome 10, 601–605
4. Yant, L. J., Ran, Q., Rao, L., Van Remmen, H., Shibatani, T., Belter, J. G., Motta, L., Richardson, A., and Prolla, T. A. (2003) Free Radic. Biol. Med. 34, 496–502
5. Thomas, J. P., Maiorino, M., Ursini, F., and Girotti, A. W. (1999) J. Biol. Chem. 265, 454–461
6. Chen, L., Na, R., Gu, M., Richardson, A., and Ran, Q. (2008) J. Neurochem. 107, 197–207
7. Liang, H., Van Remmen, H., Frohlich, V., Lechleiter, J., Richardson, A., and Ran, Q. (2007) Biochem. Biophys. Res. Commun. 356, 893–898
8. Ran, Q., Liang, H., Gu, M., Qi, W., Walter, C. A., Roberts, L. J., 2nd, Herman, B., Richardson, A., and Van Remmen, H. (2004) J. Biol. Chem. 279, 55137–55146
9. Seiler, A., Schneider, M., Förster, H., Roth, S., Wirth, E. K., Culumcse, C., Plesnila, N., Kremmer, E., Rädmark, O., Wurst, W., Bornkamm, G. W., Schweizer, U., and Conrad, M. (2008) Cell Metab. 8, 237–248
10. Imai, H., Narashima, K., Ariai, M., Sakamoto, H., Chiba, N., and Nakagawa, Y. (1998) J. Biol. Chem. 273, 1990–1997
11. Flohé, L. (2007) Biol. Chem. 388, 987–995
12. Ursini, F., Heim, S., Kiess, M., Maiorino, M., Roveri, A., Wissing, J., and Flohé, L. (1999) Science 285, 1393–1396
13. Ariai, M., Imai, H., Sumi, D., Imanaka, T., Takano, T., Chiba, N., and Nakagawa, Y. (1996) Biochem. Biophys. Res. Commun. 227, 433–439
14. Conrad, M., Moreno, S. G., Sinowatz, F., Ursini, F., Kölble, S., Roveri, A., Brielmeier, M., Wurst, W., Maiorino, M., and Bornkamm, G. W. (2005) Mol. Cell. Biol. 25, 7637–7644
15. Liang, H., Ran, Q., Jang, Y. C., Holstein, T., Musatov, A., Song, W., Van Remmen, H., and Richardson, A. (2009) Free Radic. Biol. Med. 47, 312–320
16. Okado-Matsuno, A., and Fridovich, I. (2001) J. Biol. Chem. 276, 38388–38393
17. Tanaka, M., Nakae, S., Terry, R. D., Mokhtari, G. K., Gunawan, F., Balsam, L. B., Kaneda, H., Kofidis, T., Tsao, P. S., and Robbins, R. C. (1999) Blood 104, 3789–3796
18. Ariai, F., Karama, F., and Baba, F. (2008) Res. Vet. Sci. 89, 95–99
19. Pushpa-Rekha, T. R., Burdsall, A. L., Oleksa, L. M., Chisolm, G. M., and Driscoll, D. M. (1995) J. Biol. Chem. 270, 26993–26999
20. Nomura, K., Imai, H., Kouruma, T., Ariai, M., and Nakagawa, Y. (1999) J. Biol. Chem. 274, 29294–29302
21. Imai, H., Hirao, F., Sakamoto, T., Sekine, K., Mizukura, Y., Saito, M., Kitamoto, T., Hayasaka, M., Hanaoka, K., and Nakagawa, Y. (2003) Biochem. Biophys. Res. Commun. 305, 278–286
22. Schneider, M., Vogt Weisenhorn, D. M., Seiler, A., Bornkamm, G. W., Brielmeier, M., and Conrad, M. (2006) Gene Expr. Patterns. 6, 489–494
23. Borchert, A., Wang, C. C., Ufer, C., Schiebel, H., Savaskan, N. E., and Kuhn, H. (2006) J. Biol. Chem. 281, 19655–19664
24. Arai, M., Imai, H., Koumura, T., Yoshida, M., Emoto, K., Úmeda, M., Chiba, N., and Nakagawa, Y. (1999) J. Biol. Chem. 274, 4924–4933
25. Legault, J., Carrier, C., Petrov, P., Renard, P., Remacle, J., and Mirault, M. E. (2000) Biochem. Biophys. Res. Commun. 272, 416–422
26. Schneider, M., Förster, H., Boersma, A., Seiler, A., Wehnes, H., Sinowatz, F., Neumuller, C., Deutsch, M. J., Walch, A., de Angelis, M. H., Wurst, W., Ursini, F., Roveri, A., Maleszewski, M., Maiorino, M., and Conrad, M. (2009) FASEB J. 23, 3233–3242