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Murine phospholipid hydroperoxide glutathione peroxidase: cDNA sequence, tissue expression, and mapping

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Abstract. Phospholipid hydroperoxide glutathione peroxidase (PHGPx), also known as glutathione peroxidase 4 (GPX4), is a 19-kDa, monomeric enzyme that protects cells from lipid peroxide-mediated damage by catalyzing the reduction of lipid peroxides. PHGPx is synthesized in two forms, as a 194-amino acid peptide that predominates in gonadal tissue and localizes to mitochondria, and as a 170-amino acid protein that predominates in most somatic tissues and localizes to the cytoplasm. With the rapid amplification of cDNA ends (RACE) procedure, an 876-bp PHGPx cDNA was amplified from mouse testis, and a 767-bp PHGPx cDNA was amplified from mouse heart. The cDNA sequences were identical except that the testis cDNA contained an additional 109 bp at its 5′ end. With a partial cDNA with complete homology to both the testis and myocardial PHGPx cDNAs, the murine tissue distribution of PHGPx mRNA expression was determined by Northern blotting. Highest level of PHGPx expression was found in the testis, followed by the kidney, heart and skeletal muscle, liver, brain, lung, and spleen. Northern blotting performed with a cDNA specific for the longer PHGPx transcript demonstrated that this longer PHGPx transcript was present only in the testis. A 1.4-kb PHGPx genomic fragment was amplified from murine kidney DNA and used to map the PHGPx gene by linkage analysis of restriction fragment length variants (RFLVs). The murine PHGPx gene (*Gpx4*) was mapped to a region of murine Chromosome (Chr) 10, located 43 cM from the centromere, that is syntenic with the human locus, which is located at the terminus of the short arm of human Chr 19. This information may be valuable in characterizing the role of PHGPx in modulating susceptibility to lipid peroxide-mediated injury in inbred murine strains and for targeted disruption of the gene.

Introduction

Recent studies demonstrating that lipid peroxides may influence a wide variety of cellular functions (Liao et al. 1993, 1994) have geneted growing interest in identifying and characterizing cellular enzymes that reduce lipid peroxides. Candidate enzymes that may be effective in reducing lipid peroxides include the four structurally distinct glutathione peroxidases, which consist of the three tetrameric glutathione peroxidases (GPxs 1–3) and of the monomeric phospholipid hydroperoxide glutathione peroxidase (PHGPx). Of these, PHGPx holds particular interest as a cellular defense against oxidant-mediated damage because it reduces hydroperoxides of phospholipids (Ursini et al. 1982, 1985) and cholesterol and cholesterol esters (Thomas et al. 1990) and because recent in vitro studies have strongly suggested a role for PHGPx in protection of cells against the cytotoxic effects of lipid peroxides (Imai et al. 1996; Yagi et al. 1996; Schnurr et al. 1996).

Mature PHGPx is present predominantly in mitochondria, the nucleus, and the cytoplasm as a 170-amino acid protein. However, there is evidence that the protein in mitochondrial and nuclear sites is first synthesized as a 197-amino acid precursor, from which the N-terminal 27 amino acids are cleaved after directing the protein to mitochondrial and nuclear membranes (Pushpa Rekha et al. 1995). Mitochondrial localization of the protein predominates in the testis, in which PHGPx expression is regulated by gonadotropins (Roveri et al. 1992), while cytosolic localization of the protein predominates in somatic tissues, in which little is known about the factors that regulate PHGPx expression (Pushpa Rekha et al. 1995). Recently, cDNAs for porcine (Brigelius-Flohe et al. 1994), rat (Pushpa Rekha et al. 1995), and human (Esworthy et al. 1994) PHGPx have been cloned and the porcine (Brigelius-Flohe et al. 1994) and human (Kelner and Montoya 1998) gene structures determined.

Knowledge of the murine cDNA sequence is needed for studies aimed at determining the functional importance of the gene product through either overexpression or targeted disruption of the gene. Thus, this study was undertaken to determine the cDNA sequence and chromosomal location of PHGPx in mice. The murine PHGPx sequence was compared with those of other mammalian species, and the relative levels of PHGPx mRNA expression were determined for several murine tissues. The chromosomal mapping shows the PHGPx gene (gene symbol *Gpx4*) to be located on Chr 10. This information may be valuable in characterizing the role of PHGPx in modulating susceptibility to lipid peroxide-mediated injury in inbred murine strains.

Materials and methods

Rapid amplification of cDNA ends (RACE). BALB/c Marathon-Ready® heart and testis cDNAs, which contain 5′ and 3′ adaptors, were purchased from Clonetech Laboratories (Palo Alto, Calif.). The 3′ and 5′ ends of heart and testis BALB/c cDNA were amplified according to the RACE protocol by use of the provided Adaptor Primer 1 (AP1) and internal, PHGPx-specific primers, GSP1 (5′-CGGCTGCAAACTCCTTGATTTC-3′, corresponding to bases 403–424 of the rat cDNA sequence (Pushpa Rekha et al. 1995)) and GSP2 (5′-GTGATAGAGAAGGACCTGCC-3′), corresponding to bases 622–642 of the rat cDNA sequence (Pushpa Rekha et al. 1995). Expand® Long Template PCR, a high-fidelity

The sequences reported in this paper have been submitted to GenBank (Accession numbers AF044056, AF045768, and AF045769) and the murine PHPGx gene symbol and chromosomal map position have been approved by the Mouse Genome Database Nomenclature Committee (*Gpx4*, MGD Accession ID: MGD-MRK-28058).

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Polymerase Mixture System (Boehringer-Mannheim, Indianapolis, Ind.), was used to minimize base substitution errors during amplification (Barnes 1994). With the AP1/GSP1 primer pair, 472-bp products were amplified from both the heart and testis cDNAs. With the AP1/GSP2 primer pair, a 534-bp product was amplified from heart cDNA, and a 643-bp product was amplified from testis cDNA. These cDNA fragments then were gel-purified with the QIAquick Gel Extraction Kit (QIAGEN), cloned into the pGEM-T Easy Vector (Promega Corp., Madison, Wis.), and transformed into *E. coli* strain DH5-α cells. All clones were sequenced by the dideoxy chain-termination method (Sanger et al. 1977) and confirmed by a PCR-based dye termination method using the *Taq-FS* polymerase (ABI Prism Dye Terminator Cycle Sequencing Kit, Perkin-Elmer, Foster City, Calif; Parker et al. 1996). Full-length heart and testis PHGPx cDNAs were generated by ligation of 5′ and 3′ clones after digestion at a common *Bsr*GI restriction site.

Northern blotting. A multiple-tissue Northern blot, containing 2 μg of poly A RNA from murine heart, brain, spleen, lung, liver, muscle, kidney, and testis, was purchased from Clonetech Laboratories. A cDNA probe, representing the first 534 bp of the murine heart PHGPx cDNA, and therefore completely homologous to both the heart and testis cDNAs, was ^{32}P-labeled using the Redi-Prime® random primer labeling system (Amersham Life Science, Arlington Heights, Ill.).

The filter was hybridized with the ^{32}P-labeled PHGPx probe for 2 h at 65°C, washed, and then exposed to radiographic film (Kodak) at −70°C for 4 h. Equivalent loading of polyA RNA was confirmed by hybridization of the blot with a ^{32}P-labeled probe corresponding to bp 405–434 of the human β-actin cDNA. This hybridization confirmed equivalent polyA RNA loading for each tissue except skeletal muscle, for which the polyA RNA appeared to be partially degraded. An autoradiograph of the PHGPx blot then was digitized with a Hewlett Packard ScanJet IIcx scanner, and densitometry was performed with the ImageQuant program (Molecular Dynamics, Sunnyvale, CA).

To determine which tissues expressed the longer PHGPx transcript, a cDNA fragment containing the first 111 bases of the testis PHGPx cDNA was amplified from the testis cDNA by PCR, using AP-2 and a primer (5′-CGCACAGCAGTGCTGGCTTAAG-3′) complementary to bases 90–111 of the testis cDNA. This PCR product was gel-purified and ^{32}P-labeled as described above, then used to probe the murine tissue Northern blot.

Genomic fragment amplification. A 1.4-kb murine genomic PHGPx fragment was generated by PCR amplification of genomic DNA isolated from C57BL/6J kidney, with primers (forward: 5′-CATTGGTCGG-CTGCGTGAGG-3′; reverse: 5′-CGGCTGCAAACTCCTTGATTTC-3′) designed to recognize regions that are conserved across PHGPx cDNAs from rat (Pushpa Rekha et al. 1995), pig (Brigelius-Flohe et al. 1994), and human (Esworthy et al. 1994). PCR was performed with the high-fidelity *Taq* polymerase system (Expand®, Boehringer-Mannheim) to minimize PCR-related base substitutions (Barnes 1994). The PHGPx genomic fragment sequence (GenBank Accession # AF044056) was determined with the dideoxy chain termination method (Sanger et al. 1977) and confirmed with a PCR-based dye termination method using the *Taq-FS* polymerase (ABI Prism Dye Terminator Cycle Sequencing Kit, Perkin-Elmer; Parker et al. 1996).

Chromosomal mapping. The chromosomal location of PHGPx was determined by linkage analysis of restriction fragment length variants (RFLVs) in genomic DNA isolated from a panel of 94 F_1 progeny of an interspecific cross (C57BL/6JEi × SPRET/Ei; Rowe et al. 1994). RFLVs were determined by DNA hybridization with the 1.4-kb PHGPx genomic fragment after the digestion of genomic DNA from parental and F_1 mice with *Bam*HI, *Bgl*II, *Eco*RI, *Hin*dIII, *Msp*I, *Pst*I, *Pvu*II, *Sst*I, *Taq*I, and *Xba*I. Hybridizations were performed in 0.5 M sodium phosphate, pH 7.0, 7% sodium dodecyl sulfate (SDS), 1% bovine serum albumin, and 1 mM EDTA at 65°C for 20 h as described (LeBoeuf et al. 1994). Filters were washed twice for 20 min at 55°C in 75 mM NaCl, 7.5 mM sodium citrate, at pH 7.0, containing 0.1% SDS. Several RFLVs were revealed (data not shown). *Pvu*II was chosen to type the backcross mice, since hybridizations with *Pvu*II-digested parental DNA revealed a single band of 4.5 kb for C57BL/6JEi DNA and a single band of 3.8 kb for SPRET/Ei DNA. The variant 4.5- and 3.8-kb bands were scored in 94 backcross progeny.

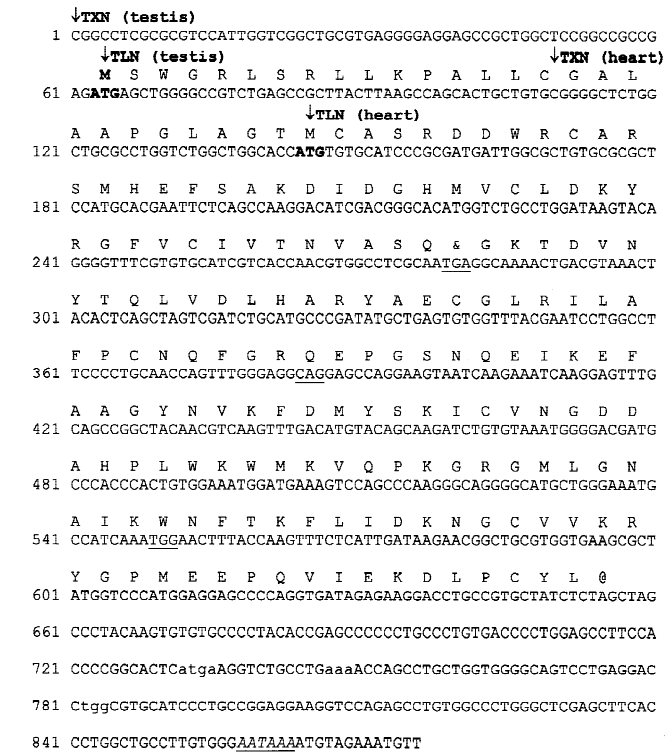

Fig. 1. Murine PHGPx cDNA sequences. Shown are the sequences for both the murine testis cDNA and murine heart PHGPx cDNA. The putative transcription start sites for both testis and heart cDNAs are indicated (↓TXN) above the sequence, as are the start codons for translation of the testis and heart PHGPx proteins (↓TLN). Codons for the three amino acids of the PHGPx catalytic site, that is, selenocysteine, glutamine, and tryptophan, are underlined. The nucleotide sequences in the 3′ UTR thought to be important for cotranslational insertion of selenocysteine at the opal codon are shown in lower-case letters. The potential polyadenylation signal is both italicized and underlined.

Results

BALB/C testis and myocardial PHGPx cDNA sequences and deduced amino acid sequences. An 876-bp cDNA was cloned from murine testis (Fig. 1; GenBank Accession # AF045768), and a 767-bp cDNA was cloned from murine heart (Fig. 1; GenBank Accession # AF045769). Sequencing revealed that the 767-bp murine heart cDNA was identical to bp 109–876 of the murine testis cDNA. That the transcript for the shorter form of PHGPx was cloned from heart is not surprising, since the 170-amino acid protein is the predominant form expressed in somatic tissues (Esworthy et al. 1994; Pushpa Rekha et al. 1995).

Mammalian PHGPx amino acid homologies. Comparison of the deduced amino acid sequences for human, porcine, rat, and murine PHGPx demonstrates a very high degree of homology (Fig. 2). The conservation of the PHGPx amino acid sequences between these species [mouse vs. rat (99%), vs. pig (96%), and vs. human (94%)] is consistent with the hypothesis that PHGPx plays a crucial role in cellular antioxidant defenses.

Tissue distribution of murine PHGPx expression. To determine the relative levels of PHGPx expression in murine tissues, we used a 534-bp, ^{32}P-labeled cDNA that was completely homologous to both heart and testis PHGPx cDNAs to probe a blot with 2μg/lane of polyA RNA from murine heart, brain, spleen, lung, liver, skel-

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cons. MSWGRLSRLLKPALLCGALAAPGLAGTMCASRDDWRCARSMHEFSAKDIDGHMVNLDKYR
human --L---C-----------------------------------------------------
pig   --FS--F----------T--V-----------------------------------------
rat   --------------------V-------------------------------------C-----
mouse ---------------------------------------------------------C-----

cons. GFVCIVTNVASQ&GKTDVNYTQLVDLHARYAECGLRILAFPCNQFGRQEPGSNQEIKEFA
human ---------------E-------------------------------------E------
pig   -Y--------------E-----------------------------------DA------
rat   -C-----------------------------------------------------------
mouse -----------------------------------------------------------

cons. AGYNVKFDMYSKICVNGDDAHPLWKWMKVQPKGRGMLGNAIKWNFTKFLIDKNGCVVKRY
human ---------F-----------------I----K-I-------------------------
pig   ---------F-----------------------------------------------------
rat   -----R-----------------------------------------------------------
mouse -----------------------------------------------------------

cons. GPMEEPQVIEKDLPCYL@
human ------L-------H-F-
pig   ------------------
rat   ------------------
mouse   ------------------
```

Fig. 2. Mammalian PHGPx protein homologies. The consensus amino acid sequence (= cons.) for 4 mammalian PGHPx sequences is shown on the top line, with single-letter amino acid codes.

etal muscle, kidney, and testis (Fig. 3A). The PHGPx transcript from murine testis appeared to be slightly larger than those of the other tissues (Fig. 3A), consistent with the observations of others for rat testis (Pushpa Rekha et al. 1995) and with the findings of the present study that the testis PHGPx cDNA is 109 bp longer than that isolated from heart. The highest levels of PHGPx expression were seen in the testis, kidney, liver, and heart, while lower levels were detected in the lung and brain (Fig. 3B). The spleen had the lowest level of PHGPx expression of all murine tissues examined (Fig. 3B).

Tissue distribution of the longer PHGPx transcript. Using the cDNA probe corresponding to bases 1–111 of the testis cDNA in Northern blot analysis, we detected the longer PHGPx transcript only in murine testis (Fig. 3C). These findings are largely consistent with those published for the rat, in which, with the more sensitive solution hybridization method, the longer PHGPx transcript was predominantly expressed in the testis and was detected in the intestine, cerebral cortex, and kidney only after long autoradiographic exposure (Pushpa Rekha et al. 1995).

Partial structure of the murine PHGPx gene. A 1.4-kb fragment of the PHGPx gene (GenBank Accession # AF044056) was amplified by PCR from murine strain C57BL/6 genomic DNA. This fragment comprises the 3′ end of exon 1, complete exons 2 and 3, the 5′ end of exon 4, and the complete introns 1–3. Comparison with the porcine and human gene structures (Fig. 4) demonstrates similar intron sizes and intron/exon junctions.

Chromosomal map location of the murine PHGPx gene (Gpx4). Comparison with genetic markers typed in the backcross mice showed co-segregation of PHGPx with markers on central Chr 10 in a cluster of other loci located 43 centimorgans (cM) from the centromere (Fig. 5A). Included among the loci mapped to this region of mouse Chr 10 are the genes for lamin B2 (*Lmnb2*), megakaryocyte-associated tyrosine kinase (*Matk*), and proprotein convertase subtilisin/kexin type 4 (*Pcsk4*). In other crosses, however, these loci map over several cM, suggesting that there is some crossover suppression in this region in the C57BL/6JEi × SPRET/Ei backcross panel. The human homologs of these genes (LMNB2, MATK, and PCSK4) are tightly linked to the human PHGPx gene (GPX4) at the terminus of the short arm of human Chr 19 [Mouse Genome Database 1995 and Fig. 5B], indicating the existence of syntenic homology in this region. Taken together with the single band polymorphism, this suggests strongly that the murine Chr 10

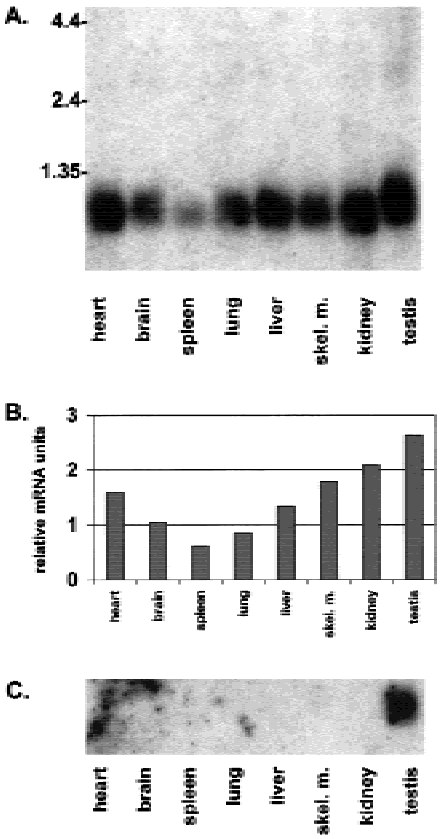

Fig. 3. Relative PHGPx mRNA expression in murine tissues. **(A)** An autoradiograph demonstrates PHGPx mRNA expression in the mouse tissues indicated (2 μg polyA RNA/lane). Highest expression of the gene is seen in the testis, kidney, liver, and heart. Also, the predominant testis transcript appears to be slightly larger than that found in other tissues, consistent with the finding that the testis cDNA (Fig. 1) is 109 bp longer than the heart cDNA (Fig. 2). **(B)** Relative expression of PHGPx mRNA in murine tissues, as determined by densitometry and normalized for splenic PHGPx mRNA, is shown graphically. **(C)** Northern blot analysis, performed with a cDNA probe that is specific for "long" PHGPx mRNA, demonstrates expression in the mouse tissues indicated (2 μg polyA RNA/lane). The "long" PHGPx mRNA is detected only in the testis.

map position represents that of the structural gene for PHGPx. A gene symbol (*Gpx4*) for the murine PHGPx gene has been assigned and the map location confirmed by the Mouse Genome Database Nomenclature Committee (MGD Accession ID: MGD-MRK-28058).

Discussion

As compared with other glutathione peroxidases, PHGPx has many unique properties that suggest that it may play a particularly important role in the reduction of intracellular lipid peroxides (Ursini et al. 1982; Pushpa Rekha et al. 1995). Lipid peroxides have been implicated in the pathogenesis of a variety of diseases, including atherosclerosis (Chait and Heinecke 1994; Itabe et al. 1994; O'Brien et al. 1996) and neurodegenerative diseases (Martin et al. 1996), for which a variety of murine models have been created (Zhang et al. 1992; Plump et al. 1992; Ishibashi et al. 1994). To facilitate study of the potential role of PHGPx in these models, this study was undertaken to determine the cDNA sequence and tissue distribution of murine PHGPx, as well as the chromosomal location of the gene.

	Exon	Size (bp)	Sequence	Intron	Size (bp)	Sequence
mouse	1	>112	AGGCACCATGgtgggctact	1	798	tgttttacagTGTGCATCCC
pig	1	>44	CGGCACCATGgtgagccatg	1	980	tgccttgcagTGCGCGTCCC
human	1	161	CGGGACCATGgtgagctagc	1	1068	tgccttgcagTGCGCGTCCC
mouse	2	95	ATAAGTACAGgtgggttcta	2	88	tctctcccagGGGTTTCGTC
pig	2	94	ACAAGTACCGgtgggtacct	2	78	ccgcctccagGGGCTACGTG
human	2	95	ACAAGTACCGgtgggcgctc	2	86	atcctcgcagGGGCTTCGTG
mouse	3	145	TGGGAGGCAGgtgtgtggct	3	123	cttcctgcagGAGCCAGGAA
pig	3	145	TGGGAGGCAGgtacgtcttg	3	124	ttctgcccagGAGCCAGGGA
human	3	145	CGGGAAGCAGgtgggctgct	3	147	gccgccacagGAGCCAGGGA
mouse	4	>18				
pig	4	152				
human	4	152				

Fig. 4. Intron/exon junctions of a murine *Gpx4* genomic fragment and comparison with the porcine and human gene structures. As compared with the corresponding regions of the porcine and human PGHPx genes, the murine PHGPx gene (*Gpx4*) fragment has similar intron and exon sizes, except that the porcine and human first introns are longer.

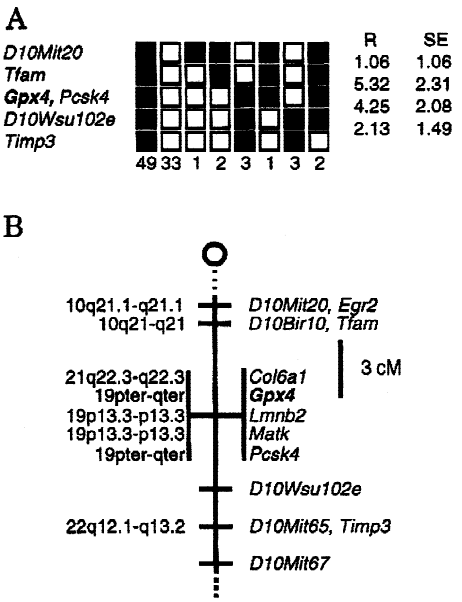

Fig. 5. Chromosome location of the mouse Gpx4 gene. **(A)** The segregation pattern of *Gpx4* and flanking markers. Loci are listed in order with the most proximal at the top. The black boxes represent the C57BL/6JEi allele, and the white boxes the SPRET/Ei allele. The number of offspring inheriting each type of chromosome is given at the bottom of each column. The percentage recombination (R) between adjacent loci is given to the right of the figure in centimorgans (cM), with the standard error (SE) for each R. Missing typings were inferred from surrounding data where assignment was unambiguous. **(B)** Linkage map of mouse Chr 10 around the *Gpx4* locus. To the left of the chromosome bar are human map positions for homologous genes. A 3-cM scale bar is shown to the right of the figure. Loci mapping to the same position are listed in alphabetical order. Missing typings were inferred from surrounding data where assignment was unambiguous. Data for mouse and human markers were collected through the Mouse Genome Database maintained by The Jackson Laboratory Mouse Genome Database (URL: http://www.informatics.jax.org).

Two different length transcripts were cloned, sequenced, and determined to have structural properties that confirm their identity as PHGPx cDNAs. Both transcripts encode for the catalytic triad of selenocysteine (testis cDNA bases 279–281; heart cDNA bases 171–173), glutamine (testis cDNA bases 384–386; heart cDNA bases 276–278), and tryptophan (testis cDNA bases 549–551; heart cDNA bases 441–443; Pushpa Rekha et al. 1995) that is essential to the function of PHGPx. For all mammalian species studied, including the mouse, all three amino acids of the catalytic triad are conserved and are present in the relative positions thought necessary to allow enzymatic reduction of peroxides.

Also, conserved within the murine PHGPx sequences are the same selenocysteine coding systems present in the porcine, human, and rat PHGPxs, as well as in other eukaryotic selenoproteins. The murine cDNAs contain an "opal" codon (testis cDNA bases 279–281; myocardial cDNA bases 171–173) that encodes for selenocysteine, but also contain a series of three short sequences in the 3′ UTR (ATGA, AAA, and TGG, indicated by small caps in Fig. 1) necessary for formation of the stable stem-loop structure used by eukaryotic cells to facilitate suppression of the opal codon and co-translational insertion of selenocysteine. These sequences in the 3′ UTR of the murine PHGPx cDNAs are identical to those of the rat PHGPx cDNA (Pushpa Rekha et al. 1995) sequence and are similar to those of other selenoproteins, including type I 5′ deiodinase and GPXs 1–3 (Brigelius-Flohe et al. 1994; Pushpa Rekha et al. 1995). The importance of these sequences may explain the unusually high degree of homology (approximately 91.5%) between the mouse PHGPx 3′ UTRs and that of the consensus mammalian PHGPx cDNA sequence.

There is a remarkably high degree of homology between the deduced amino acid sequences for human, porcine, rat, and murine PHGPx, consistent with the hypothesis that PHGPx plays a crucial role in cellular antioxidant defenses. In contrast, while the homology between the amino acid sequences of murine PHGPx and other mammalian PHGPxs is nearly 99%, there is limited resemblance between murine PHGPx and the consensus mammalian GPx amino acid sequence (Brigelius-Flohe et al. 1994; Pushpa Rekha et al. 1995). This disparity between PHGPx and GPX amino acid sequences highlights the distinct nature of PHGPx as compared with GPx and suggests that the cellular roles of these enzymes are fundamentally different. For example, it has been proposed that the primary role of GPx is to scavenge lipid peroxides in the cytosol, while PHGPx instead interacts with cellular and lipoprotein membranes to prevent lipid peroxide-mediated damage to these structures (Ursini et al. 1982; Brigelius-Flohe et al. 1994).

The longer PHGPx protein synthesized in the testis contains a putative mitochondrial targeting signal (Pushpa Rekha et al. 1995; Kelner and Montoya 1998) that directs this protein to the mitochondrial inner membrane. Expression of this protein is upregulated by gonadotropins (Roveri et al. 1992). It is not clear why high levels of PHGPx are required in the mitochondria of testicular cells. However, mitochondrial DNA, owing to its lack of histones and proximity to oxidant-generating mitochondrial enzymes, is more susceptible to oxidative damage than is nuclear DNA. Oxidant-mediated mutations in germ-line cell DNA would have particularly grave consequences. The fact that PHGPx is expressed at particularly high levels in the testis suggests the longer, mitochondrial form of the enzyme may play a critical role in prevention of oxidative damage to DNA. In contrast, the primary role of the shorter, cytosolic form of PHGPx that predominates in somatic tissue may be to prevent oxidative damage to membranes.

The relative levels of PHGPx expression were determined for a variety of murine tissues. Of note, the longer transcript was detected by Northern blotting only in murine testis and not in any of the somatic tissues studied. These findings are largely consistent with previously reported data in the rat, in which the longest rat PHGPx transcript was found to be present at high levels only in the testis, and very low levels of the longer transcript were detected in the intestine, cerebral cortex, and kidney (Pushpa Rekha et al.

1995). The reason for high level of expression of the longer PHGPx transcript in testis is not known.

Finally, this study demonstrates close linkage of PHGPx and central mouse Chr 10 markers. This mapping information will contribute to studies of the expression and function of PHGPx in the mouse.

Taken as a whole, these findings should prove to be of value to studies designed to assess the role of PHGPx in murine models of a variety of diseases, such as atherosclerosis, in which lipid peroxides have been implicated.

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Note added in proof. During the copyediting phase, the authors became aware of an article describing findings similar to a portion of the findings reported in this paper.

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