Rat Phospholipid-hydroperoxide Glutathione Peroxidase

cDNA Cloning and Identification of Multiple Transcription and Translation Start Sites*

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Phospholipid-hydroperoxide glutathione peroxidase (PhGPx) is a selenoenzyme that reduces hydroperoxides of phospholipid, cholesterol, and cholesteryl ester. Previous studies suggested that both the mitochondrial and nonmitochondrial forms of PhGPx are ~170 amino acids long. In this study, we isolated a full-length cDNA clone encoding rat testis PhGPx. Based on sequence analysis, the cDNA encodes a protein of 197 amino acids, with translation initiating at AUG61. The additional 27 amino acids at the N terminus contain the features of a mitochondrial targeting sequence. In vitro translation of the full-length PhGPx mRNA initiated predominantly at AUG61. However, translation initiated at AUG42 when AUG61 was deleted. An RNase protection assay was used to map the 5'-ends of PhGPx mRNAs in rat tissues. We identified two major windows of transcription initiation that are tissue-specific. Rat testis predominantly expresses larger transcripts that encode the 197-amino acid protein containing the potential mitochondrial targeting signal. The predominant smaller transcripts in somatic tissues lack AUG61 and encode a 170-amino acid protein, which may represent the nonmitochondrial forms of PhGPx. Our results suggest that the use of alternative transcription and translation start sites determines the subcellular localization of PhGPx in different tissues.

PhGPx1 is a unique selenoenzyme that reduces phospholipid, cholesterol, and cholesteryl ester hydroperoxides at the expense of glutathione (1-3). PhGPx can also reduce 7β-hydroxycholesterol, which our laboratory has shown to be the principal cytotoxic in oxidized lipoproteins (4). These lipid hydroperoxides are resistant to the action of the classical glutathione peroxidase, which reduces hydrogen peroxide and free fatty acid hydroperoxides (5). It has been proposed that while glutathione peroxidase is important in removing cytosolic hydroperoxides, PhGPx catalyzes the reductive inactivation of lipid hydroperoxides in membranes and lipoproteins and thus protects cellular membranes against oxidative damage (5). PhGPx has been purified to homogeneity from pig heart, liver, and brain (6, 7), rat testis cytosol and mitochondria (8), and human liver (9). Biochemical analyses of the purified protein have shown that PhGPx is a 170-amino acid protein of ~20 kDa (1, 10). cDNA clones that encode PhGPx have been isolated from pig heart and blastocyst and from human testis (10-12). Although structurally and functionally distinct, PhGPx and glutathione peroxidase share 40% homology at the amino acid level. Several of the active-site residues of glutathione peroxidase are conserved in PhGPx, which suggests that the two enzymes have similar mechanisms of action (10, 11, 13). Like other selenoproteins, the selenocysteine (Sec) in PhGPx is encoded by a UGA codon, which normally functions as a translation stop codon.

PhGPx is widely expressed, and its enzymatic activity has been detected in all rat tissues examined (14) as well as in several human tumor cell lines (15). The highest levels of activity are present in rat testis, where PhGPx expression is regulated by gonadotropins in maturing spermatogenic cells (16). PhGPx has been detected in cytoplasm, mitochondria, and plasma and nuclear membranes, but the structural basis for this subcellular localization has not been determined. Enzymological and immunochemical data suggest that PhGPx is not an integral membrane protein (1, 8). The cytosolic and membrane-associated forms of the enzyme from rat testis appear to be identical based on their molecular masses, cross-reactivity with antisera, and protein fragmentation pattern (8). However, the molecular mass of pig PhGPx, predicted from the nucleotide sequence, differed from that determined by laser desorption spectroscopy (10). It has been suggested that this difference in the molecular mass may be due to a post-translational modification that is necessary for the association of the protein with membranes (10). Recently, it was demonstrated in rat testis mitochondria that PhGPx is localized in the intermembrane space, possibly at the contact sites of the two membranes (17). Since PhGPx is a nuclear encoded gene product, this suggests that the protein may be synthesized as a larger precursor containing a mitochondrial targeting signal, which may be cleaved after import into mitochondria.

In this study, we isolated a full-length cDNA clone encoding rat PhGPx and identified two windows of transcription start sites that are tissue-specific. Our results suggest that the predominant full-length transcripts in testis direct the synthesis of a 197-amino acid protein containing a potential mitochondrial targeting signal. Somatic tissues primarily express shorter transcripts that encode a 170-amino acid protein,
which may represent the cytosolic and membrane-associated forms of the protein.

**EXPERIMENTAL PROCEDURES**

Plasmids—Plasmid RP-1 contains the full-length rat PhGPx cDNA (nucleotides 1–280) under the control of the vector DNA (Invitrogen). Plasmid RP-2 is a 5′-deletion mutant of rat PhGPx (nucleotides 131–871). Plasmids CRP-1 and CRP-2 are mutant plasmids in which Sec23 was changed to Cys in RP-1 and RP-2, respectively.

Oligonucleotides—Oligonucleotides were purchased from Genosys Biotechnologies, Inc. (The Woodlands, TX). The nucleotide positions refer to the nomenclature for the rat PhGPx cDNA (see Fig. 1). The degenerate sequences for oligonucleotides LO-1 and LO-2 were designed based on the published pig PhGPx protein sequence (13). The underlined regions indicate differences from the wild-type rat PhGPx sequence. Oligonucleotides LO-1, LO-2, PHG-1, J3, and J-5 were used for cDNA synthesis and PCR amplification. Oligonucleotides PHG-1 and PHG-2 were used for primer extension and mutagenesis, respectively. LO-1, ATGCGATATCCCTGACAAAATGCTAT (complementary to nucleotides 628–654); J3-S, GACCTGATGTCACTGATGCT (3′-primer); J-5, GACCTGATGTCACTGATGCT (3′-primer) used in primer extension analysis to map the 5′-end of rat PhGPx mRNA in rat testis. The J-5 primer was radiolabeled and used in primer extension analysis to map the 5′-end of rat PhGPx mRNA in rat testis. The J-5 primer was radiolabeled and used. The 5′-end of rat PhGPx mRNA was determined by compering the RNA sequence ladders and 32P-labeled cDNA 3′-UTR fragments.

Complementary DNA (cDNA) Cloning and Sequence Analysis of Rat PhGPx—To obtain a full-length cDNA clone that encodes rat PhGPx, cDNA was synthesized from total rat testis RNA using an oligo(dt) anchor primer. The cDNAs were amplified by PCR using a 3′-anchor primer and a degenerate oligonucleotide based on the published protein sequence of pig PhGPx (13). The PCR products were cloned into the plasmid pcDNAI/Amp, and a partial cDNA clone of 590 nucleotides was isolated. To determine the size of the full-length transcript, we performed primer extension analysis to map the 5′-end of rat PhGPx mRNA in rat testis. The results indicated that the isolated cDNA clone lacked –280 nucleotides of 5′-sequence (data not shown). The missing 5′-region was obtained using RACE technology to yield a full-length cDNA clone of 871 nucleotides. Three independent cDNA clones were analyzed by DNA sequencing to eliminate the possibility of artifacts due to misincorporation of nucleotides during PCR. The cDNA sequence and the deduced amino acid sequence of rat PhGPx are shown in Fig. 1.

Analysis of the nucleotide sequence revealed an open reading frame of 197 amino acids, with translation initiating at the AUG codon at nucleotides 61–63 (AUG<sup>61</sup>). As shown in Fig. 2, PhGPx is highly conserved across species, and rat PhGPx shares 93% and 91% identity at the amino acid level with pig and human PhGPx, respectively. Tyr<sup>123</sup>, which was shown to be phosphorylated in pig PhGPx in vivo (13), is conserved in the rat sequence. The amino acid sequence of rat PhGPx also shares –40% homology with rat glutathione peroxidase. Several of the active-site residues of glutathione peroxidase are conserved at homologous positions in rat PhGPx, including Thr<sup>32</sup>, Glu<sup>157</sup>, and the triplet Trp<sup>163</sup>-Asn<sup>164</sup>-Phe<sup>165</sup>. Amino acids conserved at homologous positions in rat PhGPx include Glu<sup>72</sup>, Ser<sup>75</sup>, Gly<sup>74</sup>, Gln<sup>108</sup>, and the triplet Trp<sup>163</sup>-Asn<sup>164</sup>-Phe<sup>165</sup>. As in other selenoproteins, Sec<sup>73</sup> in rat PhGPx is encoded by an in-frame UGA codon (T<sup>C</sup>T<sup>G</sup>CA<sup>T</sup>AA<sup>A</sup>) in the cDNA sequence shown by the asterisk in Fig. 1.)

The 3′-untranslated region (UTR) of rat PhGPx is 217 nucleotides long, and the sequence shares 80% homology with the 3′-UTRs of human and pig PhGPx, which is a high degree of conservation for a noncoding region. In the pig blastocyst, PhGPx mRNA, two polyadenylation signals were found in the 3′-UTR, indicating that the distal polyadenylation signal is utilized in this tissue (11). However, our three independent rat testis cDNA clones terminated after the proximal polyadenylation signal at nucleotides 852–857 (Fig. 1). cDNAs isolated from pig heart (13) and human testis (12) also contained only the proximal polyadenylation signal.

The 3′-UTRs of other eukaryotic selenoprotein mRNAs are necessary for the cotranslational insertion of Sec at the UGA codon, which is normally a translation stop codon (21, 22). The decoding of UGA as Sec requires a stable stem-loop structure as well as specific nucleotide sequences in the 3′-UTR (21, 22). The 3′-UTR of rat PhGPx is predicted to form a stable stem-loop structure with a high negative free energy (ΔG = –62.4 kcal) based on computer analysis with the program MulFold (data not shown). The rat (this study) and pig (10) PhGPx 3′-UTRs also contain the motifs required for Sec incorporation.
To identify the translation start site of rat PhGPx, the full-length cDNA (construct RP-1) was transcribed in vitro, and the synthetic RNA was translated in a rabbit reticulocyte lysate system. When the translation products were analyzed by SDS-polyacrylamide gel electrophoresis, only a truncated protein was detected due to premature termination of translation at UGA\textsuperscript{277}, which encodes Sec\textsuperscript{73} (Fig. 3, first lane). This is consistent with previous studies that showed that selenocysteine incorporation is inefficient in reticulocyte lysate (21). To avoid the premature termination, we used site-directed mutagenesis to convert UGA\textsuperscript{277} to UGU, which encodes cysteine (construct CRP-1). We also constructed a deletion mutant of RP-1 by deleting 134 nucleotides from the 5'-end (construct RP-2) as well as the cysteine mutant of this deletion construct (construct CRP-2). These two deletion mutants lacked AUG\textsuperscript{61}, but contained AUG\textsuperscript{141}. As shown in Fig. 3, in vitro translation of the CRP-1 RNA produced predominantly a protein of 24 kDa (second lane), whereas a 21-kDa protein was obtained when the CRP-2 deletion mutant was translated (fourth lane). We also observed a minor protein of 21 kDa in the CRP-1 translation assays, which may represent a low level of initiation at AUG\textsuperscript{141} in the full-length transcript. The 3-kDa size difference between the CRP-1 and CRP-2 translation products is equivalent to the predicted molecular mass of amino acids 1–27. These results suggest that translation initiates predominantly at AUG\textsuperscript{61} in the full-length PhGPx mRNA and that AUG\textsuperscript{141} can function as an efficient translation start site in the absence of AUG\textsuperscript{61}. Previous studies in other systems have shown that the choice of translation start sites can be influenced by the secondary structure of the 5'-UTR or by capping of the mRNA (23, 24). The sequence upstream of AUG\textsuperscript{141} is highly GC-rich (–70%) and has the potential to form a stable secondary structure \(\Delta G = -50.6 \text{ kcal}\) based on computer analysis with the program MulFold. However, we found that initiation of translation at AUG\textsuperscript{61} in vitro was not affected by heating of the RNA (65 °C, 10 min) prior to translation. There was also no difference in the pattern of translation initiation when capped or uncapped RNAs were translated for 30 or 60 min (data not shown).

Function of the N-terminal 27-Amino Acid Sequence—The 27 amino acids at the N terminus of PhGPx are highly conserved across species (Fig. 2), which suggests a functional role for this sequence. Because PhGPx has been shown to be associated with both plasma and nuclear membranes (16), the N-terminal sequence may represent a signal peptide that is cotranslationally cleaved. To test this hypothesis, we performed in vitro translation experiments in the presence of canine pancreatic microsomal membranes. The microsomal membranes efficiently cleaved the signal peptide of a control protein, \(\beta\)-lactamase. However, the full-length 24-kDa PhGPx translation product was not cleaved to a shorter form when translation was performed in the presence of microsomal membranes (data not shown), indicating that the N-terminal 27-amino acid sequence of PhGPx may not represent a signal peptide.

PhGPx has also been localized to the intermembrane space in mitochondria isolated from rat testis (17). Most nuclear-coded mitochondrial proteins are synthesized as larger precursors containing N-terminal presequences that target the protein for mitochondrial import. In the case of proteins localized to the intermembrane space, a bipartite targeting signal is required (25). After cleavage by a matrix protease to remove the N-terminal basic domain, the remaining hydrophobic sequence directs the protein to the intermembrane space, where cleavage by a membrane-associated peptidase yields the mature protein. As shown in Fig. 4, the N-terminal sequence of PhGPx has the features of such a mitochondrial targeting signal. The 27-amino acid length conforms to the typical size of leader sequences, which are 10–70 amino acids long. The PhGPx sequence is leucine-rich (7/27 amino acids), contains three hydroxyl amino acids, and lacks acidic residues. Three basic amino acids in the N-terminal portion of the sequence are
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Multiple Transcription Start Sites—Our results suggest that translation of the full-length PhGPx mRNA initiates at AUG to produce a precursor protein containing a potential mitochondrial targeting sequence. The fact that PhGPx has also been detected in the cytosol and in nuclear and plasma membranes suggests that some forms of the protein may be synthesized without the N-terminal 27 amino acids. This could be achieved in vivo by the generation of different mRNA species that encode the cytosolic and mitochondrial forms of the protein. To test this hypothesis, we performed Northern blot analysis of total RNA isolated from various rat tissues. As shown in Fig. 5, PhGPx mRNA was detected as a single species of similar size in all tissues examined, including intestine, liver, spleen, kidney, lung, heart, cerebellum, cerebral cortex, and testis. Since small differences in mRNA size may not be detected by this method, we also developed a RNase protection assay to map the 5' ends of the PhGPx transcripts. A 32P-labeled antisense RNA probe, which was complementary to nucleotides 1-192 of the full-length rat PhGPx cDNA, was hybridized to total RNA from rat tissues. After digestion with RNases A and T1, the protected fragments were separated on a sequencing gel.

As shown in Fig. 6A, multiple protected bands were detected in all rat tissues, suggesting that transcription of the PhGPx gene initiates at multiple sites in vivo. Based on the sizes of the protected bands, we identified two major windows of transcription initiation, which differed between testis and somatic tissues (Fig. 6B). In testis, the major window of transcription initiation lies between nucleotides 1 and 27, which is upstream of AUG. These transcripts would encode a 197-amino acid protein that contains the putative mitochondrial targeting signal. A second, minor window of transcription initiation between nucleotides 87 and 102 was also detected in this tissue. Translation of these shorter mRNAs would initiate at AUG to produce a 170-amino acid protein. In addition, two to three protected bands that mapped to nucleotides 134–138 were consistently observed in testis RNA (Fig. 6A, asterisk). It is not known whether these transcripts are translated in vivo since they contain a very short 5'-UTR of only three to seven nucleotides. The pattern was reversed in somatic tissues. In kidney, spleen, lung, cerebral cortex, intestine, liver, and heart, transcription initiated predominantly at a second window between nucleotides 71 and 91 (Fig. 6A). A minor band corresponding to initiation at the first window of transcription was also detected in intestine (Fig. 6A, arrow) and in kidney and cerebral cortex after long exposure of the autoradiogram (data not shown).

Several experiments were performed to confirm that the
different protected bands corresponded to true transcription start sites. Multiple RNA samples isolated from several animals and processed separately gave identical results, which suggests that the shorter transcripts were not generated by RNase degradation. RNase protection assays were performed over a wide range of temperatures (45–55 °C) and RNase digestion conditions to eliminate the possibility of nonspecific hybridization to other mRNAs. In addition, the CRP-1 and CRP-2 synthetic RNAs generated discrete protected fragments of the appropriate sizes (Fig. 6A), which suggests that the shorter transcripts in the somatic tissues were not generated by nonspecific cleavage by RNases A and T1. The presence of multiple transcription start sites was also confirmed by an independent method using RACE technology to map the 5'-ends of the mRNAs (data not shown).

**DISCUSSION**

We have demonstrated that multiple transcription start sites generate two populations of PhGPx mRNAs that have different translation start sites. Mapping of the transcription start sites localized two major windows of transcription initiation, one upstream of AUG$^{61}$, which was predominantly used in testis, and another located between AUG$^{61}$ and AUG$^{341}$, which was used primarily in somatic tissues. The presence of multiple transcription start sites is characteristic of housekeeping genes that lack a TATA box. PhGPx has been shown to be a single copy gene in humans (28). Sequence analysis of a pig genomic clone revealed that the PhGPx promoter lacks a TATA box, is GC-rich, and contains a potential Sp1-binding site (10). These features are similar to those of the promoter of the rat aspartate aminotransferase gene, which also contains multiple transcription start sites (29). The genomic clone for rat PhGPx has not been isolated, but the high conservation of the pig, human, and rat cDNAs suggests that the structure of the gene may be conserved across species. Thus, it is likely that the rat PhGPx promoter also lacks a TATA box.

The predominant form of PhGPx mRNA in rat testis was ~100 nucleotides longer at the 5'-terminus than the mRNAs from somatic tissues. Consistent with our results is the finding that the 5'-sequence of the pig parathyroid gland cDNA (10) starts from a region that corresponds to the transcription window downstream of AUG$^{61}$, while human testis (12) and pig blastocyst (11) cDNAs start upstream of AUG$^{61}$. Although these 5'-sequences were derived from the longest cDNA clones that were obtained, it is not known whether they represent the major form of the mRNA in these tissues. In an analogous system, the rat farnesyl-pyrophosphate synthetase gene promoter contains testis-specific transcription start sites that are located 25–100 nucleotides upstream of the somatic start sites (30). The somatic start sites are clustered into two groups that are preceded by TATA boxes. In contrast, the testis-specific start sites were spread over a region of 90 nucleotides with no obvious initiation sequence. Thus, the somatic and testis sites were apparently controlled by overlapping promoters with different properties. Testis-specific transcription start sites have also been detected in genes coding angiotensin-converting enzyme (31), proenkephalin (32), cytochrome c (33), and α-tubulin (34). As in the case of PhGPx, these proteins are expressed in other organs.

Based on sequence analysis, the rat testis PhGPx cDNA
contains two potential translation start sites, AUG\textsuperscript{61} and AUG\textsuperscript{141}, which were also present in the PhGPx cDNA clones isolated from human testis and pig blastocyst (11, 12). Esworthy et al. (12) recently proposed that translation of the human testis PhGPx mRNA may initiate at the upstream AUG codon, although no functional evidence was provided. We have shown that both AUG\textsuperscript{61} and AUG\textsuperscript{141} in rat PhGPx occur in a favorable context for translation initiation and that both function as efficient translation start sites in vitro when they are the first AUG codon in the mRNA. This conforms with the ribosome scanning model for translation initiation proposed by Kosak (23). In addition to the predominant 24-kDa protein, translation of the full-length PhGPx mRNA in vitro also produced a minor protein of 21 kDa, which appeared to initiate at AUG\textsuperscript{141} based on its molecular mass. A low level of initiation at the second AUG codon in the PhGPx mRNA may be due to leaky scanning of the ribosome or to internal entry of the 40 S ribosomal subunit, as has been proposed for other mRNAs (35). Studies using artificial bicistronic mRNAs showed that translation initiation at the 5’-proximal or distal AUG codon was dependent on the cell type and the concentration of eukaryotic initiation factor 4F (36). Alternatively, the 21-kDa protein may dependent on the cell type and the concentration of eukaryotic elongation factor 2.

The positively charged N-terminal sequence is cleaved by a mitochondrial processing peptidase after import of the protein into the matrix. The remaining hydrophobic stretch directs the export of the protein back across the inner membrane, where it is cleaved to the mature protein by a membrane-associated peptidase.

Our results suggest that PhGPx may be synthesized in rat testis as a 197-amino acid precursor protein that is cleaved to the mature form during import into the mitochondrial intermembrane space. PhGPx contains the sequence RLSRL\textsuperscript{5–10}, which is identical to the sequence in rat aldehyde dehydrogenase that is cleaved by the matrix processing peptidase (27). This sequence is also similar to a pentapeptide sequence (SRLLK) in the leader sequence of another mitochondrial protein, yeast KAD2 (37).

Taken together, our results suggest that a single PhGPx gene encodes both cytosolic and mitochondrial forms of the protein through differential transcription and translation start sites. This type of mechanism has been reported for several nuclear genes that encode cytosolic and mitochondrial proteins in yeast, Neurospora crassa, and mammals (38–45). Our model is consistent with current knowledge on the subcellular distribution of PhGPx in rat tissues. Studies by Ursini and co-workers (16) have shown that the majority of PhGPx activity is localized in the mitochondria in rat testis, whereas the predominant form of PhGPx in rat liver is cytosolic. However, the subcellular distribution of PhGPx in other tissues has not been analyzed in detail. Although PhGPx was detected in both the soluble and membrane fractions from various rat tissues, the membrane fractions included nuclear and plasma membranes as well as mitochondria (34). Our results suggest that PhGPx will primarily be nonmitochondrial in somatic tissues since the predominant transcripts in these tissues lack AUG\textsuperscript{61}.

Although the physiological significance of high level expression of PhGPx in testis mitochondria is not understood, this enzyme may play an important role in protecting mitochondrial DNA against oxidative damage. This hypothesis is supported by the fact that mitochondrial DNA is more susceptible to oxidative damage than nuclear DNA due to a lack of histones protecting the mitochondrial DNA, a lack of DNA repair enzymes in mitochondria, and the proximity of mitochondrial DNA to oxidants generated during oxidative phosphorylation (46).

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