Cloning of the mouse phospholipid hydroperoxide glutathione peroxidase gene

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Abstract 15-Lipoxygenases and phospholipid hydroperoxide glutathione peroxidases (PH-GPx) are counterparts in the metabolism of hydroperoxy lipids and a balanced regulation of both enzymes appears to be important for the cellular peroxide tone regulating the expression of redox sensitive genes. In contrast to lipoxygenases the molecular biology of PH-GPx is less well investigated. In this study we cloned the PH-GPx cDNA from a mouse fibroblast cDNA library and the PH-GPx gene from a mouse genomic library. The gene spans approximately 4 kb which includes 1 kb of 5′-flanking region and consists of seven exons and six introns. The immediate promoter region does not contain a TATA box but there are binding sites for several transcription factors which also occur in the porcine gene. Our investigations provide useful tools for future targeted gene disruption studies.

Key words: Atherogenesis; Inflammation; Lipid peroxidation; Oxidative stress; Selenium

1. Introduction

The phospholipid hydroperoxide glutathione peroxidase (PH-GPx) has been characterized as a peculiar isoform of selenium dependent glutathione peroxidases [1] which is capable of reducing ester lipid hydroperoxides such as hydroperoxy phospholipids or hydroperoxy cholesterol esters [2–4]. Like other glutathione peroxidases [5,6], the PH-GPx has been implicated in defense mechanisms protecting cells from oxidative stress and there are several lines of experimental evidence supporting this hypothesis [7–10]. On the other hand, the tissue distribution [11] and the regulation of PH-GPx expression [12,13] may point to a more specific role of PH-GPx in cell development and/or differentiation, particularly in spermatogenesis [14–16]. In addition, the enzyme may be of regulatory importance for the activity of enzymes involved in the arachidonic acid cascade [17–20]. An endogenous lipoxygenase inhibitor was recently purified from the human epidermoid carcinoma cell line A431 and was identified as PH-GPx [21].

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Abbreviations: SECIS, selenocysteine insertion sequence; PH-GPx, phospholipid hydroperoxide glutathione peroxidase; GPx, glutathione peroxidase

The nucleotide sequence of the mouse PH-GPx gene reported here has been submitted to the EMBL nucleotide sequence database with accession number AJ012104. A partial sequence of the mouse PH-GPx gene (30% of the full length gene) has be deposited before (accession number AF044056) in the DDBJ database.

More detailed investigations on the biological importance of mammalian PH-GPx have been hampered so far because of two reasons: (i) there is no specific PH-GPx inhibitor and (ii) targeted gene disruption is impossible so far, as the mouse PH-GPx gene has not been cloned. Although cDNAs for various mammalian PH-GPx isoforms have already been sequenced [22–25], the only complete PH-GPx gene sequenced so far is that from pigs [26]. In addition, a partial sequence for the human PH-GPx gene was recently reported in which the promoter region is lacking [27].

To provide the tools for future gene disruption studies we cloned the gene of the mouse PH-GPx, including 1 kb of the 5′-flanking region. The structures of the mouse, porcine and human PH-GPx genes were compared with respect to their overall structure, and conserved regulatory elements in the promoter region were identified.

2. Materials and methods

2.1. Chemicals

The chemicals used were from the following sources: trisodium citrate dihydrate, magnesium sulfate heptahydrate from Sigma (Deisenhofen, Germany); tris(hydroxymethyl)aminomethane (Tris), sodium hydroxide and sodium chloride from Merck (Darmstadt, Germany); the pGEM-T cloning vector system from Promega (Mannheim, Germany); sodium dodecylsulfate from Serva (Heidelberg, Germany); PWO DNA polymerase, agarose and ampicillin from Boehringer Mannheim (Mannheim, Germany); restriction endonucleases from New England Biolabs GmbH (Schwalbach, Germany); bacto yeast extract, bacto agar, and bacto tryptone from Difco (Detroit, MI, USA); Hybond-N blotting membrane from Amersham Life Science (Braunschweig, Germany). The human PH-GPx cDNA (pBSGPX4 construct) was purchased from ATCC (Rockville, MD, USA).

2.2. Cloning procedure

A mouse fibroblast cDNA library (5′-STRECH PLUS, Clontech, Palo Alto, California, USA) was screened by hybridization using a *Sma*I-*Ppu*MI fragment (480 bp) of the human PH-GPx cDNA as probe. Among some 300 000 recombinants we isolated a homogeneous PH-GPx positive plaque during three rounds of hybridization and PCR screens. Sequencing of the DNA isolated from this plaque indicated that it contained the entire coding region of the mouse PH-GPx, the complete 3′-untranslated region including the poly A signal as well as 25 bp of the 5′-untranslated region.

In order to obtain sequence information on the mouse PH-GPx gene we screened a once amplified mouse genomic library which was constructed by inserting *Bam*HI fragments of genomic DNA into the phage EMBL3 SP6/T7 (Clontech, Palo Alto, CA, USA). For hydridization screening two probes were prepared from the mouse PH-GPx cDNA (an *Eco*RI-*Nhe*I fragment of 467 bp and a *Nhe*I-*Eco*RI fragment of 258 bp). During the primary screen eight PH-GPx positive plaques were identified by hybridization among some 210 000 recombinants. These clones were picked and tested for PH-GPx by PCR. For construction of the primers we assumed that the exon/intron organization of the mouse PH-GPx gene was similar to that of the porcine enzyme. For the forward primer a sequence in the second exon and for the reverse primer a sequence in the third

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exon were selected and thus, the PCR fragment was expected to migrate in the region of 500 bp. Out of the eight plaques shown to be PH-GPx positive only one clone gave a PCR product of the expected size. This PCR fragment was cloned into the pGEM-T cloning vector and was sequenced completely. Since the sequence data suggested that the construct contains PH-GPx genomic DNA this plaque was replated at lower density. By DNA hybridization it was shown that 50% of the resulting plaques were PH-GPx positive (secondary screen). One of these positive recombinants was picked, replated again and 10 well isolated plaques were checked for the PH-GPx gene by PCR (tertiary screen). Since all of the 10 clones gave the correct PCR product one clone was picked, the DNA was isolated and sequenced. Sequence alignments were carried out with the DNA Star software package and the search for potential transcription factor binding sites was performed with the Matinspector program [28].

3. Results

Three rounds of plaque hybridization of a mouse fibroblast cDNA library with a probe derived from the human PH-GPx cDNA led to the isolation of a recombinant which contained the mouse PH-GPx cDNA. Sequencing of the final construct (in pBluescript II SK$^+$) indicated a cDNA size of 770 bp with a 21 bp 5′-untranslated region and a 236 bp 3′-untranslated region ending with a poly A signal. Nucleotides 157–159 of our cDNA were identified as the TGA opal codon which is suppressed in translation by the selenocysteine insertion sequence (SECIS) in the 3′-untranslated region of the cDNA [23,29,30]. Since the sequence of fibroblast PH-GPx cDNA was identical to the PH-GPx cDNA recently isolated from mouse testis [25], it will not be described here in more detail. It should, however, be mentioned that the 5′-untranslated region of our cDNA was some 200 bp shorter than the testis cDNA but the missing part was obtained by analyzing the PH-GPx gene (see below). Doing this we found a second ATG start codon (5′-ATG) which was in frame with the previously identified ATG (3′-ATG). A similar constellation of two in frame starting ATGs has been reported for the PH-GPx cDNAs isolated from testis of rats, mice and humans [22–25]. It has been suggested before that this additional ATG (5′-ATG) constitutes the start codon for a putative signal peptide which may direct the protein into the mitochondria [31].

During the first round of hybridization of a mouse genomic library using two different probes derived from the cloned mouse PH-GPx cDNA we identified eight PH-GPx positive recombinants. These plaques were rescreened by PCR with different primer combinations. PCR products of the expected molecular weight were detected only for one of these eight clones (Fig. 1). Three other recombinants (clones A, D and F) gave PCR signals which were of the same molecular weight as the PCR products obtained with the PH-GPx cDNA as template. Size comparison and sequence data suggested that these particular recombinants contained pseudogene(s) which lack at least the second intron. The existence of pseudogenes has already been reported for the classical GPx [32]. Four other plaques (clones B, C, E and G) were PCR negative.

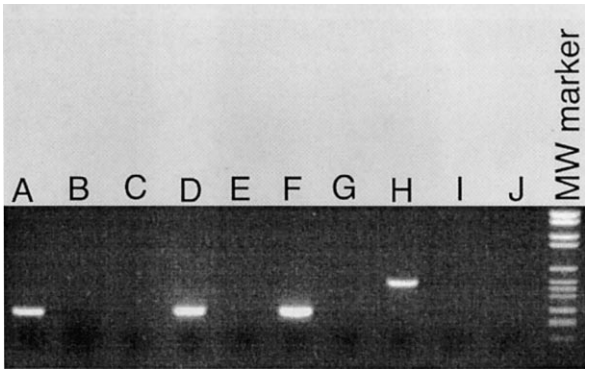

Fig. 1. PCR analysis of genomic PH-GPx positive recombinants. Among 210 000 recombinants tested by hybridization 10 plaques were shown to be PH-GPx positive. These clones were isolated, the DNA was prepared and PCR was carried out using the following primers constructed from the sequence of the PH-GPx cDNA which was cloned in this study: ACA TCG ACG GGC ACA TGG TCT GCC TGG AT (forward) and CCA ACG TGG GTG GGC ATC GTC CCC ATT TAC (reverse). Since these primers are localized in exon 2 and exon 3 a PCR fragment of about 500 bp was expected provided the exon/intron organizations of the mouse and porcine PH-GPx gene are comparable. In contrast, when intron 2 is lacking (pseudogenes), a 300 bp fragment is expected. PCR conditions: annealing temperature 67°C, denaturation temperature 94°C, synthesis temperature 72°C, 30 cycles. PCR products were analyzed in a 2% agarose gel. The sizes of the MW markers are as follows: 2176 bp, 1766 bp, 1230 bp, 1033 bp, 653 bp, 517 bp, 453 bp, 396 bp, 298 bp, 224/220 bp, 154 bp.

Table 1
Exon/intron organization of mouse, porcine and human PH-GPx genes

Structural element	Length (bp)		
	mouse gene	porcine gene [26]	human gene [27]
5′-Flanking region (sequenced so far)	1013	615	n.p.
Exon 1	**228**	**237**[a]	**163**
Intron 1	797	980	1059
Exon 2	**95**	**95**	**95**
Intron 2	91	77	85
Exon 3	**145**	**145**	**145**
Intron 3	123	124	147
Exon 4	**152**	**152**	**152**
Intron 4	804	86	433
Exon 5	**25**	**25**	**25**
Intron 5	92	153	133
Exon 6	**60**	**60**	**60**
Intron 6	86	69	80
Exon 7	**269**[b]	**252**[b]	**239**[b]

[a]Incomplete cDNA, cap site concluded from sequence homology (Fig. 3).
[b]To the poly A signal of the cDNA.
n.p., not published.

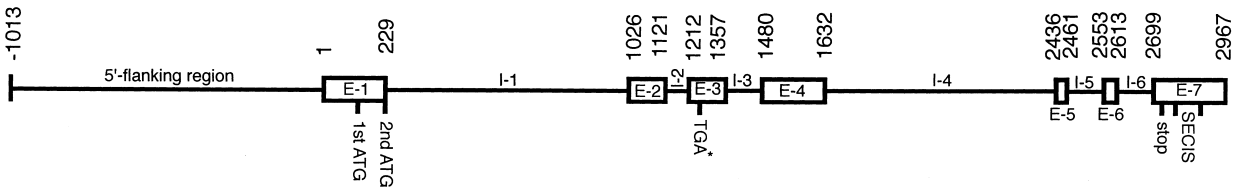

Fig. 2. Structure of the mouse PH-GPx gene. The numbers above the boxes indicate the beginning of the structural elements (exons, introns, flanking region). TGA* identifies the position of the opal TGA which encodes the catalytically active selenocysteine. The positions of the two starting ATGs (exon 1), the stop codon and the SECIS (exon 7) are also indicated. The cap site was labeled nucleotide 1.

Clone H was replated at lower density and rescreening by hybridization indicated that about 50% of the resulting plaques were PH-GPx positive (secondary screen). One of the positive clones was picked and replated again. Ten of the resulting plaques which were well separated from each other were picked and all of them were PH-GPx positive by PCR (tertiary screen). One of these clones was selected, the DNA was isolated and sequencing indicated that it contained a full length mouse PH-GPx gene. This gene spans a total length of 3980 bp and is divided into seven exons and six introns (Fig. 2). The exon/intron organization is very similar to the porcine and human PH-GPx genes [26,27] with the exception that intron 4 of the mouse gene is much larger (Table 1). As mentioned above, we found a second ATG starting codon (5′-ATG) in exon 1 which is in frame with the ATG starting the translation of the cytosolic PH-GPx

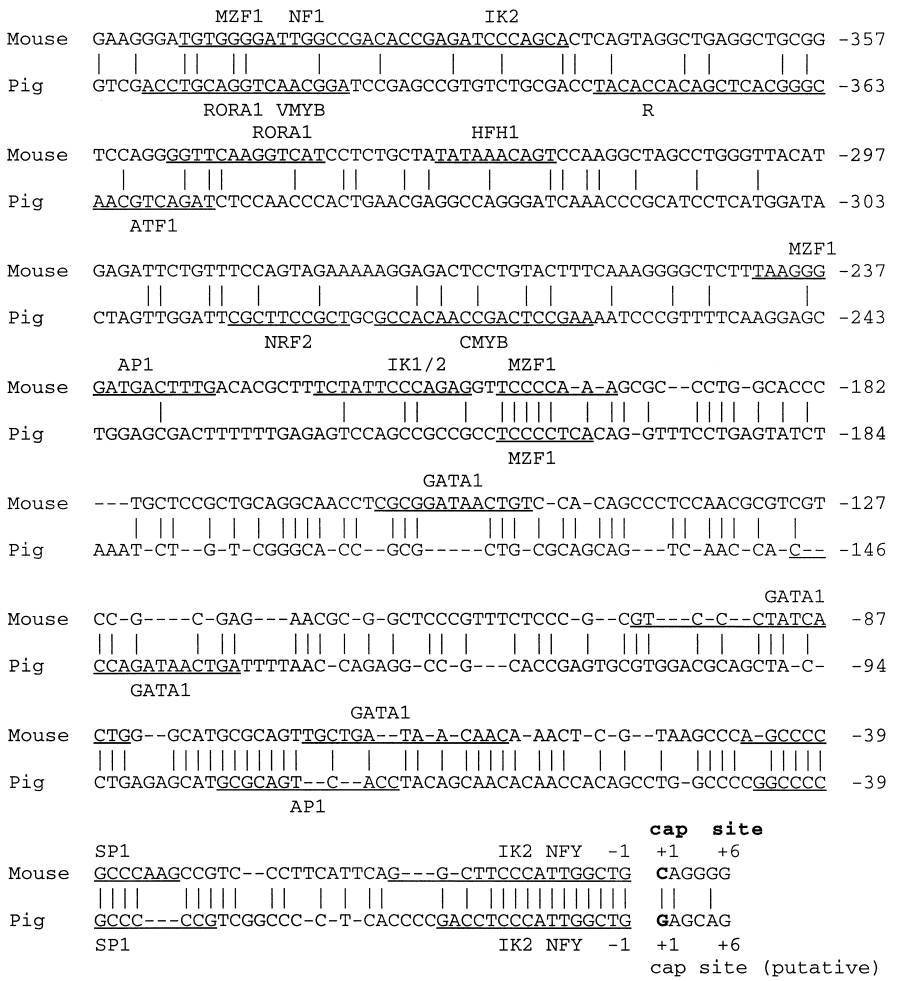

Fig. 3. Structural analysis of the 5′-flanking region of the PH-GPx genes. The 5′-flanking regions of the mouse (this paper) and porcine PH-GPx genes [26] were aligned for maximal homology using the DNA Star software package (Macintosh version). Putative binding sites for transcription factors (underlined) were analyzed with the Matinspector program [28]. Abbreviations: NFY (Y-box binding factor), IK2 (ikaros 2), SP 1 (stimulating protein 1), GATA1 (GATA 1 binding protein), MZF1 (myeloic zinc finger protein 1), NF1 (nuclear factor 1), AP1 (activating protein 1), CMYB (protooncogene product CMYB), RORA1 (orphan hormone nuclear receptor), HFH1 (fork head domain factor), R (Epstein-Barr virus transcription factor) or NRF2 (nuclear respiratory factor 2), NF-kappaB (transcription factor NF-κB), CREB (cAMP response element), ATF1 (activating transcription factor 1). It should be stressed that the size of the full length cDNA (cap site) has only been determined experimentally in mice. However, the fact that the first 12 nucleotides in front of the mouse cap site are identical in the porcine gene suggests that the porcine promoter also starts here (putative cap site).

(3′-ATG). Exon 3 contains the opal codon which encodes the selenocysteine. The stop codon as well as the SECIS was mapped to exon 7 (Fig. 2). The transcription initiation site of the mouse PH-GPx gene was determined by comparing its sequence with the structure of the full length cDNA [25] obtained using the RACE technique (rapid amplification of cDNA ends). In addition, we sequenced 1013 nucleotides of the 5′-flanking region which may contain the promoter. The sequence between positions −422 and −1 was aligned to the corresponding region of the porcine PH-GPx gene [26] and we found a moderate degree of conservation (47% homology) among the first 200 nucleotides proximal to the cap site (Fig. 3). In contrast, the more distal parts of both genes showed a lower degree of conservation (24% homology). Neither the mouse nor porcine 5′-flanking regions contains a TATA box at the canonical site of about −30. The binding sites for NFY (Y-box binding factor), IK2 (ikaros 2), SP1 (stimulating protein 1) and MZF1 (myeloic zinc finger protein 1) found in the mouse promoter have counterparts in the porcine gene. A cluster of three GATA 1 elements (GATA 1 binding protein) was found in the mouse 5′-flanking region (one GATA 1 element is present in the porcine promoter) suggesting a role of PH-GPx expression in red cell biology. Potential binding sites in the distal part of the porcine and mouse 5′-flanking region do not share a high degree of similarity, and thus it remains to be investigated whether the sites indicated in Fig. 3 may be of functional relevance. Intron 1 of the mouse gene contains binding sites for NF-κB and SP 1 which are also present in the porcine gene. Moreover, two cAMP response elements (CREB) were found in exons 1 and 3 but they are not conserved in the porcine gene.

4. Discussion

Selenoproteins which contain selenium either as cofactor or as selenocysteine amino acid are widespread in all life kingdoms [33]. Among the 11 genes encoding different selenocysteine containing proteins identified thus far in mammals four encode various glutathione peroxidases [34] and three encode different thyroid hormone deiodinases [29,35]. Others encode thioredoxin reductase [36], selenophosphate synthase 2 [37] and selenoproteins P and W [38,39]. Recently, a new selenium containing protein has been identified in humans which does not have a high degree of homology to other mammalian selenoproteins. The biological relevance of this new protein is currently unknown [33].

Glutathione peroxidases constitute an enzyme family which can be divided into four different subtypes: the classical intracellular glutathione peroxidase (GPx), the plasma glutathione peroxidase (pGPx), the gastrointestinal glutathione peroxidase (GI-GPx) and the phospholipid hydroperoxide glutathione peroxidase (PH-GPx). These enzymes have attracted the attention of scientists because of their potential antioxidative capability. PH-GPx, which was discovered as second glutathione peroxidase subtype in 1982 [40], is unique among these peroxidases because it is capable of reducing hydroperoxy ester lipids. Although the existence of a PH-GPx has been known for more than 15 years [40,41], the molecular biology of this enzyme subfamily has not been studied until recently. In 1994 the first gene for a mammalian PH-GPx was sequenced [26] and in the same year the cDNA for the human testis PH-GPx was cloned [24]. Later on PH-GPx cDNAs from other mammalian tissues were isolated [22,23]. After we had started this project three sequences for the mouse PH-GPx cDNAs were released by the EMBL data bank (AF045769, AF045768, D87896). These sequences were identical to each other and match exactly the mouse fibroblast PH-GPx cDNA sequenced in this study. The mouse PH-GPx gene cloned in this study constitutes the second complete mammalian PH-GPx gene sequenced so far and alignments of the 5′-flanking region with that of the porcine gene suggested structural elements which might be of regulatory importance.

The biological importance of the PH-GPx subfamily has not been clarified yet, but for the time being there are three major hypotheses [42]: (i) protection against oxidative stress [7–10], (ii) involvement in spermatogenesis [14–16], (iii) regulator of the arachidonic acid cascade [17–20]. In addition, there is circumstantial evidence for the implication of PH-GPx in post-translational protein modification [41]. Most of the experimental evidence suggesting the biological role of glutathione peroxidases originates from animal studies carried out under selenium deficiency. However, since glutathione peroxidases are not the only selenoproteins in mammals the results of such studies must be interpreted with care. Moreover, PH-GPx activity persists even under extreme selenium deficiency [42,43], and thus selenium deficiency experiments may not provide conclusive experimental data on the role of PH-GPx. As a more convincing approach to obtain more detailed information on the physiological relevance of the PH-GPx, targeted gene disruption may be considered. Since the stem cell technology for targeted gene disruption has mainly been worked out in mice, cloning of the mouse PH-GPx gene provided important tools for future knockout experiments.

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