Genetic evidence for an androgen-regulated epididymal secretory glutathione peroxidase whose transcript does not contain a selenocysteine codon

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Epididymal glutathione peroxidase (GPX) has been suggested as a major factor in combating loss of fertility of spermatozoa due to lipid peroxidation. We report here the isolation and sequence of putative GPX cDNAs from rat (*Rattus rattus*) and cynomolgus-monkey (*Macaca fascicularis*) epididymis, which exhibit marked sequence identity with known GPXs. In both species the cDNAs encode predicted preproteins containing 221 amino acid residues. Unlike other characterized GPX sequences, epididymal GPX mRNA does not contain a selenocysteine codon (UGA). However, sequence comparison and molecular-modelling studies suggest a high degree of structural conservation between epididymal and other GPXs. Transcripts corresponding to epididymal GPX are not detected in a variety of other tissues (liver, spleen, kidney and testis) and appear to be androgen-regulated in the epididymis.

INTRODUCTION

Mammalian spermatozoa are unusually rich in polyunsaturated fatty acids, a property that predisposes them to the deleterious effects of oxygen free radicals such as superoxides, hydroxyl radicals and singlet oxygen (Mann & Lutwak-Mann, 1981). These free radicals react with unsaturated acyl or alkenyl moieties in membranes, leading to the formation of cytotoxic hydroperoxides and destruction of phospholipids. In human spermatozoa, lipid peroxidation has been correlated with increased membrane permeability, loss of motility, morphological abnormalities and various pathological conditions that lead to a low level of fertilizing capacity (Aitken & Clarkson, 1987; Alvarez & Storey, 1989; Rao *et al.*, 1989).

Normally, peroxidative damage to spermatozoa is contained by two enzyme systems, superoxide dismutase and glutathione peroxidase (GPX)/reductase (mammalian spermatozoa lack catalase activity; Mann, 1964). The relative protective effect conferred by these two enzyme systems varies between species; rabbit spermatozoa, for example, rely largely on superoxide dismutase to destroy superoxides, whereas mouse and human spermatozoa are more dependent on GPX to inactivate hydrogen peroxide and organic hydroperoxides (Alvarez & Storey, 1989). Recently, the protective role of GPX in spermatozoa has received attention in relation to fertility and survival of spermatozoa in the cauda epididymis (Aitken & Clarkson, 1987; Alvarez & Storey, 1989).

GPX is primarily a cytoplasmic enzyme that contains at its catalytic centre a redox-active selenocysteine residue encoded by a TGA codon; we shall refer to these well-characterized cytoplasmic enzymes as 'cytosolic GPXs'. However, secreted GPXs have recently been described from human and rat placenta (Takahashi *et al.*, 1990; see also the Genbank Nucleotide Sequence Database). These enzymes also possess a selenocysteine residue and show a high degree of sequence similarity to

cytosolic GPXs, indicating that GPXs exist as a gene family. At least two lines of evidence suggest that the enzyme(s) responsible for GPX activity in semen may have properties more akin to those of a secreted GPX than a cytosolic GPX. First, human seminal plasma contains an activity that effectively counteracts the toxic effects of exogenous peroxidized fatty acids on sperm membranes in a manner consistent with its corresponding to a secreted GPX (Jones *et al.*, 1979). Second, a partial cDNA clone has been identified for an epididymis-specific GPX-related mouse protein that is apparently secreted (Ghyselinck & Dufaure, 1990; Ghyselinck *et al.*, 1990; Faure *et al.*, 1991).

Here we describe the cDNA cloning, sequencing and hormonal regulation of cynomolgus monkey (*Macaca fascicularis*) and rat (*Rattus rattus*) epididymal transcripts which appear to encode an epididymis-specific form of secreted GPX distinct from both cytosolic GPX and placental secreted GPX.

MATERIALS AND METHODS

Materials

Restriction endonucleases, cDNA synthesis kit, polynucleotide kinase, RNAase-free DNAase, deoxynucleotides and pre-cut cloning vector DNAs were obtained from Pharmacia, Milton Keynes, U.K. Oligo(dT)–cellulose (Type 3) was from Collaborative Research, Bedford, MA, U.S.A. T4 DNA ligase, the Klenow fragment of DNA polymerase I and Hybond N nylon membrane were purchased from Amersham International, Bucks., U.K. $[\alpha^{-32}P]dATP$ (> 800 Ci·mmol⁻¹) and $[\gamma^{-32}P]ATP$ (> 3000 Ci·mmol⁻¹) were from du Pont–NEN, Stevenage, Herts., U.K. Nitrocellulose filters and micro-dialysis membranes were from Schleicher und Schüll and Millipore respectively. Calf intestinal alkaline phosphatase (Molecular Biology grade) and proteinase K were from Boehringer Mannheim G.m.b.H., Lewes, East Sussex, U.K. and low-melting-temperature agarose from Gibco–BRL, Paisley, Renfrewshire, Scotland. All other

Abbreviations used: GPX, glutathione peroxidase; PH-GPX, phospholipid hydroperoxide glutathione peroxidase; 1 × SCC, 0.15 M-NaCl/0.015 M-sodium citrate.

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The nucleotide sequence data reported appear in the EMBL Nucleotide Sequence Database under the accession numbers X62403 (Macaca fascicularis epididymal glutathione peroxidase) and X62404 (Rattus rattus epididymal glutathione peroxidase).

chemicals were of AnalaR grade or the purest grade available. Fresh tissue samples were obtained from adult male rats (Wistar strain) and monkeys (*M. fascicularis*).

Oligonucleotides for sequencing and hybridization were synthesized on a du Pont Coder 300 synthesizer using phosphoramidite chemistry and were used without subsequent purification.

RNA isolation and Northern-blot analysis

Castration and testosterone treatment of rats and the isolation of RNA from frozen tissues were as described previously (Girotti *et al.*, 1992). Where appropriate, poly(A)-containing RNA was purified from 1.5–3 mg of total RNA (Craig *et al.*, 1976).

Total RNA or poly(A)-containing RNA was fractionated by electrophoresis through a 1.1% (w/v) agarose gel containing formaldehyde, blotted on to a Hybond-N nylon membrane, prehybridized and hybridized as described previously (Walker *et al.*, 1990), using random-primed (Feinberg & Vogelstein, 1984) ³²P-labelled cDNA plasmid inserts as probes. All Northern blots were reprobed with a cloned mouse actin cDNA, pAM91 (Humphries *et al.*, 1981) to confirm equivalent track loadings and the integrity of the RNA preparations.

Construction and storage of epididymal cDNA libraries

A 5 μ g portion of poly(A)-containing RNA was used to direct cDNA synthesis using a Pharmacia kit, according to the recommendations of the manufacturer. Briefly, this method employs oligo(dT)₁₂₋₁₈ for priming first-strand cDNA synthesis and an *Eco*RI-*Not*I linker-adaptor cloning strategy that enables cDNA inserts to be cloned into the unique *Eco*RI site of plasmid pAT153 (Twigg & Sherratt, 1980). The ionic strength of the resultant ligations was reduced by micro-dialysis to enable efficient electroporation of *Escherichia coli* TG2 cells (Sambrook *et al.*, 1989) using a Bio-Rad Gene Pulser (Dower *et al.*, 1988). After overnight growth as discrete colonies, approx. 10⁵ transformants (corresponding to 6 ng of pAT153 DNA per ligation) were harvested in glycerol broth, snap-frozen in aliquots and stored at -70 °C until required.

Isolation of GPX cDNA clones and DNA sequence analysis

Approx. 3×10^4 monkey or rat epididymal cDNA clones were transferred to nitrocellulose filters (Grunstein & Hogness, 1975) and screened for inserts that hybridized to an oligonucleotide (⁵CCCACCAGGAACTTCTCAAAGTTCCAG³) corresponding to a conserved region of cytosolic GPX proteins. Hybridization was for 24–48 h under conditions of moderate stringency (6 × SSC, 45 °C) (1 × SSC is 0.15 M-NaCl/0.015 M-sodium citrate). After two rounds of clone purification and rescreening, plasmid DNA was isolated from positive clones and subjected to preliminary characterization by partial sequencing and/or restriction analysis with *Rsa*I. Monkey cDNA clone pmE-GPX and rat clone prE-GPX were chosen for complete DNA sequence determination.

Sequencing of recombinant plasmid cDNA clones was performed using a custom primer walking strategy on a du Pont Genesis 2000 automated sequencer utilizing fluorescently labelled dideoxynucleotides. The sequencing of pmE-GPX and prE-GPX were complete on both DNA strands.

Computer methods

DNA sequences were compiled and aligned using the program LASERGENE (DNASTAR, West Ealing, London, U.K.). Optimal eukaryotic signal-peptide cleavage sites were predicted using the von Heijne (1983) rules.

Computer modelling of monkey epididymal GPX was performed using the co-ordinates of the bovine cytosolic GPX dimer (Epp et al., 1983) from the Brookhaven Database. The programs INSIGHT, MOLEDT and DELPHI (Biosym Technologies, San Diego, CA, U.S.A.) were used to view, build and perform electrostatic calculations respectively on a Silicon Graphics Personal IRIS 4D-20 workstation. Side chains that differed at corresponding positions in the two aligned structures were replaced using MOLEDT for both subunits in the dimer. Torsion angles were maintained where possible, but where no guide atoms existed (for longer side chains), idealized geometrics were used. From this point, only side chains within a 1.5 nm (15 Å) radius of Cys-52 of the A-subunit were considered in detail. Where heavy atoms of new side chains came within 0.25 nm (2.5 \AA) of another heavy atom, their torsion angles were adjusted. The single deletion in this region (between Thr-55 and Ala-56; see Fig. 5 below) was modelled employing a 'spare parts' method (Claessens et al., 1989) using a library of fragments from structures in the Brookhaven Protein Databank. A C_a distance matrix method was used to select the ten best-fit structures for three residue splicers at both the N- and C-termini of a tworesidue variable-geometry region. Four of the fragments chosen in this way gave good backbone root-mean-square fits in the splicer regions and all corresponded to a two residue 3¹⁰ helix configuration in residues either side of the deletion. The side chains of the two residues projected in the same direction as those in the crystal structure of bovine cytosolic GPX. A threeresidue section of the fragment with the best root-mean-square fit in the splicer region was built into the structure.

The electrostatic potential field of bovine cytosolic GPX and modelled monkey epididymal GPX were calculated using the program DELPHI, utilizing a finite difference solution to solve the linearized Poisson–Boltzmann equation. Protein and solvent are represented as a two-continuum dielectric model. Protein and solvent dielectrics were 2 and 80 respectively. Charges were assigned to potentially charged residues assuming a pH of 7 (a charge of +0.5 was assigned to His residues) and potential contours were displayed using the program INSIGHT.

RESULTS AND DISCUSSION

Cloning and preliminary characterization of rat and monkey epididymis-specific GPX cDNAs

Approx. 3×10^4 rat and monkey epididymal cDNA clones were independently screened with a non-redundant oligonucleo-



Fig. 1. Size and tissue-specific expression of rat and monkey epididymal GPX transcripts

RNA samples were electrophoresed on an agarose gel under denaturing conditions, blotted and probed with an epididymal GPX cDNA insert as described in the Materials and methods section. (a) Epididymal polyadenylated RNA (1 μ g) isolated from cynomolgus monkey (lane 1) or rat (lane 2), and probed with mixed monkey (pmE-GPX) and rat (prE-GPX) epididymal GPX cDNA inserts. (b) Total RNA (15 μ g) isolated from rat epididymis (lane 1), testis (lane 2), spleen (lane 3), kidney (lane 4) and liver (lane 5), probed with rat epididymal GPX (prE-GPX) cDNA insert.



Fig. 2. Effect of castration and testosterone treatment on the steady-state levels of GPX transcripts in the rat epididymis

Epididymal total RNA samples $(15 \ \mu g)$ were electrophoresed on an agarose gel under denaturing conditions, blotted and probed with a rat epididymal GPX (prE-GPX) cDNA insert, as described in the Materials and methods section. (a) Epididymal RNA isolated from normal rats (lane 1) or rats castrated for 2 days (lane 2), 4 days (lane 3), 7 days (lane 4) or 14 days (lane 5). (b) Epididymal RNA isolated from 14-day castrated rats (lane 1) or from rats castrated for 14 days then treated with testosterone for 1 day (lane 2), 2 days (lane 3), 4 days (lane 4) or 7 days (lane 5). Lane 6 contains epididymal RNA from normal rats.

tide corresponding to a region that is highly conserved among GPX amino acid sequences from several mammalian species (WNFEKFLVG; position 160–168 in Fig. 5 below). These screens yielded 11 and 10 independent strongly hybridizing clones from rat and monkey cDNA libraries respectively.

Northern-blot analysis using the cDNA insert from one such rat clone, prE-GPX, revealed a single band corresponding to a transcript of 1.9 kb that is expressed in the rat epididymis but not in testis, liver, kidney or spleen (Fig. 1). Parallel analysis with the monkey-derived clone, pmE-GPX, revealed a hybridizing transcript of approx. 1.8 kb in the monkey epididymis (Fig. 1).

Androgen regulation of epididymis-specific GPX

The expression of several epididymal proteins is known to be sensitive to the depletion of androgens and other testicular factors that follows castration. To investigate the dependence of steady-state levels of rat epididymal GPX transcripts on testicular function, Northern-blot analyses were performed on total RNA from epididymides removed from rats at various times after castration (Fig. 2a) or after castration and subsequent testosterone administration (Fig. 2b).

Hybridizing mRNA could not be demonstrated in epididymal tissue from rats 2 or more days after castration (Fig. 2a), although its expression was rapidly restored, ultimately to levels near to those before castration, in castrated animals that had subsequently been administered testosterone (Fig. 2b). These findings suggest that prE-GPX- (and presumably pmE-GPX-) hybridizing mRNAs are markedly androgen-sensitive and epididymis-specific and are broadly consistent with those previously reported for a murine epididymal GPX where a partial cDNA clone was used as probe (Ghyselinck *et al.*, 1990; Faure *et al.*, 1991).

DNA sequence analysis of rat and monkey epididymal GPX

Monkey clone pmE-GPX and rat clone prE-GPX were initially selected for automated DNA sequencing as they each possessed relatively large cDNA inserts. The sequences of the cDNA inserts in these clones are presented in Figs. 3 and 4.

Rat prE-GPX cDNA (Fig. 4) is significantly shorter than its corresponding mRNA (1.15 kb as opposed to approx. 1.9 kb)

and is apparently truncated at its 3' end. The absence of a polyadenylation/cleavage signal from the 3' end of monkey pmE-GPX (Fig. 3) suggests that it, too, is truncated, although its insert size (1.52 kb) is close to that of its transcript (1.8 kb) when allowance is made for the poly(A) tail on the latter.

The cDNA inserts of pmE-GPX and prE-GPX cDNA each harbour a 663-nucleotide open reading frame encoding a predicted protein of 221 amino acid residues (M_r 25 300). The proposed initiating ATG codon is associated with sequence motifs known to be conserved in some eukaryotic translational initiation sites (Kozak, 1983) and marks the start of protein synthesis by analogy with human placental secretory GPX (Takahashi *et al.*, 1990; see Fig. 5).

Alignment of the deduced amino acid sequences encoded by either pmE-GPX or prE-GPX cDNAs with the sequences of human, rat or bovine cytosolic GPXs reveals a high degree of similarity, with 45-53% of the residues in the latter being conserved (Fig. 5). However, compared with these cytosolic GPXs, each epididymal sequence possesses an N-terminal extension of 26 amino acid residues, the first 21 of which are predicted as a potential signal peptide in each case. An Nterminal extension of 26 residues is also present in the sequence of the human placental secreted GPX (Fig. 5). Indeed, the degree of conservation between the placental GPX sequence and epididymal sequences discussed here is high (for example, they share approx. 70% identity) compared with the degree of conservation with their cytosolic GPX counterparts. This pattern of similarity suggests that secreted and cytosolic GPXs constitute distinct families and that the proteins encoded by pmE-GPX and prE-GPX cDNAs are members of the former. A more distantly related GPX family (phospholipid hydroperoxide glutathione peroxidase, PH-GPX), whose members are probably cytosolic, has also been reported (Schuckelt et al., 1991). PH-GPX exhibits less than 40% sequence identity with epididymal, placental and other cytosolic GPXs.

Analysis of the DNA sequences of monkey and rat epididymal GPX indicates that neither possesses the selenocysteine TGA codon found in all cytosolic and secreted placental GPXs sequenced to date. Instead, the corresponding position in the rat and monkey epididymal proteins (and that of the murine epididymal GPX; Ghyselinck et al., 1990) is occupied by a Cys residue (Cys-52) encoded by TGY. This finding was unexpected, as selenocysteine is an integral part of the GPX redox reaction centre (Epp et al., 1983). The sequences of a 5' portion of a further four independent monkey- and four independent ratderived clones (corresponding to a region that included Cys-52 and all, or part, of the putative signal peptide) were identical with their pmE-GPX and prE-GPX counterparts (results not shown). This suggests that, in contrast with placental GPX and cytosolic GPX, epididymal GPX is not a selenoenzyme (or, at least, does not contain a selenocysteine TGA codon) and argues that epididymal and placental secreted GPXs are each representatives of a further GPX subgroup. Additionally, cytosolic GPX residues implicated in substrate binding (Arg-56 and Arg-179 in Fig. 5; Epp et al., 1983) are not conserved in epididymal GPXs (but are also notably absent from placental GPX and PH-GPX).

Given these differences, we undertook preliminary modelling studies in order to determine whether epididymal GPX is conserved with respect to cytosolic GPX at the level of tertiary structure, particularly in the vicinity of its (potential) catalytic domain.

Modelling the tertiary structure of monkey epididymal GPX

A preliminary investigation of the tertiary structure of monkey epididymal GPX was undertaken by modelling the predicted monkey sequence on the 0.2 nm (2 Å) structure of bovine

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Fig. 5. Alignment of epididymal, cytosolic and placental GPXs

Gaps were introduced by eye to maximize the alignment. | indicates identity between aligned sequences. # denotes a TGA-encoded selenocysteine residue. Residue numbering refers to the monkey epididymal GPX sequence. Residues -21 to -1 denote the putative signal sequence. Sequences are: mouse epididymal GPX (Ghyselinck & Dufaure, 1990), human cytosolic GPX (Sukenaga *et al.*, 1987), bovine cytosolic GPX (Mullenbach *et al.*, 1988), pig PH-GPX (Schuckelt *et al.*, 1991) and human placental GPX (Takahashi *et al.*, 1990).



Fig. 6. Structure of the active site of bovine cytosolic GPX and modelled monkey epididymal GPX

Stereo views of residues within a 0.6 nm (6 Å) sphere of the active-site selenocysteine selenium atom of bovine GPX (a) and cysteine sulphur atom of epididymal GPX (b) are shown, together with the peptide backbone of the co-linear active-site helix. Residue side chains involved in hydrogenbonding (represented by broken lines) are numbered according to Fig. 5. 'SE' denotes selenocysteine. Note that the single residue deletion in the helix of epididymal GPX still allows the two main-chain hydrogen bonds to the sulphur of Cys-52. In addition, His-179 in epididymal GPX can hydrogen-bond to the cysteine sulphur atom.

cytosolic GPX (Epp et al., 1983). These studies suggest that overall the structures are similar, presumably reflecting the high degree of amino acid sequence conservation between them (51%)identity). Additionally, the electrostatic potential fields around bovine GPX and the modelled epididymal GPX are qualitatively similar at low isopotential contour level (kT/e = -0.05 and)+0.05), independently of whether the epididymal enzyme is assumed to be monomeric, dimeric or tetrameric (results not shown). However, amino acid sequences adjacent to, and colinear with, the redox-active selenocysteine residue in the primary structure of bovine GPX are less well conserved; only 16 of the 45 residues (36%) in bovine GPX are identical in the corresponding region of monkey epididymal GPX (residues 28-71; Fig. 5). We therefore examined in greater detail the predicted tertiary structure of the epididymal GPX within 1.5 nm (15 Å) radius of Cys-52 (that corresponds to the selenocysteine residue of bovine GPX) to investigate whether this region was also structurally less well conserved. The cardinal feature of the derived model is the high degree of conservation between the arrangement of atoms in the two structures, with Cys-52 of epididymal GPX and SeCys of cytosolic GPX residing at the Ntermini of long α -helices (Fig. 6). (It is likely that the resultant helix dipole in bovine GPX stabilizes the active form of the enzyme, R-Se⁻). In contrast, Pro-60 of epididymal GPX could not be built into the α -helix without unacceptable steric hindrance of its C_s and C_y side-chain atoms with main-chain atoms of the helix. This suggests that the Pro Ψ angle is opened to produce a 3^{10} helix conformation, thereby introducing a kink into the modelled epididymal GPX helix. The apparent deletion of a single residue in epididymal GPX (between Thr-55 and Ala-56) gives rise to a tighter helix turn in the same region (Fig. 6). Nevertheless, this model preserves the overall active-site geometry of bovine GPX, with the immediate environment of Cys-52 in the modelled epididymal GPX essentially retaining the hydrogenbonding pattern of the analogous selenocysteine residue in cytosolic GPX.

Epididymal GPX possesses a His residue (His-179) whose side chain can adopt a *trans* (χ_1) conformation without steric clashes with other protein atoms, enabling the formation of a hydrogen bond with S_y of Cys-52. Such an interaction would lower the p K_a of the thiol group of Cys-52 to a value closer to that of the selenol group of bovine GPX, with a predicted stabilization of the R-S⁻ form of the enzyme. In the crystal structure of bovine GPX, the Arg residue that corresponds to His-179 in epididymal GPX adopts a *gauche* side-chain torsion angle and cannot interact with selenocysteine by virtue of its size. The replacement of the redox-active selenium by a sulphur atom in epididymal GPX may therefore permit the resulting enzyme to function via the same catalytic mechanism, reflecting the similar redox chemistries of selenium and sulphur.

The subtle structural perturbations that exist between bovine GPX and its modelled epididymal counterpart are arguably consistent with their utilization of slightly different substrates (for example, membrane-bound peroxidized phospholipid derivatives in the case of epididymal GPX). However, these studies confirm that similarities between cytosolic GPX and epididymal GPX sequences result in a highly conserved active-site architecture and support the notion that both enzymes operate via a closely related catalytic mechanism.

Our finding that the epididymis is a rich source of GPX supports the hypothesis that the enzyme has a function in preserving spermatozoa in a viable form during storage in the cauda epididymis. By preventing the accumulation of lipid hydroperoxides in plasma membranes, secreted GPX in the epididymis would protect spermatozoa from the deleterious effects of oxygen free radicals.

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