RESEARCH COMMUNICATION

The majority of human glutathione peroxidase type 5 (GPX5) transcripts are incorrectly spliced: implications for the role of GPX5 in the male reproductive tract

Len HALL[1], Karin WILLIAMS, Anthony C. F. PERRY, Jan FRAYNE and Jennifer A. JURY
Department of Biochemistry, University of Bristol, School of Medical Sciences, Bristol BS8 1TD, U.K.

An epididymis-specific, secretory glutathione peroxidase (GPX5) has been proposed previously to play a role in protecting mammalian sperm membranes from the deleterious effects of lipid peroxidation, which, if not contained, can lead to reduced fertilizing capacity. Here we report the cDNA cloning of human GPX5 and show that the majority of transcripts contain a 118 nt frame-shifting deletion, arising, most likely, from inappropriate excision of exon 3 during processing. Antisera raised against recombinant human GPX5 cross-reacted with rat and macaque (*Macaca fascicularis*) epididymal proteins of the size expected for full-length, active GPX5. However, no similar reactivity could be demonstrated in any of the human samples tested.

INTRODUCTION

The presence of reactive oxygen species (ROS) in the mammalian male reproductive tract is well documented. In humans, elevated levels of ROS lead to an increase in sperm membrane permeability, loss of sperm motility and a concomitant reduction in fertilizing ability [1–3]. In addition to non-enzymic scavenging systems, several enzymic systems have been identified within the epididymis that may contribute to scavenging and detoxification of free radicals and ROS. The high levels of extracellular superoxide dismutase within the rat and macaque epididymis [4,5] and in human seminal plasma [5] suggests that, in man also, the epididymis is likely to be a major source of this secreted enzyme. The presence of an active superoxide dismutase within epididymal fluid, in turn, requires a hydrogen peroxide scavenging system. The extreme toxicity of hydrogen peroxide to sperm [1,3], and indeed to any cell, is counteracted by several enzymic and non-enzymic systems, thereby providing ample backup to this vital complex. Such a backup is found within the epididymis; the extremely efficient scavenging of hydrogen peroxide is brought about by catalase and glutathione peroxidase (GPX)/glutathione reductase, and by the suicide scavenging properties of albumin and other large, globular secreted proteins [3].

Within the rat epididymis, two GPX isoenzymes have been identified at both the mRNA and protein level; plasma GPX (GPX3), which is a selenocysteine-containing enzyme that is fully active within epididymal fluid and is capable of using several electron donors, and epididymal glutathione peroxidase (GPX5), which is an epididymis-specific, selenium-independent isoenzyme that has GPX activity in transfected cells [6], but its physiological electron donor has yet to be identified. The association of GPX5 with the sperm membrane overlying the acrosomal cap [7] would seem to be ideally located to inactivate hydrogen peroxide traversing this membrane from the epididymal fluid and thereby prevent membrane lipid peroxidation. However, recent studies [8] on purified porcine GPX5 suggest that this enzyme has very little activity towards hydrogen peroxide or organic hydroperoxides. Instead, it has been proposed [8] that acrosome-bound GPX5 may protect sperm from premature acrosome reaction in the epididymis by binding to lipid peroxides, which might otherwise interact with phospholipase A_2 and induce the acrosome reaction.

In order to study the expression of human GPX5, establish its presence in human seminal plasma and to look for a possible correlation with elevated ROS levels or other indicators of reduced fertility, it was first necessary to identify and clone human GPX5 cDNA before raising specific antisera. With this in mind, a reverse transcription (RT)-PCR-based approach was used to clone human GPX5 cDNA using oligonucleotides based on the known macaque (*Macaca fascicularis*) cDNA sequence [9]. Polyclonal antisera were then raised against a human GPX5 fusion protein generated in recombinant bacterial cells, and were used for Western-blot analysis of human, macaque and rat protein extracts.

MATERIALS AND METHODS

RT-PCR

Total RNA (2 μg) from human testis (obtained from Clontech Labs., Palo Alto, CA, U.S.A.) was used as the template for Expand™ RT-directed cDNA synthesis (Boehringer Mannheim, Lewes, East Sussex, U.K.) using oligo $(dT)_{12-18}$ as a primer. Aliquots of cDNA were then used with appropriate primers (see the Results section) in PCR reactions using the following parameters: denaturation at 94 °C for 1 min, annealing at 58 °C for 1.5 min, extension at 72 °C for 1 min (30 cycles) using the

Abbreviations used: GPX, glutathione peroxidase; RT, reverse transcription; MBP, maltose-binding protein; ROS, reactive oxygen species.

[1] To whom correspondence should be addressed (e-mail L.Hall@bris.ac.uk).

The nucleotide sequence data reported in this paper appear in the EMBL Nucleotide Sequence Database under the accession number AJ005277.

Expand™ High Fidelity PCR System (Boehringer Mannheim). The resulting PCR products were resolved on low-melting-temperature agarose gels, the appropriate bands were excised, purified and cloned into plasmid pUC18 and completely sequenced on both DNA strands using an ABI 377 automated DNA sequencer.

Production of polyclonal antisera to recombinant maltose-binding protein (MBP)–GPX5 fusion protein

The section of human GPX5 cDNA (derived from correctly spliced mRNA) encoding the first 70 amino acid residues of mature GPX5 was selected for bacterial expression. This region was amplified by PCR, cloned into the pMAL-c2 expression vector (New England Biolabs, Beverly, MA, U.S.A.), expressed in *Escherichia coli* and the fusion protein was affinity-purified on an amylose-agarose resin column (New England Biolabs). The polyclonal antisera were raised in two New Zealand White rabbits, essentially as described previously [10].

Western-blot analysis of GPX5

Macaque and rat caput and cauda epididymidal extracts were prepared by homogenization of tissues in PBS (154 mM NaCl/1.9 mM NaH_2PO_4/8.1 mM Na_2HPO_4, pH 7.2) containing 1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride (ICN Biomedicals Ltd., Thame, U.K.), followed by incubation on ice in the same buffer containing 1 % (v/v) Triton X-100 (Sigma, Poole, Dorset, U.K.). The homogenates were centrifuged at 10000 ***g*** and the supernatants were stored at −20 °C until required.

Plasma-membrane-enriched fractions of macaque, rat cauda epididymidal sperm and human ejaculated sperm were prepared by detergent extraction with 1 % (v/v) Triton X-100 and vortex mixing [11]; 1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride was included in the preparations.

Proteins (100 μg/lane) were separated by SDS/PAGE [12 % (w/v) gels] electroblotted on to poly(vinylidene difluoride) membranes (PolyScreen™, NEN Life Science Products, Brussels, Belgium). Blots were probed with anti-human GPX5 polyclonal antiserum for 1 h, washed, and incubated with horse-radish-peroxidase-conjugated porcine anti-rabbit IgG (Dako Ltd, High Wycombe Bucks, U.K.) for 1 h. Detection of bound antibodies was achieved by enhanced chemiluminescence (Amersham International, Little Chalfont, Bucks., U.K.) and exposure of the membranes to Hyperfilm™ (Amersham International).

RESULTS AND DISCUSSION

Cloning human epididymal GPX5

A commercial preparation of human testis total RNA (a pooled sample from 29 sudden-death victims, aged 23 to 65) was used as a template for oligo-dT-directed cDNA synthesis. Previous work from our laboratory had established that this source of RNA also contained epididymis-specific sequences, indicating that the epididymides had not been removed from the testes before RNA isolation.

In initial attempts to clone the entire coding region of human GPX5 cDNA, a PCR-based approach with specific primers (5′-CAG ACT AGC ATC TAC AAA CAC TAG-3′ and 5′-CCT GCC CTT AAC TCC ACC TT-3′) based on the cloned macaque GPX5 sequence was used [9]. Whereas PCR products were readily obtained, they were consistently about 120 bp shorter than predicted from the known macaque sequence. Similar results were obtained using a variety of primer pairs. To determine the identity of the apparently truncated PCR product, it was gel-purified, cloned and several of the resulting clones were subjected to DNA sequence analysis. Alignment of the sequences of these putative human GPX5 clones with that of macaque GPX5 cDNA confirmed that they were GPX5 orthologues, but that the cloned human PCR products all possessed a frame-shifting deletion of 118 bp within the coding region when compared with the macaque sequence.

In order to establish whether all GPX5 transcripts possessed a deletion in humans, attempts were made to ‘force’ the PCR amplification of a full-length human GPX5 transcript using a primer (5′-GCG CAA TAT CCT GAA CTA AAT GCA-3′) which straddled the site of deletion and contained, at its 3′ end, 11 bases derived from the deleted region (and based on the macaque sequence). Hence this primer could only bind to, and produce an amplified product from, a full-length cDNA template. When this primer was used in conjunction with a suitable downstream primer, a very faint PCR product of the expected (macaque) size was obtained with human cDNA, indicating the presence of some correctly sized transcripts. This PCR product was subsequently cloned and sequenced.

By combining overlapping sequence data from the deleted PCR products and the ‘forced’ PCR product, a full-length human GPX5 sequence could be derived, but this did not provide incontrovertible proof that a full-length transcript existed. It was therefore important to establish that a complete, full-length human GPX5 transcript could be amplified as a single product by PCR. This was achieved with a more concentrated, better quality cDNA template, using the macaque-based primers, which flank the entire GPX5 coding region, and fractionating the resulting PCR products on a low-melting-temperature agarose gel. This resulted in the appearance of two bands (Figure 1); a major band representing the deleted product and a larger, but very minor band, consistent with the expected size of the full-length human GPX5 coding sequence. Neither band was obtained using human genomic DNA as the template, indicating that both bands were amplified from cDNA transcripts rather than contaminating genomic DNA. The larger PCR product was excised, cloned and sequenced, and was found to contain the expected full-length human GPX5 cDNA coding sequence (Figure 2).

Sequence analysis of deleted and full-length human GPX5 cDNA clones

Amplification of human GPX5 transcripts from a commercial testis RNA preparation by RT-PCR has indicated that the majority of transcripts contain a 118 bp deletion within the coding region, which would render them incapable of encoding an active GPX. Nevertheless, some full-length functional transcripts do appear to be produced, albeit at a very much lower level.

The above results were obtained with ‘testicular’ RNA pooled from several individuals, therefore it was important to establish whether the full-length and deleted GPX5 transcripts were both present within a single individual. With this in mind, RT-PCR experiments were carried out on an epididymal RNA sample prepared from a single patient who had undergone surgery. As with the pooled RNA sample, the majority of GPX5 PCR products contained the 118 bp deletion but a small amount of full-length GPX5 PCR product could be ‘forced’ using the deletion-straddling primer (see above), indicating that both deleted and full-length transcripts were present in epididymal RNA from one individual. In contrast, comparable RT-PCR analyses of macaque and rat testis RNA, as well as Northern-blot analyses [9], indicated the absence of deleted forms of GPX5

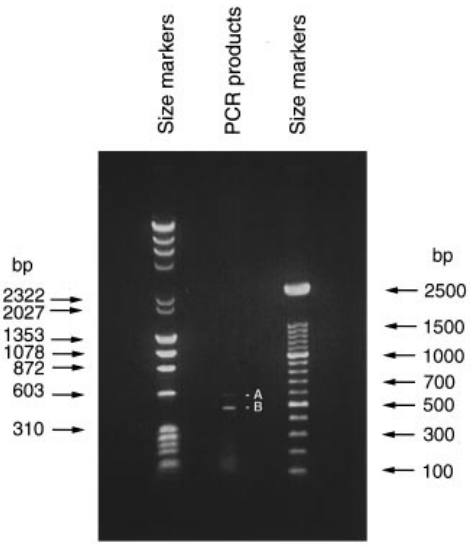

Figure 1 Amplification of full-length and deleted human GPX5 transcripts by RT-PCR

Attempts were made to amplify the entire coding region of human GPX5 by RT-PCR (see the Results section) using primers based on the published macaque GPX5 sequence [9]. The resulting PCR products were resolved by agarose-gel electrophoresis in the presence of ethidium bromide and visualized by UV transillumination. Band A, representing the expected size of the full-length human GPX5 PCR product, was a very minor component of the amplification reaction. Band B, which was approx. 120 bp shorter than band A, represents the major amplified product.

in these species, and all of the GPX5 transcripts were of the expected full-length size.

In the mouse, the GPX5 gene is split between five exons [12], the equivalent positions of the introns in the human sequence is indicated in Figure 2. We have recently isolated the human GPX5 gene from a genomic library and the macaque GPX5 introns have been amplified and cloned using a PCR-based approach (K. Whittington and L. Hall, unpublished work). DNA sequence analyses have confirmed an identical exon/intron organization in the mouse, macaque and human GPX5 genes. From this organization it can be seen that the region of human GPX5 cDNA deleted from the majority of transcripts corresponds precisely to exon 3, strongly suggesting that the human GPX5 deleted transcripts arise by incorrect splicing of the corresponding hnRNA, with exon 2 being spliced on to exon 4 and a concomitant elimination of exon 3.

Detection of GPX5 by Western-blot analysis

The findings described in the section above imply that the level of functional (i.e. full-length) GPX5 protein in the human epididymis must be extremely low in comparison with that in other species such as rat, mouse and macaque. Clearly this raises important questions about its contribution to free radical scavenging in the human epididymis.

In order to confirm that GPX5 protein levels are indeed substantially decreased in the human, polyclonal antibodies were raised against an MBP–GPX5 recombinant fusion protein containing the first 70 amino acid residues of the mature human GPX5 protein, deduced from its nucleotide sequence. By limiting the immunogen to the first 70 amino acid residues, cross-reactivity with other GPX isoforms (see Figure 3) could be minimized.

The DNA construct for expression of recombinant MBP–GPX5 was prepared by PCR amplification, using the full-length human GPX5 cDNA transcript as the template. The resulting fusion protein was used to immunize two rabbits for the production of polyclonal antisera. Antisera from both rabbits (L16 and L17) recognized the MBP–GPX5 fusion protein on Western blots, whereas the pre-immune sera gave no reaction (results not shown).

Because of the scarcity of normal human epididymal tissue, Western blots of macaque and rat epididymidal homogenates

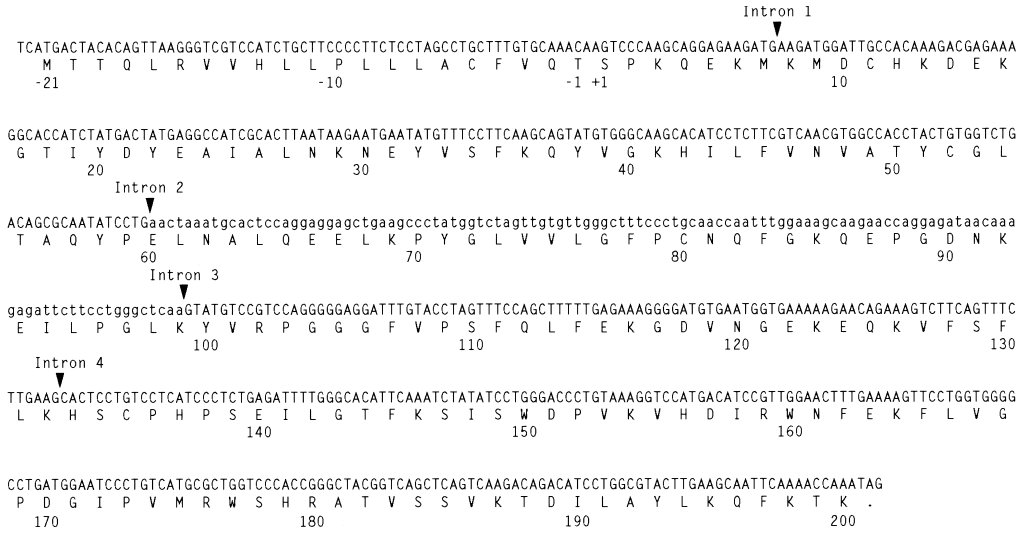

Figure 2 Nucleotide sequence of the entire coding region of full-length human GPX5 cDNA

The coding region of human GPX5 cDNA was amplified by RT-PCR using primers based on the published macaque GPX5 sequence [9]. The complete nucleotide sequence was confirmed by analysis of clones derived from at least two independent PCR reactions. The positions of introns in mouse [12], human and macaque GPX5 (K. Whittington and L. Hall, unpublished results) genomic sequences are indicated. Nucleotides shown in lower case (corresponding exactly to exon 3) are deleted in the majority of human GPX5 transcripts (see Figure 1), which is indicative of aberrant splicing.

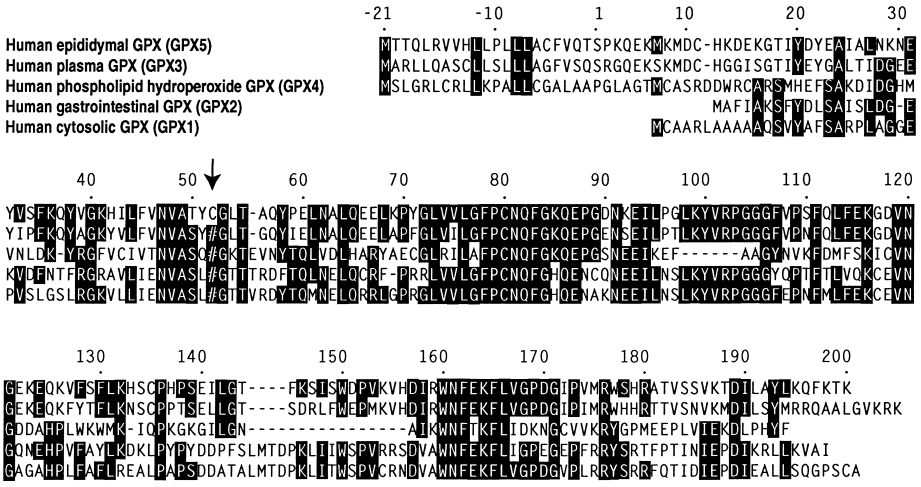

Figure 3 Alignment of human GPX isoforms

The five characterized isoforms (GPX1 to GPX5) of human glutathione peroxidase were aligned with gaps introduced to maximize homology. Highly conserved residues (present in at least three of the five isoforms) are white-on-black. The TGA-encoded selenocysteine residue (#), present in GPX1, GPX2, GPX3 and GPX4 but not GPX5, is indicated with an arrow. Sequences of human GPX1 to GPX4 were obtained from sequence databases.

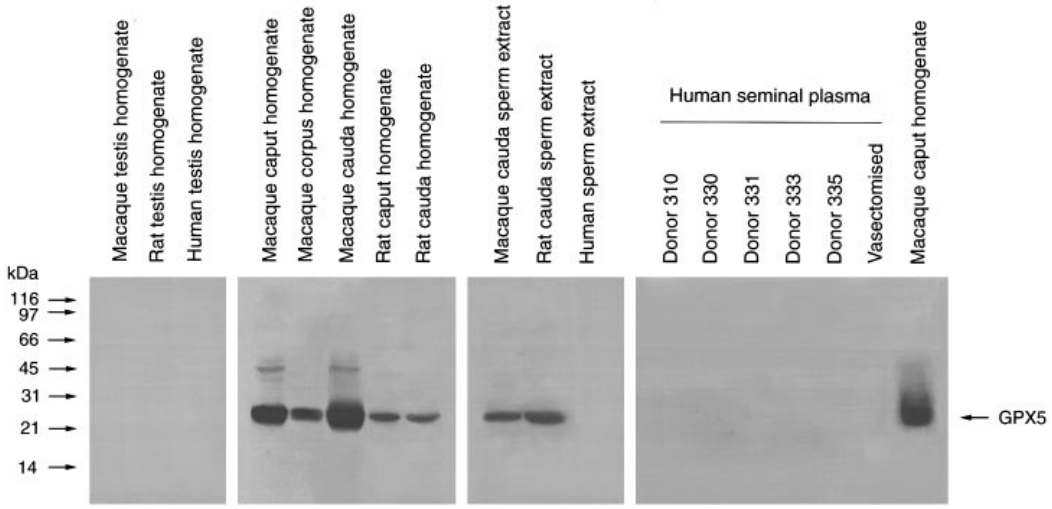

Figure 4 Detection of GPX5 by Western-blot analysis

Tissue homogenates (macaque and rat), sperm membrane extracts (macaque, rat and human) and seminal plasma samples (human) were separated by SDS/PAGE (12% gels), blotted and probed with an anti-human GPX5 polyclonal antiserum (see the Materials and methods section).

and sperm membrane extracts were carried out alongside human seminal plasma and human sperm membrane extracts in order to investigate the presence of GPX5 within these species (Figure 4). It was found that the antiserum raised against the human GPX5 fusion protein cross-reacted with rat GPX5 and that GPX5 was abundant in rat epididymis (both caput and cauda), epididymal fluid and cauda sperm membrane extracts as observed previously (K. Williams, J. Frayne and L. Hall, unpublished work). GPX5 was also abundant in macaque epididymis (caput, corpus and cauda) as well as in macaque cauda sperm membrane extracts. In contrast, GPX5 was undetectable in human ejaculated sperm membrane extracts or in human seminal plasma from five fertile semen donors or one vasectomized donor, indicating a very low abundance in these samples and consistent with the observed low level of correctly processed human GPX5 transcripts. However, the unavailability of normal, fresh human epididymal tissue precluded comparative analyses of epididymal homogenates or cauda epididymidal sperm. No immunoreactive bands of the size expected for GPX5 were obtained with extracts from human and macaque tissues known to express other GPX isoforms, confirming the specificity of the antiserum towards GPX5.

The consequences of reduced levels of GPX5 in the human epididymis, and the effect on fertility, must await definitive characterization of the function of this selenium-independent isoform. The recent proposal that membrane-bound GPX5 may play a role in preventing premature acrosome reaction [8] seems unlikely as human sperm, which are clearly deficient in this enzyme, are not prone to this condition.

This work was supported by the Biotechnology and Biological Science Research Council, U.K. and the Wellcome Trust, U.K. We thank Rhiannon Murray for assistance with automated DNA sequencing.

REFERENCES

1 Aitken, R. J., Clarkson, J. S. and Fischel, S. (1989) Biol. Reprod. **41**, 183–187
2 Cummins, J. M., Jequier, A. M. and Kan, R. (1994) Mol. Reprod. Dev. **37**, 345–362
3 Storey, B. T. (1997) Mol. Human Reprod. **3**, 203–213
4 Perry, A. C. F., Jones, R. and Hall, L. (1993) Biochem. J. **293**, 21–25
5 Williams, K., Frayne, J., McLaughlin, E. A. and Hall, L. (1998) Mol. Human Reprod. **4**, 235–242
6 Vernet, P., Rigaudiere, N., Ghyselinck, N., Dufaure, J. P. and Drevet, J. R. (1996) Biochem. Cell Biol. **74**, 125–131
7 Vernet, P., Faure, J., Dufaure, J. P. and Drevet, J. R. (1997) Mol. Reprod. Dev. **47**, 87–98
8 Okamura, N., Iwaki, Y., Hiramoto, S., Tamba, M., Bannai, S., Sugita, Y., Syntin, P., Dacheux, F. and Dacheux, J.-L. (1997) Biochim. Biophys. Acta, **1336**, 99–109
9 Perry, A. C. F., Jones, R., Niang, L. S. P., Jackson, R. M. and Hall, L. (1992) Biochem. J. **285**, 863–870
10 Frayne, J., Jury, J. A., Barker, H. L., Perry, A. C. F., Jones, R. and Hall, L. (1998) Mol. Human Reprod. **4**, 429–437
11 Jones, R. (1986) J. Reprod. Fertil. **77**, 435–449
12 Ghyselinck, N. B., Dufaure, I., Rigaudiere, N., Mattei, M. and Dufaure, J. P. (1993) Mol. Endocrinol. **7**, 258–272

Received 20 April 1998/8 May 1998; accepted 11 May 1998