Identification of a specific sperm nuclei selenoenzyme necessary for protamine thiol cross-linking during sperm maturation[1]

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SPECIFIC AIMS

We investigated the properties of a 34 kDa selenoprotein that is present only in the sperm nuclei and is highly expressed in the nuclei of late spermatids, where the reorganization and condensation of DNA take place. We performed experiments to determine the primary structure of the selenoprotein and obtain information on its functions in sperm maturation and sperm quality, especially with regard to its role in the processes of DNA condensation.

PRINCIPAL FINDINGS

1. A 34 kDa selenoprotein was identified as a specific sperm nuclei glutathione peroxidase (snGPx) with properties similar to phospholipid hydroperoxide glutathione peroxidase (PHGPx)

By labeling rats in vivo with ^{75}Se and separating the proteins in the tissue homogenates and subcellular fractions by SDS-PAGE, a 34 kDa selenoprotein was detected that is present only in the sperm nuclei and is highly expressed in the late spermatids. After isolation of the sonication-resistant spermatid nuclei (SRS nuclei) and treatment of their surfaces with the detergent cetyl-trimethylammonium bromide, it was found to be the only selenoprotein within these nuclei.

Fragment analysis by MALDI-MS showed a considerable similarity between the 34 kDa selenoprotein purified from rat testis and the selenoenzyme PHGPx, one of the four glutathione peroxidases so far identified that catalyze the reduction of peroxides by oxidation of glutathione. It also reacted with a PHGPx antibody and, like PHGPx, catalyzed the reduction of various peroxides. Its specific activity was about 3000 U/mg protein when phosphatidylcholine hydroperoxide was used as the substrate. Due to its location, we called it sperm nuclei glutathione peroxidase.

During transfer of the spermatozoa from the testis to the epididymis, about two-thirds of the 34 kDa selenoprotein are processed into smaller proteins with molecular masses of 24, 22, and 20 kDa. This truncation is accompanied by a shift in the pI from an extreme basic value of > 10 for the 34 kDa protein to a value of about 7.5 for the 20 kDa product. The reduction in mass, however, has no effect on the enzymatic properties of the processed proteins.

2. snGPx differs from PHGPx in its amino-terminal sequence, which is encoded for by an alternative exon situated in the first intron of the PHGPx gene and contains a nuclear localization signal

The primary structure of snGPx was determined by analysis of its first amino-terminal amino acids, database search, polymerase chain reaction, and sequencing of the cDNA from mouse and rat. An alternative exon responsible for formation of the novel selenoprotein was identified in the first intron of the PHGPx gene (**Fig. 1*A***). The sequence of this exon in mouse and rat and of the putative corresponding exon in humans and pig is shown in Fig. 1*B*. It encodes for an arginine-rich sequence immediately after the first methionine.

Information on the function of this sequence was obtained in an experiment in which two green fluorescent protein (GFP) fusion genes were constructed. For the first, the entire alternative exon of the mouse sequence was fused to GFP; for the second, the sequence after the second methionine was fused. After transfection of both constructs into two mammalian cell lines, the subcellular localization of the two fusion proteins was determined by GFP fluorescence. Only the larger GFP fusion protein had entered the nuclei, indicating that the amino-terminal sequence contains a nuclear localization signal.

[1] To read the full text of this article, go to http://www.fasebj.org/cgi/doi/10.1096/fj.00–0655fje; to cite this article, use *FASEB J.* (March 5, 2001) 10.1096/fj.00-0655fje

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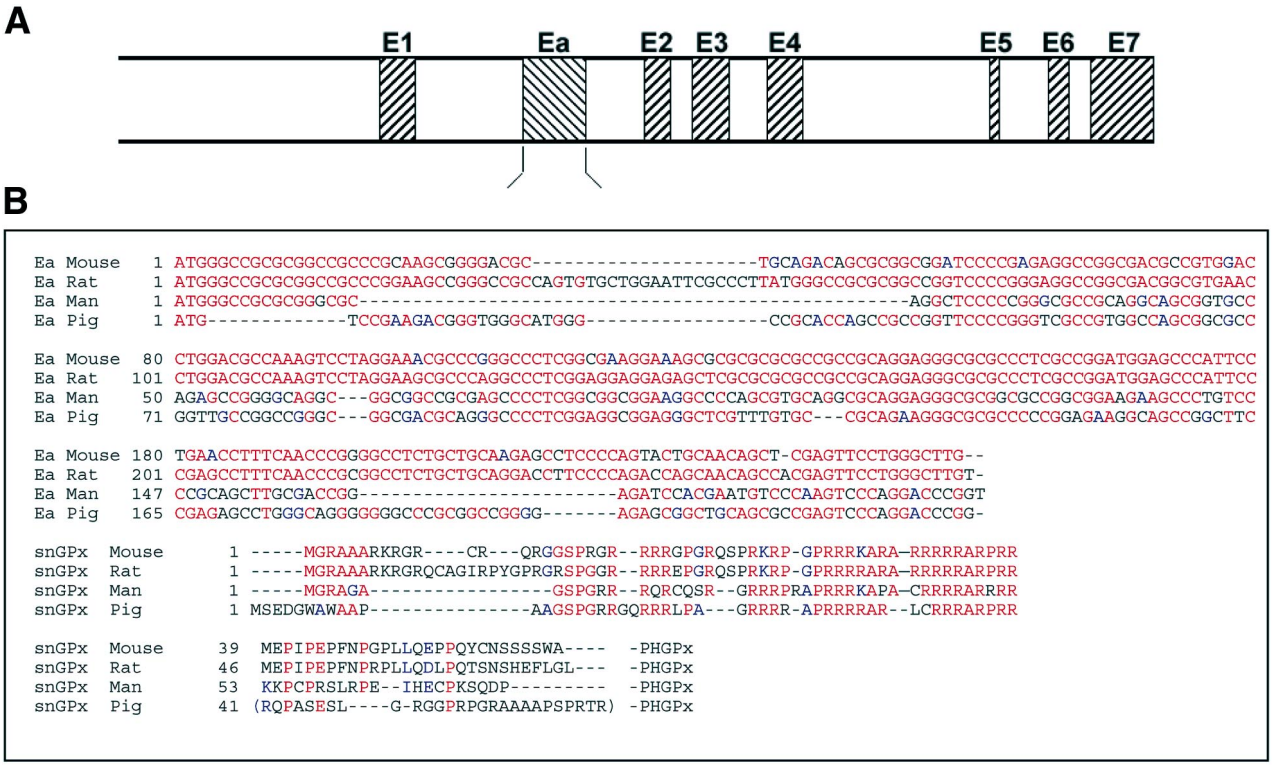

Figure 1. Composition of the alternative exon Ea encoding for the amino-terminal sequence of sperm nuclei glutathione peroxidase (snGPx). *A)* Differences in the primary structure of PHGPx and snGPx: PHGPx is encoded by seven exons E1 to E 7 whereas, due to alternative splicing, snGPx is encoded by the exons Ea to E7. *B)* Sequence of Ea and the corresponding amino-terminal sequence of snGPx in mouse (accession no. AF 274027), rat (accession no. AF 274028), human, and pig.

This sequence showed more than 50% homology to that of the protamines, small basic proteins rich in arginine and cysteine that replace the histones during sperm maturation. They are known to bind to DNA via their polyarginine region, and it is most likely that snGPx is attached to DNA in a similar way.

3. The 34 kDa selenoenzyme acts as a protamine thiol peroxidase, which is responsible for stabilizing the condensed chromatin by cross-linked protamine disulfides and thus necessary to ensure male fertility

The oxidation of protamine thiols plays an important role in sperm maturation. After repackaging of the sperm DNA with protamines, the resulting highly condensed chromatin is stabilized by cross-linking of protamine disulfides. This process is induced by reactive oxygen species (ROS) and is thus analogous to glutathione oxidation and peroxide reduction catalyzed by glutathione peroxidases. This, together with the finding that the glutathione concentration in spermatids decreases largely during the late stages of spermatid development, suggested that the selenoenzyme, located in the nuclei near the protamines, might be able to use the protamine cysteine residues as reductants, thereby acting as a protamine thiol peroxidase.

We therefore investigated the sperm nuclei condensation in selenium-deficient rats in which the concentration of snGPx in the SRS nuclei was decreased to about one-third of the value of selenium-adequate control animals. Staining with acridine orange enables discrimination between double- and single-stranded nucleic acids. As sperm DNA can be denatured by acid or heat only before but not after protamine disulfide cross-linking, acridine orange staining after acid treatment makes it possible to monitor the thiol disulfide status during chromatin condensation of mammalian sperm nuclei. The staining revealed that nearly all of the sperm cells taken from the vas deferens were incompletely condensed (**Fig. 2**). In vitro experiments with sperm nuclei from selenium-adequate rats showed that the condensed state was lost during reduction of protamine disulfides with dithiothreitol and could be restored by adding hydrogen peroxide. Recondensation was blocked by adding bromosulfophthalein, an inhibitor of PHGPx, or a surplus of another thiol in the form of GSH. From these findings, it can be concluded that snGPx is involved in protamine disulfide cross-linking.

CONCLUSIONS AND SIGNIFICANCE

The process of chromatin condensation seems to be crucial not only for maturation of sperm cells, but also for fertility and genesis of offspring. In humans, a high correlation between regular sperm chromatin condensation and the in vitro fertilization rate has been observed. In vitro experiments with mice showed that a

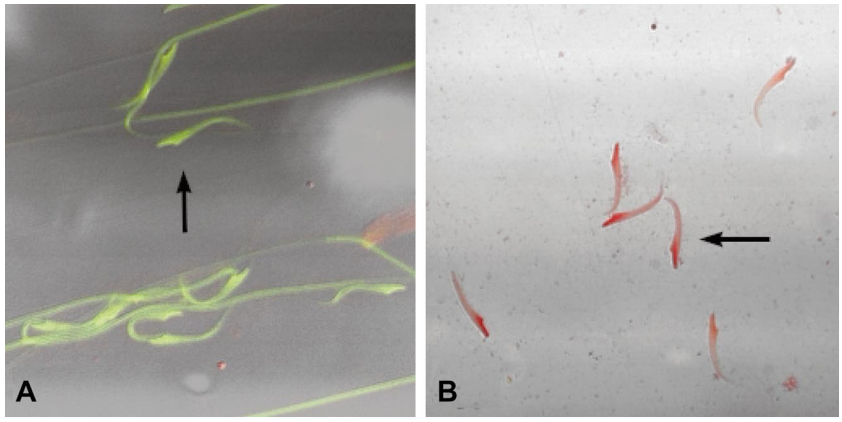

Figure 2. Effect of selenium deficiency on the sperm nuclei condensation in the rat. Spermatozoa from rats supplied with adequate amounts of selenium (*A*) and selenium-deficient animals (*B*) were collected from the vas deferens and stained with acridine orange. Acridine orange stains double-stranded DNA green and single-stranded red. As sperm DNA is resistant to acid denaturation only if the protamines are cross-linked by disulfides, the method allows chromatin condensation and the protamine disulfide status to be determined. Nearly all of the sperm nuclei in selenium-deficient rats showed abnormal condensation.

condensed sperm nucleus with a stable matrix is needed to ensure normal embryonic development. Thus, the snGPx-dependent protamine thiol oxidation appears to play a pivotal role in male fertility and reproduction.

When the spermatozoa reach the caput epididymis and the condensation process is completed, snGPx is partly processed into smaller proteins with the same enzymatic activity but with neutral pI values, indicating that the basic arginine-rich, amino-terminal sequence has been lost. We propose that snGPx and the processed proteins fulfill two functions: *1)* oxidation of protamines by the full-length DNA-bound snGPx and *2)* protection of sperm DNA against oxidative damage by this enzyme and the truncated forms which, as they are not bound to the DNA, might be more efficient in ROS degradation.

Several studies have shown that the quality of human sperm has been deteriorating, and it has been shown that one of the causes is an increase in oxidative DNA damage. Whereas limited generation of ROS is required for protamine thiol cross-linking, DNA damage due to excessive amounts of ROS affects the health of the offspring. The determination of the snGPx status of the sperm nuclei may therefore be of importance in sperm quality assessment.

It has been suggested that PHGPx plays a role in chromatin condensation and protection of sperm DNA against oxidative damage. However, PHGPx is expressed only in a cytosolic or mitochondrial form and in the spermatozoa was found to be mainly membrane bound. With the identification of snGPx as the only selenoprotein present in the spermatid nuclei and its characterization as a protamine thiol peroxidase (**Fig. 3**), this discrepancy has now been resolved.

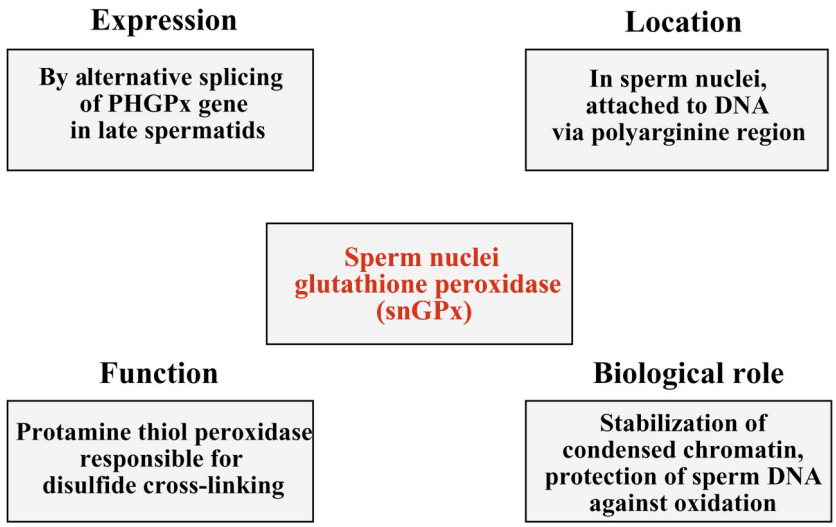

Figure 3. Schematic diagram of the characteristics and functions of the selenocysteine-containing sperm nuclei glutathione peroxidase.

Selenium is known to play a role in various processes in the male reproductive system. PHGPx has recently been shown to act as a structural component of the mitochondrial capsule and thus is needed in the formation of the flagellum, whereas in the nuclei, snGPx and its processed products are involved in chromatin condensation and protection of the germ line against oxidative damage. Selenium was also shown to be necessary for testosterone biosynthesis in a manner not yet identified. It will be of great interest to find out to what extent disturbances in the formation and function of selenoproteins contribute to the etiology of male fertility disorders. FJ