Forum Review

The Mammalian Testis-Specific Thioredoxin System

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ABSTRACT

Redox control of cell physiology is one of the most important regulatory mechanisms in all living organisms. The thioredoxin system, composed of thioredoxin and thioredoxin reductase, has emerged as a key player in cellular redox-mediated reactions. For many years, only one thioredoxin system had been described in higher organisms, ubiquitously expressed in the cytoplasm of eukaryotic cells. However, during the last decade, we and others have identified and characterized novel thioredoxin systems with unique properties, such as organelle-specific localization in mitochondria or endoplasmic reticulum, tissue-specific distribution mostly in the testis, and features novel for thioredoxins, such as microtubule-binding properties. In this review, we will focus on the mammalian testis-specific thioredoxin system that comprises three thioredoxins exclusively expressed in spermatids (named Sptrx-1, Sptrx-2, and Sptrx-3) and an additional thioredoxin highly expressed in testis, but also present in lung and other ciliated tissues (Txl-2). The implications of these findings in the context of male fertility and testicular cancer, as well as evolutionary aspects, will be discussed. *Antioxid. Redox Signal.* 6, 25–40.

THIOREDOXIN SYSTEMS AND THE HUMAN GENOME

THIOREDOXINS (Trx) are a family of proteins that are conserved in all organisms from lower prokaryotes to human and function as general protein disulfide reductases. The redox activity of thioredoxins resides in the sequence of their conserved active site Cys-Gly-Pro-Cys (CGPC), which undergoes reversible oxidation of the two cysteine residues from a dithiol to a disulfide form (4). The maintenance of thioredoxin in its active reduced form is carried out by the flavoenzyme thioredoxin reductase at the expense of the reducing power of NADPH, which forms the so-called thioredoxin system [as depicted in Fig. 1 (4)]. The functions assigned to the different thioredoxin systems rely mostly on their redox properties and are continuously increasing, practically covering all the different facets of cellular metabolism, as well as many diseases and pathological conditions. Readers are

referred to recent reviews on these issues, because it is beyond of the scope of this study to enumerate all of them (4, 62, 64, 74, 110). Traditionally, thioredoxins are monomeric proteins consisting of five central stranded β-sheets externally surrounded by four α-helices with the active site located in a protrusion of the protein between the β-2 strand and the α-2 helix, and this three-dimensional structure is conserved through evolution (23). In contrast, two different classes of thioredoxin reductases are found. Bacteria, yeasts, lower plants, and fungi have thioredoxin reductases that are homodimers composed of 35-kDa subunits, do not contain selenocysteine residues, and undergo a large conformational change in which, after reduction of the active-site disulfide by FAD, the NADPH domain must rotate with respect to the FAD domain to expose the nascent dithiol for reaction with thioredoxin (106). In higher eukaryotes, the thioredoxin reductases are homodimers of 55-kDa subunits arranged in a head-to-tail conformation (83) that contain selenocysteine as

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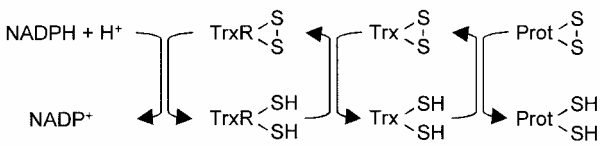
FIG. 1. Scheme of electron flow in the thioredoxin system. Electrons from NADPH are used to reduce thioredoxin reductase (TrxR), which in turn reduces thioredoxin (Trx). Reduced thioredoxin is able to reduce disulfide bonds in a wide array of protein substrates.

the penultimate residue (30). In this class of thioredoxin reductases, the electrons are transferred sequentially from NADPH and FAD to the active site of the same subunit and finally to the selenylsulfide of the other subunit (83). An exhaustive review in this issue, by A.-K. Rundlöf and E.S.J. Arnér, covers various aspects of thioredoxin reductases.

Thioredoxin and thioredoxin reductase were discovered in 1964 in *Escherichia coli* as the electron donor system for ribonucleotide reductase, an essential enzyme that converts ribonucleotides to deoxyribonucleotides (44, 59). Later, the same system was identified in yeast (31) and subsequently in practically all organisms investigated, including plants, which have additional thioredoxin systems involved in light energy transduction (8). However, it required almost 25 years to identify the human thioredoxin orthologue (107), which was found to be an adult-cell leukemia-derived factor responsible for interleukin-2 receptor induction (100). Soon after, a sec-

ond thioredoxin was identified in yeast (61), thus setting the basis for the concept of a thioredoxin family of proteins. Since then, and owing mostly to the advent of major genome sequencing projects, the thioredoxin family has expanded in numbers in all classes of organisms from bacteria to mammals. More importantly, one can further place thioredoxins into a superfamily that groups those families of proteins that share a common fold and a similar sequence of the active site CXXC, where the residues flanked by the two cysteines are changed from Gly-Pro in thioredoxins to various other combinations. Thus, in this superfamily, we find glutaredoxins (35), protein disulfide isomerase (63), nucleoredoxin (42), and thioredoxin-related transmembrane protein (52), for example.

The completion of the human genome sequence has fostered the identification of novel members of practically all protein families, including the thioredoxin family (43, 102). Considering the human thioredoxin systems as the model for mammalian organisms, there are eight different thioredoxins (assuming active sequence CGPC) and three thioredoxin reductases genes (Table 1). Apart from all the protein-coding thioredoxin genes, we have also identified seven pseudogenes scattered along several human chromosomes in an attempt to identify all the thioredoxin sequences in the human genome (91).

Based on protein domain organization, two distinct groups of thioredoxins can be distinguished (Fig. 2). Group I includes those proteins that exclusively code for one thioredoxin domain, whereas group II is composed of fusion proteins of thioredoxin domains with additional non-thioredoxin

TABLE 1.   CLASSIFICATION OF HUMAN THIOREDOXINS AND THIOREDOXIN REDUCTASES

Name	NCBI accession no.	Chromosomal localization	Size (kDa)	Tissue specificity	Subcellular localization	References
Thioredoxins						
Trx-1*	NP_003320	9q31	11.71	Ubiquitous	Mainly cytosolic, nuclear upon certain stimuli	107
Trx-2	NP_036605	22q13.1	11.87	Ubiquitous	Mitochondrial	21
Txl-1/Trp32	NP_004777	18q21.2	32.25	Ubiquitous	Cytosolic	45, 54
ERdj5/JPDI*	NP_061854	2p22.1–23.1	91.08	Ubiquitous	Endoplasmic reticulum	19, 36
Sptrx-1	NP_115619	18p11.2–11.31	53.27	Testis/spermatid	Sperm fibrous sheath	57
Sptrx-2	NP_057700	7p14.1	67.27	Testis/spermatid	Sperm fibrous sheath	81
Sptrx-3*	Not assigned	Not determined	14.57	Testis/spermatid	Golgi	Jiménez *et al.*, unpublished observations
Txl-2*	AAG28497	3q22.3–23	36.85	Ubiquitous, but highly prominent in testis and lung	Associated with microtubules in cilia and flagella	82
Thioredoxin reductases						
TrxR1*	NP_003321	12q23–24.1	54.71	Ubiquitous	Cytosolic	28
TrxR2*	NP_006431	22q11.21	53.06	Ubiquitous	Mitochondrial	29, 55, 93
TGR†	AAD51325	3p13–q13.33	63.63	Ubiquitous, but highly expressed in testis	Cytosolic	93

*Splicing variants have been reported.
†The starting methionine has not been determined.

FIG. 2. Domain organization of human thioredoxins. The proteins are drawn to scale based on the Trx-1 structure. Note that Txl-1/Trp32 and Sptrx-3 have the tryptophan residue at the active site changed by a glycine or arginine, respectively. MTS, mitochondrial targeting sequence; NDPk, NDP kinase. The names in black color denote the ubiquitous proteins, and those in gray the testis/spermatid-specific ones.
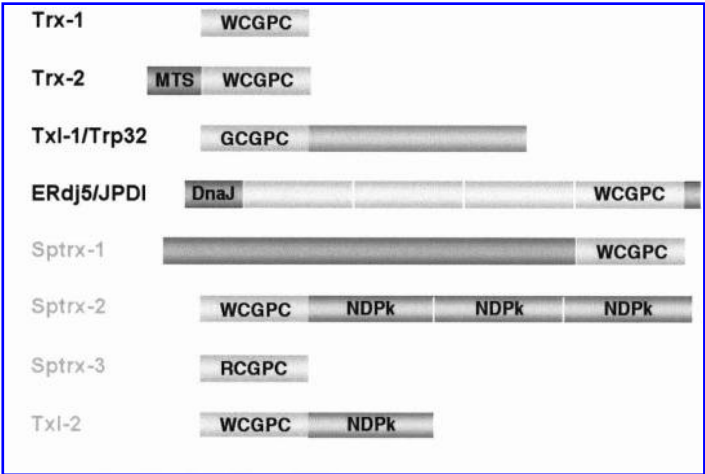
domains. Members of both groups can be found in all organisms investigated thus far (see Introduction of 82). In humans, group I consists of Trx-1, Trx-2 (21, 107), and spermatid/sperm specific thioredoxin 3 (Sptrx-3) (Jiménez *et al.*, unpublished observations), whereas group II of human thioredoxins comprises thioredoxin-like 1 (Txl-1)/Trp32, ERdj5/JPDI, Sptrx-1, Sptrx-2, and Txl-2 (19, 36, 45, 54, 57, 81, 82). Of these, ERdj5/JPDI, Sptrx-2, and Txl-2 are composed of two different known protein domains present in the same polypeptide. Thus, ERdj5/JPDI is composed of an N-terminal DnaJ domain followed by four thioredoxin-like domains, whereas Txl-2 and Sptrx-2 are fusion proteins of an N-terminal thioredoxin domain followed by one or three nucleoside diphosphate (NDP) kinase domains, respectively. Interestingly, the additional domains of Txl-1/Trp32 and Sptrx-1 are novel and have no other homology in humans. In the case of the human (mammalian) proteins, an additional classification of thioredoxins can be proposed based on their expression pattern: Trx-1, Trx-2, Txl-1/Trp32, and ERdj5/JPDI are ubiquitously expressed although they can be found in different subcellular compartments. In contrast, Sptrx-1, Sptrx-2, and Sptrx-3 are testis/germ cell/spermatid-specific proteins. Txl-2 can be placed as an intermediate between the two groups as its expression is not confined to one particular tissue, but is mainly represented in close association with microtubules within tissues with cilia and flagella such as seminiferous epithelium (spermatids) and lung airway epithelium (82).

Three forms of thioredoxin reductases are found in humans (Table 1): thioredoxin reductase 1 (TrxR-1) is found in the cytosol [although it has been speculated that it can also translocate into the nucleus to maintain Trx-1 reduced (28)], TrxR-2 is a mitochondrial enzyme (29, 55, 93), and thioredoxin glutathione reductase (TGR) is a fusion protein of an N-terminal glutaredoxin-like domain followed by a typical thioredoxin reductase domain. TGR can reduce thioredoxin, GSSG, and GSH-mixed disulfides *in vitro* (93, 94) (Fig. 3). Cytosolic TrxR-1 and mitochondrial TrxR-2 are ubiquitously expressed, whereas TGR is highly expressed in testis, in parallel with the three major locations for the previously described cytosolic, mitochondrial, or testis-specific thioredoxins. ERdj5/JPDI is the only member of the thioredoxin family for which no colocalizing reductase has been reported, sug-

gesting a different reaction mechanism due to the highly oxidizing environment in the endoplasmic reticulum.

THE TESTIS/SPERMATID-SPECIFIC THIOREDOXIN SYSTEM

Spermiogenesis and spermatozoa

The primary function of the spermatozoon is to provide the male pronucleus, and in many species the centrosome, for the fertilized egg (95). To achieve this objective, the mammalian spermatozoon has developed a highly specialized morphology that allows it to protect its DNA during migration to the fertilization site and to recognize, penetrate, fuse with, and activate the receptive ovum (20). Spermatogenesis is an extraordinarily intricate process of cellular differentiation that takes place in the seminiferous tubules of the testis, resulting in the production of spermatozoa. The male testis seminiferous tubule is composed of two cell types: Sertoli cells, which function mainly to create a favorable environment for the differentiation of the other cell type, the germ cells, which give rise to the mature spermatozoon (22). The
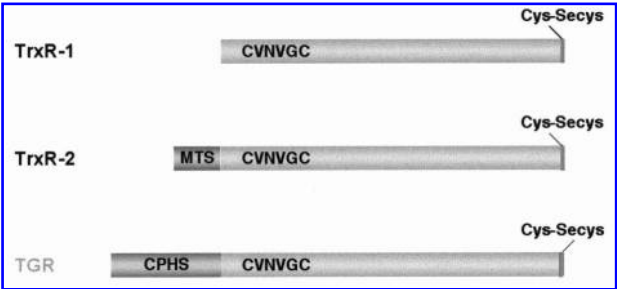
**FIG. 3. Domain organization of human thioredoxin reductase.** The proteins are drawn to scale based on the TrxR-1 structure. The active site CVNVGC, the C-terminal Cys-Secys pair, and the CPHS active site of the glutaredoxin-like module are indicated. MTS, mitochondrial targeting sequence. The names in black color indicate ubiquitous proteins, and that in gray color denotes highly expressed in testis.

primordial germ cells are the spermatogonia, which are diploid and located in close association with the basement membrane inside the seminiferous tubules, embedded between the Sertoli cells. Spermatogonia divide mitotically either to replenish themselves or to produce spermatocytes. Spermatocytes undergo meiosis and produce haploid cells named spermatids. During this meiotic division, random separation of homologous chromosomes and crossing over of genetic material occur, events that contribute to the diversity required for the survival of the species (7). Spermiogenesis is the final step of spermatogenesis and consists of a dramatic biochemical and morphological differentiation process in which the haploid round spermatid is transformed into the highly polarized spermatozoon, a process that takes ~22 days in humans. Spermiogenesis is a tightly synchronized process involving many physiological changes that are unique to this cell type. For a more detailed review, as well as diagrams on mammalian spermatogenesis, the reader is referred to Barratt (7) and de Kretser and Kerr (22).

The mammalian spermatozoon is organized into two major parts, the flagellum concerned with energy production and motility, and the head containing the paternal DNA and the structures required for ovum recognition, sperm-zona penetration, sperm-oolemma fusion, and activation (20, 103, 104). Each of these two elements has distinct structures required for the spermatozoon function. Thus, in the apical part of the head we find the acrosome, a cap-like vesicular formation that contains a number of proteolytic and glycolytic enzymes and holoenzymes necessary to assist the spermatozoon in its passage through the oocyte vestments (1, 103). Also in the head, the perinuclear theca is a condensed layer of selected cytoplasmic and nuclear proteins that is sandwiched between the nuclear envelope and the inner acrosomal membrane apically, and between the nuclear envelope and the plasma membrane caudally. It is assumed to play a pivotal role in acrosomal-nuclear docking and nuclear shaping during spermiogenesis, and in the sperm-oocyte interactions at fertilization (6, 99). As shown in Fig. 4, the sperm tail can be divided into four major regions: the connecting piece provides anchoring of the flagellum to the sperm head and houses the sperm proximal centriole in nonrodent mammals; the middle piece contains the helically wrapped mitochondria that supply the energy for flagellar movement; the principal piece is the longest portion of the tail; and finally, the end piece consists basically of a plasma membrane surrounding the microtubule doublets of the axoneme. The mammalian spermatozoa tail has also developed a series of cytoskeletal elements, namely the outer dense fibers (ODF) and the fibrous sheath (FS) designed to regulate motility and provide structural support to the sperm tail during its movement (67, 105). ODF surround the axoneme in the middle piece and principal piece of the sperm tail. In the middle piece, nine ODF associate with corresponding microtubule doublets of the axoneme. In the principal piece, ODF 3 and 8 are substituted by or converted into two longitudinal columns of the FS that are bridged by FS ribs and together surround the seven remaining ODF (24, 66).
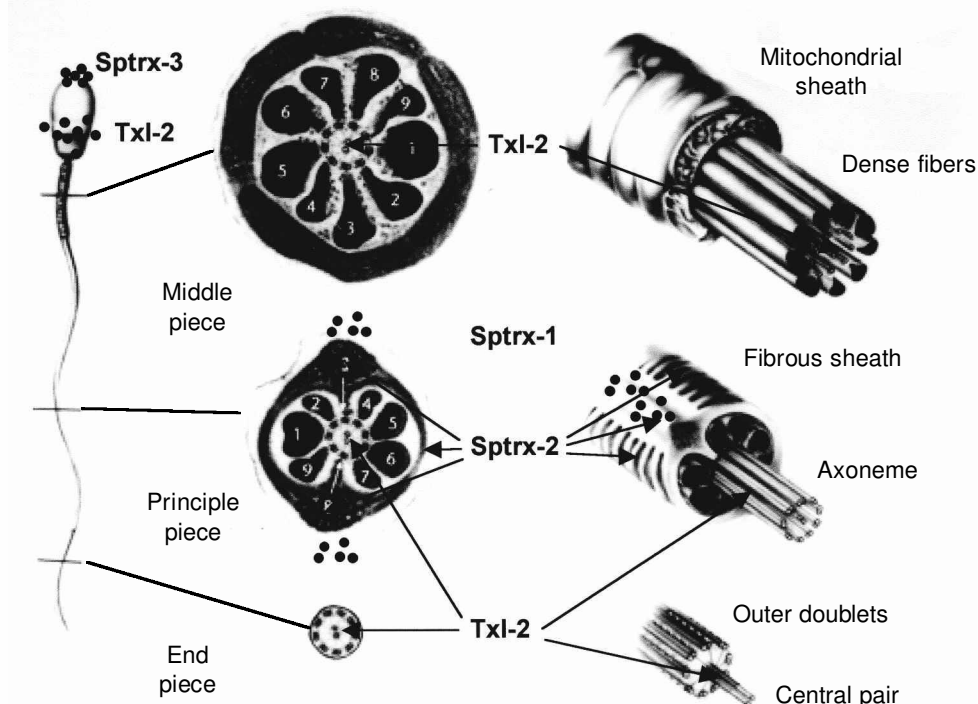
FIG. 4. Diagram of Sptrx-1, Sptrx-2, Sptrx-3, and Txl-2 localization in human spermatozoa. Sptrx-1 is represented by circles to indicate its transient association with the longitudinal columns of the FS. Sptrx-2 is a structural component of the mature FS in both the longitudinal columns and the ribs. Txl-2 is associated with the microtubules of the sperm axoneme and also in the transient manchette (represented by circles). Sptrx-3 is represented by circles to indicate its transient expression during acrosome biogenesis. Reprinted from reference 24, with permission from Elsevier.

Ubiquitous thioredoxin systems in testis

Before discussing on the individual spermatid-specific thioredoxins, it is very important to detail what is known about the ubiquitous thioredoxin systems in testis. The expression of the cytosolic thioredoxin system has been examined in rat testis by immunocytochemistry, and it was found that Trx-1 is highly expressed in all Leydig cells, but only in a small fraction of spermatogonia (10–15%) and very little in Sertoli cells. The more differentiated germ cells, such as spermatocytes or spermatids, do not express it (33, 80). Surprisingly, TrxR-1 expression was found mainly in those cell types negative for Trx-1, namely Sertoli cells, spermatocytes, and spermatids (80). This result is somehow intriguing, as it was expected that both proteins should colocalize to constitute an active thioredoxin system, and prompted us to attempt a more thorough developmental analysis of the expression of these two proteins during spermatogenesis. Our preliminary data indicate that both Trx-1 and TrxR-1 are found mostly in the cytoplasm and tail of elongating spermatids, a more consistent localization as expected for a coordinated system (Oko et al., unpublished observations).

The mitochondrial thioredoxin system in testis has been studied by northern analysis showing a relatively high level of both Trx-2 and TrxR-2 as expected for a high-metabolic rate tissue (21, 55, 56, 90). Immunolocalization data are only available for Trx-2 where it is predominantly found in the germ line (21). Finally, both Txl-1/Trp32 and ERdj5/JPDI mRNA levels have been found to be moderate to high in testis, but no immunolocalization in this tissue has been reported (19, 36, 45, 54).

Spermatid-specific thioredoxins

During the last few years, our group has characterized a number of thioredoxins encoded by the human genome. One of the most exciting results of this quest has been the discovery of a group of thioredoxins with a tissue-specific expression in spermatids and mature spermatozoa, the reason for which they have been named Sptrx-1, Sptrx-2, and Sptrx-3 (for sperm thioredoxins) (57, 81, and Jiménez et al., unpublished observations). In addition, we have recently reported another member of this family, Txl-2, a microtubule binding protein highly expressed not only in sperm manchette and axoneme, but also in other ciliated tissues (82). These novel thioredoxins were identified by in silico search for human expressed sequence tags (EST) with the conserved active site CGPC (Fig. 2).

The first identified spermatid-specific thioredoxin was human Sptrx-1, a polypeptide of 486 amino acids organized into two distinct domains: an N-terminal domain consisting of 23 repeats of a 15-residue motif and a C-terminal domain typical of thioredoxins (57). Mouse and rat Sptrx-1 orthologues have an identical structure to that of human Sptrx-1. The N-terminal repetitive domain has no homology with any other protein in the databases and is predicted to organize as a coiled-coil protein (38). Human Sptrx-1 elutes as a 400-kDa protein in gel filtration chromatography, consistent with either an oligomeric form and/or a highly asymmetrical structure. Furthermore, Sptrx-1 behaves as both reductant and oxi-

dant in vitro, and crystallization/circular dichroism experiments indicate that the N-terminal repetitive domain of Sptrx-1 is largely unstructured and labile (37, 57). By northern blot and in situ hybridization, we have found that Sptrx-1 mRNA is exclusively expressed in testis, with transcripts localized to round and elongating spermatids. Consistent with the mRNA expression, Sptrx-1 protein is synthesized within elongating spermatids, localized in close association with the assembling longitudinal columns of the FS, but not the ribs that connect these two columns, during tail elongation (Figs. 5 and 6) (37, 111). This particular expression pattern, to our knowledge not described for any other protein so far, strongly supports the possibility that Sptrx-1 could be a part of a nucleation center for the formation of the longitudinal columns and the transverse ribs of the FS (111). The spermiogenesis process in general, and the sperm tail formation in particular, are characterized by a progressive increase of disulfide bonding, which starts at the spermatid stage and continues during epididymal transit (9, 12, 87). Together with its unique spatial and temporal expression pattern in the tail of elongating spermatids, the dual reducing/oxidizing activity of Sptrx-1 in vitro suggests that Sptrx-1 could indeed participate in the regulation of FS assembly by supporting the formation of disulfide bonds during sperm tail morphogenesis. In addition, its reducing activity might be required to rectify incorrect disulfide pairing and generate suitable pairs between the different FS constituents (37). Finally, the reduction of the disulfide bonds in the flagella of abnormal spermatozoa could serve as a quality control signal for their marking and elimination during epididymal passage (98).

Human Sptrx-2 is a 588-amino acid protein that is also organized in two different domains, which have been characterized in more detail than those of Sptrx-1. Thus, Sptrx-2 consists of an N-terminal thioredoxin domain followed by three repeats of NDP kinase domain, the first of which is not complete (81). Mouse and rat orthologues have also been cloned, which share similar organization (57a). NDP kinases (also known as nm23) constitute another well known family of structurally and functionally conserved proteins identified across a wide range of species from bacteria to human. NDP kinases catalyze the transfer of γ-phosphates between nucleosides and deoxynucleoside di- and triphosphates, playing a pivotal role in maintaining a balanced pool of nucleotides. In addition to the kinase function, nm23 proteins have been implicated in cell growth, cancer progression, and development (34, 49, 73). Northern blot and in situ hybridization show that Sptrx-2 mRNA, similar to Sptrx-1 mRNA, is only expressed in testis at the round and elongating spermatid stages (57a, 81). Although recombinant human Sptrx-2 expressed in bacteria can be easily purified, it failed to show any enzymatic activity as a thioredoxin using NADPH and thioredoxin reductase, or dithiothreitol as electron donors. Furthermore, no NDP kinase activity was detected (A. Karlsson, personal communication). By light immunocytochemistry and immunofluorescence analyses, we detected Sptrx-2 protein in the principal piece of the spermatid and spermatozoon flagellum, suggesting that Sptrx-2 is a structural component of the FS. We confirmed this observation by immunogold-electron microscopy in rat seminiferous tubules where Sptrx-2 was found in both the longitudinal columns and the ribs of the FS (57a). A detailed
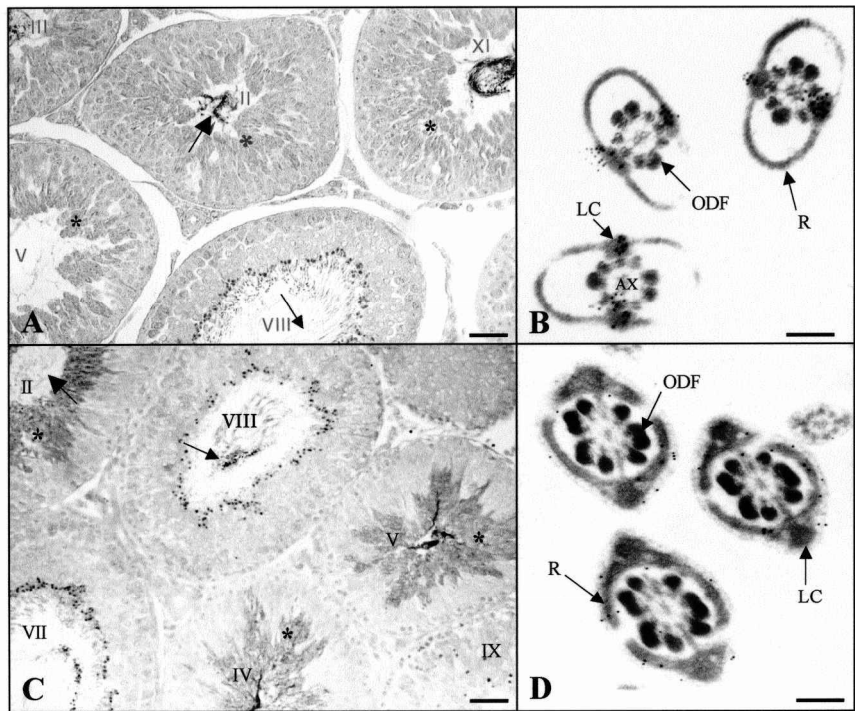
FIG. 5.   Developmental expression of Sptrx-1 (A and B) and Sptrx-2 (C and D). In comparing immunoperoxidase-stained testicular sections A and C, note that the tails of step 16 spermatids (large arrows) in stage II of the cycle are strongly immunoreactive for Sptrx-1, but not for Sptrx-2. However, as elongated spermatids progressively mature from steps 16 (stage II) to 19 (stage VIII), Sptrx-1 immunoreactivity in the tails gradually disappears, whereas Sptrx-2 reactivity strengthens (compare small arrows in sections A and C). Thus, at the time of spermiation (stage VIII), only Sptrx-2 remains in the tail as an integral component of the FS. In comparing immunogold-labeled tail cross-sections in B and D, note that Sptrx-1 only transiently associates with the longitudinal columns (LC) of the FS (as seen here in step 15 spermatids), whereas Sptrx-2 becomes an integral component of both the LC and ribs (R) of the FS (as seen in step 19 spermatids). AX, axoneme; ODF, outer dense fibers. Roman numerals denote the stages of the cycle of the rat seminiferous epithelium; asterisks point to the outermost layer of elongated spermatids closest to the tubular lumen. Bars in A and C, 20 μm; bars in B and D, 0.2 μm.

developmental analysis of both Sptrx-1 and Sptrx-2 expression detected differences in their respective patterns. Whereas Sptrx-1 expression peaks at steps 14–16 of the rat spermiogenesis cycle, Sptrx-2 is incorporated into the FS at a later stage, peaking from step 15 to 19 (57a, 111). Yet another important difference between these two proteins is that whereas Sptrx-1 appears to be required during FS assembly but not in fully differentiated mature sperm FS, Sptrx-2 remains a structural component of the mature FS and can be detected in cauda epididymal or ejaculated spermatozoa. Given this pattern, Sptrx-2 may be necessary for the posttesticular events such as epididymal sperm maturation, hyperactivation/capacitation, or even fertilization and zygotic development. In this regard, we have shown previously that the sperm tail FS, where Sptrx-2 resides, is one of the first sperm structures degraded in the zygotic cytoplasm at fertilization. The solubilization of FS precedes the degradation of paternal mitochondria and ODF, and coincides with the early stages of male pronuclear development (96). Extensive studies have demonstrated the requirement of disulfide bond reduction for the successful processing of the sperm nucleus and sperm accessory structures during mammalian fertilization (95), and Sptrx-2 may be involved in regulating this process. Furthermore, although we could not detect any kinase activity in recombinant Sptrx-2, its well conserved NDP kinase domain supports the idea that Sptrx-2 could be a phosphate donor for the phosphorylation of other FS proteins. Phosphorylation is the major regulatory mechanism in spermatozoa and underlies important processes during the acquisition of fertilizing capability by the spermatozoon, such as capacitation and hyperactivation (25, 60, 109).

Our continuous search for novel members of the thioredoxin family yielded a close homologue of Sptrx-2, which we later named Txl-2. Txl-2 has similar yet distinct domain organization to that of Sptrx-2, namely an N-terminal thioredoxin domain followed by one NDP kinase domain (82) instead of three as in Sptrx-2. While cloning human Txl-2 from a testis library, we found that in addition to a full-length variant coding for a 330-amino acid residue, an alternative splicing variant lacking exon number five was present, resulting in a shorter protein of 291 amino acid residues. Due to the striking similarity in both sequence and domain organization and the fact that we cloned Txl-2 from a testis cDNA library, we initially assumed Txl-2 to be a novel testis-specific protein. However, mRNA analysis indicated that indeed Txl-2 should be placed between the ubiquitous and the tissue-specific
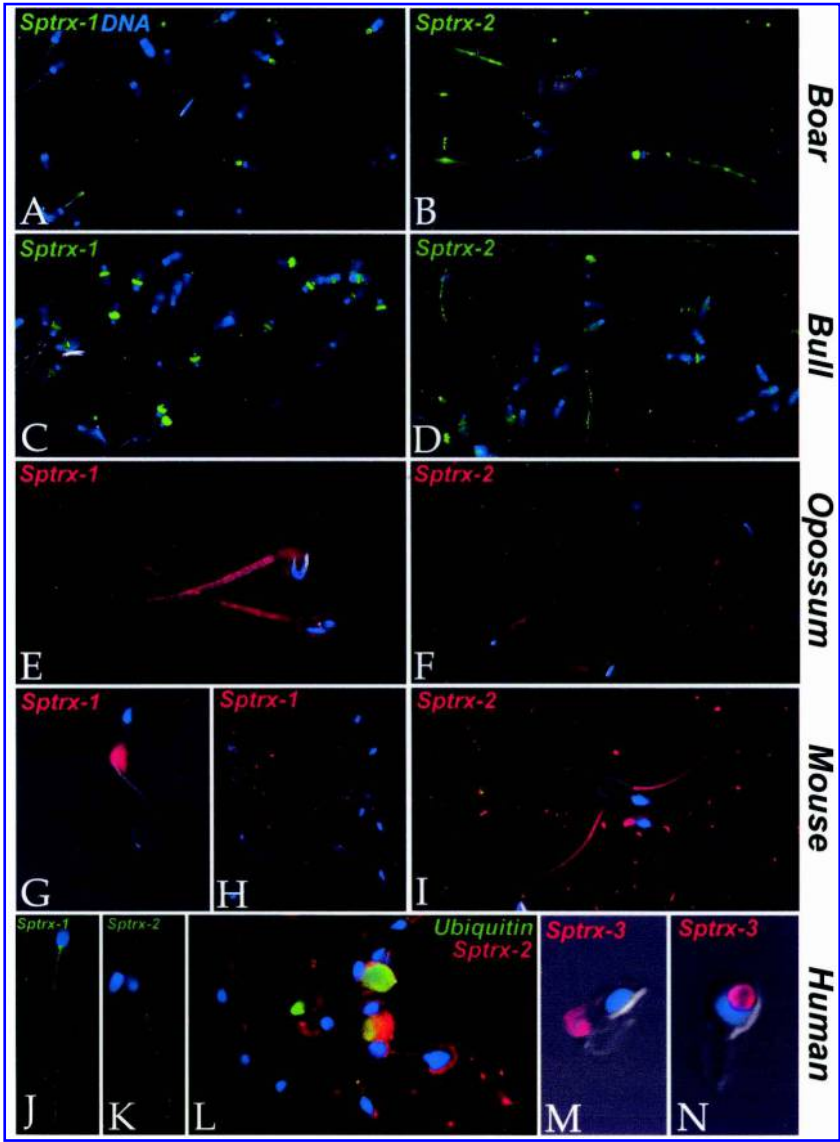
FIG. 6.   Evolutionary comparison of sperm thioredoxins in the spermatozoa of nonruminant (A and B; domestic boar *Sus scrofa*) **and ruminant (C and D; domestic bull** *Bos taurus*) **ungulates, marsupials (E and F; opossum** *Didelphis virgini-* *ana*) **rodents (G–I; domestic mouse** *Mus musculus*), **and primates (J–N; human).** Ejaculated boar spermatozoa express Sptrx-1 in cytoplasmic droplets (A) and Sptrx-2 in the FS of the sperm tail principal piece (B). In ejaculated bull spermatozoa, both Sptrx-1 (C) and Sptrx-2 (D) are present in the postacrosomal sheath of the sperm heads, whereas Sptrx-2 (D) is also seen in the FS. In opossum, both Sptrx1 (E) and Sptrx2 (F) are present in the FS, whereas some Sptrx-1 immunoreactivity can also be seen in the residual cytoplasm surrounding the midpiece and connecting piece (E). In mouse testicular spermatozoa (G), Sptrx-1 can be found in the residual bodies and cytoplasmic droplets, and the remnants of immunoreactive protein are also seen in the cytoplas-mic droplets of epididymal spermatozoa (H). Sptrx-2 in the epididymal mouse spermatozoa is restricted mainly to FS (I), whereas some residual immunoreactivity can be detected in the acrosome. Human spermatozoa express both Sptrx-1 (J) and Sptrx-2 (K) in sperm tail principal piece, as well as in the midpiece. In the semen samples of teratospermic, infertile men (L–N), Sptrx-2 (L; red) colocalizes prominently with proteolytic-degradation chaperone ubiquitin (L; green) in both sperm head and flagellum, whereas Sptrx-3 is found mainly in the redundant cytoplasm (M) and trapped inside the nuclear vacuoles (N).

thioredoxins. When attempting to determine the size and tis-sue distribution of Txl-2 mRNA by northern blot analysis, we consistently failed to detect any signal despite the use of low stringency conditions, extended time of exposure, or use of different probes, suggesting that Txl-2 mRNA might be ex-pressed at very low levels. To improve the sensitivity, we used

real-time PCR, which confirmed that expression of Txl-2 mRNA in adult tissues is very low with highest levels found in testis and lung and lower levels in a variety of additional tissues (82). In clear parallel with Sptrx-2, we were unable to detect any thioredoxin or kinase enzymatic activity of recom-binant Txl-2 expressed in bacteria. This lack of activity de-

spite the presence of two well characterized enzymatic domains in both proteins raises the possibility that posttranslational modifications or interaction with other proteins or cofactors might be required for their function. Specific antibodies against recombinant human Txl-2 detected Txl-2 in close association with the cilia of the lung airway epithelium and the microtubule-based spermatid manchette and axoneme (82) (Fig. 7). This particular localization strongly suggested that Txl-2 was a microtubule-binding protein. To prove this point, we performed *in vitro* microtubule binding assays using recombinant full-length and Δ5Txl-2 splicing variant. The result clearly demonstrated that the Δ5Txl-2 variant binds microtubules with very high affinity, whereas full-length Txl-2 binding is weak (82). This is the first report of a member of the thioredoxin family with a microtubule-binding activity. However, there are reports of other members of the NDP kinase family with such an activity (48, 72, 79). Consistent with this result, we have also identified Txl-2 in the cilia of the brain ependymal cells (Pelto-Huikko and Miranda-Vizuete, unpublished observations).

The subcellular localization of Txl-2 in spermatid manchette deserves further examination. The manchette is a transient microtubule-based structure that caudally surrounds the spermatid nucleus (58). The manchette recently has been proposed to be a transient storage location for both signaling proteins involved in nucleocytoplasmatic trafficking and structural proteins that are eventually sorted to the centrosome and the developing spermatid tail (40, 41). However, in this case, Txl-2 seems to associate with the spermatid manchette and axoneme simultaneously during spermatid elongation (82).

Although we still do not know the function of Txl-2, its localization in close association with microtubules of cilia and flagella suggests that Txl-2 could control microtubule stability and maintenance. Its putative disulfide reducing activity, by virtue of the thioredoxin domain, might regulate ligand interactions and microtubule assembly as it has been reported that cysteine residues in tubulin are critical for those events (13). More importantly, *Chlamydomonas* flagellar protein p72 and sea urchin sperm axoneme protein IC1 have been reported to have NDP kinase activity and suggested to be the suppliers of GTP for microtubule assembly (65, 70). Thus, it is conceivable that Txl-2 plays this role in the formation, functioning, and maintenance of mammalian axonemes.

Finally, we have very recently identified a third testis-specific member of the thioredoxin family, Sptrx-3 (Fig. 2). By *in situ* hybridization, Sptrx-3 mRNA displays an expression pattern similar to Sptrx-1 and Sptrx-2 mRNA, mainly present in round and early elongating spermatids (Jiménez *et al.*, unpublished observations). Sptrx-3 differs from the other testis/spermatid-specific proteins in that Sptrx-3 is composed of only one thioredoxin domain. In addition, multiple splicing variants have been identified. The fact that its mRNA is expressed at the spermatid level indicates that the protein is most probably required in the later steps of spermiogenesis or in the mature spermatozoa. This localization is consistent with our more recent data on immunolocalization of Sptrx-3 in the developing acrosome and Golgi (Oko *et al.,* unpublished observations). A scheme showing the localization of
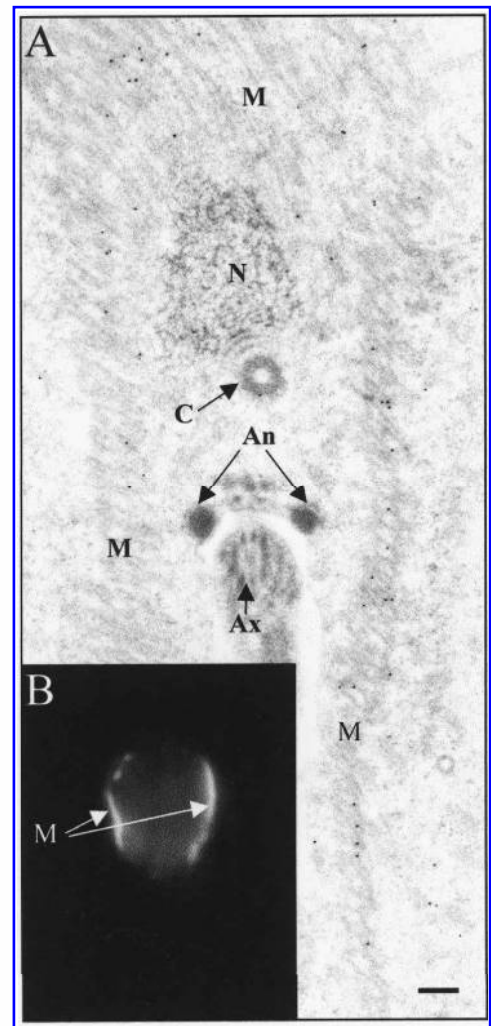
FIG. 7. Txl-2 associates with the microtubules of the manchette. Nuclear condensation and elongation of spermatids are concomitant with the formation of a microtubular girdle, the manchette, which surrounds the caudal half of the nucleus and reaches deep into the distal cytoplasm. It most likely is involved in sperm head shaping and protein transport. **(A)** Immunogold labeling of the manchette (M). N, nucleus; C, proximal centriole; An, annulus; Ax, axoneme. Bar = 0.2 μm. **(B)** Immunofluorescent colocalization of Txl-2 and tubulin on the manchette of an early elongating spermatid.

the different thioredoxins in spermatozoa is depicted in Fig. 4. Perinuclear and Golgi localization of Sptrx-3 during spermiogenesis is consistent with immunodetection of Sptrx-3 protein in the redundant cytoplasm and nuclear vacuoles of infertile men's semen samples (Fig. 6), suggestive of incomplete spermatogenesis and retention of cytoplasmic droplets and residual bodies.

Spermatid-specific thioredoxin reductases

The unexpected finding of several spermatid-specific thioredoxins immediately raised the question of whether additional thioredoxin reductases might also exist in testis to

maintain the different thioredoxins in their reduced form. The human genome contains only three thioredoxin reductases genes (Table 1): TrxR-1, TrxR-2, and TGR (28, 29, 55, 93, 94) (Fig. 3). Of these, TrxR-1 and TGR are of particular interest regarding testis-specific thioredoxin activity.

TrxR-1, a cytosolic enzyme, is expressed highly in Sertoli cells, spermatocytes, and spermatids, cells that express little or no Trx-1 (33, 80). This lack of colocalization suggests that reductase substrates other than Trx-1 might exist in these cells, and the spermatid-specific thioredoxins are obvious candidates. However, using recombinant protein expressed in bacteria, only Sptrx-1 (57), but not Sptrx-2 and Txl-2 (81, 82), is a substrate for TrxR-1. As mentioned above, the lack of activity of both proteins might be due to the requirement of additional cofactors or interacting proteins not present in the *in vitro* assay, or to posttranslational modifications that are not achieved when the proteins are expressed in bacteria. To investigate in more detail the complexity of the different thioredoxin systems in testis, we have initiated a developmental study of Trx-1 and TrxR-1 during spermatogenesis.

TGR deserves special attention in the context of testis and spermatogenesis. TGR was initially identified in human and mouse databases in the course of a genomic screening for novel thioredoxin reductases (93). Evaluation of TGR mRNA expression pattern by northern blot analysis indicated a prominent expression in testis that has been confirmed at the protein level using specific antibodies (93). A subsequent cloning of the full-length mouse cDNA showed that TGR is composed of two defined domains: The N-terminal domain is similar to that of glutaredoxins, although the active site contains only one cysteine residue, and the C-terminal domain is typical of thioredoxin reductases, including the SeCys insertion as penultimate residue (Fig. 3). Studies using mouse TGR protein confirmed that both domains are enzymatically active, and TGR is able to reduce not only Trx-1, but also GSSG and GSH-linked disulfides *in vitro*, providing specificity for both the thioredoxin and the glutathione system, and broadening even more the substrate specificity shown by thioredoxin reductases (94). Recent data indicate that TGR is expressed mostly in spermatocytes and elongating spermatids (V. Gladyshev, personal communication), strongly supporting its role as reductant for the spermatid-specific thioredoxins, a possibility that we are currently exploring.

EVOLUTIONARY ASPECTS

The striking finding that out of eight "classical" thioredoxins in the human genome half are either highly expressed in testis or spermatid-specific invites speculations about why, from an evolutionary point of view, it was necessary for these proteins to arise in the male reproductive tract. No definitive answer to this question can be given, but an overview of the biochemical mechanisms that underlie the formation and maturation of the male gamete, as well as comparative genomics, can be of some help finding plausible explanations.

A phylogenetic analysis of the eight human thioredoxins clearly positions the spermatid-thioredoxins into two clusters

(Fig. 8): Sptrx-2 and Txl-2 are in the same branch, which indicates that could have arisen as consequence of a genomic duplication event from a common ancestor as the intron/exon organization of their respective genes (at least in the thioredoxin domain) is identical (81, 82). The acquisition of thioredoxin and NDP kinase domains in the same polypeptide is a relatively recent event in evolution as the first organisms to present such a protein are sea urchins (echinoderms) and ascidian *Ciona intestinalis* (tunicate) (65, 69). On the other hand, Sptrx-1 and Sptrx-3 are clustered in another branch together with Trx-1. Analysis of the genomic organization of these three genes is conclusive in determining their common origin. The Sptrx-1 open reading frame does not contain introns (38, 57), indicating that it originated as a retrotransposition of the Trx-1 gene. Earlier, Trx-1 ancestor also underwent a genomic duplication that gave rise to Sptrx-3, retaining identical genomic organization to that of human Trx-1 (Jiménez *et al.*, unpublished observations). The remaining members of the family are derived from ancestors already present in lower eukaryotes, such as *Drosophila melanogaster, Caenorhabditis elegans*, and *Saccharomyces cerevisiae* (19, 53, 71). Thus, the phylogenetic analysis supports a late but rapid evolution of the thioredoxin family that can be traced to the emergence of a higher complexity of the internal fertilization process and a requirement for the spermatozoa to acquire additional cytoskeletal structures.

One might wonder why tissue specificity of thioredoxins occurs only in testis, but not any other organ or tissue, including the ovary. A likely explanation might be the evolution of
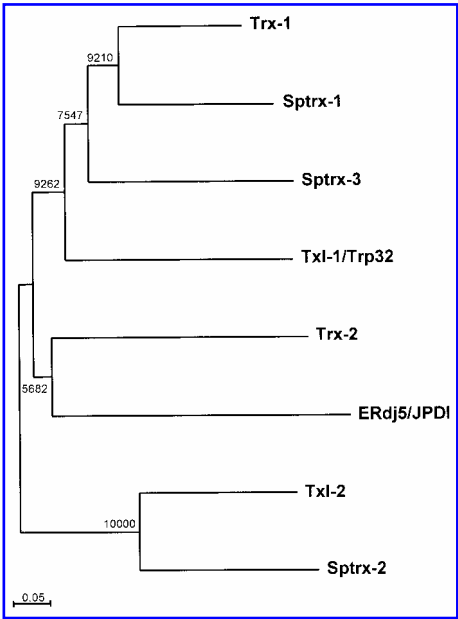
FIG. 8. Unrooted phylogenetic tree of the deduced amino acid sequences of all human thioredoxins. The alignment of the sequences was performed using the ClustalX program, and the tree was generated with 10,000 bootstrap trials. The bootstrap values are indicated at the nodes. All the sequences were obtained from the NCBI database except that of Sptrx-3 (Jiménez *et al.,* unpublished results).

the fertilization process, which has made it increasingly difficult for the spermatozoon to reach the oocyte, as a consequence of internal fertilization and functional adaptations of the oocyte vestments (20, 104). To overcome these difficulties, higher vertebrates evolved the spermiogenesis mechanism, which is basically a metamorphosis in which a somatic cell-like germ cell (haploid round spermatid) is converted into a highly specialized and differentiated gamete (spermatozoon) in an orchestrated and complex sequence of events that involves morphological, physiological, and biochemical changes (7). These changes are unique events that do not happen in any other cell and can be grouped into (a) formation of the acrosome and sperm head skeleton, (b) nuclear condensation, (c) development of the flagellum and its accessory structures, and (d) reorganization/reduction of the cytoplasm and cellular organelles (22). It is therefore reasonable to speculate that spermatid-specific thioredoxins were acquired through evolution to accomplish the above-mentioned changes required for spermiogenesis to take place. Supporting this hypothesis is the fact that no orthologues of any spermatid-specific thioredoxins or Txl-2 are present in the genome of lower eukaryotes, such as nematodes (*C. elegans*) or insects (*Drosophila*), phyla whose spermatozoa are much simpler. In contrast, spermatid-specific thioredoxins are ubiquitously expressed in spermatozoa of mammals, ranging from marsupials and rodents to ungulates and primates (Fig. 6). Marsupials and humans are the only studied species in which both Sptrx-1 and Sptrx-2 can be detected in the flagellum (Fig. 6). The first orthologues we find in the evolutionary scale are IC1 proteins of sea urchin and the ascidian *Ciona intestinalis* (65, 69), which have identical domain organization to that of Sptrx-2 and are components of the dynein machinery of the sperm axoneme. Interestingly, Sptrx-2 is not associated with the axoneme, but with the FS of the mammalian spermatozoa. Instead, Txl-2 with its microtubule-binding capacity is found in the sperm axoneme. Thus, it seems that the association of thioredoxin and kinase domains is required for the function of the sperm axoneme and most likely necessary to supply energy and modulate the microtubule thread-milling. The role of Sptrx-2 in the FS is still unknown. As nonmammalian orthologues have not been identified for Sptrx-1 or Sptrx-3, studies to determine the testis-specific role of these proteins in mammals are under way.

THE TESTIS/SPERMATID THIOREDOXIN SYSTEM IN MALE REPRODUCTIVE DISORDERS

There are two major pathological situations resulting as a consequence of failure or malfunctioning of male germ cells: infertility and testicular cancer. Sadly, human infertility is a fairly common condition that, by various estimates, affects 15–20% of couples, with approximately equal contribution from both partners (18). Historically, failures related to germ-cell formation have been studied more intensively in males than in females (68). One reason of this bias is the fact that the production of germ cells from the undifferentiated spermatogonia to the mature spermatozoa occurs during adult-

hood, whereas the production of the oocyte takes place during fetal development. In addition, the extracorporal position of the testis and external gamete discharge during ejaculation make studies of gametogenesis in male more feasible than in female. Many environmental, behavioral, and genetic factors affect male infertility, and it has been estimated that the genetic factor accounts for up to 60% of the causes underlying this phenotype, mostly due to autosomal-recessive genes (46). A reduction in sperm count and infertility has been found to be associated with an increased rate of chromosomal abnormalities (101). The rate of these abnormalities spans from 4.1% in men with oligozoospermia to 15.4% in azoospermic men. The causes can be diverse and might be related to failure of chromosome pairing and crossing-over in meiosis, as well as chromosomal break points in genes important for testicular development and function (68). Furthermore, saturation mutagenesis studies carried out in *Drosophila* indicate that the combined effect of many genes is likely to contribute in a much higher proportion to defects of spermatogenesis than effect of single genes (32).

Despite the fact that the Y chromosome has acquired a large number of testis-specific genes during recent evolution (89), none of the spermatid-specific thioredoxins or Txl-2 genes are localized in this chromosome (Table 1). Although we do not have evidence yet that any of the spermatid-specific thioredoxins are involved in male infertility phenotypes as a consequence of chromosomal abnormalities, their expression pattern supports a potential role in this pathology.

Among the pathologies affecting spermatozoa, dysplasia of the fibrous sheath (DFS) is an anomaly found in spermatozoa of severe asthenozoospermic patients characterized by a marked hypertrophy and hyperplasia of the FS (76, 77). In addition to causing an abnormal configuration of FS, DFS affects various cytoskeletal components, including axonemal microtubule doublets, ODF, and the mitochondrial sheath (15). As mentioned above, Sptrx-1 and Sptrx-2 are two components of the sperm tail FS, and Txl-2 is present in the transient spermatid manchette and in the tail axoneme. The localization of these thioredoxins in the spermatid tail makes them potential candidates to be involved in the development of DFS as it has been reported that a strong genetic component underlies this pathology (14). It will be very interesting to ascertain whether any mutation, polymorphism, or other genetic anomaly affecting any of the genes coding for the spermatid thioredoxins is correlated with DFS.

Primary ciliary dyskinesia (PCD), also known as immotile cilia syndrome (ICS), is a disorder affecting ciliary movement with an incidence of 1 in 20,000–30,000. Genetic studies demonstrate an extensive locus heterogeneity of this trait where the majority of affected families transmit PCD as an autosomal recessive disease (11). In PCD patients, cilia and sperm flagella demonstrate reduced motility due to diverse molecular pathologies often involving the dynein arms of axonemal microtubule doublets, resulting in chronic respiratory problems, dextrocardia and *situs inversus*, hydrocephalus, and male infertility (11). Indeed, DFS has also been considered a variant of PCD as the absence of dynein arms in axonemes is a common symptom (16). The FS location of Sptrx-1 and Sptrx-2 and its potential involvement in DFS make it possible, although less likely, that they participate in

PCD. However, Txl-2 is a serious candidate gene for PCD due to its axonemal localization and its microtubule-binding activity (82).

Sperm-flagellar pathology is often associated with the retention of redundant cytoplasm that would otherwise be rejected as residual body and cytoplasmic droplet during the final stages of spermiogenesis. This is well illustrated by the association of Sptrx-3 with the redundant cytoplasm and nuclear vacuoles in sperm from teratospermic infertility patients (Fig. 6; Sutovsky and Miranda-Vizuete, unpublished observations).

Another anomaly affecting male reproductive function is the development of autoimmune antibodies to spermatozoa (antisperm antibodies). Being associated with the impaired sperm function at various stages of the reproductive process, autoimmune disease is another important cause of male infertility (17). There are two types of antisperm antibodies: those induced as a consequence of obstruction of the male reproductive tract by disease, trauma, or surgical procedures such as vasectomy (also denominated sperm autoantibodies) (26, 27), and those produced by the female partner, interfering with the normal transit through the female reproductive tract and sperm–oocyte recognition (17). The production of male sperm autoantibodies is assumed to be a consequence of stimulation of the immune response when the spermatozoa or their components are no longer sequestered behind the blood–testis and blood–epididymal barriers. The male duct system suffers frequent ruptures after obstruction, forming spermatic granulomas in which sperm come in contact with macrophages, lymphocytes, and other immune cells (27). Not much is known about the causes by which the female body induces the production of antisperm antibodies. Most often, it is assumed that the immune response is mounted as a consequence of previous exposure to sperm antigens when the female mechanisms that normally tolerate the haploid "foreign" spermatozoa do not function properly or become hypersensitive (88). Other explanations have been considered, such as molecular mimicry by which epitopes from invading pathogens bear similarity to those of spermatozoa, thereby leading to antibody cross-reaction (17).

As sperm tail ODF have been reported to be the dominant postobstructive autoantigens (27), we wondered whether any of the spermatid-specific thioredoxins (which are located in the FS of the spermatid tail externally surrounding ODF) can also be categorized in this group. This approach has resulted in identifying Sptrx-2 as a novel sperm autoantigen, whereas antibodies recognizing Sptrx-1 or Txl-2 were not detected in the postvasectomy rat sera (57a). This result indicates that not only ODF, but also some FS proteins, are able to elicit sperm autoantigens and therefore should be taken into consideration when screening for novel components of the postobstructive autoimmune response.

The other major pathology affecting the male reproductive system is testicular germ cell tumors (TGCTs), which are not a direct cause of male infertility, but their treatment and management can impair or eradicate spermatogenesis. Although germ-cell tumors are rare in the general male population as a whole, accounting for <1% of all cancers, they are the most common malignancy in young adult Caucasian males. They are found mainly during the third to the fourth decade of life

with an incidence of 6 to 11 per 100,000, and there is a continuous increasing trend (10, 51). TGCTs are classified into three groups by epidemiological, clinical, and histological studies: (a) teratomas and yolk sac tumors, which always manifest before puberty, (b) seminomas and nonseminomas that appear after puberty, and (c) spermatocytic seminomas, which usually appear in elderly men (50). Seminomas and nonseminomas account for the vast majority of the TGCTs, whereas yolk sac tumors, teratomas, and spermatocytic seminomas are rare. It is now generally agreed that both seminomas and nonseminomas originate from carcinoma *in situ* (CIS), whereas the origins for the other two, rare types of testicular tumors are still not clear, but definitively not CIS (78). CIS cells are localized within the seminiferous tubules between the basal membrane and the Sertoli cell layer and resemble early primordial germ cells. It is assumed that the initiating event leading to the development of CIS originates during intrauterine development (39, 78). TGCTs are uniquely sensitive to cisplatin-based chemotherapy with >90% of newly diagnosed cases cured (75). This property makes the TGCTs an ideal system to study cell death pathways and their relevance to the treatment of other types of cancer (75). The major cytotoxic effect of cisplatin is generally attributed to the formation of DNA–platinum adducts, which cause cell cycle arrest and trigger apoptosis (5). Cisplatin is an efficient inhibitor of both Trx-1 and TrxR-1, as well as glutaredoxins, and the cytosolic thioredoxin and glutaredoxin systems have been implicated in the cellular pathways leading to cisplatin detoxification (5, 85, 86). Thus, increased expression and activity of the thioredoxin system has been correlated with resistance against cisplatin-induced cytotoxicity by tumor cells (84, 108). Moreover, some studies have identified chromosomal amplifications in resistant TGCTs affecting Trx-1 and TrxR-1 locus (75, 92). In this context, the presence of four testis/spermatid-specific thioredoxins raises the possibility that abnormal levels of one or several of these novel thioredoxins might be involved in the 10% of the TGCTs that are resistant to cisplatin treatment. To examine this hypothesis, we have initiated a study aiming to determine the mRNA and protein levels of the four spermatid-specific thioredoxins in all types of testicular tumors, which is expected to shed more light into the biochemical mechanisms that result in drug resistance.

CONCLUDING REMARKS

The finding that four novel thioredoxins are either exclusively or predominantly expressed in testis is fascinating and opens a new area of research. Our current knowledge merely scratches the surface, and there are indeed more questions than answers regarding thioredoxin function in mammalian spermatogenesis. Clearly, the development of animal models will be the primary tool to elucidate the role of these proteins as no *in vitro* system has been established thus far that would faithfully recapitulate spermatogenesis. Other approaches, such as two-hybrid screening, will also be useful to identify potential substrates and partners in FS, axoneme, or spermatid manchette. There is also a strong need of deeper knowledge regarding the distribution and role of the cytosolic thioredoxin system during spermatogenesis, particularly TGR, which

bridges two of the major redox systems in the cell, the thioredoxin and the glutaredoxin systems. Redox regulation is a major issue in spermatogenesis, and oxidative stress has been underpinned as a major causative factor of male infertility (2, 3). Defective sperm function has been associated with the retention of excess residual cytoplasm and increased free radical production by mechanisms that are poorly understood. We still do not know whether any of these spermatid-specific thioredoxins act as antioxidant defense to counteract the overproduction of reactive oxygen species in spermatozoa, although the conservation of the thioredoxin domain might indicate that this could indeed be the case in at least some of them. Other potential functions of the testis thioredoxin system might not even be anticipated. Such is the case of ubiquitin-dependent epididymal sperm quality control (97), because it was recently shown that Trx-1 functions as cofactor during protein ubiquitination (47). Clearly, the complete picture of the thioredoxin systems in the context of testis function is needed to determine why this class of proteins has become necessary for mammalian spermatogenesis, which has been regarded as the engine of evolution.

ACKNOWLEDGMENTS

ABBREVIATIONS

CIS, carcinoma *in situ*; DFS, dysplasia of the fibrous sheath; FS, fibrous sheath; NDP, nucleoside diphosphate; ODF, outer dense fibers; PCD, primary ciliary dyskinesia; Sptrx-1, Sptrx-2, and Sptrx-3, spermatid/sperm specific thioredoxins 1, 2, and 3; TGCT, testis germ cell tumor; Sptrx, spermatid/sperm specific thioredoxin; TGR, thioredoxin glutathione reductase; Trx-1 and Trx-2, thioredoxins 1 and 2; TrxR-1 and TrxR-2, thioredoxin reductases 1 and 2; TXl-1 and Txl-2, thioredoxin-like 1 and 2.

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