Characterization of Sptrx, a novel member of the thioredoxin family specifically expressed in human spermatozoa.

Antonio Miranda-Vizuete[1,5], Johanna Ljung[2], Anastasios E. Damdimopoulos[1], Jan-Åke Gustafsson[1], Richard Oko[3], Markku Pelto-Huikko[4] and Giannis Spyrou[1].

[1] Department of Biosciences at Novum, Center for Biotechnology, Karolinska Institute, S-141 57 Huddinge, Sweden, [2] Department of Medical Biochemistry and Biophysics, Karolinska Institute, S-17177 Stockholm, Sweden, [3] Department of Anatomy and Cell Biology, Queen´s University, Kingston, Ontario K7L 3N6, Canada and [4] Department of Developmental Biology, Tampere University Medical School and the Department of Pathology, Tampere University Hospital, Fin-33101 Tampere, Finland.

[5] Corresponding author: Tel.: 46-8-6083338; Fax: 46-8-7745538; Email: anmi@biosci.ki.se

Abbreviations: EST, expressed sequence tag; DFS, dysplasia of the fibrous sheath; FS, fibrous sheath; ODF, outer dense fibers; ORF, open reading frame; PDI, protein disulfide isomerase; PHGPx, phospholipid hydroperoxide glutathione peroxidase; RACE, rapid amplification of cDNA ends; ROS, reactive oxygen species; Trx, thioredoxin; UTR, untranslated region.

Human Sptrx mRNA GenBank accession number: AF080095.
Human Sptrx 5´-UTR intron GenBank accession number: AF288156

SUMMARY

Thioredoxins (Trx) are small ubiquitous proteins that participate in different cellular processes via redox-mediated reactions. We report here the identification and characterization of a novel member of the thioredoxin family in human, named Sptrx (**s**perm-**sp**ecific **trx**), the first with a tissue-specific distribution, located exclusively in spermatozoa. Sptrx ORF encodes for a protein of 486 amino acids comprised of two clear domains: an N-terminal domain consisting of 23 highly conserved repetitions of a 15-residue motif and a C-terminal domain typical of thioredoxins. Northern analysis and *in situ* hybridization shows that Sptrx mRNA is only expressed in human testis, specifically in round and elongating spermatids. Immunostaining of human testis sections identified Sptrx protein in spermatids while immunofluorescence and immunogold electron microscopy analysis demonstrated Sptrx localization in the cytoplasmic droplet of ejaculated sperm. Sptrx appears to have a multimeric structure in native conditions and is able to reduce insulin disulfide bonds in the presence of NADPH and thioredoxin reductase. During mammalian spermiogenesis in testis seminiferous tubules and later maturation in epididymis, extensive reorganization of disulfide bonds is required to stabilize cytoskeletal sperm structures. However, the molecular mechanisms that control these processes are not known. The identification of Sptrx with an expression pattern restricted to the post meiotic phase of spermatogenesis, when sperm tail is organized, suggests that Sptrx might be an important factor in regulating critical steps of human spermiogenesis.

INTRODUCTION

Thioredoxins (Trx) are low-molecular weight proteins (12 kDa) that catalyze thiol-disulfide redox reactions by the reversible oxidation of the cysteine residues of their conserved active site WCGPC (1). Thioredoxins are maintained in their active reduced form by the flavoenzyme thioredoxin reductase (TrxR) that uses the reducing power of NADPH, which constitutes the so-called thioredoxin system (2). All the organisms from bacteria to human appear to have at least one complete thioredoxin system and the progressive complexity of eukaryotic organisms is also reflected in the increasing number of thioredoxin systems. Thus, *E. coli* contains two thioredoxins and one thioredoxin reductase; yeast has two thioredoxin systems, one in cytosol and the other one in mitochondria, and photosynthetic organisms have several thioredoxin systems in different cellular compartments including chloroplasts. Finally, mammalian cells have, at least, two thioredoxin systems located in the cytosol and mitochondria, respectively (3). Different functions have been assigned to thioredoxins relying mostly on their general disulfide-reductase activity. In mammals, cytosolic Trx has been shown to be an antioxidant, a modulator of apoptosis, cell growth and differentiation and also a regulator of the DNA-binding activity of several transcription factors (following translocation into the nucleus), while the function of the mitochondrial thioredoxin system has not been yet elucidated although it is thought to constitute a major antioxidant defense mechanism. A common characteristic of both mammalian systems is their ubiquitous presence in all the tissues investigated within the same organism (3,4).

The human spermatozoon provides the male pronucleus for fertilization and to achieve this objective it has developed a highly specialized morphology organized into two major structures: the head and the tail or flagellum (5). The head contains the

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highly condensed DNA contributed by the male and all the cellular components that ensure egg recognition and sperm-egg fusion while the flagellum is responsible for sperm motility and energy production for the propulsive apparatus (6,7). Mammalian sperm tail is divided into three main regions running in a proximal to distal direction away from the sperm head: the mid-piece (that is attached to the sperm head by the neck or connecting piece), the principal piece and the end piece (for a scheme on sperm tail organization see (7) and (8)). The flagellum of the mammalian spermatozoa is organized around the central axoneme which consists of 9+2 microtubules, similar to that seen in the cilia and flagella of all eukaryotic cells and extends through the full length of the flagellum (7). In addition, mammalian spermatozoa contain characteristic cytoskeletal elements associated with the axoneme, namely outer dense fibers (ODF) and fibrous sheath (FS), that do not have any counterparts in any other cell type (5). ODF are adjacent to the axoneme and extend from the neck to the posterior end of the principal piece. In the middle piece, nine ODF (each one associated with one axonemal microtubule doublet) are surrounded by a helically wrapped mitochondrial sheath, that supplies the energy required for motility. This distribution pattern changes in the principal piece where FS replaces the mitochondrial sheath and the ODF number is reduced from nine to seven that progressively decrease in size. FS is composed of two longitudinal columns, running opposite to doublets 3 and 8 in replacement of the two missing ODF, connected by a series of ribs formed of closely packed filaments (5). The function of ODF and FS is not fully elucidated but it seems to be related with the control of the flagellar motion (8) and protection against shearing forces during epididymal transit (9). In recent years, the number of sperm-specific proteins has increased considerably although it is estimated that only 10-20% of the sperm polypeptides have been identified. The present work adds a new member to the list of sperm-specific proteins, the first belonging to the thioredoxin family.

EXPERIMENTAL PROCEDURES

cDNA cloning of human Sptrx gene. The Basic Local Alignment Search Tool (BLAST) (10) was used to identify human EST clone-encoded proteins similar to the ORF of human Trx1 or Trx2 (11,12). One of these EST entries (AA431210) was found to encode a putative novel human thioredoxin like protein. Based on this sequence, the nested primers hSptrx-R1/hSptrx-R2 (nucleotides 1653-1627 and 1605-1579, all nucleotide numbers referred to GenBank entry AF080095) and hSptrx-F4/hSptrx-F5 (nucleotides 1297-1320 and 1335-1359, respectively) were used for 5´- and 3´-RACE in a human testis cDNA library (Clontech) to isolate human Sptrx cDNA. The resulting sequences were used to amplify by PCR the full-length cDNA from the same library.

Northern blot analysis: Human multiple tissue Northern blots and human RNA Master blot with poly(A)$^+$ RNA from different tissues were purchased from Clontech. The human Sptrx ORF was labeled with [$-^{32}$P]dCTP (Rediprime random primer labeling kit, Amersham) and hybridized at 65°C overnight in ExpressHyb Solution following the protocol provided by Clontech. The blots were also hybridized with human GAPDH as control. The blots were scanned and quantified with the Gel Pro Analyzer program (Media Cybernetics).

Expression and purification of human Sptrx. The ORF encoding human Sptrx was cloned into the *BamHI-EcoRI* sites of the pGEX-4T-1 expression vector (Pharmacia) and transformed in *E. coli* BL21(DE3). The induction of the fusion protein was performed as previously described (13). Overexpressing cells were disrupted by 10 min sonication and the supernatant was cleared by centrifugation at 15,000 x *g* for 30 min and loaded onto a glutathione sepharose 4B column (Pharmacia Biotech). Binding to the

matrix was allowed to occur during 2 h at room temperature. Thrombin (5U per mg fusion protein) was used to remove GST by incubation overnight at 4°C. The resulting protein preparation was then subjected to ion exchange chromatography using a HiTrap Q column (Pharmacia Biotech) and human Sptrx was eluted using a gradient of NaCl. Protein concentration was determined from the absorbance at 280 nm assuming a molar extinction coefficient of 7690 M^{-1} cm^{-1} that was calculated using the Protean Program included in the DNASTAR Software Package (DNASTAR Inc., Madison, USA). The cloning, overexpression and purification of the truncated form of human Sptrx (h Sptrx) was identical to that described for the full-length protein except that the ion exchange purification step was not required as the protein eluted in a pure form following thrombin cleavage.

Enzymatic activity assays. Two different enzymatic assays were used to determine the ability of human Sptrx to reduce insulin *in vitro*. In the so-called DTT assay, DTT is used as reductant and the assay was carried out as previously described (11). The other assay used thioredoxin reductase and NADPH as electron donors for thioredoxin and was performed essentially as described elsewhere (13). In both cases human Trx1 was used as control.

Antibody production and immunoblotting analysis. Purified GST-hSptrx was used to immunize rabbits (Zeneca Research Biochemicals). After six immunizations, serum from rabbits was purified by ammonium sulfate precipitation. Affinity purified antibodies were prepared using a cyanogen bromide-activated Sepharose 4B column, onto which 0.5 mg recombinant N-terminal Sptrx fragment (lacking the thioredoxin domain) had been coupled using the procedure recommended by the manufacturer (Pharmacia). Specificity of the antibodies was tested by western blotting using

recombinant hSptrx and total cell extracts. Immunodetection was performed with horseradish peroxidase-conjugated donkey anti-rabbit IgG diluted 1:5000 following the ECL protocol (Amersham Corp.).

Preparation of spermatozoa and extraction of sperm proteins. Semen samples from healthy donors were allowed to liquefy at room temperature and separated from seminal plasma by centrifugation (1000 x g) for 10 min at room temperature. After two washes in PBS the pelleted spermatozoa were frozen at -20°C until use. The sperm pellet was solubilized in a lysis buffer containing Tris-HCl 0.1 M pH 8.0, NaCl 0.15 M, protease inhibitor cocktail (Boehringer Mannheim) and phosphatase inhibitor cocktail (SIGMA) at the concentration recommended by the manufacturers. Samples were then subjected to three cycles of freezing/thawing in dry ice-ethanol, incubated for 30 min on ice and centrifuged at 14000 rpm for 30 min. The soluble fraction was used for further analysis.

In situ hybridization, immunocytochemistry and immunoelectron microscopy. For *in situ* hybridization, human testes were obtained from orchiectomies performed due to prostate cancers. The samples were frozen on dry ice, sectioned with Microm HM 500 cryostat at 14 m and thaw-mounted onto polylysine glass slides. The sections were stored at –20C until use. Four oligonucleotide probes (nucleotides 181-220, 957-996, 1208-1257 and 1519-1558 of human Sptrx cDNA) were used. All probes produced similar results when used separately and were usually used simultaneously to intensify the signal. Several control probes with the same length and similar GC content and specific activity were used to determine the specificity of the hybridization. *In situ* hybridization was carried out as described previously (14).

For immunocytochemistry, paraffin sections containing multiple human tissues (T1065, Lot: 9994A) were obtained from Dako (Copenhagen). In addition routine paraffin sections of human testis were used. For immunofluorescence analysis human sperm samples were obtained from healthy volunteers. Immunocytochemistry was performed as described previously (15) either by the ABC-method or by indirect immunofluorescence method using goat anti-rabbit-FITC (1:100, 30 min, Boehringer-Mannhein) as a secondary antibody . The fluorescence samples were embedded in PBS-glycerol mixture containing 0.1% *p*-phenylenediamine. The sections were examined with Nikon Microphot-FXA microscope equipped with proper fluorescent filters. For electron microscopy immunocytochemistry, see procedures previously described (5,16). Affinity purified anti Sptrx antibodies were used at 1/20 dilution and colloidal gold-conjugated goat anti-rabbit IgG at 1/20 dilution was the secondary antibody.

RESULTS

cDNA cloning and expression, sequence analysis, genomic organization and chromosomal localization of human Sptrx gene. By sequence comparison we found that Genbank EST entry AA431210 encoded a putative novel human thioredoxin sequence. Based on this sequence, we designed specific primers and performed 5'- and 3'-RACE PCR analysis in a human testis cDNA library to clone the full-length cDNA sequence of this novel protein. The complete sequence of the cDNA consists of an ORF of 1461 bp, a 5'-UTR of 180 bp including three stop codons in frame and a very short 3'-UTR of 17 bp before the poly(A)$^+$ tail (Fig. 1). Human Sptrx ORF encodes a protein of 486 amino acids with an estimated molecular mass of 53.3 kDa and a pI of 4.82. Despite another methionine residue located seven positions downstream, we have chosen the first methionine as translation start point since it is located in a better Kozak-context for initiation of translation (17). Analysis of human Sptrx sequence identified two distinct domains: a N-terminal domain (comprising the first 375 residues) displaying only a very weak similarity with wheat proline-rich protein (18) and mouse neurofilament H protein (19), and a C-terminal domain (comprising the last 111 residues) similar to the previously described Trx1 and Trx2 (11,12). The N-terminal domain is characterized by a unique arrangement of residues organized as 23 repeats of a 15-residue motif shortly after the start methionine. As shown in Figure 1, this motif has a general pattern (QPK(X)GDIPKS(P/S)E(K/E)(X)I) which is very conserved among the different repetitions and very rich in both basic (Lys) and acidic residues (Glu, Asp) that are responsible for the low pI of 4.82 and a net charge at pH 7.0 of –26.32. Regarding the C-terminal thioredoxin domain, many of the structural amino acids that are conserved in mammalian thioredoxins like Phe-11, Asp-26, Ala-29, Pro-40, Asp-58 or Lys-81 (numbers referred to those of human Trx1) are also conserved in Sptrx (Fig. 2). The

major characteristic of the C-terminal thioredoxin domain of Sptrx is the presence of four additional cysteines besides the two at the active site (Fig. 1). More strikingly, the three additional cysteine residues in human Trx1 (positions 62, 69 and 74) are conserved in human Sptrx (residues 443, 450 and 454 respectively) while the fourth Sptrx cysteine residue, at position 471, corresponds to Ser-90 in Trx1 (Fig. 2) (20). Comparison of the protein sequence with PROSITE database (21) identified, along with the above mentioned thioredoxin domain, several potential phosphorylation sites for protein kinases CKI, CKII, GSK3, p34cdc2 and PKC and two highly scored PEST sequences for proteasome-dependent degradation centered at positions 16 and 375, respectively (Fig. 1). In addition, the protein sequence does not reveal any signal peptide to target the protein to any subcellular compartment. This is further confirmed by the expression of a fusion protein of human Sptrx and GFP at its C-terminus resulting in a cytosolic localization (data not shown). Sptrx mRNA 3´-UTR is composed of only 17 nucleotides and inspection of the sequence immediately upstream from the stop codon identifies a putative non-canonical poly(A)$^+$ signal (ATTAAA) that might indeed drive polyadenylation (22).

Multiple-tissue Northern blots were used to determine the size and tissue distribution of human Sptrx mRNAs using the ORF as the probe. Human Sptrx mRNA was detected only in human testis as a single band of 1.7 kb in good agreement with the size of the cloned cDNA (Fig. 3A). No signal was obtained in any other tissue after longer exposure. To evaluate the possibility that Sptrx mRNA could be expressed in other tissues not present in these blots, we also screened an RNA dot blot containing poly(A)$^+$ RNAs from 50 different human tissues. Among the tissues examined, hybridization signal was observed only in testis mRNA (Fig. 3B).

A homology search in GenBank identified Sptrx genomic region in the genomic BAC clone AC006238. The main feature of human Sptrx gene is the lack of introns

within the coding region and the presence of only one intron of 508 bp in the 5′-UTR (Fig. 4). This intron, that interrupts the first in-frame 5′-UTR stop codon (Fig. 1), displays all the typical intron features as it conforms to the GT/AG rule and contains the splicing branch point consensus TGCTAAT and the downstream pyrimidine tract (Fig. 4). Finally, the BAC clone AC006238 has been mapped to human chromosome 18p11.2-11.31, between the markers D18S459 and D18S482, by PCR screening of a human-rodent radiation panel (Fig. 4). To our knowledge, no male germ cell deficiency or any other type of reproductive anomaly has been reported to map in this region.

Expression of recombinant human Sptrx and enzymatic activity. Recombinant human Sptrx migrated in SDS-PAGE at 91 kDa size (Fig. 5A) while its theoretical size is 53 kDa. This retardation in the migration of the protein could be explained by its low pI and net negative charge at neutral pH. In addition, computer analysis of the Sptrx sequence predicts that the N-terminal domain of the protein is organized in -helical secondary structure which might also contribute to this anomalous migration as described for other testis proteins (23,24). Next, we performed PAGE under non-denaturing conditions and human Sptrx migrated at approximately 180 kDa thus suggesting that it might be multimeric in its native state (Fig. 5B). Preincubation of the protein with DTT did not modify this pattern of migration implying that the multimeric conformation is maintained by non-covalent forces.

Enzymatic activity of thioredoxins is usually assayed by their capacity to reduce the disulfide bonds of insulin using either DTT as artificial reductant or NADPH and thioredoxin reductase as a more physiological reducing system (2). Figure 6A shows the activity of human recombinant Sptrx with DTT as reducing agent. Surprisingly, the truncated form of Sptrx expressing only the thioredoxin domain did not display a significant activity over the background, suggesting that the active site is buried in this

truncated form and only accessible in the full-length form. In another assay where NADPH and calf thymus thioredoxin reductase are used as electron donors for Sptrx, both the full-length and the truncated form are active. Similarly, the truncated form of Sptrx is also less active than the full-length protein indicating that the accessibility of the active site is maintained, at least in part, by the N-terminal domain of Sptrx. However, the enzymatic activity is higher in the oxidized forms than in the reduced ones in contrast with Trx1 used as control (Fig. 6B).

Tissue expression, cellular and subcellular localization of human Sptrx. To investigate the expression pattern of Sptrx mRNA, *in situ* hybridization was performed in human testis sections and showed that it is expressed in round and elongating spermatids with no signal in the rest of the testicular cells (Fig. 7). Similar results have been obtained in mouse testis sections (M. Pelto-Huikko and A Miranda-Vizuete, unpublished results). Affinity purified antibodies were used to study the expression pattern of Sptrx protein in different human tissues and cell lines. As shown in Figure 8A, only human testis and sperm cell extracts expressed Sptrx as a band of approx. 91 kDa in agreement with the migration of the recombinant protein in SDS gels. No signal was detected in any other tissue or cell line used as control. We also studied the expression of Sptrx in a blot of different human testis and prostate tumours and found no difference with respect to normal tissues (data not shown). Next, we used these antibodies to investigate whether we could identify Sptrx homologues in testis extracts from other mammals. Figure 8B shows that they recognize a similar band in mouse, rat and bovine testis extracts.

The unforeseen result of the sperm-specific localization of human Sptrx prompted us to investigate whether other members of the two thioredoxin systems (cytosolic and mitochondrial) (3,25) as well as a newly identified thioredoxin reductase highly

expressed in testis (26) are present in these cells. We used specific antibodies against all these proteins and by Western blot analysis we were able to identify all of them in both human testis and sperm extracts (Fig. 8C). To address the question whether Sptrx protein distribution paralleled the mRNA expression, we performed immunohistochemical analysis in human testicular sections that revealed that Sptrx expression was restricted to spermiogenesis and most prominently expressed in round and elongating spermatids. Other cellular types like spermatogonia, primary spermatocytes, Leydig and Sertoli cells were not stained (Fig. 8D). Identical Sptrx distribution pattern has been observed in rat and mouse testis (R. Oko, M. Pelto-Huikko and A. Miranda-Vizuete, unpublished results). Immunofluorescence of human ejaculated spermatozoa showed that Sptrx was localized from the caudal region of the head to the end of the principal piece (Fig 8E). However, when using Triton during the fixation protocol this signal was removed thus suggesting that Sptrx is contained (loose) within the tail cytoplasm and the cytoplasmic droplet. To further confirm this point, we performed immunogold electron microscopy analysis in human ejaculated spermatozoa and found that Sptrx is distributed mostly in the cytoplasmic droplet although some scattering of residual labeling was present throughout the sperm tail (Fig 8F-G). These results in human ejaculated sperm have been also validated for rat, mouse and bull sperm (R. Oko and A. Miranda-Vizuete, unpublished results).

DISCUSSION

During the last five years an increasing number of genes which expression is restricted to spermatogenesis have been reported and the fact that most of their encoded proteins display enzymatic or regulatory properties suggests that they might play a more active role in sperm function than merely structural. In this context, we report here the cloning and characterization of Sptrx, a novel member of the thioredoxin family exclusively expressed in human spermatozoa representing the first mammalian member of the thioredoxin family with a tissue-specific distribution.

Sptrx cDNA was cloned from a human testis cDNA library and both ORF and UTRs display important features. Analysis of Sptrx ORF identifies a two-domain structure consisting of a N-terminal part organized in 23 repeats of 15 amino acid residues with no homology to any other protein in the databases and a C-terminal domain similar to thioredoxin. Sptrx N-terminus is rich in charged residues and several putative sites for phosphorylation by different kinases can be identified. Among these sites, PKC and casein kinase II appear to be the most likely sites of phosphorylation sites in serine residues. Phosphorylation is one of the major regulatory mechanisms in sperm physiology and practically all the different cellular processes that sperm undergoes *e. g.* acrosome reaction, capacitation, hyperactivation, motility or binding to egg zona pellucida are mediated by this mechanism (7). In addition, a complete set of different kinases has been identified in mammalian sperm cells both ubiquitous and sperm-specific (27). Another important feature of the N-terminal domain of Sptrx is the presence of two PEST sequences, which have been reported to be involved in rapid degradation of proteins by the ubiquitin 26S proteasome machinery (28). Ubiquitin-dependent degradation has recently been put forward as an important general mechanism for selective degradation of defective sperm along with other contaminants

of cellular origin, during epididymal transit (29). Preliminary experiments show that Sptrx is phosphorylated by PKC and ubiquitinated *in vitro* (A. Miranda-Vizuete, unpublished results).

The Sptrx gene is organized in two exons separated by one intron located in the 5´-UTR that might be involved in Sptrx regulation as it has been described for other testis-specific proteins (30). On the other hand, the 3´-UTR is unexpectedly short with only 17 nucleotides and lacking polyadenylation signal, although inspection of the ORF sequence immediately upstream from the stop codon identifies a putative non-canonical poly(A)$^+$ signal (ATTAAA). To our knowledge, there are only two cases where a polyadenylation signal is located within the ORF of the corresponding gene (31,32). Although further experiments are required to confirm this point, the distance of 20 nucleotides between the polyadenylation signal to the poly(A)$^+$ site and a GU-rich element 30 nucleotides downstream from the poly(A)$^+$ site supports this possibility (22). Taken together, Sptrx gene organization reveals an unusual case within thioredoxin family. While other members like Trx1 or Trx2 are composed of five and three exons, respectively (33 and A. E. Damdimopoulos, unpublished results) neither the Sptrx genomic segment corresponding to the thioredoxin domain nor the N-terminal repetitive domain are interrupted by any intron. It is then reasonable to speculate that the Sptrx thioredoxin domain might have arisen as a retroposition event from the mature original mRNA ancestor and integrated randomly in the genome (34). Whether the N-terminal domain was already at the integration locus as a pre-existing gene or was acquired after the thioredoxin domain integration can not be inferred as no homologue has been yet identified. Most of the expressed intronless retroposons, including Sptrx, are testis specific forms. This apparent tissue-specificity may be due to transcriptional promiscuity in testes as the retroposition event is required to occur in the germ line to be fixed in the population (35).

Sptrx migrates at 180 kDa under non-reducing conditions suggesting a multimeric native conformation. When assayed with calf thymus thioredoxin reductase it can reduce insulin disulfide bridges but this activity is higher in the oxidized form than in the reduced one. This is the first report where a thioredoxin has higher activity in its oxidized form. As noted previously, Sptrx contains four additional cysteine residues that might form intra- or inter-monomeric disulfide bonds therefore stabilizing the protein. Although Sptrx native conformation is not maintained by disulfide bonds as shown by non-denaturing gel assay, the decrease of the enzymatic activity after preincubation with a reducing agent indicates that additional cysteine residues in Sptrx modulate its activity. A truncated form of Sptrx lacking the N-terminal domain only displays residual activity implying that the N-terminus is necessary to maintain the accessibility of the active site.

The synthesis of the sperm cytoskeletal polypeptides has been shown to occur in the cytoplasm of elongating spermatids, which is consistent with the expression pattern obtained for Sptrx and corresponds to the growth of the structures that will organize the sperm tail. We have found that all the thioredoxins and thioredoxin reductases so far identified are present in human ejaculated sperm. Thus, although the specific function that they play in spermatozoa is not known, their presence in the mature cell suggests an important role in sperm physiology. Particularly interesting is the presence in sperm of a novel form of thioredoxin reductase that has been found to be highly expressed in testis (26). This novel TrxR together with Sptrx might constitute the first tissue-specific thioredoxin system, although colocalization of both proteins has not been addressed.

Disulfide bond formation is a very important issue in sperm physiology. The nuclear and accessory structures of mammalian sperm tail become stabilized by extensive disulfide bonding during spermiogenesis and epididymal maturation and

this stabilization is reversed during fertilization by the reduction of these disulfide bonds (36,37). As we have demonstrated, Sptrx is able to function as a reductant *in vitro* and displays a spatial and temporal expression in the tail of elongating spermatids which occurs simultaneously with the assembly of ODF and FS. Our data suggests that Sptrx could indeed participate in the regulation of this assembly process by reducing non-correct disulfide pairings thus favouring the generation of the correct disulfide bonds between the different sperm tail constitutents.

In an attempt to identify possible functions of Sptrx in sperm physiology we studied the involvement of Sptrx in the formation of the sperm capsule, a structure that embeds the helix of mitochondria in the middle piece of sperm tail. PHGPx protein represents at least 50% of the capsule material and it is found in an enzymatically inactive, oxidatively cross-linked, unsoluble form (38). The switch of PHGPx from a soluble active enzyme to an enzymatically inactive structural protein has been shown to occur during differentiation of spermatids into spermatozoa (once the mitochondria have been helically arranged around the axoneme and ODF in the sperm tail middle piece) and to be dependent on thiol groups in proteins distinct from PHGPx (38). Despite Sptrx meeting these two criteria, it is not involved in the process as neither the recombinant protein could oxidize soluble PHGPx nor the Sptrx antibodies could prevent aggregation in the presence of hydrogen peroxide (L. Flohé, personal communication).

In conclusion, the identification of Sptrx in human spermatozoa opens up a new dimension within the thioredoxin field. Its expression during sperm tail formation strongly indicates an important role in this process and therefore a potential target for male factor infertility studies. There are numerous reports describing sperm flagellum pathologies for which the molecular bases in unknown. Among those, the best characterized is dysplasia of the fibrous sheath (DFS), a human sperm defect resulting

in male sterility that consists of a complete disorganization of this structure which is already evident in spermatids by the loose arrangement of the FS ribs dispersed in the cytoplasmic matrix (39). The simultaneous expression of Sptrx at the time that FS assembles suggests that there might be a correlation between an anomalous expression of Sptrx and the development of DFS. If so, diagnosis of patients affected with DFS or any other sperm tail pathology might be helped by screening of Sptrx status.

ACKNOWLEDGEMENTS

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LEGENDS TO THE FIGURES

Fig. 1. Nucleotide and amino acid sequence of human Sptrx. The nucleotide numbers are displayed on the right and the amino acid numbers on the left. The three stop codons in frame within the 5´-UTR are underlined. The up arrow indicates the 5´-UTR intron site while the down arrow indicates the thioredoxin domain start. The two putative sites for ubiquitination are double underlined. The conserved thioredoxin active site and the four additional cysteine residues are boxed.

Fig. 2. Alignment of the predicted amino acid sequence of Sptrx thioredoxin domain with human Trx1 (11) **and the mature form of human Trx2** (12). Identical residues are displayed in black boxes. The arrows indicate the position of the four additional cysteines in Sptrx and the active site is boxed.

Fig. 3. Expression pattern of human Sptrx mRNA. (A) Human multiple tissue northern blot. The human Sptrx probe hybridized with one mRNA species at 1.7 kb only in testis. (B) Human RNA master blot. The human Sptrx probe hybridized with testis mRNA (arrow). For a complete list of tissues see Clontech catalogue (www.clontech.com). In both cases GADPH was used as control to determine the relative amount of mRNA from each tissue. P.B.L., peripheral blood leukocytes.

Fig. 4. Genomic organization and chromosomal localization of human Sptrx gene. The sequence of the 5´-UTR intron is shown, the conserved GT/AT dinucleotides at the intron junction are in bold and the splicing branch point is boxed. Human Sptrx gene is located at 24.3-32.4 cM from the top of the linkage group of human chromosome

18. By comparing this location with other genes in the region we have mapped it to 18p11.2-11.31.

Fig. 5. Expression and purification of human recombinant Sptrx protein. (A) SDS-PAGE analysis. After IPTG induction human Sptrx was purified as GST fusion protein and thrombin cleavaged with 5 U/mg protein (Lane 1) and after ion exchange column (Lane 2). 5 μg of protein were loaded onto a 10% gel. (B) Native-PAGE analysis of human recombinant Sptrx protein. 2 μg of purified Sptrx were loaded in a 4-15% gradient phast system gel (Pharmacia-Biotech).

Fig. 6. Enzymatic activity of human Sptrx. Purified Sptrx was assayed for its ability to reduce insulin disulfide bonds. (A) DTT was used as electron donor: (O) Trx1, (❏) Sptrx, ( ) Truncated Sptrx, (◇) DTT alone. Proteins were used at a final concentration of 5 μM and the reaction was initiated adding 1 μl of 100 mM DTT. (B) NADPH and thioredoxin reductase were used as electron donors: (O) Trx1 reduced, (●) Trx1 oxidized, (❏) Sptrx reduced, (■) Sptrx oxidized, ( ) Truncated Sptrx reduced, (▲) Truncated Sptrx oxidized. Reduced proteins were obtained by DTT buffer preincubation (13). The reaction was initiated adding 5 μl of calf thymus thioredoxin reductase ($50A_{412}$ unit) and stopped after 20 min by the addition of 6 M guanidine HCl, 1 mM DTNB. All experiments were repeated three times and a representative experiment is shown.

Fig. 7. *In situ* hybridization analysis of human Sptrx mRNA distribution. Dipped sections of human testis were hybridized with Sptrx probe. Grains demonstrating Sptrx mRNA can be seen over round and elongating spermatids (black arrowheads). Sertoli cells, pachytene spermatocytes (white arrowheads) and other

spermatogenic cells at earlier stages of differentiation and interstitial Leydig cells (white arrows) lack Sptrx mRNA. Bar represents 30 μm.

Fig. 8. Tissue, cellular and subcellular distribution of human Sptrx protein. (A) Sptrx expression in different human tissues and cell lines. Lanes 1) MCF7, 2) HEK293, 3) Jurkat, 4) Testis, 5) Prostate, 6) Sperm, 7) Liver. All extracts were at 10 μg except testis 1 μg and sperm 0.1 μg. (B) Interspecies cross-reactivity of human Sptrx antibodies in testis extracts from different mammalian samples. (Hu) human 1 μg, (Mo) mouse 10 μg, (Bo) bovine 10 μg and (Ra) rat 10 μg. (C) Identification of all known thioredoxin and thioredoxin reductases in human sperm. Recombinant TrxR3 is not available and human liver extract was used as control. Trx2 and TrxR2 are used as His-tagged protein explaining the slightly higher size. (Re) Recombinant 5 ng, (Li) Liver 10 μg, (Te) Testis 10 μg and (Sp) Sperm 10 μg. (D) Immunocytochemistry of human testis showing strong Sptrx labeling in the apically localized spermatids, spermatozoa and residual bodies (arrowheads). Spermatogenic cells at earlier stages of development and Leydig cells are devoid of staining (arrows). Bar represents 50 μm. (E) Immunofluorescent demonstration of Sptrx in human ejaculated spermatozoa. Labeling is present from the postacrosomal region and the neck (arrowhead) through the middle and principal piece (small arrows). The anterior part of the head is unlabeled (arrow). Bar represents 4 μm. (F) Electron micrographs of ejaculated sperm middle piece section and (G) tail longitudinal section showing accumulation of Sptrx labeling throughout the cytoplasmic droplet (CD) with most of the gold label found outside the saccules (S). Labeling at much lower incidence, if any, is found randomly distributed in sperm axoneme (arrowhead). Bars represent 0.2 μm.

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CTCAGCACAGGGGAGCCACCTTGAAGCTCTCAAATATCACTGTTG  45
TGAATACAGAGAGGGAAAACCAACTGTAACGTGCCACCCAAATAA  90
AGCTCATTACTAGTCCTGTCCAGCAACGTGCCTCTCCTGGCCCTA  135
GAGTTCTTGGAAATAGCCCAGGCCAAAGAGAAGGCCTTTCTCCCC  180
ATGGTCAGCCACACGTTCCACATGCGCACAGAGGAGTCTGATGCC  225
  1  M  V  S  H  T  F  H  M  R  T  E  E  S  D  A

TCACAGGAGGGCGATGACCTACCCAAGTCCTCAGCAAACACCAGC  270
 16  S  Q  E  G  D  D  L  P  K  S  S  A  N  T  S

CATCCCAAGCAGGATGACAGCCCCAAGTCCTCAGAAGAAACCATC  315
 31  H  P  K  Q  D  D  S  P  K  S  S  E  E  T  I

CAGCCCAAGGAGGGTGACATCCCCAAGGCCCCAGAAGAAACCATC  360
 46  Q  P  K  E  G  D  I  P  K  A  P  E  E  T  I

CAATCCAAGAAGGAGGACCTCCCCAAGTCCTCAGAAAAGCCATC   405
 61  Q  S  K  K  E  D  L  P  K  S  S  E  K  A  I

CAGCCCAAAGAGAGTAACATCCCCAAGTCCTCAGCAAAACCCATC  450
 76  Q  P  K  E  S  N  I  P  K  S  S  A  K  P  I

CAGCCCAAGCTGGGCAATATTCCCAAGGCCTCAGTGAAGCCCAGC  495
 91  Q  P  K  L  G  N  I  P  K  A  S  V  K  P  S

CAGCCCAAGGAGGGTGACATCCCCAAGGCCCCAGAAGAAACCATC  540
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CAATCCAAGAAGGAGGACCTCCCCAAGTCCTCAGAAGAAGCCATC  585
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CAGCCCAAAGAGGGTGACATCCCCAAGTCCTCAGCAAAACCCATC  630
136  Q  P  K  E  G  D  I  P  K  S  S  A  K  P  I

CAGCCCAAGCTGGGCAATATTGCCAAGACCTCAGTGAAGCCCAGC  675
151  Q  P  K  L  G  N  I  A  K  T  S  V  K  P  S

CAGCCCAAGGAGAGTGATATCCCCAAGTCCCCAGAAGAAACCATC  720
166  Q  P  K  E  S  D  I  P  K  S  P  E  E  T  I

CAGCCCAAGGAGGGTGACATCCCCAAGTCCTCAGCAAAGCCCATC  765
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CAGCCCAAGCTGGGCAATATTCCCAAGGCCTCAGTGAAGCCCAGC  810
196  Q  P  K  L  G  N  I  P  K  A  S  V  K  P  S

CAGCCCAAGGAGGGTGACATCTCCAAGTCCCCAGAAGAAGCCATC  855
211  Q  P  K  E  G  D  I  S  K  S  P  E  E  A  I
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Figure 1 (Continued)

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226     Q   P   K   E   G   D   L   P   K   S   L   E   E   A   I

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241     Q   P   K   E   G   D   I   P   K   S   P   E   E   A   I

       CAGCCCAAGGAGGGTGACATCCCCAAGTCCCTAGAGGAAGCCATC 990
256     Q   P   K   E   G   D   I   P   K   S   L   E   E   A   I

       CAGCCTAAGGAGGGTGACATCCCCAAGTCCCCAGAAGAAACCATC 1035
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       CAGCCCAAGAAGGGTGACATCCCCAAGTCCCCAGAAGAAGCCATC 1080
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       CCATCCAAGGAGGGTGACATCCTAAAGCCTGAAGAAGAAACAATG 1305
361     P   S   K   E   G   D   I   L   K̲   P̲   E̲   E̲   E̲   T̲   M̲

       GAGTTCCCGGAGGGGGACAAGGTGAAAGTGATCCTGAGCAAGGAG 1350
376     E̲   F̲   P̲   E̲   G̲   D̲   K   V   K   V   I   L   S   K   E

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       GTGGACTTCTCGGCCACG TGGTGTGGGCCCTGC AGGACCATCAGA 1440
406     V   D   F   S   A   T   W   C   G   P   C   R   T   I   R

       CCATTCTTCCATGCCCTGTCTGTGAAGCATGAGGATGTGGTGTTC 1485
421     P   F   F   H   A   L   S   V   K   H   E   D   V   V   F

       CTGGAGGTGGACGCTGACAAC TGT GAGGAGGTGGTGAGAGA TGC 1530
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451     A   I   M   C   V   P   T   F   Q   F   Y   K   K   E   E

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481     V   I   A   E   L   K   ***
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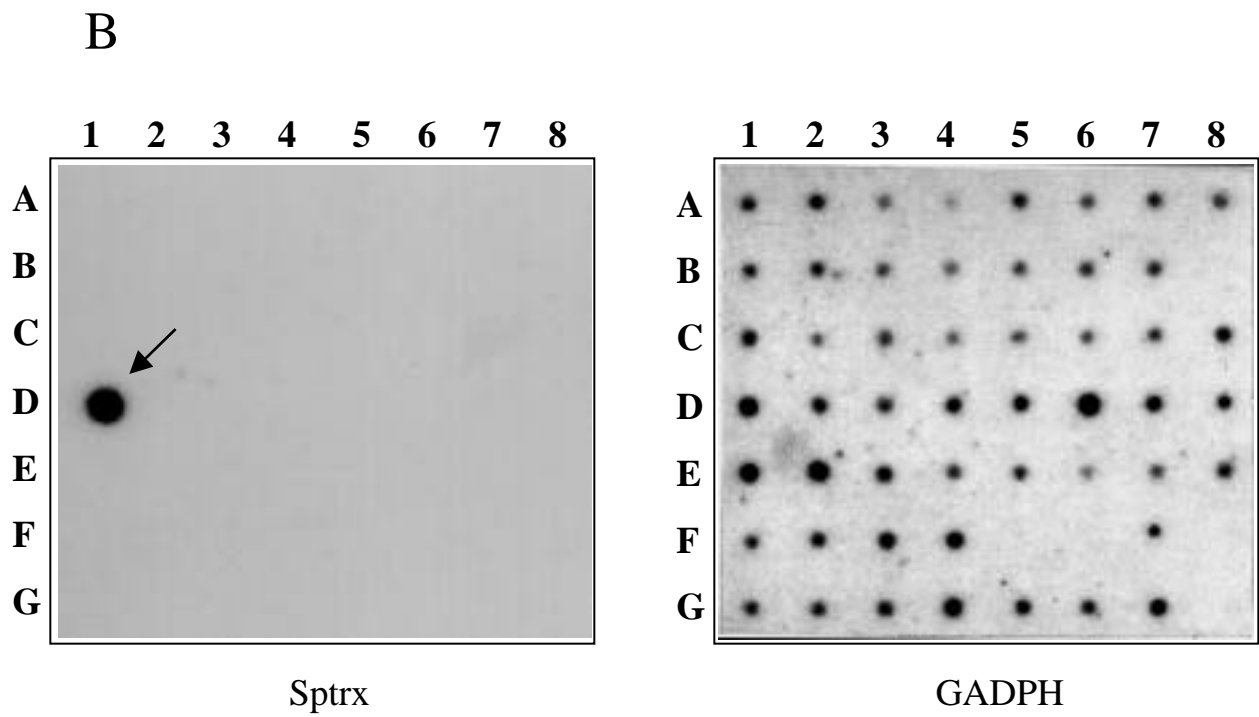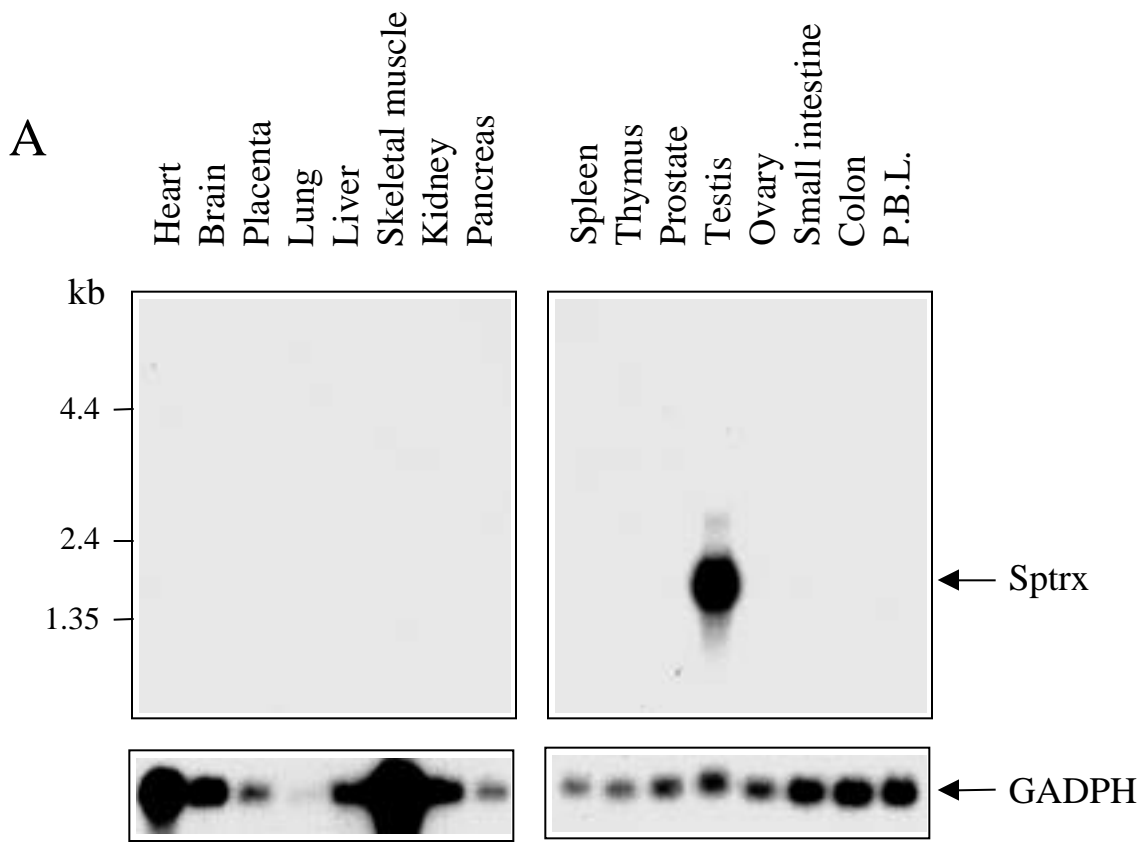
Figure 1

Figure 2

A

Heart · Brain · Placenta · Lung · Liver · Skeletal muscle · Kidney · Pancreas

Spleen · Thymus · Prostate · Testis · Ovary · Small intestine · Colon · P.B.L.

kb

4.4

2.4

1.35

← Sptrx

← GADPH

B

1 2 3 4 5 6 7 8

A
B
C
D
E
F
G

1 2 3 4 5 6 7 8

A
B
C
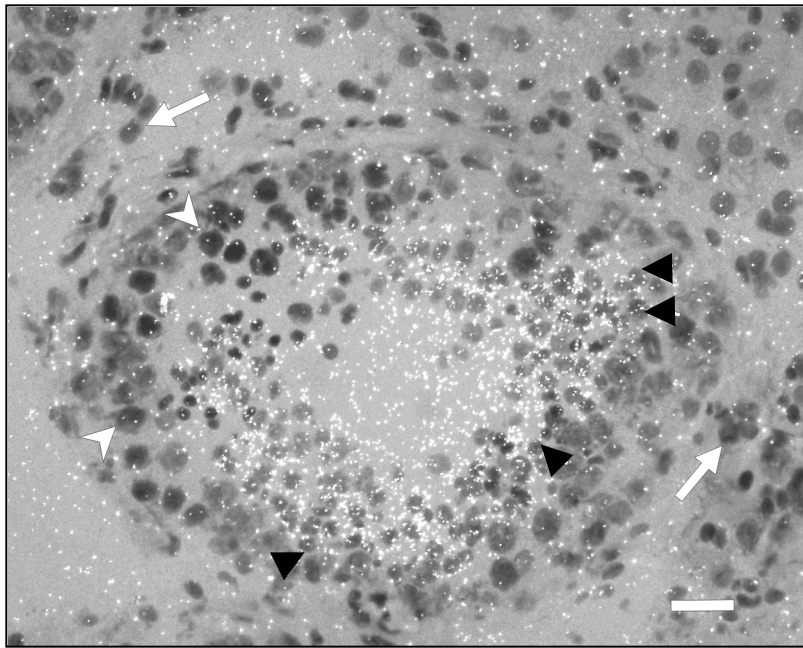D
E
F
G

Sptrx

GADPH
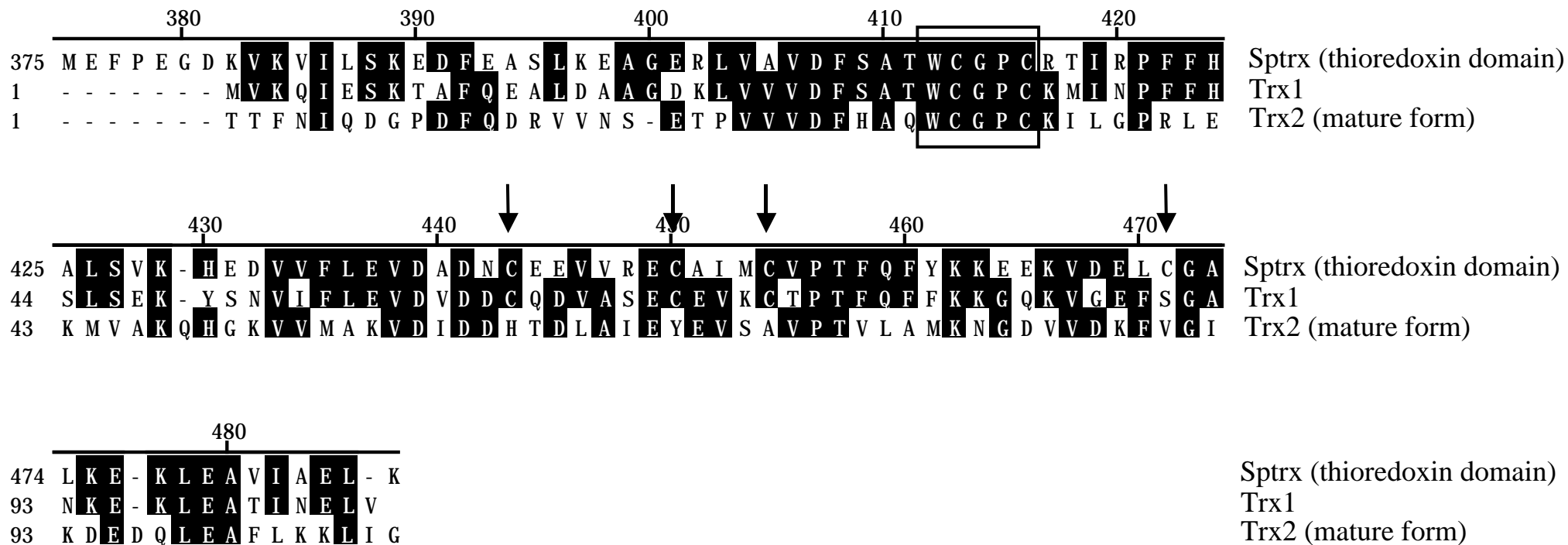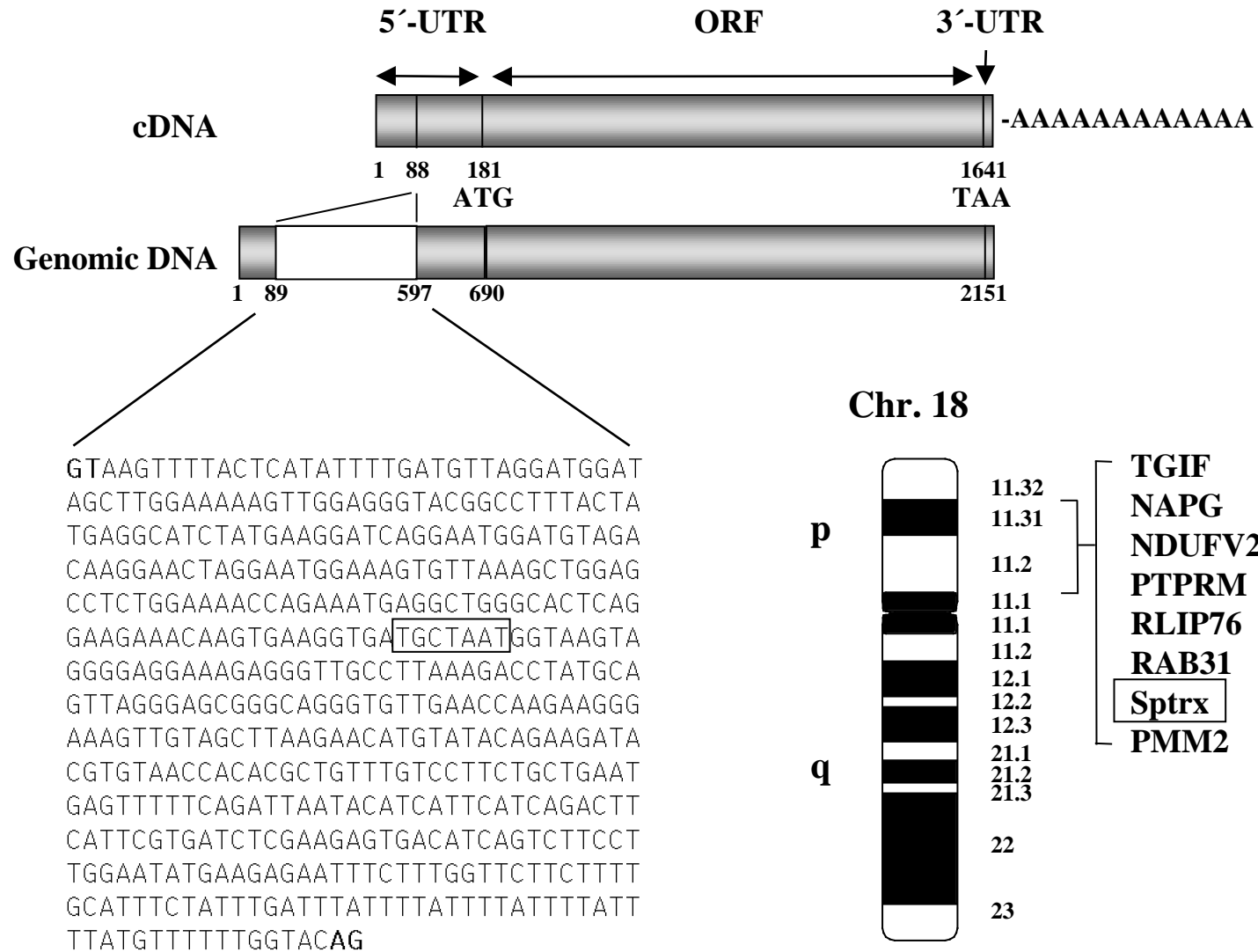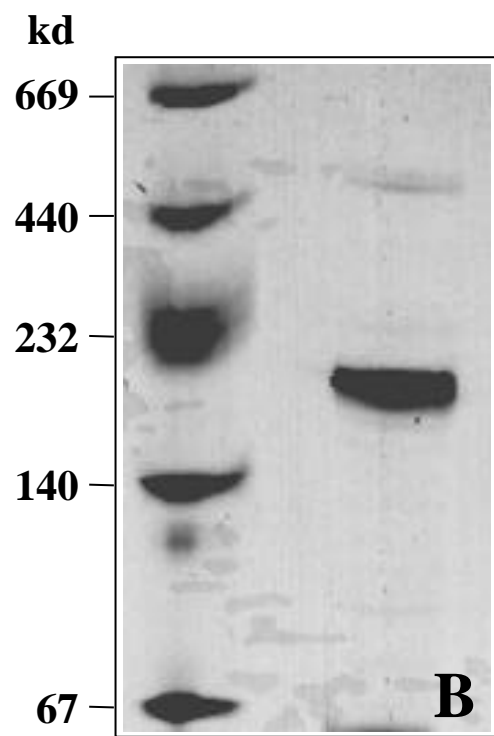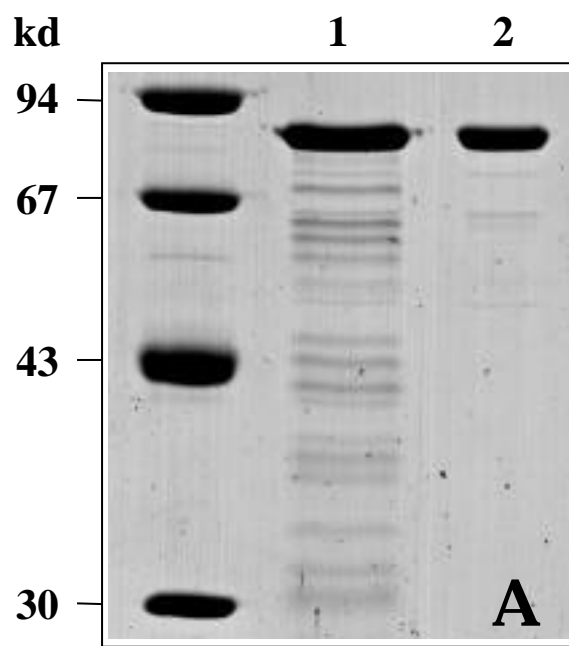
Figure 3

Figure 4

Figure 5
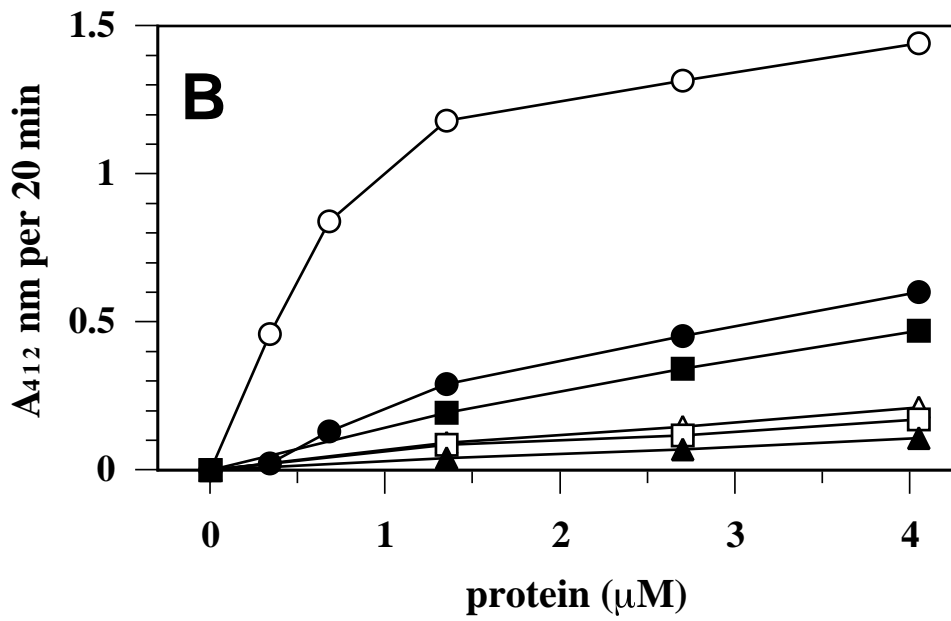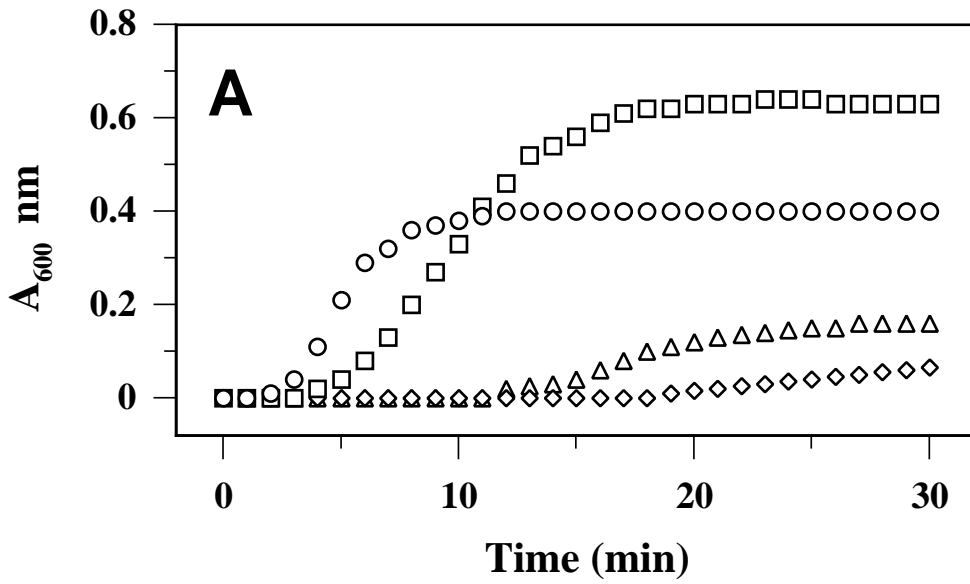
Figure 6

Figure 7
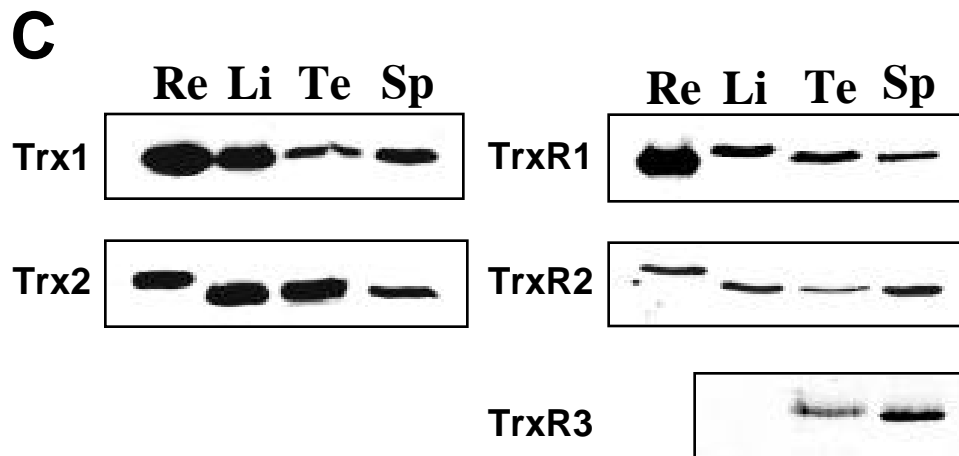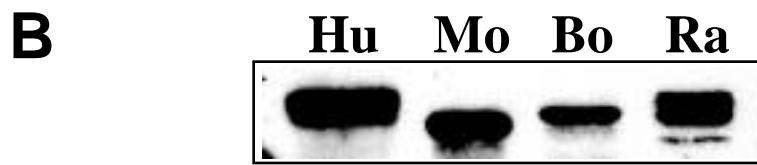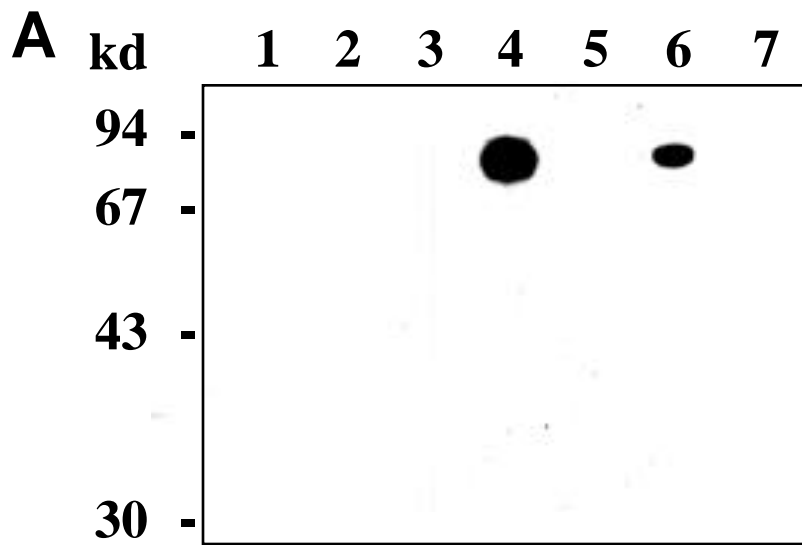
Figure 8 (Continued)

Figure 8