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Expression of SPANX proteins in human-ejaculated spermatozoa and sperm precursors

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Summary

The sperm protein associated with nucleus in the X chromosome (SPANX) gene family is constituted by only a few members, clustered at Xq27, encoding small proteins which range from 15 to 20 kDa. These proteins have been shown to be present both in mature spermatozoa and in tumours, such as melanoma and some leukaemias. We developed polyclonal sera in order to study the distribution of the protein in human-ejaculated spermatozoa and their precursors. A synthetic peptide was designed from a domain common to the SPANX protein family and polyclonal sera were raised in mice. Seven healthy volunteer men with normal sperm parameters were recruited and the expression of SPANX proteins was evaluated in spermatozoa and ejaculated sperm precursors by immunocytochemistry and immunofluorescence analyses. SPANX proteins, present in a large fraction (96%) of mature spermatozoa, were localized in the sperm head (39.2%), midpiece (22.8%) or in both sites (34.4%). Spermatids also showed the presence of SPANX proteins in their cytoplasm, although a significantly higher number of spermatids were SPANX-negative compared with spermatozoa. In conclusion, SPANX proteins are expressed in an elevated percentage of spermatids and mature spermatozoa. In the latter, they are preferentially located in the sperm head. The greater number of SPANX-negative spermatids observed could relate to their easier exfoliation from the seminiferous tubules.

Keywords: polyclonal sera, SPANX gene family, SPANX proteins, spermatids, spermatozoa, synthetic peptide

Introduction

Spermatogenesis is a complex developmental process which involves amplification of germinal stem cells, their differentiation into spermatocytes, meiotic division and finally transformation into mature spermatozoa. Therefore, spermatogenesis provides an interesting system for examining the regulation of gene expression during development and differentiation. The genes expressed during spermatogenesis can be divided into two main groups: diploid and haploid expressed genes (Nayernia *et al.*, 1996). Many of the haploid, post-meiotically expressed genes have been mapped to autosomal chromosomes (Golden *et al.*, 1993; Nelson &

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Krawetz, 1993; Tarnasky *et al.*, 1998), whereas only few X-linked genes have been described, for example, the sperm protein associated with nucleus in the X chromosome (SPANX) gene family (Moss *et al.*, 1997; Morales *et al.*, 1998; Turner *et al.*, 1998).

Recent studies have characterized several molecular and biochemical properties of SPANX gene family. It is constituted by only a few members clustered at Xq27. The genes, commonly organized in two exons, encode very small proteins 97–103 amino acid long, whose molecular weight ranges from 15 to 18 kDa, and are expressed post-meiotically (Westbrook *et al.*, 2000). As many as eight SPANX loci (named SPANX A1, A2, B1, B2, C, D, E and F1) have been reported in the Human Genome Map (build 33) spanning about 600 kb at Xq27.

SPANX proteins are present in testicular and ejaculated spermatozoa and in both sources they were observed in nuclear 'craters' (Baccetti *et al.*, 1989) and in cytoplasmic remnants, while SPANX mRNAs have been shown in haploid (both elongated and round) spermatids, using in situ hybridization of human testicular sections (Westbrook *et al.*, 2000).

The function of SPANX gene-encoded proteins is currently unknown. It is not known whether they are all present in the spermatozoa or some members are preferentially expressed. In fact, their strong sequence similarity, both at the amino acid and nucleotide levels, precludes such an analysis. Recent evidence suggests that one member of this family, referred to as CTp11, is expressed in tumours such as melanoma (Zendman *et al.*, 1999), and in myeloma and other haematological malignancies (Wang *et al.*, 2003; Zendman *et al.*, 2003).

The purpose of this study was to evaluate the expression pattern of this gene family in human-ejaculated spermatozoa and spermatids. To accomplish this, we raised polyclonal antibodies in mice.

Materials and methods

Semen samples

Semen samples were obtained from seven healthy volunteer subjects (aged 22–40 years old) with normal sperm parameters, as suggested by the World Health Organization guidelines (WHO, 1999), and normal (<10%) percentage of ejaculated sperm precursors. They underwent physical urogenital examination, seminal physicochemical evaluation and spermoculture to evaluate the presence of male accessory gland infection. All normal volunteers included in this study had physical examination within the normal range, a normal seminal physicochemical properties (i.e. volume, pH, etc.) and a negative spermoculture. Morphometric criteria were used to differentiate spermatids from other round cells present in the ejaculate. Spermatids have a larger cytoplasm and a lower nucleus/cytoplasm ratio than lymphocytes. In contrast, macrophages have a larger cytoplasm and a kidney-shaped pale nucleus. The different nuclear shapes allow easy differentiation between spermatids and granulocytes. Another additional criterion used for identifying leucocytes was peroxidase staining (WHO, 1999). Leucocytes appeared brown (peroxidase-positive), whereas spermatids remained unstained (peroxidase-negative). The protocol was approved by the Institutional Review Board and an informed written consent was obtained by each subject.

Designing synthetic peptide

The synthetic peptide TPTGDSDPQP was designed on the second exon of the SPANXA genes. The sequence considered is common to SPANXA1/A2, SPANXC, CTp11, SPANXB1/B2 and SPANXF1, but some variations occur in other members. BLAST analysis did not show any occurrence of this sequence outside the SPANX family group (Fig. 1).

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                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                            10         20         30         40         50         60

Spanx A1        MDKQSSAGGV KRSVPCDSN- -----EANEM MPETPTGDSD PQPAPKKMKT SESSTILVVR
Spanx A2        MDKQSSAGGV KRSVPCDSN- -----EANEM MPETPTGDSD PQPAPKKMKT SESSTILVVR
Spanx B1        MGQQSSVRRL KRSVPCESNE ANEANEANKT MPETPTGDSD PQPAPKKMKT SESSTILVVR
Spanx B2        MGQQSSVRRL KRSVPCESNE ANEANEANKT MPETPTGDSD PQPAPKKMKT SESSTILVVR
Spanc C         MDKQSSAGGV KRSVPCESN- -----EVNET MPETPTGDSD PQPAPKKMKT SESSTILVVR
CTp11           MDKQSSAGGV KRSVPCESN- -----EVNET MPETPTGDSD PQPAPKKMKT SESSTILVVR
Spanx D         MDKQSSAGGV KRSVPCDSN- -----EANEM MPETSSGYSD PQPAPKKLKT SESSTILVVR
Spanx E         MDKQSSAGGV KRSVPCDSN- -----EANEM MPETSSGYSD PQPAPKKLKT SESSTILVVR
Spanx F1        MGQQSSVRRL KRSVPCESNE ANEANEAMKT MPETPTGDSD PQPAPKKMKT SESSTILVVR

                    ....|....| ....|....| ....|....| ....|....| ...
                            70         80         90        100

Spanx A1        YRRNFKRTSP EELLNDHARE NRINPLQMEE EEFMEIMVEI PAK
Spanx A2        YRRNFKRTSP EELLNDHARE NRINPLQMEE EEFMEIMVEI PAK
Spanx B1        YRRNVKRTSP EELVNDHARE NRINPDQMEE EEFIEITTER PKK
Spanx B2        YRRNVKRTSP EELVNDHARE NRINPDQMEE EEFIEITTER PKK
Spanc C         YRRNVKRTSP EELLNDHARE NRINPLQMEE EEFMEIMVEI PAK
CTp11           YRRNVKRTSP EELLNDHARE NRINPLQMEE EEFMEIMVEI PAK
Spanx D         YRRNFKRTSP EELVNDHARK NRINPLQMEE EEFMEIMVEI PAK
Spanx E         YRRNVKRTSP EELVNDHARE NRINPLQMEE EEFMEIMVEI PAK
Spanx F1        YRRMVKRTSP EELLNDHARE NRINPDQMEE EEFIEITTER PKK
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Figure 1. Alignment of SPANX proteins, including those found in the build 33 of the human genome and the previously described protein CTp11 (Zendman *et al.*, 1999). The sequence corresponding to the peptide used in the present study are highlighted in grey.

Immunization of mice

Peptide conjugation to ovalbumin was carried out through the peptide amino terminal using glutaraldehyde, as previously described (Reichlin, 1980). Four mice were inoculated intraperitoneally using alumina powder as adjuvant (Chase, 1967). After 3 weeks, mice were boosted with the same antigen and killed after one more week by intracardiac perfusion, in agreement with national and local legislations regulating animal health care. Sera were checked by dot blot analysis for affinity towards the peptide.

Immunoassays

Immunocytochemistry was carried out on whole, glutaraldehyde-fixed seminal specimens. For immunofluorescence studies, a suspension containing 20×10^6 spermatozoa/mL was incubated with a 1/500 dilution of the primary antibody in Tris-buffered saline (TBS) containing 3% bovine serum albumin for 1 h at room temperature. Following several washes in TBS containing 0.05% Tween-20, the suspension was incubated with a 1/30 dilution of TRITC-labelled goat anti-mouse IgG (Sigma-Aldrich Corp., St Louis, MO, USA). A pool of pre-immune sera, from a control group of mice, and only the secondary antibody, served as negative controls. Before microscopic examination, nuclei were counter-stained with 100 ng/mL 4′,6-diamidino-2-phenylindole (DAPI) (Cytocell, Bambury, UK). For immunoenzymatic reactions, 1 mL of a suspension containing 20×10^6 fixed spermatozoa/mL was first splashed on slides using the ThinPrep apparatus (Cytyc Corp., Boston, MA, USA), rehydrated and treated for 5 min with 3% hydrogen peroxide. The primary antibody was used at the same dilution as above, and then biotinylated secondary antibody and streptavidin-peroxidase, LSAB 2 System, Peroxidase (DAKO Corp., Carpinteria, CA, USA), were used consecutively, after thoroughly washing with TBS containing 0.05% Tween-20. The slides were revealed in 0.5 mg/mL 3,3′-diaminobenzidine-4HCl solution (DAKO Corp.), for 5 min and then counter-stained with haematoxylin, dehydrated and mounted in DPX mountant (BDH, Poole, UK). A pool of pre-immune sera, from a control group of mice, treated 2×5 min in $1 \times$ SSC and then for 5 min in 3% hydrogen peroxide in $1 \times$ SSC, then treated as described above for ThinPrep immunocytochemistry, served as negative controls.

Western blot analysis

Sperm proteins from normal donors were solubilized in Laemmli buffer (Laemmli, 1970), at the concentration of about $1–2 \times 10^2$ spermatozoa/μL in the presence of 5% β-mercaptoethanol at 100 °C for 3 min, electrophoresed on 15% polyacrylamide/SDS gel, then electroblotted onto nitrocellulose membrane (Bio-Rad, Philadelphia, PA, USA) for 2 h at 0.24 mA/cm^2. Protein bands were detected on the membrane using the same antibody as for immunoassays (see above).

Statistical analysis

Results are shown as median and range. The data were analysed using the Mann–Whitney test. SPSS 9.0 was used for statistical calculation. A significant statistical difference was accepted where the p-value was <0.05.

Results

Localization of SPANX proteins in spermatozoa

Polyclonal antibodies raised against the synthetic peptide TPTGDSDPQP were expected to exhibit affinity for most, if not all, SPANX proteins, even for those variants, such as SPANXD and E, which show variations with respect to the designed sequence (Fig. 1). We thus considered SPANX proteins as a whole, even if SPANX A, B, C and F were significantly better than the other variants. In agreement with these considerations, Western blot analysis, carried out on sperm proteins, gave two main positive bands of approximately 15–18 kDa (Fig. 2).

Experiments on both sperm suspensions and thinpreps of spermatozoa showed positive cytoplasmic signals in most of the 250 spermatozoa examined from each of the seven donors. SPANX proteins signals were found predominantly in cytoplasmic remnants of mature spermatozoa (Table 1): 39.2% (36.9–40.4%) at head level, in close contact with the nuclear envelop and/or beneath the acrosomal region; 22.8% (21.5–24.6%) associated with the sperm midpiece; moreover,

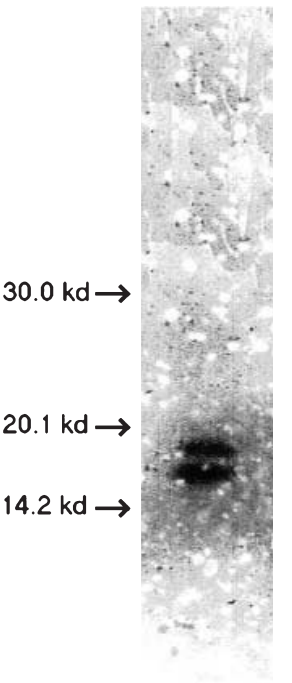

Figure 2. Western blot of a semen sample from a normal donor showing SPANX protein bands in the 15–18 kDa range. The R_f of carbonic anhydrase, soybean trypsin inhibitor and cytochrome C, respectively, are indicated by the arrows on the left.

Table 1. Sperm parameters of the seven healthy volunteers with normal sperm parameters and pattern of SPANX protein presence in spermatozoa and spermatids

ID	Density ($\times 10^6$/mL)	Total motility (%)	Progressive motility (%)	Normal forms (%)	Spermatids (%)	SPANX-positive spermatozoa				SPANX-proteins in spermatids	
						Head positive, *n* (%)	Midpiece positive, *n* (%)	Head + midpiece positive, *n* (%)	Total positive, *n* (%)	Negative, *n* (%)	Positive, *n* (%)	Negative, *n* (%)
Donor 1	80	89	29	30	3	100 (40.0)	56 (22.4)	87 (34.8)	243 (97.2)	7 (2.8)	109 (90.8)	11 (9.2)
Donor 2	114	58	32	39	1	98 (39.2)	52 (20.8)	86 (34.4)	236 (94.4)	14 (5.6)	111 (92.5)	9 (7.5)
Donor 3	105	51	24	29	1	103 (41.2)	61 (24.4)	79 (31.6)	243 (97.2)	7 (2.8)	108 (90.0)	12 (10.0)
Donor 4	82	53	26	32	2	99 (39.6)	57 (22.8)	76 (30.4)	232 (92.8)	18 (7.2)	105 (87.5)	15 (12.5)
Donor 5	90	55	23	42	1	88 (35.2)	59 (23.6)	91 (36.4)	238 (95.2)	12 (4.8)	113 (94.2)	7 (5.8)
Donor 6	110	68	38	36	3	95 (38.0)	62 (24.8)	83 (33.2)	240 (96.0)	10 (4.0)	110 (91.7)	10 (8.3)
Donor 7	70	70	35	65	4	97 (38.8)	55 (22.0)	89 (35.6)	241 (96.4)	9 (3.6)	108 (90.0)	12 (10.0)
Median	90	58	29	36	2	98 (39.2)	57 (22.8)	86 (34.4)	240 (96)	10 (4)	109 (90.8)	11 (9.2)
10th percentile	76	52.2	23.6	29.6	1	92.2 (36.9)	53.8 (21.5)	77.8 (31.1)	234.4 (93.8)	7 (2.8)	106.8 (89.0)	8.2 (6.8)
90th percentile	112	69	36.5	53.5	3.5	101 (40.4)	61.5 (24.6)	90.0 (36)	242.0 (96.8)	16 (6.4)	112 (93.3)	13.5 (11.3)

SPANX proteins were found both in the sperm head and midpiece in the 34.4% (31.1–36%) of specimens (Fig. 3, panels a–c). Altogether, SPANX proteins were found in the 96% (93.8–96.8%) of the mature spermatozoa examined. Comparing the sites of SPANX staining, we found that the percentage of spermatozoa with SPANX-positive heads was substantially greater than that of spermatozoa with SPANX-positive midpiece or head + midpiece. The percentage of SPANX-positive head + midpieces was also significantly ($p < 0.05$) greater than that observed in the midpiece alone.

Distribution of SPANX proteins in ejaculated spermatid

The presence and distribution of SPANX proteins were evaluated in round and elongated spermatids found in semen samples (Table 1). Analysis was carried out on 120 spermatids from each of the seven normal donors by means of immunofluorescence. Many spermatids (90.8%, range: 89–93.3%) presented a uniformly fluorescent cytoplasm, with complete exclusion of the nucleus (Fig. 3, panel d). A significantly higher percentage of spermatids showed no SPANX protein expression compared with spermatozoa. As this analysis was performed simultaneously both on spermatozoa and on spermatids, we could exclude artefacts given by insufficient reaction of the immune serum. Control sera from pre-immunized mice gave complete absence of signal; moreover, concordant results were obtained in control experiments with sera from different mice.

Discussion

Although the number of SPANX genes present and those possibly expressed in spermatids and/or spermatozoa has not been the object of a specific study so far, in silico genome analysis, repeatedly done during the course of the present work, revealed a growing number (up to eight) of gene loci potentially encoding SPANX proteins. As SPANX genes map on the X chromosome and are expressed post-meiotically, one would expect that only half of the sperm population was SPANX-positive. However, this does not seem to be the case, as 96% of the spermatozoa examined showed a positive staining to SPANX in one or two principal segments. To explain the presence of SPANX in most spermatozoa, Westbrook *et al.* (2000) proposed a combination of post-meiotic synthesis in X-bearing spermatozoa and active transport to Y-bearing germ cells. This hypothesis would also explain the uneven distribution of SPANX proteins among positive spermatozoa. However, Westbrook *et al.* (2001), in another paper, showed that nearly 50% of spermatozoa possesses SPANX protein(s); more interestingly, this percentage was found irrespective of the presence of the X chromosome. Thus the uneven distribution of SPANX among spermatozoa both in amount and in location suggests that some unpredictable events occurred during sperm morphogenesis concerning the synthesis and/or the post-translational processing of these proteins.

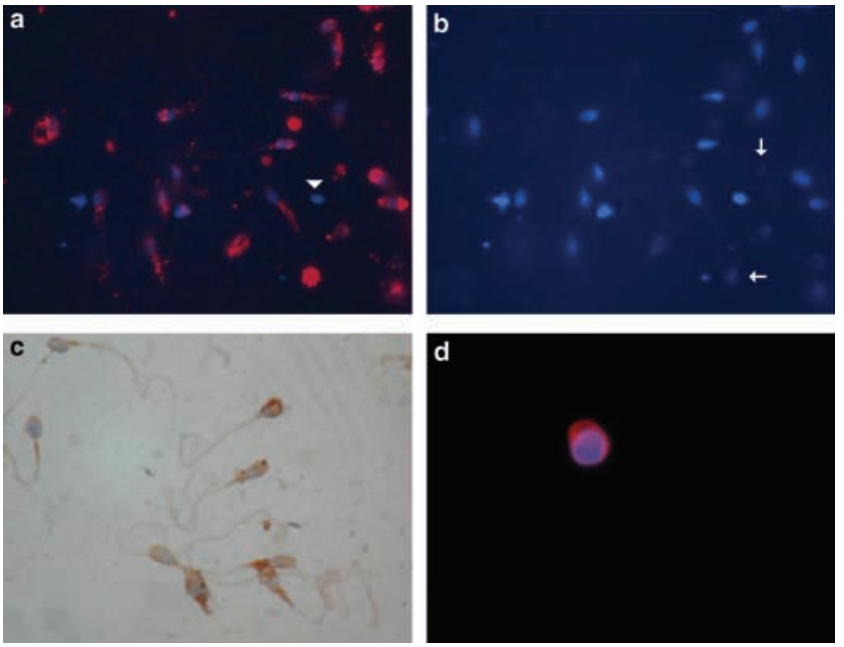

Figure 3. Immunofluorescence (a, b and d) and immunocytochemical (c) analysis of semen samples, labelled as detailed in 'Materials and methods' (original magnification ×400). (a) Double immunofluorescence (TRITC and DAPI, for SPANX and nuclear DNA fluorescence, respectively) showing both SPANX-positive and SPANX-negative cells, mainly spermatozoa, with a heterogeneous distribution of SPANX throughout mature spermatozoa. Some spermatids, either positive or negative (white arrowhead), are present. (b) DAPI staining, shown for clarity, of the same field. Sometimes, the focus varies among adjacent cells so that the fluorescence intensity is different (white arrow). (c) Spermatozoa on ThinPrep preparations showing a different degree of positivity. (d) SPANX-positive spermatid showing homogeneous labelling of the cytoplasm, but not of the nucleus.

In order to achieve more accurate results, we raised an antibody against a peptide which overlaps most of the SPANX sequences known to date, including CTp11 (Zendman *et al.*, 1999), which is 100% homologous to SPANXC, instead of using a recombinant protein and analysed a number of healthy volunteers to provide statistical power for the observed values. We found that a significantly higher number of spermatozoa showed a positive SPANX-staining in the head; however, midpiece and head + midpiece localization were remarkably constant in all men examined. This suggests that SPANX proteins, once synthesized, are distributed randomly in spermatozoa, but show a strong preference for the sperm head. Our antibody was found to give similar results both by immunofluorescence and immunocytochemistry on thinpreps. The reliability of thinprep results, demonstrated in the present study by parallel analysis with immunofluorescence, will allow us doing routine studies either of normal or pathologic specimens without the problems associated with routine immunofluorescence studies (such as costly reagents and equipments necessary for immediate image acquisition).

As reported also by Westbrook *et al.* (2000), a small percentage of spermatozoa did not show any detectable SPANX protein in their cytoplasm. In the present study, moreover, we have shown that different volunteers harbour a roughly constant fraction of negative spermatozoa. We suggest that differences in the distribution pattern are indicative of the different origin (X chromosome- vs. Y chromosome-bearing spermatozoa) of the SPANX complement. X chromosome-bearing spermatozoa should label stronger than Y chromosome-bearing ones, which would show only trace of positivity. Alternatively, SPANX proteins may be lost in resting spermatozoa or sperm survival could be associated with different amounts of SPANX present in the cytoplasm. In order to elucidate this discrepancy, we extended the analysis to the spermatids found in the same semen samples. A greater number of spermatids did not show SPANX-staining compared with spermatozoa. This excludes the possibility that SPANX proteins are being lost in resting spermatozoa and we hypothesize that the higher percentage of negative spermatids relates to their easier exfoliation from the seminiferous tubules than positive ones, which should be joined together by inter-cytoplasmic bridges (Morales *et al.*, 1998). In fact the genetically haploid spermatids are functionally diploid as a result of the sharing of gene products through their intercellular bridges. This movement of molecules among haploid cells is crucial for the production of spermatozoa as numerous essential proteins are encoded on sex chromosomes. As an example testis–brain RNA-binding protein (TB-RBP) is a single-stranded DNA- and RNA-binding protein prominent in the nuclei and cytoplasm of specific stages of differentiating male germ cells. TB-RBP moves from the nucleus to the cytoplasm and through intercellular bridges of male germ cells (Morales *et al.*, 1998, 2002). Another example is the role of citron kinase in cytokinesis of spermatogenic precursors. In fact during spermatogenesis, the first morphological indication of spermatogonia differentiation is incomplete cytokinesis, followed by the assembly of stable intercellular cytoplasmic communications. This distinctive feature of differentiating male germ cells has been highly conserved during evolution, suggesting that regulation of the cytokinesis endgame is a crucial aspect of spermatogenesis (Cunto *et al.*, 2002).

The biological properties of SPANX are poorly understood. In particular, their cytoplasmic localization is in

contrast with the presence of bipartite nuclear localization signals (NLS) found on their sequence (Westbrook *et al.*, 2000) and the nuclear localization of the transfected protein in cultured cells (Westbrook *et al.*, 2001). Spermatids present a large cytoplasm where SPANX proteins appear, by immunofluorescence, to be uniformly diffused. Although ultrastructural studies showed that spermatid cytoplasm is organized into lamellar structures (Smith & Berlin, 1977; Sun *et al.*, 1977; Bird & Seiler, 1986), we could not evidence them by fluorescence microscopy, and there were no sign of SPANX clustering around the nuclei. This suggests the absence of a functional nuclear import mechanism recognizing NLS in the sperm cell lineage.

The implications of these observations may shed new light on the comprehension of SPANX biology. In addition, the observation that SPANX loci fall within the physical interval harbouring the testicular germ cells tumour (*TGCT1*) locus (Rapley *et al.*, 2000) makes them important candidates for studying the pathogenesis of testicular tumours.

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