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Human testicular protein TPX1/CRISP-2: localization in spermatozoa, fate after capacitation and relevance for gamete interaction

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Testicular protein Tpx-1, also known as CRISP-2, is a cysteine-rich secretory protein specifically expressed in the male reproductive tract. Since the information available on the human protein is limited to the identification and expression of its gene, in this work we have studied the presence and localization of human Tpx-1 (TPX1) in sperm, its fate after capacitation and acrosome reaction (AR), and its possible involvement in gamete interaction. Indirect immunofluorescence studies revealed the absence of significant staining in live or fixed non-permeabilized sperm, in contrast to a clear labelling in the acrosomal region of permeabilized sperm. These results, together with complementary evidence from protein extraction procedures strongly support that TPX1 would be mainly an intra-acrosomal protein in fresh sperm. After *in vitro* capacitation and ionophore-induced AR, TPX1 remained associated with the equatorial segment of the acrosome. The lack of differences in the electrophoretic mobility of TPX1 before and after capacitation and AR indicates that the protein would not undergo proteolytical modifications during these processes. The possible involvement of TPX1 in gamete interaction was evaluated by the hamster oocyte penetration test. The presence of anti-TPX1 during gamete co-incubation produced a significant and dose-dependent inhibition in the percentage of penetrated zona-free hamster oocytes without affecting sperm motility, the AR or sperm binding to the oolema. Together, these results indicate that human TPX1 would be a component of the sperm acrosome that remains associated with sperm after capacitation and AR, and is relevant for sperm–oocyte interaction.

Key words: acrosome reaction/CRISP family/gamete interaction/sperm/testes

Introduction

The *tpx-1* gene was originally isolated as a testis-specific expression sequence in the course of characterizing genes from mouse chromosome 17 (Kasahara *et al.*, 1987). The predicted Tpx-1 protein appeared as a secreted, cysteine-rich molecule, 54% homologous to rat epididymal glycoprotein DE (Brooks *et al.*, 1986; Kasahara *et al.*, 1989), first identified in our laboratory (Cameo and Blaquier, 1976). Although it was originally suggested that Tpx-1 and DE could correspond to species-specific products of the same gene, different evidence determined that they were distinct proteins (Kasahara *et al.*, 1989). The identification of genes for DE in mouse (Haendler *et al.*, 1993) and human (Hayashi *et al.*, 1996; Krätzschmar *et al.*, 1996), and for Tpx-1 in human (Kasahara *et al.*, 1989), mouse (Mizuki *et al.*, 1992), guinea pig (Foster and Gerton, 1996) and rat (O'Bryan *et al.*, 1998), confirmed the existence of a family of proteins denominated the 'cysteine-rich secretory protein' (CRISP) family. In recent years, besides DE and Tpx-1, also called CRISP-1 and CRISP-2, respectively, other CRISP proteins were identified in different mammalian tissues (Haendler *et al.*, 1993; Kjeldsen *et al.*, 1996; Krätzschmar *et al.*, 1996; Pfisterer *et al.*, 1996; Rich *et al.*, 1996; Yudin *et al.*, 2002). Non-mammalian proteins belonging to the CRISP family have been found in venoms from lizards and snakes (Morrissette *et al.*, 1995; Chang *et al.*, 1997; Yamazaki *et al.*, 2003), and in oocytes and embryos from *Xenopus* (Olson *et al.*, 2001; Schambony *et al.*, 2003). Significant molecular similarities have also been found between CRISP proteins, allergens from insect venoms and pathogenesis-related proteins from plants (Lu *et al.*, 1993; Schreiber *et al.*, 1997). Altogether, these proteins comprise the cysteine-rich antigen 5 pathogenesis-related (CAP) protein superfamily.

Among this widely distributed family, only DE and Tpx-1 have been detected specifically in the mammalian male reproductive tract. Rat epididymal protein DE associates with the sperm surface during maturation (Kohane *et al.*, 1980a,b), migrates from the dorsal region of the acrosome to the equatorial segment with the acrosome reaction (AR) (Rochwerger and Cuasnicu, 1992a) and participates in sperm–oocyte fusion through complementary sites on the oocyte surface (Rochwerger *et al.*, 1992b; Cohen *et al.*, 1996). Results from our group indicated the participation of both mouse and human homologues of DE in gamete fusion through binding sites on the surface of the corresponding oocytes (Cohen *et al.*, 2000; Cohen *et al.*, 2001).

Tpx-1 was originally described as one of the primary autoantigens abundantly present within the guinea pig acrosome (Hardy *et al.*, 1988). In rat sperm, in addition to its presence in the acrosome, Tpx-1 was found to exist as a component of the outer dense fibres

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of the tail (O'Bryan *et al.*, 1998, 2001), and as a surface molecule responsible for the specific interaction between spermatogenic and Sertoli cells (Maeda *et al.*, 1998, 1999). Thus, although there is consensus on the specific expression of Tpx-1 in rodent male germ cells and its presence in mature sperm, the results on its subcellular localization are still controversial, and the possible function of the sperm protein has not yet been reported. Regarding human Tpx-1 (TPX1), the information available so far is limited to the identification and testicular expression of the gene (Kasahara *et al.*, 1989; Krätzschmar *et al.*, 1996).

In view of this, the aim of the present work has been to study the presence and localization of TPX1 in human spermatozoa and its possible involvement in gamete interaction.

Materials and methods

Human sperm capacitation

Ejaculates were obtained after a 48 h period of sexual abstinence from adult (21–35 years old) healthy donors whose initial semen characteristics fell within the World Health Organization criteria for normality (WHO, 1999). After complete liquefaction, the semen was diluted with six volumes of Biggers–Whitten–Whittingham (BWW) medium (Biggers *et al.*, 1971), centrifuged (10 min at 300 × *g*), and washed again with 1 ml of BWW containing 3.5% human serum albumin (BWW-HSA) (Sigma Chemical Co., St Louis, MO, USA).

Induction and evaluation of AR

Sperm samples were allowed to swim-up for 1 h at 37 °C in 1 ml of BWW-HSA, and the selected motile spermatozoa were diluted to a final concentration of 0.5–1 × 10^7 cells/ml and incubated 18–22 h at 37 °C in BWW-HSA in an atmosphere of 5% CO_2. For induction of the AR, ionophore A23187 (Sigma) was added (10 μM final concentration) to sperm 30 min before the end of the capacitation period. For evaluation of the AR sperm were fixed in 2% paraformaldehyde (Sigma) in phosphate-buffered saline (PBS), mounted on slides and air-dried. Slides were then immersed for 5 min in ice-cold methanol for cell permeabilization, and incubated with 50 μg/ml of fluorescein isothiocyanate (FITC)-conjugated *Pisum sativum* agglutinin (PSA, Sigma) in PBS for 30 min at room temperature, in the dark. Slides were rinsed in PBS, mounted in 90% glycerol in PBS, and observed under an epifluorescence microscope (Nikon Optiphot, Nikon, Japan) to determine the acrosomal status of sperm, as previously described (Cross *et al.*, 1986). Sperm were scored as acrosome intact when a bright staining was observed on the acrosome, or as acrosome reacted when either fluorescence staining was restricted to the equatorial segment or no labelling was observed.

Antibodies

The PCR product of a cDNA clone for human TPX1 (Kasahara *et al.*, 1989) was ligated into pMAL-C2 vector (New England BioLabs Inc., Beverly, MA), which was used to transform *Escherichia coli* bacteria. Recombinant TPX1 (recTPX1) was expressed as a fusion protein coupled to maltose binding protein (MBP), by induction with isopropyl-1-thio-β-galactoside (Promega, Madison, WI) and then purified by an amylose resin column (New England BioLabs Inc., Beverly, MA). Anti-TPX1 (4.94 mg/ml) was developed by immunization of rabbits with recTPX1 followed by immunoglobulin G (IgG) purification as described previously (Kasahara *et al.*, 1989). Rabbit antisera raised against AA1 (autoantigen 1), the guinea pig homologue of TPX1, was a kind gift from Dr Daniel Hardy (Hardy *et al.*, 1988).

Sperm indirect immunofluorescence (IIF)

Labelling of live sperm in suspension

Human sperm aliquots were washed twice in PBS containing 4 mg/ml BSA (PBS-BSA4), and incubated with anti-TPX1 (1:10 in PBS-BSA4) for 30 min at 37 °C. After washing with 10 volumes of PBS-BSA4, sperm were incubated with FITC-conjugated anti-rabbit IgG (1:50 in PBS-BSA4) (Sigma) for 30 min at 37 °C. Following washing, motile sperm were mounted on slides at 37 °C and observed under an epifluorescence microscope.

Labelling of fixed sperm

Sperm were fixed for 10 min in 2% paraformaldehyde in PBS at room temperature. After extensive washing with PBS, sperm were air-dried on slides and methanol-permeabilized as described above. Slides were then incubated in 5% normal goat serum in PBS (NGS-PBS) for 30 min at 37 °C and then exposed to the primary antibody (anti-TPX1 or anti-AA1) (1:100 in 1% NGS-PBS) for 2 h at 37 °C. After washing three times in PBS, sperm were incubated for 30 min at 37 °C with FITC-IgG (1:100 in PBS), washed and mounted on slides for observation under an epifluorescence microscope. To remove possible molecules masking TPX1 from the antibody on the sperm surface of non-permeabilized sperm, the cells were incubated for 30 min in 0.6 M NaCl in PBS previous to IIF. In all cases, normal rabbit IgG (Sigma, 5 mg/ml) was used as a negative control for anti-TPX1.

Preparation of sperm extracts

After complete liquefaction, the semen was washed in PBS by centrifugation at 700 g and then incubated for 30 min in either PBS, 0.6 M NaCl in PBS, or 1% Triton X-100 (Sigma) in PBS. After centrifugation of the suspension 10 min at 700 g, the pellet was stored at −20 °C until use, and the supernatant was centrifuged again at 13 000 g to remove sperm particles remaining in that fraction. Proteins in the supernatants were precipitated by addition of one volume of a 10% solution of trichloroacetic acid (Merck Química Argentina, Buenos Aires, Argentina), incubation at −20 °C for 20 min and centrifugation at 13 000 × *g*, at 4 °C for 20 min. Sperm pellets and protein precipitates were suspended in non-reducing Laemmli sample buffer (Laemmli, 1970) and subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot.

Western blot

Protein samples were separated in non-reducing 15% SDS-polyacrylamide gels, according to the method of Laemmli (Laemmli, 1970) and then electrotransferred to nitrocellulose membranes (Towbin *et al.*, 1979). To ensure correct loading of proteins in each lane, the membranes were stained with Ponceau reagent [1 mg/ml Ponceau S (Sigma) in 5% acetic acid] previous to immunodetection. The membranes were blocked for 1 h with powdered skim milk (2% in PBS) and incubated 1–2 h with a 1:1000 dilution of anti-TPX1. Membranes were washed thoroughly and incubated for 1 h with biotin-conjugated anti-rabbit IgG (1:500) (Sigma). After extensive washing, the membranes were incubated for 1 h with ExtrAvidin–horse-radish peroxidase (1:1000, Sigma), and reactive bands were visualized with 3,3′-diaminobenzidine [40 μg/ml (Sigma) in Tris 0.1 M (Sigma) pH 7.5 and 0.01% H_2O_2]. All incubations were carried out at room temperature.

Zona-free hamster oocyte penetration test (HOPT)

This test was performed as described in the WHO Laboratory Manual for the Examination of Human Semen (WHO, 1999). Immature (4–6 weeks old) hamster (*Mesocrycetus aureatus*) females were ovulation inducted by i.p. injection of 25 IU of equine Chorionic Gonadotropin (Syntex, Buenos Aires, Argentina), followed by i.p. administration of 25 IU of human chorionic gonadotropin (HCG) (Sigma) 48 h later. Oocytes were recovered from the oviducts of ovulation inducted animals 14–16 h after human chorionic gonadotropin (HCG) administration. Cumulus cells were dispersed with 0.1% hyaluronidase (Sigma, type IV) prepared in PBS-BSA4. After washing in BWW-HSA, the zona pellucidae (ZP) were removed by treatment with 0.1% trypsin (Sigma) in BWW. Finally, zona-free oocytes were thoroughly washed in BWW-HSA and distributed among treatment groups. Only human sperm samples presenting more than 70% motile sperm after capacitation were used for insemination. Aliquots containing 3.5 × 10^5 motile cells were diluted to 100 μl with BWW-HSA, and incubated with anti-TPX1 (1:10–1:250) or rabbit normal IgG at 37 °C under paraffin oil (Saybolt viscocity 75/340; Sanitas, S.A. Buenos Aires, Argentina). After 30 min, 15–20 zona-free hamster oocytes were added to each drop and gametes co-incubated for 2–3 h at 37 °C in an atmosphere of 5% CO_2. The oocytes were then freed from

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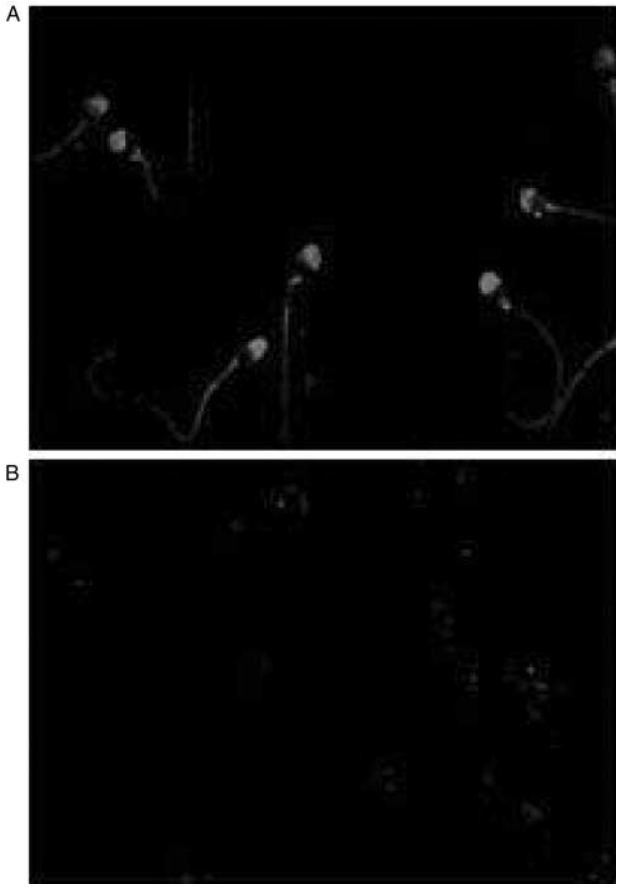

Figure 1. Localization of TPX1 in human sperm. Human fresh sperm were fixed, permeabilized and then subjected to IIF. (**A**) Sperm incubated with either anti-TPX1 or anti-AA1 as primary antibodies. (**B**) Sperm incubated with normal rabbit IgG as primary antibody. Magnification × 400.

unbound and loosely bound spermatozoa by serial aspiration through a finely drawn pipette, fixed in 4% glutaraldehyde, mounted on slides and stained with 1% acetocarmine solution. The number of oocytes presenting decondensing sperm heads or pronuclei and sperm tails in the oocyte cytoplasm, as well as the number of spermatozoa bound per oocyte, were determined by observation under the microscope (400×) and recorded. Aliquots of sperm remaining in incubation drops were placed in slides previously warmed at 37 °C, and the motility was evaluated subjectively for each treatment. The remaining sperm were fixed and subjected to PSA staining for acrosomal status evaluation.

Statistical analysis

Results are expressed as mean values ± SEM for each series of experiments. Statistical significance of the data was analysed using chi-square (χ^2) test for the percentages of sperm motility and AR, and of oocytes with bound and penetrated sperm. The numbers of sperm bound and fused per oocyte were analysed using the Student's *t*-test. Results were considered significantly different at a P value < 0.05.

Results

Localization of TPX1 in fresh, non-capacitated sperm

The localization of TPX1 in human fresh sperm was analysed by IIF on live and both non-permeabilized and methanol-permeabilized fixed spermatozoa using anti-TPX1 as primary antibody. No staining or a very faint labelling in the acrosome was observed in live and fixed non-permeabilized sperm, respectively (data not shown). To investigate the possibility that TPX1 were a peripheral protein hindered from antibody accessibility by other molecules bound to the sperm surface (i.e. coating proteins or decapacitating factors), fresh human sperm were treated with 0.6 M NaCl and then subjected to IIF. No differences were observed between treated and non-treated cells. By contrast, most fixed methanol-permeabilized sperm showed a strong fluorescent labelling in the acrosomal cap accompanied by a weak staining in the neck and tail that were not observed in control, IgG-incubated sperm samples (Figure 1A,B). Taken together, these observations indicate that human TPX1 would be mainly an intracellular protein localized to the acrosomal region of non-capacitated sperm.

In parallel, experiments were also undertaken in which human sperm were subjected to differential protein extraction procedures, and the presence of TPX1 in the supernatant and pellet fractions were analysed by Western blot. Results showed that while TPX1 (mol·wt 25 kDa) could not be released from sperm by treatment with PBS or ionic strength (0.6 M NaCl), it was completely removed by exposure to 1% Triton X-100, as judged by its detection only in the supernatant fraction of these samples (Figure 2).

Fate of TPX1 during sperm capacitation and AR

In order to investigate the behaviour of TPX1 during capacitation and AR, fresh sperm and sperm incubated under capacitating conditions and induced to undergo the AR by exposure to calcium ionophore were fixed, permeabilized and subjected to IIF as previously described. The analysis and quantification of the fluorescent patterns obtained indicated that while most fresh sperm showed strong fluorescence in the acrosomal cap (pattern A, Figure 3A), the percentage of cells with this pattern in capacitated and ionophore-treated sperm decreased concomitantly with an increase in the proportion of sperm showing labelling in the equatorial segment (pattern ES). Similar numbers of sperm either unstained or weakly labelled in the acrosomal region (pattern W) were present in all populations. As observed in Figure 3B, the percentage of acrosome reacted sperm in the three

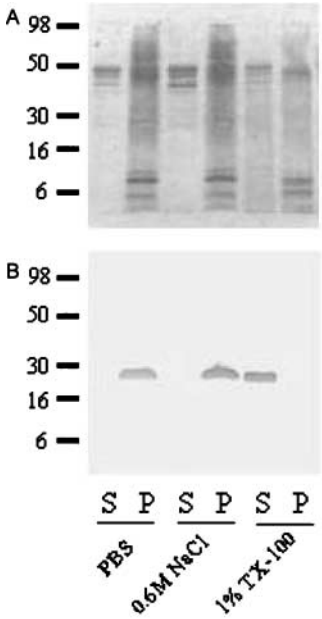

Figure 2. Differential extraction of TPX1 from sperm. Supernatants (S) and pellets (P) obtained by extraction of sperm proteins with PBS, 0.6 M NaCl or 1% Triton X-100 (TX-100) were separated by SDS-PAGE and transferred to nitrocellulose membranes. (**A**) Protein content detected by Ponceau staining. (**B**) Immunodetection of TPX1 by Western blot using anti-TPX1 as primary antibody.

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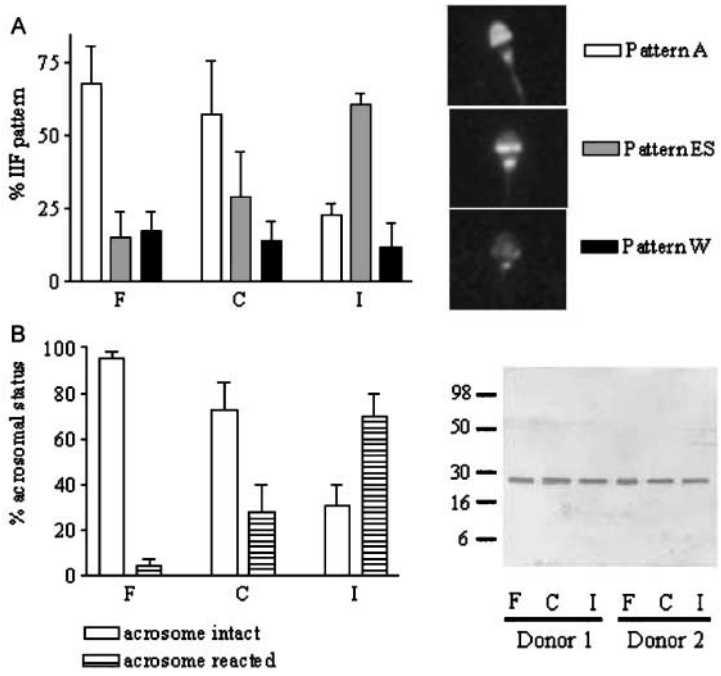

Figure 3. Fate of protein TPX1 after capacitation and ionophore-induced AR. (**A**) Microphotographs representative of fluorescent patterns obtained by IIF: acrosomal region (A), equatorial segment (ES) and weak labelling (W) (magnification ×1000), and quantification of each pattern in fresh (F), capacitated (C) and ionophore-treated (I) sperm. (**B**) Acrosomal status in each sperm population determined by FITC-PSA staining. Results in (**A** and **B**) represent the mean value ± SEM of four independent experiments. (**C**) Total protein extracts from equal amounts of fresh (F), capacitated (C) and ionophore-treated (I) sperm, from two different donors, were analysed by SDS-PAGE and Western blot.

populations correlated with the percentage of cells "showing pattern ES" suggesting that the TPX1 would be localized to the equatorial segment of sperm after the occurrence of the AR.

To study whether TPX1 was proteolytically processed as a result of capacitation and/or AR, total protein extracts from equal amounts of fresh, capacitated and ionophore-treated sperm were subjected to SDS-PAGE and examined by Western blot using anti-TPX1 as first antibody. Results revealed no differences in the electrophoretic mobility of the protein among the three sperm populations, arguing against a processing of TPX1 during capacitation and/or AR (Figure 3C).

Effect of anti-TPX1 on gamete interaction

The possible relevance of TPX1 for gamete interaction was analysed by means of the HOPT, which evaluates the ability of human capacitated sperm to penetrate zona-free hamster oocytes (Yanagimachi *et al.*, 1976). Capacitated human sperm were pre-incubated with different dilutions of anti-TPX1 and then exposed to zona-free hamster oocytes. Gametes co-incubated in the absence of the antibody or in the presence of rabbit IgG were used as controls. Results showed that the exposure of sperm to anti-TPX1 at a dilution $\leq 1/50$ produced a significant inhibition in both the percentage of penetrated oocytes and the average number of sperm penetrated per oocyte compared to controls (Figure 4A).

The inhibition in oocyte penetration would not be due to detrimental effects of anti-TPX1 on sperm cells, as the antibody did not induce sperm agglutination at any of the dilutions assayed and the motility of the antibody-treated cells were not different from controls (Table I). As the occurrence of the AR is a prerequisite for fusion of human sperm with the hamster oolema (Yanagimachi, 1984), the inhibition in oocyte penetration observed in the presence of anti-TPX1 could have been due to an effect of the antibody on the occurrence of this functional event. However, results indicated that the sperm exposed to anti-TPX1 exhibited percentages of spontaneous AR not significantly different from those observed in cells incubated under control conditions (Table I).

Considering that gamete fusion comprises a first step of binding of sperm to the oolema followed by a subsequent step of gamete fusion itself, the effect of anti-TPX1 on sperm ability to bind to the zona-free hamster oocytes was also evaluated. Results shown in Figure 4B revealed that the antibody did not affect this stage of gamete interaction as judged by the fact that neither the percentage of oocytes with bound sperm nor the number of sperm bound per oocyte were different from the corresponding controls.

Discussion

The *tpx-1* gene was first described by Kasahara and co-workers more than 15 years ago (Kasahara *et al.*, 1987, 1989). However, to our knowledge, this is the first report describing the presence and localization of protein TPX1 in human sperm, its fate after capacitation and AR, and its putative functional role in gamete interaction.

IIF experiments using anti-human TPX1 antibody suggest the intracellular localization of the protein, as judged by the lack of significant labelling in live and fixed non-permeabilized fresh cells in contrast to the clear labelling observed in the acrosomal cap of permeabilized sperm. Although the weak labelling observed in the tail of these sperm might be reflecting the presence of the protein in the outer dense fibres of the tail as reported for the rat (O'Bryan *et al.*, 1998, 2001), our results indicate that TPX1 is mainly localized in the acrosomal region of human sperm.

Complementary evidence provided by differential protein extraction procedures also argues against a surface localization of TPX1 in human sperm. First, high ionic strength was incapable of removing

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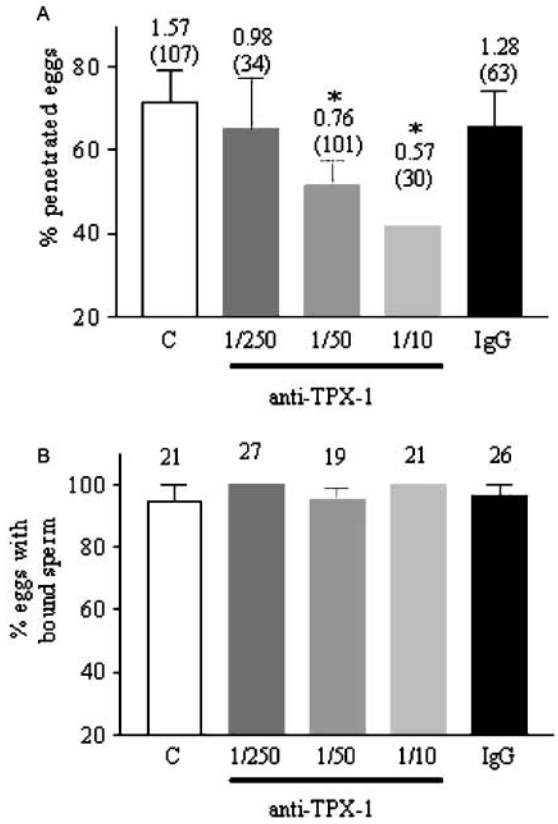

Figure 4. Effect of anti-TPX1 on the ability of human sperm to penetrate zona-free hamster oocytes. Capacitated sperm were incubated in drops of medium alone (C), or medium containing either anti-TPX1 (1/250–1/10) or rabbit IgG (1/50). ZP-free hamster oocytes were then added to drops and co-incubated with sperm for 3 h. Oocytes were recovered, fixed and stained with acetocarmine solution. Both oocyte penetration and sperm–oocyte binding were evaluated. (**A**) Bars represent the percentage of penetrated oocytes. The average number of sperm penetrated per oocyte and the total number of oocytes observed (in parenthesis) are shown at the top of each bar. (**B**) Bars represent the percentage of oocytes with bound sperm. At the top of each bar, the average number of sperm bound per oocyte is shown. Results represent the mean value ± SEM of eight independent experiments. $*P < 0.05$ versus controls.

or unmasking TPX1, indicating that the protein would neither be associated with the plasma membrane by ionic interactions nor be masked by an ionically-associated surface component. Second, although the extraction of TPX1 from sperm by detergent might suggest a strong association of TPX1 to the sperm surface, the lack of hydrophobic domains in the mature protein does not support its insertion into the sperm plasma membrane (Kasahara *et al.*, 1989). Finally, while the high abundance of cysteines in the molecule opens the possibility that TPX1 is bound to a sperm surface component by disulfide bonds, the finding that the detergent treatment removed the protein alone, uncoupled to a covalently-bound partner, would not favour this type of association.

Together, the results of IIF and protein extraction procedures strongly support the intracellular nature of the protein. Moreover, electron microscopy studies in rat and guinea pig have confirmed the localization of the protein to the acrosomal vesicle (Hardy *et al.*, 1991; O'Bryan *et al.*, 2001) supporting the idea that human TPX1 would be, as in other species, an intra-acrosomal component. The N-terminal secretory peptide present in TPX1 might be responsible for directing the protein to this organelle during spermatogenesis (O'Bryan *et al.*, 2001).

Table I. Effect of anti-TPX1 on sperm motility and AR

	% motile sperm	% acrosome reacted sperm
Control	65 ± 8	17 ± 1
Anti-TPX1 1/250	56 ± 11	14 ± 2.5
Anti-TPX1 1/50	62 ± 12	18 ± 0.5
Anti-TPX1 1/10	56 ± 10	12 ± 4
IgG 1/50	59 ± 9	16 ± 7

No significant differences were detected on sperm motility or AR among groups.

While a surface population of TPX1 does not seem to be present in mature sperm cells, it has been suggested that the germ cells are capable of secreting TPX1 to the media and that the secreted protein would bind to the surface of spermatogenic cells to participate in their interaction with Sertoli cells (Maeda *et al.*, 1998, 1999). Therefore, rather than being controversial, the different localizations reported for TPX1 might be indicative of different functions of the protein at different stages of cell development.

The fate of TPX1 after capacitation and AR was examined by evaluating both the presence and localization of the protein on fresh, capacitated and ionophore-treated sperm and the occurrence of AR in each of these sperm populations. The correlation observed between the percentage of sperm showing TPX1 in the equatorial segment and the percentage of acrosome-reacted cells in the three groups indicates that TPX1 would be localized in the equatorial segment of sperm after the AR. Several possibilities could account for this localization of TPX1 in acrosome reacted sperm. While TPX1 might be released from the acrosomal cap with the AR, part of the protein could remain within the equatorial segment region of the acrosomal vesicle after the occurrence of this event. However, this localization would not be consistent with the role of the protein in

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gamete fusion indicated by our functional studies. Alternatively, TPX1 could be released from the acrosome during the AR and bind to the surface of the equatorial segment. This mechanism of release and subsequent association with the cell surface has already been suggested for Tpx-1 in rat germ cells (Maeda *et al.*, 1999), and for acrosin, an acrosomal component detected in the equatorial segment of sperm after the AR (Takano *et al.*, 1993). Although less probable considering the soluble nature of the protein reported for other species (Hardy *et al.*, 1991; Kim *et al.*, 2001), TPX1 might also reach the plasma membrane over the equatorial segment by migration from the acrosomal membranes after the AR. Electron microscopy studies will provide the information required to discriminate among these possibilities.

As previously reported for other sperm proteins (Moos *et al.*, 1993; Hunnicutt *et al.*, 1996; Wen *et al.*, 1999), TPX1 present in sperm after capacitation and AR might have undergone post-translational modifications as a consequence of these functional events. However, the finding that the electrophoretic mobility of the protein extracted from capacitated and acrosome reacted sperm remained unchanged suggests that this might not be the case for TPX1.

The potential relevance of TPX1 for human gamete interaction was examined by evaluating the effect of anti-TPX1 on the sperm ability to penetrate zona-free hamster oocytes. Results showed that the presence of the antibody during gamete co-incubation produced a significant and dose-dependent decrease in the percentage of penetrated oocytes. The lack of effect of anti-TPX1 on sperm motility, the occurrence of the AR, and sperm binding to the oolema, indicated that the observed inhibition would not be due to steric hindrance or detrimental effects of the antibody on the cells, but rather to its specific effect on sperm–oocyte interaction. Since TPX1 exhibits significant homology (58%) with ARP/CRISP-1 (Kasahara *et al.*, 1989; Krätzschmar *et al.*, 1996), a human epididymal protein involved in gamete fusion (Cohen *et al.*, 2001), the inhibitory effect of anti-TPX1 might be attributed to its cross-reaction with ARP. However, the specific recognition of TPX1 and ARP by their corresponding antibodies (Cohen *et al.*, 2001) excludes this possibility.

The analysis of the results obtained led us to speculate that TPX1 present in the equatorial segment after the AR could mediate gamete fusion by interacting with complementary sites on the oocyte surface, as previously reported for ARP (Cohen *et al.*, 2001). Recent observations from our group indicating that mouse recombinant Tpx-1 both binds to the surface and inhibits penetration of zona-free rat oocytes (unpublished data), support this possibility. In this regard, it is important to note that Tpx-1 has also been implicated in another cell–cell interaction event such as the association between rat spermatogenic and Sertoli cells (Maeda *et al.*, 1998, 1999).

It is known that sperm with intact acrosomes can bind to but not fuse with the oocyte plasma membrane suggesting that key components of the sperm fusion apparatus seem to be either absent from the surface of intact sperm or require some kind of activation for fusion to occur (Yanagimachi, 1988; Arts *et al.*, 1993, 1997). Strong evidence has been provided indicating that acrosin, one of the best characterized enzymes from the mammalian acrosome, is involved in the development of hamster sperm fusibility (Takano *et al.*, 1993) and that human sperm metalloendoprotease(s) released during the AR would be important for the fusion of sperm and zona-free hamster oocytes (Diaz-Perez *et al.*, 1988; Diaz-Perez and Meizel, 1992). According to this, TPX1 might also be one of the acrosomal components released during the AR and involved in the acquisition of fusion competence of human sperm.

The molecular mechanisms underlying the potential functional roles of TPX1 are still unknown. TPX1 belongs to the CRISP family of proteins composed of more than 70 members grouped together because of a unique and common structure that suggests a conserved functional domain (Krätzschmar *et al.*, 1996; Szyperski *et al.*, 1998). In this regard, it is interesting to note that TPX1 has more than 60% homology with CRISP ion-channel regulating proteins isolated from reptile venoms (Morrissette *et al.*, 1995; Yamazaki *et al.*, 2003), an observation of particular interest given the importance of ions for fusion events. Significant homology (50%) has also been found between TPX1 and CRISP proteins exhibiting either enzymatic or protease inhibitory activities (Yamakawa *et al.*, 1998; Milne *et al.*, 2003; Schambony *et al.*, 2003). TPX1 could directly or indirectly participate in gamete fusion through any of these activities, as recently suggested for MAK248, a novel CRISP protein located in the equatorial segment of macaque sperm (Yudin *et al.*, 2002).

In summary, evidence provided in the present work indicates that human TPX1 would be an intra-acrosomal protein that remains associated to the equatorial segment of acrosome reacted sperm, and is relevant for human gamete interaction. The molecular mechanisms involved in the function of TPX1 are currently under investigation in our laboratory.

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