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Investigation of the expression and functional significance of the novel mouse sperm protein, a disintegrin and metalloprotease with thrombospondin type 1 motifs number 10 (ADAMTS10)

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Abstract

Fertilization represents the culmination of a series of complex interactions between male and female gametes. Despite advances in our understanding, the precise molecular mechanisms underlying these fundamental interactions remain largely uncharacterized. There is however growing recognition that this process requires the concerted action of multiple sperm receptors that possess affinity for complementary zona pellucida ligands and those that reside on the surface of the oolemma. Among the candidate sperm proteins that have been implicated in fertilization, those belonging to the ADAM (a disintegrin and metalloprotease) family of proteases have received considerable attention. The focus of the studies described herein has been the characterization of a closely related member of this protease family, ADAMTS10 (a disintegrin and metalloprotease with thrombospondin type 1 motifs number 10). We have demonstrated that ADAMTS10 is expressed during the later stages of mouse spermatogenesis and incorporated into the acrosomal domain of developing spermatids. During sperm maturation, the protein appears to be processed before being expressed on the surface of the peri-acrosomal region of the head. Our collective data suggests that from this position ADAMTS10 participates in sperm adhesion to the zona pellucida. Indeed, preincubation of capacitated spermatozoa with either galardin, a broad spectrum inhibitor of metalloprotease activity, or anti-ADAMTS10 antisera elicited a significant reduction in their ability to engage in zona adhesion. Overall, these studies support the notion that sperm-oocyte interactions involve considerable functional redundancy and identify ADAMTS10 as a novel candidate in the mediation of these fundamentally important events.

INTRODUCTION

Mammalian fertilization is a highly complex process, the molecular basis of which has proven extremely difficult to resolve. Despite decades of research, the paucity of our knowledge in this field continues to overshadow attempts to resolve the aetiology of defective sperm function observed in a significant proportion of infertile males and to progress the development of novel, male-oriented methods for fertility regulation (McLaughlin & Aitken, 2011). This problem stems in part from widely accepted models that depict sperm-oocyte recognition as being attributed to a single receptor-ligand interaction. Notwithstanding the appeal of this simple lock and key mechanism, it fails to account for both the myriad of putative receptors and their complementary ligands that have been identified to date and mounting evidence that gamete adhesion can be resolved into multiple interactions of both low and high affinity (Thaler & Cardullo, 1996; Thaler & Cardullo, 2002; Dun, *et al.*, 2010). Against this background, it has been argued that the sperm receptor may in fact be a composite structure containing multiple recognition molecules that are

assembled into a functional complex during the latter stages of sperm maturation (Nixon, *et al.*, 2005; Nixon, *et al.*, 2007; Tanphaichitr, *et al.*, 2007; Van Gestel, *et al.*, 2007; Gadella, 2008; Gadella, *et al.*, 2008). Among the challenges posed by this new model are the mechanisms by which the activity of such a large cohort of proteins is coordinated to ensure both productive interaction with the egg and integration with downstream cell signaling events. An intriguing possibility is that specialized membrane microdomains, or rafts, may serve as platforms to sequester and/or mediate the assembly of, multimeric zona receptor complexes on the outer leaflet of the sperm surface (Van Gestel, *et al.*, 2005; Bou Khalil, *et al.*, 2006; Tanphaichitr, *et al.*, 2007; Boerke, *et al.*, 2008; Gadella, *et al.*, 2008; Nixon, *et al.*, 2009; Nixon, *et al.*, 2010).

Taking advantage of unprecedented technological developments in high-throughput mass spectrometry and enhanced methods for protein pre-fractionation, we have begun to explore the proteomic profile of sperm membrane rafts. Such studies have generated extensive protein inventories, revealing the anticipated presence of a number of proteins that have been implicated in sperm-zona pellucida binding, in addition to those involved in downstream interaction with the oolemma (Nixon, *et al.*, 2009; Nixon, *et al.*, 2010). In addition they have identified a number of previously unknown and / or uncharacterized sperm proteins and provided the impetus for defining which specific elements of the raft proteome are of functional significance to the fertilizing spermatozoon. With this goal in mind we have focused on the characterization of one such novel protein, the metalloprotease ADAMTS10 (a disintegrin and metalloprotease with thrombospondin type 1 motifs number 10).

The rationale for targeting such a protein stems in part from pilot studies in which ADAMTS10 was identified as a putative client protein of heat shock protein 1 (HSPD1, formerly HSP60; Dun, unpublished), a molecular chaperone protein implicated in assembly of multimeric sperm receptor complexes (Asquith, *et al.*, 2004; Asquith, *et al.*, 2005). Furthermore, ADAMTS10 shares a similar modular architecture (Somerville, *et al.*, 2004) and a high degree of sequence identity to the ADAM family of metalloproteases that are known to mediate diverse cell and extracellular matrix adhesion events across a variety of cell types (Primakoff & Myles, 2000; Van Goor, *et al.*, 2009). A gamut of ADAM family proteins are expressed in the male reproductive tract, many of which represent isoforms unique to the male germ line (Evans, 2001). Perhaps the best characterized of these are fertilin α (ADAM1b) and β (ADAM2), a sperm membrane heterodimer originally implicated in sperm-oolemmal binding and fusion (Primakoff, *et al.*, 1987; Wolfsberg, *et al.*, 1995). Several lines of evidence suggest that fertilin binds to integrin(s) on the oocyte plasma membrane and it has been suggested that a short hydrophobic amino acid sequence reminiscent of viral fusion proteins is responsible for its proposed role in sperm-oocyte fusion (Blobel, *et al.*, 1992; Almeida, *et al.*, 1995). However, knockout studies failed to confirm these assertions in an in vivo system (Cho, *et al.*, 1998). Somewhat surprisingly, the sperm from ADAM2 null mice showed only modest reductions in their ability to engage in oolemmal binding and fusion but a far more prominent defect in their ability to bind to the zona pellucida and navigate through the uterotubal junction (Cho, *et al.*, 1998). A similar phenotype has also been documented following targeted deletion of other sperm ADAMs (ADAM1a and ADAM3) (Shamsadin, *et al.*, 1999; Nishimura, *et al.*, 2004) in addition to the testis specific molecular chaperones, calmeglin and calsperin (Ikawa, *et al.*, 2001; Ikawa, *et al.*, 2010), that appear necessary for ADAM protein assembly and / or presentation on the sperm surface. These data highlight a fascinating causal link between ADAM protein expression and fertilization (Ikawa, *et al.*, 2010). It is therefore of considerable interest to determine if the closely related family of ADAMTS proteins are also critical determinants of sperm function.

MATERIALS AND METHODS

Chemicals and Reagents

Unless otherwise specified, chemical reagents were obtained from Sigma (St. Louis, MO) and were of molecular or research grade. Polyclonal antibodies raised against a synthetic peptide based on the catalytic domain of ADAMTS10 were purchased from Abcam (catalogue # ab59813; Cambridge, MA). Monoclonal anti-CD59 (cat # MCA1927) and polyclonal anti-HSPD1 (cat # sc1052) antibodies were purchased from AbD Serotec (Raleigh, NC) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Alexa Fluor 555-labeled cholera toxin B subunit (CTB-AF555), Alexa Fluor 594-labeled *Arachis hypogaea* lectin (PNA), appropriate Alexa Fluor 488 (green) and 594 (red) conjugated secondary antibodies, and SYTOX green were all obtained from Invitrogen (Carlsbad, CA). Horseradish peroxidase (HRP) conjugated secondary antibodies were from Upstate Biotechnology (Lake Placid, NY).

Preparation of polyclonal antisera to mouse ADAMTS10

Total RNA was isolated from adult mouse testes using Trizol (Invitrogen, Carlsbad, CA) and reverse transcribed with reverse transcriptase III (Invitrogen). *Adamts10* cDNA incorporating the metalloprotease, disintegrin, cysteine-rich and spacer domains (amino acids 257–886, Fig. S1) was amplified using Taq polymerase with proofreading activity (Advantage 2 PCR Enzyme System, BD Biosciences, Sparks, MD) with synthetic oligonucleotide primers (forward: 5'-CGGAGAGATGTGGAGCAGTATGT-3'; reverse: 5'-TGGTGGACATGGTTCTGTGTTGC-3') using standard procedures to generate an expected product size of 1890 bp. Amplified PCR products were gel purified and sequenced using standard methods. They were then ligated into the pENTR/D-TOPO (Invitrogen) cloning vector and used to transform chemically competent *E. coli* (OneShot TOP10, Invitrogen). Bacterial colonies containing the desired insert were identified by colony PCR screening and amplified by overnight culture in LB media containing 100 µg/ml kanamycin. Recombinant plasmid DNA was isolated from overnight cultures (FastPlasmid Mini kit, Eppendorf, Hauppauge, NY) and used to perform an LR recombination reaction to generate an expression clone in the pDEST17 vector (Invitrogen). This vector is designed to produce recombinant protein in a bacterial expression system with an N-terminal 6 × His tag. Recombinant expression vectors were subsequently transformed into DH5α competent *E. coli* cells and clones containing the correct insert identified as above. Purified DNA from the expression clones was finally transformed into BL21-AI chemically competent *E. coli* (Invitrogen) for recombinant protein expression.

Overexpression of the recombinant ADAMTS10 protein was induced by growth of the BL21-AI cells in the presence of 2% arabinose. Bacterial lysates containing such proteins were affinity-purified on a nickel resin column (Ni-NTA Superflow, Qiagen, Valencia, CA) and fractions containing recombinant protein were identified on SDS–polyacrylamide gels. Excised bands corresponding to the purified ADAMTS10 protein were then used to immunize a rabbit for the production of polyclonal antisera. Immunoglobulin G (IgG) from the resulting antisera was affinity purified using immobilized protein G (Protein G Dynabeads, Invitrogen) and specific ADAMTS10 antibodies were affinity purified against the recombinant ADAMTS10 immunogen.

Collection and preparation of mouse spermatozoa

All experimental procedures were carried out with the approval of the University of Newcastle's Animal Care and Ethics Committee (ACEC). Inbred Swiss mice were obtained from a breeding colony held at the institutes' Central Animal House and maintained according to the recommendations prescribed by the ACEC. Mice were housed under a controlled lighting regime

(16L:8D) at 21-22°C and supplied with food and water ad libitum. Prior to dissection, animals were euthanized via CO₂ inhalation.

Immediately after adult male mice (>8 weeks old) were euthanized, their epididymides and testes were removed and carefully dissected free of fat and overlying connective tissue. The caudal region was isolated, blotted free of blood and immersed under pre-warmed water-saturated mineral oil. Caudal spermatozoa were collected by back-flushing after which the perfusate was deposited into modified Biggers, Whitten, and Whittingham media (BWW; (Biggers, *et al.*, 1971)) composed of 91.5 mM NaCl, 4.6 mM KCl, 1.7 mM CaCl₂·2H₂O, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄·7H₂O, 25 mM NaHCO₃, 5.6 mM D-glucose, 0.27 mM sodium pyruvate, 44 mM sodium lactate, 5 U/ml penicillin, 5 µg/ml streptomycin, 20 mM HEPES buffer and 3 mg/ml bovine serum albumin (BSA), then allowed to disperse into the medium for 15 min. Where indicated, negative control (non-capacitated) incubations were conducted using medium prepared without NaHCO₃ while positive control (capacitated) incubations were conducted in media supplemented with 1 mM pentoxifylline (ptx) and 1 mM dibutyryl cyclic adenosine monophosphate (dbcAMP). These treatments have been demonstrated to both suppress and promote sperm capacitation respectively (Nixon, *et al.*, 2006). An osmolarity of 300 mOsm/kg was maintained in all media.

Following collection, sperm concentration was determined and the cells diluted as required. Sperm were then assessed for motility and the non-capacitated samples used immediately. Alternatively, populations of capacitated spermatozoa were prepared by incubation for 45 min at 37°C under an atmosphere of 5% CO₂: 95% air. At regular intervals throughout the incubation, sperm suspensions were gently mixed to prevent settling of the cells and at the end of the incubation, sperm vitality and motility were again assessed. Neither parameter was affected by any of the treatments reported in this study.

To prepare caput and corpus spermatozoa, the appropriate region of the epididymis was dissected and placed in a 500 µl droplet of BWW medium. Multiple incisions were then made in the tissue with a razor blade and spermatozoa gently washed into the medium with mild agitation. The resultant cell suspension was then layered over 27% Percoll and centrifuged (400 × g for 15 min). The pellet, consisting of >95% pure spermatozoa, was washed by gentle centrifugation (400 × g for 2 min) to remove excess Percoll and then resuspended in fresh BWW medium and counted as described above. Similarly, testicular spermatozoa were prepared by decapsulating the isolated testes, making multiple incisions in the tissue with a razor blade and allowing the cells to gently disperse into the medium with mild agitation.

Immunohistochemical localization of ADAMTS10

Formalin fixed testis tissue was embedded in paraffin and cut into 5 µm sections. Following de-waxing and rehydration, antigen retrieval was performed by microwaving (500 W) the sections for 20 min in citrate buffer (10 mM trisodium citrate, 4.4 mM HCl, pH 6.0). All subsequent incubations were performed at 37°C in a humid chamber, and all antibody dilutions and washes were conducted in PBS. Sections were blocked at 37°C for 1 h in 10% v/v normal rabbit serum supplemented with 3% w/v BSA in phosphate-buffered saline (PBS). Slides were washed and incubated sequentially in primary antibody (diluted 1:100) and an appropriate Alexa Fluor 488 conjugated secondary antibody (diluted 1:300). After washing, the sections were counterstained with 10 µg/ml propidium iodide, a nuclear dye included to aid morphological assessment. Slides were mounted in antifade reagent (13% Mowiol 4-88, 33% glycerol, 66 mM Tris (pH 8.5), 2.5% 1,4 diazobicyclo-[2.2.2]octane), and viewed using a confocal microscope (Carl Zeiss Laser Scanning Microscope 510, Thornwood, NY).

Immunocytochemical localization of ADAMTS10

Sperm suspensions were lightly fixed in 1% paraformaldehyde, solubilized in 0.2 M Triton X-100 for 20 min at 4°C, and washed three times with 0.05 M glycine in PBS before being plated onto poly-L-lysine coated glass slides. The cells were blocked with 10% rabbit serum/3% BSA for 1 h at 37°C. Slides were washed with PBS prior to overnight incubation with primary antibody (diluted 1:100) at 4°C. Slides were then subjected to 3 washes in PBS and incubated with an appropriate Alexa Fluor 488 conjugated secondary antibody (diluted 1:300) for 1 h at 37°C. Slides were again washed and mounted in antifade reagent before being viewed by confocal microscopy.

For co-localization studies with cholera toxin B subunit (CTB), live sperm suspensions were mixed with an equal volume of Alexa Fluor 555-labeled CTB (10 µg/ml) and incubated for 15 min at 37°C. The cells were then washed four times in 2 volumes of BWW medium and fixed in 4% paraformaldehyde for 15 min at 37°C. The cells were aliquoted onto poly-L-lysine coated glass slides and allowed to settle before being blocked with PBS supplemented with 3% BSA and 10% goat serum for 1 h at 37°C. They were then sequentially labeled with the appropriate primary and Alexa Fluor 488 conjugated secondary antibodies for 1 h at 37°C in a dark, humidified chamber. The slides were then mounted with anti-fade reagent and observed using a confocal microscope.

In order to assess whether ADAMTS10 localization was influenced by the acrosomal status of spermatozoa, acrosomal exocytosis was induced by incubation of capacitated cells in 2.5 µM calcium ionophore A23187 for 30 min as previously described (Asquith, *et al.*, 2005). Sperm suspensions were then washed, resuspended in hypoosmotic swelling (HOS) medium (Jeyendran, *et al.*, 1984) and incubated for an additional 1 h. Following incubation, the cells were sequentially labeled with the appropriate primary and Alexa Fluor-488 conjugated secondary antibodies as indicated above. Spermatozoa were then dual labeled with PNA conjugated to Alexa Fluor 594 and prepared for confocal microscopy as outlined above.

Electron Microscopy

Isolated spermatozoa were fixed in 4% paraformaldehyde/0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) followed by dehydration, infiltration and embedding in LR White resin. Sections (70 nm) were cut on an Ultracut S ultramicrotome (Reichert-Jung, Austria) with a diamond knife (Diatome Ltd., Bienne, Switzerland) and placed on nickel grids. All antibody dilutions and washes for immunogold labeling were in Dulbecco's phosphate buffered saline (DPBS; pH 7.4). Grids were treated with 0.05 M glycine dissolved in DPBS for 40 min followed by washing and blocking in 3 % BSA in DPBS for 1 h at 37°C. Primary antibody diluted to 1:25 was applied and incubated overnight at 4°C. Grids were washed and incubated with 1:20 dilutions of secondary antibody conjugated to 10 nm gold particles for 2 h at 37°C. After washing, sections were post-fixed in 2% glutaraldehyde, dried and stained with 1% uranyl acetate in 40% methanol. Micrographs were taken on a JEOL-100CX transmission electron microscope (JEOL, Tokyo, Japan) operating at 80 kV.

Flow cytometry analysis of ADAMTS10 surface expression

In order to assess the surface expression of ADAMTS10, live spermatozoa were sequentially incubated with anti-ADAMTS10 and Alexa Fluor 488 conjugated secondary antibodies for 15 min at 37°C in a dark humidified chamber, before being washed twice in BWW. Spermatozoa were then counterstained with the vitality stain, propidium iodide (0.5 µM). After washing, the

cells were analyzed on a fluorescence-activated cell sorting (FACS) Vantage flow cytometer (Becton Dickinson, San Jose, CA). This system collects fluorescence data in logarithmic mode and light-scatter data in linear mode. Ten thousand cells were counted in each sample at a rate of 50–500 events per sec and data were analyzed using the Cell Quest package.

Analysis of ADAMTS10 expression in sperm membrane rafts

Low-density Brij 35 insoluble membrane fractions (detergent resistant membranes, DRMs) were prepared from mouse spermatozoa as previously described (Nixon, *et al.*, 2009). Briefly, capacitated mouse spermatozoa were washed by gentle centrifugation ($400 \times g$ for 1 min) and resuspended in 300 μ l of 1% Brij 35 in 25mM HEPES buffer (pH 6.5, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, protease inhibitor cocktail). Following mechanical disruption of the cells by homogenization in a Dounce homogenizer (30 strokes), the cells were incubated for 30 min at 4°C. Cell debris was pelleted by centrifugation at $10,000 \times g$ for 5 min and the supernatant, containing Brij 35-insoluble material, was then mixed with an equal volume of 80% sucrose (w/v) in 25 mM HEPES buffer for a final sucrose concentration of 40%. This suspension was placed in the bottom of an ultracentrifuge tube as the base of a discontinuous sucrose gradient. Additional layers consisting of 30% (980 μ l) and 5% (620 μ l) sucrose were carefully overlaid and the whole gradient was centrifuged at $100,000 \times g$ for 18 h at 4°C in an SW41 rotor (Beckman Coulter, Inc. Fullerton, CA). Following centrifugation, eleven 200 μ l fractions were carefully extracted by pipette from the top of the gradients and assessed for their light scattering properties by measurement of their absorbance at 620 nm and protein concentration using a BCA assay kit (Thermo Fisher Scientific, Rockford, IL). The fractions were then stored at -20°C prior to further analysis.

Dot immunoassays were performed to examine the partitioning behaviour of GM1 gangliosides, and hence DRMs, within the sucrose gradient. Briefly, 50 μ l of each of the isolated fractions were diluted 1:1 in PBS, added to wells of a Bio-Dot apparatus (Bio-Rad, Hercules, CA), suctioned onto nitrocellulose membranes and air dried. The membrane was then blocked with 5% milk powder in TBST (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20) for 1 h at room temperature, followed by incubation with HRP-conjugated cholera toxin in TBST supplemented with 0.5% milk powder for 1 h. It was then washed five times with TBST and subsequently developed with an enhanced chemiluminescence (ECL) assay (GE Healthcare, Piscataway, NJ, USA) and exposed to Hyperfilm ECL.

The protein composition of low-density Brij 35 insoluble membrane fractions prepared from mouse spermatozoa was assessed by SDS-PAGE and by immunoblotting analysis. For the purpose of SDS-PAGE, equivalent volumes of fractionated protein suspensions were precipitated with methanol / chloroform (2:1 v/v respectively) to collect both the detergent soluble and insoluble proteins. Following centrifugation at $10,000 \times g$ for 1 min, the supernatant was discarded and an additional 300 μ l methanol was added. The samples were then re-centrifuged at $10,000 \times g$ for 15 min and the supernatant discarded. The protein pellets obtained from either technique were allowed to air dry, then resolubilized and denatured by boiling in SDS sample buffer (Laemmli, 1970) containing 2% β -mercaptoethanol for 5 min and resolved on 10% SDS polyacrylamide gels according to the methods described by Laemmli (Laemmli, 1970). Resolved proteins were electrotransferred to nitrocellulose membranes (Hybond Super-C, GE Healthcare) under a constant current of 300 mA for 1 h (Towbin, *et al.*, 1979) and prepared for immunoblotting with anti-ADAMTS10.

Functional analysis of ADAMTS10 in mouse spermatozoa

At present no functional data is available regarding ADAMTS10, thus precluding the use of a known specific inhibitor for functional studies on this protein. For this reason, two broader specificity inhibitors were employed. The first was tissue inhibitor of metalloprotease 3 (TIMP3), a physiological inhibitor of both matrix metalloproteases (MMPs) and of several ADAMTS proteins (Kashiwagi, *et al.*, 2001). In addition, sperm were treated with the broad spectrum metalloprotease inhibitor galardin (HONHCOCH₂CH(i-Bu)CO-L-TRP-NHMe isomer 6A or GM6001) (Grobelny, *et al.*, 1992). To assess the impact of such inhibitors, as well as anti-ADAMTS10 antibodies, on sperm-zona pellucida binding ability, populations of capacitating mouse spermatozoa were incubated with each reagent for 30 min at 37°C. Following incubation, the spermatozoa were washed and an aliquot of 2×10^4 cells was then deposited into a droplet of BWB containing a minimum of 8 salt stored oocytes (Nixon, *et al.*, 2006). The gametes were co-incubated under oil in 5% CO₂ for 30 min at 37°C. Following incubation the oocytes were washed three times by serial aspiration and then incubated for 5 min in a droplet of BWB supplemented with 5 µg/ml of the DNA-specific fluorochrome 4',6-diamidino-2-phenylindole (DAPI). The oocytes were then washed in BWB and mounted on glass slides under coverslips supported on pillars comprising 80% paraffin wax and 20% Vaseline gel. The number of sperm bound to each zona was subsequently counted using both phase contrast and fluorescence microscopy and expressed as a percentage of the capacitated control sample.

Blue native polyacrylamide gel electrophoresis

Following incubation under either capacitating or non-capacitating conditions, suspensions of 1×10^6 sperm / ml were lightly pelleted (300 x g for 5 min) and resuspended in native protein lysis buffer (1% n-dodecyl β-D-maltoside, 0.5% Coomassie Blue G250 and a cocktail of protease inhibitors [Roche, Mannheim, Germany]). The samples were gently mixed and then incubated at 4°C on an orbital rotator for 30 min. Following incubation, the lysate was recovered by centrifugation at $14\,000 \times g$ for 20 min at 4°C and dialyzed against the Blue native cathode buffer (Invitrogen), to remove excess salt and detergent. Following dialysis, the sample was supplemented with glycerol to a final concentration of 5% (v/v) and resolved on a pre-cast blue native polyacrylamide gel (NativePAGE Novex 4-16%, Bis-Tris; Invitrogen) using a NativePAGE cathode and anode buffer system (Invitrogen). The BN-PAGE electrophoresis apparatus was placed at 4°C and the samples separated at 100 V until the Coomassie dye front reached the bottom of the loading wells. The voltage was then increased to 200 V and the separation continued until the Coomassie dye front reached the bottom of the gel. The gels were then removed from the electrophoresis apparatus and stained with Coomassie G250. Alternatively, the gels were prepared for either immunoblotting with anti-ADMTS10 using standard procedures.

Co-immunoprecipitation of ADAMTS10 interacting proteins

Approximately 60 µl (per treatment) of protein G magnetic beads (Millipore) were washed 3 × in PBS. This was followed by conjugation with 5 µg of anti-ADAMTS10 antibody at 4°C overnight with constant mixing. Following conjugation, the antibody-bead complexes were washed 2 × before being covalently cross-linked by incubation in 15 mM DTSSP (Thermo Fisher Scientific) for 2 h at 4°C. The cross-linking reaction was quenched using 1 M Tris and the conjugated beads were washed as above. A control sample of beads was also left non-conjugated and was incubated with PBS only. Both of the bead preparations were then incubated with approximately 100 µg of native sperm lysates (prepared as described above) that had been pre-cleared against non-conjugated beads to limit non-specific interactions. After an overnight incubation at 4°C

with constant mixing, the beads were washed $3 \times$ prior to elution of bound proteins by incubation in 0.2 M glycine pH 2.5 for 15 min at room temperature. Precipitated proteins were resolved on 10% polyacrylamide gels and prepared for either silver staining or immunoblotting.

Proteomic analysis of ADAMTS10

To confirm the specificity of the predominant sperm protein recognized by anti-ADAMTS10 antibodies, the protein was excised from a duplicate gel, destained and dehydrated in acetonitrile before being rehydrated in a minimal volume of 20 mM ammonium bicarbonate containing 40 ng/ μ l trypsin for 16 h at 37°C. The resulting tryptic peptides were purified using a ZipTip and sequenced by matrix-assisted laser desorption time of flight mass spectrometry (MALDI-ToF) using an Ettan MALDI-ToF Pro mass spectrometer (Amersham Biosciences). Briefly, 1 μ l fractions of the tryptic peptides were mixed with an equal volume of matrix solution (a saturated solution of α -cyano-4-hydroxycinnamic acid in 0.1% TFA:acetonitrile, 50:50) before being applied in duplicate to the stainless steel sample target plates. The peptide mass spectra were acquired in the reflectron mode and internal mass calibration was performed with two trypsin auto-digestion fragments (842.5 and 2211.1 Da). Peptide mass fingerprints (PMFs) were used as inputs to search the National Center for Biotechnology Information nonredundant (NCBI nr) database using the MASCOT search engine (<http://www.matrixscience.com>) with a probability-based scoring algorithm. Prior to the database search, known contamination peaks such as keratin and autoproteolysis peaks were removed. Searches were performed without restriction of protein molecular weight (Mr) or pI and with mandatory carbamidomethylation of cysteines and variable oxidation of methionine residues. One trypsin miscleavage was allowed. Peptide mass tolerance and fragment mass tolerance were set to 50 ppm and ± 0.4 Da, respectively. High confidence identifications reported in Fig. S1 were those that possessed statistically significant search scores (greater than 95% confidence interval, equivalent to MASCOT expect value <0.05).

Statistics

All experiments were replicated with material collected from at least 3 different animals and the graphical data presented represent means $\pm$ SEM, the standard errors being calculated from the variance between samples. Statistical significance was determined using an analysis of variance (ANOVA).

RESULTS

ADAMTS10 is expressed in the mouse testis and epididymal spermatozoa

To facilitate characterization of ADAMTS10, a truncated recombinant protein incorporating the metalloprotease, disintegrin, cysteine-rich and spacer domains (amino acids 257–886, Fig. S1) was synthesized using an *E. coli* expression system. Unfortunately, despite attempting several variations of the expression protocol, the recombinant ADAMTS10 product proved largely insoluble. Although this precluded its use in functional assays it was able to be used for the production of polyclonal antisera. The antisera were subsequently affinity purified and assessed for their specificity by immunoblotting against cell lysates prepared from mouse lungs (positive control), testes and spermatozoa. As the results presented in Fig. 1A demonstrate, the immune sera labeled a predominant protein of approximately 65 kDa in cell lysates prepared from either the mouse testes or lung. An additional band of approximately 120 kDa, corresponding to the size predicted for the full length, glycosylated protein, was also present in the testes and lung samples, albeit of much lower intensity. In contrast, the predominant band observed in isolated epididymal spermatozoa was approximately 50 kDa. Although this protein was observed in all sperm samples analyzed, the caput and corpus cells displayed an additional band of approximately 120kDa, whereas an alternative weak band of 65kDa was detected in corpus and cauda spermatozoa. Given the disparity between the predicted molecular weight of full length and processed forms of ADAMTS10 (approximately 118kDa and 95kDa, respectively; (Somerville, *et al.*, 2004) and that observed experimentally, an additional commercial antibody raised against the catalytic domain was sourced to validate these results. Notwithstanding some minor differences, a similar profile of proteins was labeled with this additional antibody. Notably, a protein of approximately 50 kDa was again the predominant band labeled in cell lysates prepared from epididymal spermatozoa. Although such findings suggest the ADAMTS10 protein is extensively processed, the precise nature of these modification(s) remains to be fully investigated. Nevertheless, we were able to obtain partial amino acid sequence that confirmed the identity of the 50 kDa sperm protein as ADAMTS10 and demonstrated that this processed form retains the catalytic domain (Fig. S1).

ADAMTS10 is expressed in the peri-acrosomal region of developing spermatids and mature mouse spermatozoa

Having confirmed the ability of ADAMTS10 antisera to detect the target protein in cell lysates prepared from mature spermatozoa, it was used in conjunction with indirect immunofluorescence to examine the ontogeny of ADAMTS10 expression within the testis and its localization in developing spermatozoa. As illustrated in Fig. 2, ADAMTS10 was not able to be detected in developing germ cells lining the basal margin of the seminiferous tubules. Indeed, the first significant expression of the protein was not observed until the formation of round spermatids. Within these cells, ADAMTS10 staining was primarily localized to a discrete crescent pattern characteristic of that of the developing acrosomal vesicle (Fig. 2A). Intense labeling of this domain remained apparent throughout the later stages of spermatogenesis and was clearly evident in the populations of elongating spermatids lining the luminal border of the seminiferous tubules (Fig. 2B).

To confirm acrosomal localization, enriched populations of round and elongating spermatids were prepared by sediment gradient isolation. These cells were then fixed, permeabilized and labeled with anti-ADAMTS10 before being counterstained with PNA, a marker of the acrosomal membrane (Mortimer, *et al.*, 1987). Consistent with the results secured by immunohistochemistry of whole testes sections (Fig. 2), ADAMTS10 expression was initially detected in round

spermatids (Fig. 3). Although diffuse labeling of ADAMTS10 was present throughout these cells, an intense pattern of crescent shaped labeling was also apparent. Strong co-localization with PNA confirmed this labeling pattern corresponded to that of the developing acrosomal vesicle. A similar pattern of acrosomal labeling was also observed in elongating spermatids (Fig. 3), raising the possibility that ADAMTS10 is either involved in cytological remodeling of the spermatogenic cells leading to the formation of the acrosomal vesicle and / or that the protein is transported into this domain during formation of the organelle to fulfill an important function in mature spermatozoa.

To begin to investigate the latter possibility, immunolocalization studies were undertaken to examine the profile of ADAMTS10 expression in maturing spermatozoa recovered from the male reproductive tract. Initial studies conducted on populations of fixed and permeabilized spermatozoa revealed that ADAMTS10 was strongly localized within the peri-acrosomal region of these cells. This pattern of ADAMTS10 localization appeared to be similar in mouse sperm recovered from the testis and the different regions of the epididymis and it did not appear to be noticeably influenced by the capacitation status of these cells (Fig. 4A). An additional focus of protein localization was observed within the sperm tail, however this appeared to change from predominantly principal piece to a more punctate pattern during epididymal maturation. The specificity of these labeling patterns was confirmed by the absence of staining in the secondary only (prepared by substituting the primary antibody with media alone) and pre-immune controls.

The presence of the ADAMTS10 within the peri-acrosomal region of mature spermatozoa is of potential interest as it intuitively suggests that the protein may participate in the cascade of sperm-oocyte interactions that underpin fertilization. However, since the previous study was conducted on populations of permeabilized spermatozoa, these data provide limited insight into the surface expression characteristics of the protein. A flow cytometry assay was therefore conducted to facilitate the objective measurement of surface protein expression in live cells and the exclusion of dead or moribund cells (identified through incorporation of a cell viability stain). As shown in Fig. 4B, ADAMTS10 was expressed on the surface of less than 5% of live spermatozoa recovered from the testis, or caput and corpus epididymidis. The absence of surface labeling in these cells suggests that ADAMTS10 must reside in an intracellular location. This was confirmed by ultrastructural localization of ADAMTS10 which revealed the protein was predominantly expressed the outer acrosomal membrane of these cells (Fig. S2). Interestingly, however, a marked increase in surface expression of ADAMTS10 was detected following sperm entry into the caudal region of the epididymis and a further dramatic increase was again detected following the induction of capacitation in this population of cells. The analysis of these cells by immunofluorescence microscopy confirmed that surface labeling was primarily restricted to the peri-acrosomal region of the sperm head (Fig. 4C).

ADAMTS10 partitions into membrane rafts and is lost following acrosomal exocytosis

Our interpretation of the collective data secured in the previous studies is that ADAMTS10 represents a novel sperm protein with a potentially important role in the cellular interactions that underpin fertilization. Since a number of proteins that mediate these interactions have been shown to preferentially partition into membrane rafts, it was of interest to confirm that the ADAMTS10 resides within these microdomains and determine whether it complexes with additional proteins in capacitated mouse spermatozoa. Analysis of raft association was achieved via co-immunolocalization of ADAMTS10 with fluorescently labeled cholera toxin subunit B (CTB), a pentameric label that binds with high affinity to G_{M1} gangliosides, one of the key

components of sperm membrane raft microdomains. As shown in Fig. 5A, ADAMTS10 displayed strong co-localization with CTB within the peri-acrosomal region of the head of the majority of capacitated spermatozoa, thus suggesting it is expressed within membrane raft microdomains in this region of the cell. This interpretation is consistent with the results of immunoblotting experiments in which ADAMTS10 was shown to be abundantly expressed in the light-buoyant density detergent resistant membrane fractions expected of a raft-resident protein (Fig. 5B, fractions 5 and 6).

To achieve the second goal of determining whether ADAMTS10 constitutes part of a multimeric protein complex, native sperm lysates were resolved by BN-PAGE (Fig. 6A) before being immunoblotted with anti-ADAMTS10 antibodies (Fig. 6B). This analysis revealed cross-reactivity between ADAMTS10 antibodies and at least three high molecular protein bands of approximately 240 kDa, 740, and 800 kDa (Fig. 6B). The apparent molecular weight of these bands is considerably higher than that expected for monomeric ADAMTS10 thus raising the prospect that the protein is assembled into either a homo- or heteromeric complexes on the sperm surface. Since our previous studies have implicated the molecular chaperone, HSPD1, in the assembly of multimeric protein complexes on the surface of mouse spermatozoa (Asquith, *et al.*, 2004), it was of interest that we could demonstrate the presence of at least two putative complexes that harbored both proteins (Fig. 6C, arrowheads). Evidence in support an ADAMTS10 - HSPD1 interaction was provided by the demonstration that both proteins co-localize within the peri-acrosomal region of mouse spermatozoa (Fig. 6D) and HSPD1 was able to be co-precipitated from native sperm lysates using anti-ADAMTS10 antibodies (Fig. 6E).

To further characterize the properties of ADAMTS10, its expression was examined following induction of acrosomal exocytosis. The rationale for this study is based on well established evidence that proteins involved in primary sperm-zona pellucida interaction are lost, along with the apical plasma membrane in which they reside, following acrosomal exocytosis. As anticipated, the induction of the acrosome reaction with the calcium ionophore A23187 led to a complete loss of PNA staining within the peri-acrosomal region of approximately 50-60% of the capacitated sperm population (Fig. 7A). Acrosomal loss in each of these cells was also accompanied by a dramatic reduction in ADAMTS10 labeling within the sperm head (Fig. 7B).

Inhibitors of metalloprotease function decrease sperm-oocyte interactions

In view of the data presented above, inhibitory studies were undertaken to begin to investigate the function of ADAMTS10 in mouse spermatozoa. In the absence of specific pharmacological inhibitors of ADAMTS10 function, we instead employed the use of two broad specificity metalloprotease inhibitors: tissue inhibitor of metalloprotease 3 (TIMP3), a physiological inhibitor of both matrix metalloproteases (MMPs) and of several ADAMTS proteins (Hashimoto, *et al.*, 2001; Kashiwagi, *et al.*, 2001); and galardin, a hydroxamic acid known to act as a broad spectrum inhibitor of metalloprotease activity (Grobelny, *et al.*, 1992).

At the concentrations employed in the present study (5-100 ng/ml), TIMP3 failed to elicit any marked impact on zona pellucida recognition and adhesion (Fig. 8A). The use of higher concentrations of this inhibitor was negated by the severe adverse effects it had on sperm viability. In contrast, galardin affected a dose-dependent decrease in zona binding in the absence of noticeable effects on sperm motility or viability (Fig. 8B). Similarly, substitution of the inhibitors with anti-ADAMTS10 antisera (20 µg/ml) also elicited a marked reduction in sperm adhesion to homologous zonae pellucidae (Fig. 8C). Indeed, the degree of zona interaction was decreased by approximately four fold to levels that were comparable to those seen in the non-capacitated sperm controls. Importantly, this suppression was not related to a reduction in sperm

viability or motility following antibody treatment as both parameters remained at levels comparable to those seen in the untreated controls. The specificity of this inhibition was further attested to by the failure of pre-immune sera (20 µg/ml) or anti-CD59 (20 µg/ml) antibodies to elicit any impact on sperm-zona pellucida interaction. In contrast to the results secured for sperm-zona pellucida binding, anti-ADAMTS10 antibodies had no discernible effect on either sperm-oolemma binding or fusion (results not shown).

DISCUSSION

The focus of the studies described herein has been the characterization of the novel sperm protein ADAMTS10, a recently identified member of the metalloprotease superfamily. Since the first description of the ADAMTS family by Kuno and co-workers (Kuno, *et al.*, 1997) a total of nineteen mammalian ADAMTS proteins have now been identified (Apte, 2009) which possess unique and overlapping functional roles in diverse biological processes ranging from cell migration, connective tissue organization, regulation of angiogenesis and blood coagulation, arthritis and the regulation of fertility (Flannery, *et al.*, 1999; Georgiadis, *et al.*, 1999; Hurskainen, *et al.*, 1999; Shindo, *et al.*, 2000; Li, *et al.*, 2001; Zheng, *et al.*, 2001; Cal, *et al.*, 2002; Russell, *et al.*, 2003; Richards, 2005; Stanton, *et al.*, 2005). Although ADAMTS proteins possess the signature metalloprotease and ancillary domains that characterize the closely related ADAM family of proteins they are distinguished from these metalloproteases by the absence of EGF-like, transmembrane and cytoplasmic domains (Kaushal & Shah, 2000). Furthermore, unlike the ADAM proteases, ADAMTS are secreted enzymes with a prominent role in extracellular matrix (ECM) proteolysis. This activity is primarily attributed to the presence of thrombospondin type 1 repeats (TSRs), a hallmark of the ADAMTS family (Porter, *et al.*, 2005; Apte, 2009). In addition to a variable spacer domain, the TSR is thought to be instrumental in mediating ADAMTS interaction with glycosaminoglycans within the ECM (Kuno & Matsushima, 1998; Somerville, *et al.*, 2003; Apte, 2004).

As with other ADAMTS family members, ADAMTS10 is synthesized as a zymogen, possessing an N-terminal signal sequence (Met¹-Ala²⁵) followed by a short prodomain that functions to preserve enzymatic latency and aids in correct protein folding and secretion (Cao, *et al.*, 2000). Experimental evidence secured by Somerville *et al.*, (2004) has demonstrated that furin, a proprotein convertase that is expressed in the testes and epididymidis of various mammalian species (Torii, *et al.*, 1993; Thimon, *et al.*, 2006), is the enzyme most likely responsible for zymogen processing. The amino terminus of the mature protease is predicted to commence at Ser²³⁴, immediately upstream of the catalytic domain. This metalloprotease domain is highly conserved, possessing a characteristic zinc binding signature commonly found in reprotin-type metalloproteases, the disintegrin-like domain and the first of five thrombospondin type I (TSR) repeat motifs. The presence of the HEXXH consensus sequence is suggestive of catalytic activity (Tang, 2001) and indeed, the metalloprotease activity of ADAMTS10, and several other ADAMTS proteins, is now well established *in vitro* (Abbaszade, *et al.*, 1999; Kuno, *et al.*, 1999; Tortorella, *et al.*, 1999; Somerville, *et al.*, 2004). At present however, less is known regarding the *in vivo* substrates of this family. Nevertheless, as some ADAMTS proteins are capable of cleave a range of substrates (including: the proteoglycans aggrecan, versican, and brevican; the fibrillar procollagens I, II, and III; and von Willebrand factor (Porter, *et al.*, 2005), it has been proposed that *in vivo* specificity may be dependent on the physiological environment in which the enzyme is expressed (Tang, 2001). On the contrary, the ADAMTS disintegrin-like domains have not yet been shown to possess disintegrin activity (Apte, 2004) and the consensus X(D/E)ECD site responsible for integrin binding is not well conserved in the ADAMTS family. Importantly, the work of Somerville and colleagues (Somerville, *et al.*, 2004) has demonstrated that ADAMTS10 is secreted to the cell surface and is a functional metalloprotease capable of cleaving α_2 -macroglobulin.

One curiosity arising from our characterization of ADAMTS10 was that the molecular weight of the predominant protein (50 kDa) recognized in epididymal spermatozoa by anti-ADAMTS10 antibodies differs substantially from that predicted on the basis of the primary sequence for either the full length zymogen or that of the processed mature form of the protein (118 and 95 kDa, respectively). Although post-translational modifications, such as the

documented N-glycosylation of ADAMTS10, are known to influence protein resolution in SDS-PAGE gels it is considered unlikely that they alone could account for such large discrepancies in electrophoretic mobility. While exploration of the origin of this alternative sized protein proved beyond the scope of the present work, it is considered likely that it originates from additional cleavage of the parent protein. The latter explanation is consistent with experimental evidence that proteolytic cleavage does occur in the ancillary C-terminal domains of other ADAMTS proteins. Indeed, C-terminal processing has been described for ADAMTS1, ADAMTS4, ADAMTS8, ADAMTS9 and ADAMTS12 (Vazquez, *et al.*, 1999; Rodriguez-Manzaneque, *et al.*, 2000; Cal, *et al.*, 2001; Gao, *et al.*, 2002; Somerville, *et al.*, 2003). Such, cleavage events generally occur within the spacer region (Vazquez, *et al.*, 1999; Rodriguez-Manzaneque, *et al.*, 2000; Flannery, *et al.*, 2002; Gao, *et al.*, 2002; Gao, *et al.*, 2004) and have been shown to elicit a profound impact on both substrate specificity and localization of the enzymes. In the best characterized example, ADAMTS4 has been shown to undergo autocatalytic C-terminal processing from a 75 kDa full-length active form to produce isoforms of 60 and 50 kDa (Flannery, *et al.*, 2002; Gao, *et al.*, 2004). Such processing results in the release of the enzyme from the ECM and dramatic alteration of its bioactivity (Gao, *et al.*, 2002) with, for instance, the aggrecanase activity profile being reduced to 1% of normal (Kashiwagi, *et al.*, 2004). In the case of ADAMTS10, it has recently been reported that the protein is highly susceptible to C-terminal processing events, analogous to those reported for ADAMTS4, if the protein is expressed in cells cultured in the absence of serum, and hence the protective activity of the proteolytic inhibitors contained therein (Somerville, *et al.*, 2004).

Although further studies are required to investigate the nature of ADAMTS10 processing, it was of considerable interest that post-testicular sperm maturation was identified as the likely staging site for proteolytic cleavage and activation of the ADAMTS10 zymogen. This situation is analogous with that reported for several members of the related ADAM protein family. Indeed many of the ADAM proteins that have proven to be instrumental in male reproductive function are regulated by proteolytic processing during epididymal passage (Schlondorff & Blobel, 1999)]. The significance of such processing is emphasized by reports that they occur in concert with the acquisition of sperm fertilizing ability in the epididymis (Aitken, *et al.*, 2007). It was also of interest that the surface labeling of ADAMTS10 increased dramatically following sperm capacitation and that the protein co-localized with membrane raft markers in these cells. While such findings raise the possibility that the protein participates in sperm-oocyte interactions (Dun, *et al.*, 2010), the dearth of functional data regarding ADAMTS10 prohibited the use of specific inhibitors to examine this role. Instead, two broad spectrum metalloprotease inhibitors were employed, tissue inhibitor of metalloprotease 3 (TIMP3) and galardin. TIMPs are small, disulphide-bonded proteins that are broadly effective competitive inhibitors of the matrix metalloproteases, yet display much greater selectivity towards both the ADAMs and ADAMTSs (Baker, *et al.*, 2002). TIMP3 is the only known endogenous ADAMTS inhibitor, and has been shown to potently inhibit mouse gamete fusion (Correa, *et al.*, 2000). However, the current study revealed that TIMP3 does not influence the initial binding event between capacitated spermatozoa and the zona pellucida. In contrast, treatment of spermatozoa with galardin, an alternative broad spectrum metalloprotease inhibitor (Grobelyny, *et al.*, 1992), initiated a dose-dependent decrease in their affinity for the zona pellucida in the absence of an effect on sperm motility or viability. While these data do not directly implicate ADAMTS10 in the initial stages of gamete interaction, they do provide the first evidence for the involvement of metalloproteases in this process and support the contention that this enzyme activity is an important determinant of sperm-zona interaction. This claim is further substantiated by the use anti-ADAMTS10 antisera, which also proved effective in reducing sperm-ZP interaction. However, we acknowledge that caution must

be exercised in interpreting experiments using antibody based inhibition of zona interaction due to the possibility of non-specific steric hindrance.

It therefore remains to be equivocally established whether ADAMTS10 directly binds ligands within the zona pellucida. An alternative tenable hypothesis that is in keeping with the localization of this protein, its putative interaction with the molecular chaperone HSPD1, and the fact that it resides in a multimeric protein complex, is that the proteolytic 'shedase' activity of ADAMTS10 is required to facilitate the unmasking, maturation and/or activation of alternative proteins involved sperm-oocyte interactions. In this context, the identification of ADAMTS10 as a putative binding partner of a cell surface chaperone draws interesting parallels with recent reports that matrix metalloproteases, such as MMP2 and MMP9, also form dynamic associations with molecular chaperones on the surface of certain tumor cells (Eustace, *et al.*, 2004; Stellas, *et al.*, 2010). In this capacity, the molecular chaperones assist in the activation of the matrix metalloproteinase thereby promoting increased tumor invasiveness (Eustace & Jay, 2004; Eustace, *et al.*, 2004). Further, antibodies that disrupt these interactions are demonstrably capable of preventing metalloprotease activation and significantly inhibiting the metastatic potential of carcinoma cell lines (Stellas, *et al.*, 2010). In the case of spermatozoa it has been shown that targeted deletion of molecular chaperones, such as calmeglin and calsperin, results in a male infertility phenotype associated with impaired sperm transport in the female reproductive tract in vivo and the loss of sperm-zona binding ability (Ikawa, *et al.*, 1997; Ikawa, *et al.*, 2010). These defects in sperm function appear to be due to misexpression, misprocessing and/or misfolding of fertilization-dependent proteins. Indeed, these chaperones appear to be required for ADAM1A/ADAM2 dimerization and/or subsequent maturation of ADAM3 (Ikawa, *et al.*, 1997; Ikawa, *et al.*, 2001; Ikawa, *et al.*, 2010; Ikawa, *et al.*, 2010). It is therefore perhaps not surprising that these infertility phenotypes are mirrored by those reported in reciprocal knockouts of ADAM2 and ADAM3 proteins and that spermatozoa in the latter models also experience the loss of multiple gene products (Nishimura, *et al.*, 2001; Nishimura, *et al.*, 2004). While comparable knockout studies of ADAMTS10 are required to determine if this protein similarly affects the expression profile of other proteins and contributes to defective sperm function, these collective findings raise the possibility that extracellular interaction between chaperones and metalloproteases may be a widespread phenomenon that regulates a range of biological and pathological processes.

Overall, these studies support the notion that sperm-oocyte interactions involve considerable functional redundancy and identify ADAMTS10 as a novel candidate in the mediation of these fundamentally important events. Future investigation of ADAMTS10, focusing on the elucidation of its substrates and hence the proteolytic pathways in which it participates are required to determine its precise role in the regulation of sperm function.

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FIGURE LEGENDS

Figure 1. Validation of anti-ADAMTS10 antibodies. (A) Recombinant ADAMTS10 protein was used to generate rabbit polyclonal antisera. IgG was isolated by protein G chromatography and specific ADAMTS10 antibodies were affinity purified against the recombinant ADAMTS10 immunogen. The purified post-immune antibodies were assessed for cross-reactivity in immunoblots of protein lysates prepared from mouse testes and lung, in addition to spermatozoa sampled from the caput, corpus and cauda epididymidis (5 µg per lane). The latter samples were isolated from populations of both non-capacitated (NC) and capacitated (Cap) spermatozoa. After labeling, the blot was stripped and reprobed with anti-tubulin antibodies to confirm equal protein loading across all sperm samples. (B) In order to confirm antibody specificity, a replicate immunoblot was also probed with commercial antibodies directed against ADAMTS10. Each experiment was replicated three times and representative blots are depicted.

Figure 2. Expression of ADAMTS10 protein in mouse testes. (A and B) Paraffin embedded sections of mouse testes were sequentially labeled with anti-ADAMTS10 and Alexa Fluor 488 (green) conjugated secondary antibodies. The sections were then counterstained with propidium iodide (red) to assist with morphological analysis. Control sections were prepared in which the primary antibody was substituted with either (C) buffer only (secondary only) or (D) pre-immune sera. This experiment was replicated three times and representative images are shown. Scale bar = 100 µm.

Figure 3. Co-localization of ADAMTS10 with PNA in isolated populations of developing spermatozoa. Enriched populations of round and elongating spermatids were isolated via sediment gradient filtration and fixed in 4% paraformaldehyde. They were then sequentially labeled with anti-ADAMTS10 and appropriate Alexa Fluor 488-conjugated secondary antibodies (green) followed by Alexa Fluor 594-conjugated PNA (red). Immunofluorescent labeling was detected using confocal microscopy. This experiment was replicated three times and representative images are shown. Scale bar = 10 µm.

Figure 4. Expression of ADAMTS10 in mature mouse spermatozoa. (A) Populations of spermatozoa were isolated from mouse testis, caput, corpus and cauda epididymidis. The latter samples were either held in a non-capacitated state (NC) or driven to capacitate (Cap). The cells were fixed in 1% paraformaldehyde and permeabilized with 0.2 M Triton X-100 before being sequentially labeled with anti-ADAMTS10 and Alexa Fluor 488 conjugated secondary antibodies (green) and counterstained with DAPI (blue). Control samples were prepared in which the anti-ADAMTS10 antibodies were substituted with either buffer alone (secondary only) or pre-immune sera. This experiment was replicated three times and representative images are shown. Scale bar = 10 µm. (B) To examine the surface expression of ADAMTS10, live populations of spermatozoa were labeled with anti-ADAMTS10 and Alexa Fluor 488 conjugated secondary antibodies before being counterstained with the vitality stain, propidium iodide. In addition to secondary antibody only controls, a positive control population of spermatozoa were prepared by labeling the cells with anti-flotillin antibodies. Sperm surface labeling was then analyzed using a fluorescence activated cell sorter. Data represent the mean $\pm$ standard error of the mean from three separate experiments. * $P < 0.05$, ** $P < 0.01$. (C) The surface localization of ADAMTS10 in these capacitated spermatozoa was assessed by labeling live populations with anti-ADAMTS10 and Alexa Fluor 488 conjugated secondary antibodies (green) and counterstaining with DAPI (blue).

Figure 5. Co-localization of ADAMTS10 proteins with cholera toxin subunit B (CTB) in mouse spermatozoa. (A) The ability of ADAMTS10 to partition in sperm membrane rafts in situ was examined by co-localization with the raft marker, G_{M1}, in live capacitated spermatozoa. For this purpose membrane rafts were visualized by staining live spermatozoa with Alexa Fluor 555 labeled cholera toxin B subunit (red). The cells were then fixed and labeled with anti-ADAMTS10 and Alexa Fluor 488 conjugated secondary antibodies (green). This experiment was replicated three times with a minimum of 200 spermatozoa being examined in each and representative images are shown. (B) The partitioning behavior of ADAMTS10 into buoyant, low density membrane fractions was examined following fractionation of detergent resistant membranes within a sucrose gradient. Each of the eleven fractions recovered from the sucrose gradient lysate were prepared for dot-blotting with CTB or immunoblotting with anti-ADAMTS10 and HRP conjugated secondary antibodies. Each experiment was replicated three times and representative data are shown. Scale bar = 5 μ m.

Figure 6. Examination of whether ADAMTS10 constitutes part of a multimeric sperm protein complex. Native sperm lysates were resolved by (A) 1D BN-PAGE and (B,C) transferred to nitrocellulose membranes. The membranes were then probed with (B) anti-ADAMTS10 antibodies or (C) anti-HSPD1 antibodies. This experiment was replicated 3 times and a representative BN-PAGE gel and immunoblots are shown. Arrowheads indicate protein bands that were labeled with both anti-ADAMTS10 and anti-HSPD1 antibodies. (D) The putative interaction between ADAMTS10 and HSPD1 was investigated by co-localization of the proteins in mouse spermatozoa. For this purpose, spermatozoa were stained with anti-ADAMTS10 (red) followed by anti-HSD1 (green) and appropriate Alexa Fluor conjugated secondary antibodies. This experiment was replicated three times with a minimum of 200 spermatozoa being examined in each and representative images are shown. Scale bar = 5 μ m. (E) The ADAMTS10 / HSPD1 interaction was further investigated through the use of a co-immunoprecipitation strategy in which native lysates prepared from capacitated spermatozoa were immunoprecipitated with anti-ADAMTS10. Experimental controls included proteins recovered by boiling non-conjugated beads (beads only), proteins recovered from boiling non-conjugated beads following their incubation with native lysate (preclear), whole sperm native lysate (native lysate), anti-ADAMTS10 antibodies only (antibody only), and the first and final bead washes (wash 1 and wash 3, respectively). Proteins that were eluted from ADAMTS10-conjugated beads following their incubation with native sperm lysates were resolved in the final lane (elution). Following separation of the proteins they were transferred to nitrocellulose and immunoblotted with anti-ADAMTS10 to confirm the efficacy of the immunoprecipitation, before being stripped and reprobed with anti-HSPD1. The arrowheads indicate proteins corresponding to ADAMTS10 and HSPD1.

Figure 7. Examination of ADAMTS10 behavior in acrosome reacted spermatozoa. Capacitated populations of mouse spermatozoa were induced to acrosome react through incubation with the calcium ionophore, A23187. Following the induction of acrosomal exocytosis, the cells were incubated in HOS media prior to being sequentially labeled with anti-ADAMTS10 and appropriate Alexa Fluor 488-conjugated secondary antibodies (green) followed by Alexa Fluor 594-conjugated PNA (red). Immunofluorescent labeling of viable cells was detected using confocal microscopy. This experiment was replicated three times and representative images are shown. Scale bar = 5 μ m.

Figure 8. Effect of metalloprotease inhibitors on sperm-zona pellucida interactions. Cauda epididymal sperm were capacitated in the presence of varying doses metalloprotease inhibitors (TIMP3 or galardin) or anti-ADAMTS10 antibodies (20 μg / ml). (A-C) They were then assessed for the ability to adhere to homologous zonae pellucidae. (A and B) Control samples contained vehicle (DMSO) at the same dilution used in the highest inhibitor concentration or (C) pre-immune sera (20 μg / ml) and antibodies against an alternative sperm surface protein (CD59; 20 μg / ml). The mean number of sperm bound per oocyte was recorded and expressed as a percentage of the capacitated control, which typically bound at a rate of approximately 30 - 40 spermatozoa / oocyte. Data represent the results of three replicates and are expressed as mean $\pm$ standard error of the mean. * $P < 0.05$, ** $P < 0.01$

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. Structure of mouse ADAMTS10. (A) Mouse ADAMTS10 is a modular protein of 1104 amino acids comprising a number of distinct functional domains (see key). (B) In order to confirm the specificity of the predominant 50 kDa sperm protein recognized by anti-ADAMTS10 antibodies, the protein was excised and sequenced by matrix-assisted laser desorption time of flight mass spectrometry (MALDI-ToF) using an Ettan MALDI-ToF Pro mass spectrometer (Amersham Biosciences). This strategy returned three high confidence peptide matches corresponding to ADAMTS10 (red, underlined text).

Figure S2. Immunogold labeling of ADAMTS10 in caput epididymal spermatozoa. Caput epididymal spermatozoa were fixed in 4% paraformaldehyde / 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) followed by dehydration, infiltration and embedding in LR White resin. Sections were blocked in 3 % BSA in Dulbecco's phosphate buffered saline (DPBS) for 1 h at 37°C before being sequentially labeled with anti-ADAMTS10 (overnight at 4°C) and secondary antibody conjugated to 10 nm gold particles for 2 h at 37°C. After washing, sections were post-fixed in 2% glutaraldehyde, dried and stained with 1% uranyl acetate in 40% methanol. Micrographs were taken on a JEOL-100CX transmission electron microscope (JEOL, Tokyo, Japan) operating at 80 kV. Scale bar = 500 nm, A = acrosome and N = nucleus.

Figure 1.

Figure 2.

Figure 3.

Round spermatids

Elongating Spermatids

Figure 4.

A

B

C

Figure 5.

Figure 6.

Figure 7.

Figure 8.

A Figure S1.

B

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MASACQILRWALALGLGLTFKVTTHAFRSQDELLSSLESYEIAFPTRVDHNGAMLAFSPPAFRRQRRGAGATTESRLFYKVAAPSTHFLNLNTRSPRLLAG 100
HVSVEYWTREGLAWQRAARAHCLYAGHLQGQAGSSHVAVSTCGGLHGLIVADDEEYLIEPLQGPGKGRGPEESGPHVVYKRSSLRHPHLDACGVRDEK 200
PWKGRPWWRLLTKPPPARPLGNESERGLGLKRSVSRERYVETLVVADKMMVAYHGRRDVEQYVLAIMNIVAKLQDSSLGNIIVNLVTRLILLTETDQPT 300
LEITHHAGKSLDSFCKWQKSIVSHSGHGNAPENGVANHDTAVLITRYDICIYKNKPCGTLGLAPVGGMCERERSCSINEDIGLATAFTIAHEIGHTFGM 400
NHDGVGNGCGARGQDPAKLMAAHITMKTNPFWSSCSRDIYITSFLDSGLGLCLNNRPPRQDFVYPTVAPGQAYDADEQCRFQHGVKSRQCKYGEVCSSELW 500
CLSKSNRCITNSIPAAEGLTLCQTHITDKGWCYKRVCVPFGSRPEGVDGAWGPWTPWGDCSRSCGGGVSSSRHCDSRPRTIGGKYCLGERRRHRSCNTND 600
CPPGSQDFREMQCSEFDSVPFRGKFYTWKTYRGGGVKACSLTCLAEGFNFYTERAAAVVDGTPCRPDTVDICVSGECKHVGCDRVLGSDLRDCKRVCVG 700
DGSACETIEGVFSPALPGTGYEDVWVWIPKGSVHIFIQDLNLSLSHLALKGDQESLLLEGLPGTPQPHRLPLAGTTFHLRQGPDAQSLEALGPINASLI 800
MVLAQAEALPALHYRFNAPIARDALPPYSWHYAPWTKCSAQCAAGGSQVQVVECRNQLDSSAVAPHYCSGHSKLPKRQRACNTEPCPPDWVVGNSRCSRSC 900
DAGVRSRSVVCQRRVSAAEEKALDDACQPRPPVLEACQGPMCPPEWATLDWSECTPSCGPGRLRHRVVLCKSADQQRSTLPPGHCLPAKPPSTMRNCLR 1000
RCPPARWVTSEWGECSTQCGLGQQQRTVRCTSHTGQPSRECTEALRPSTMQQCEAKCDSVVPPGDGPPECKDVNKVAYCPLVLKFQFCSRAYFRQMCKKT 1100
CQGR 1104
  
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Figure S2.