Analysis of chaperone proteins associated with human spermatozoa during capacitation

L.A. Mitchell1,2, B. Nixon1 and R.J. Aitken1,2,3

1Reproductive Science Group, Discipline of Biological Sciences, School of Environmental and Life Sciences, University of Newcastle, Callaghan, NSW 2308, Australia; 2ARC Centre of Excellence in Biotechnology and Development, Discipline of Biological Sciences, School of Environmental and Life Sciences, University of Newcastle, Callaghan, NSW 2308, Australia

3Correspondence address. Tel: +61 2 4921 2082; Fax: +61 2 9216 308; E-mail: john.aitken@newcastle.edu.au

Mammalian spermatozoa must undergo a post-ejaculatory period of maturation, known as capacitation, before they can engage in the process of fertilization. Studies in the mouse have established that capacitation facilitates sperm–zona recognition via mechanisms that involve the appearance of tyrosine phosphorylated chaperone proteins on the sperm surface overlying the acrosome, the site of sperm–zona recognition. In this study, we examined whether a similar relationship existed between the tyrosine phosphorylation events associated with capacitation and sperm–zona interaction in human spermatozoa. These studies confirmed that capacitation is associated with an increase in both sperm–zona binding and an increase in tyrosine phosphorylation over the sperm tail. However, we could not detect the surface expression of phosphotyrosine residues over the sperm head, as observed with murine spermatozoa. Moreover, although we could clearly detect a number of chaperone proteins in human spermatozoa including HSPE1, DNAJB1, HSPD1, HSPA1A, HSPCA, HSPH1, HSPA5 and TRA1, none of these molecules were expressed with murine spermatozoa. Moreover, although we could clearly detect a number of chaperone proteins in human spermatozoa including HSPE1, DNAJB1, HSPD1, HSPA1A, HSPCA, HSPH1, HSPA5 and TRA1, none of these molecules were expressed on the sperm surface. On the basis of these results, it is unlikely that these proteins play an active role in the remodeling of the sperm surface during capacitation. We conclude that strong species-specific differences exist in the molecular mechanisms that drive sperm–egg recognition and that alternative, chaperone-independent, mechanisms must underpin sperm–zona interaction in the human.

Keywords: spermatozoa; capacitation; chaperone; zona pellucida

Introduction

During their passage through the male reproductive tract, mammalian spermatozoa undergo an initial phase of post-testicular maturation in the epididymis during which they acquire the potential to fertilize an oocyte. However, this potential is only realized after ejaculation as spermatozoa undergo a series of physiological and biochemical changes during their ascent of the female reproductive tract (Austin, 1951; Chang, 1951). Collectively, these changes are known as ‘capacitation’ and culminate in the generation of functionally competent sperm that are able to bind to the zona pellucida, exhibit acrosomal exocytosis and, ultimately, fuse with the vitelline membrane of the oocyte (Yanagimachi, 1988).

Sperm–zona pellucida interaction has been extensively researched, and although much is known about the importance of the zona glycoprotein ZP3 for sperm binding, little is known about the corresponding sperm surface elements that mediate this interaction. A number of candidate molecules have been suggested including, but not limited to, galactosyltransferase, zonadhesion, an α-mannosidase, sp56 and zona receptor kinase (as reviewed by McLeskey et al., 1998; Wasserman, 1999; Talbot et al., 2003). However, current research indicates that none of these putative zona receptors are solely responsible for sperm–zona interaction. Thus, it has been hypothesized that zona interaction is mediated by the coordinated action of several sperm proteins that constitute a multimeric receptor complex on the sperm surface (Nixon et al., 2005). Studies of the key components of these complexes and the mechanisms by which they are assembled have become one of the key focuses of our laboratory.

It is now well established that the tyrosine phosphorylation of sperm proteins plays an important physiological role in mammalian sperm capacitation (Aitken et al., 1995; Visconti et al., 1995a). Recent studies from our laboratory have revealed that the tyrosine phosphorylation associated with sperm capacitation is a prerequisite for sperm–zona pellucida binding in the mouse (Asquith et al. 2004). Moreover, we have demonstrated that in this species, certain tyrosine phosphorylated proteins become localized to the sperm head surface during capacitation and appear to play a key role in sperm–zona binding (Asquith et al., 2004). Subsequent proteomic analysis revealed that two of the major targets for tyrosine phosphorylation on the sperm head during capacitation were the molecular chaperones, heat shock protein 60 (Hspd1) and endoplasmin (Tra1) (Asquith et al., 2005). In light of these results, we proposed that these phosphorylated chaperones were responsible for orchestrating the assembly and expression of a multimeric zona receptor complex on the sperm surface during capacitation (Asquith et al., 2005). Intriguingly, the surface expression of phosphotyrosine residues has also recently been confirmed in populations of capacitating porcine spermatozoa (Piehler et al., 2006). In light of these results, it is possible that the surface expression of tyrosine phosphorylated chaperones represents a highly conserved mechanism for the co-ordinated presentation of zona receptors on the sperm surface during capacitation. The purpose of this investigation...
was to determine whether these mechanisms are also operative in populations of capacitating human spermatozoa.

Materials and Methods

Reagents and antibodies

Unless otherwise stated the chemicals used throughout this study were purchased from Sigma (St Louis, MO, USA) and were of research or molecular biology grade, HEPES, penicillin and streptomycin were purchased from Gibco (Paisley, UK). Sodium chloride, sodium hydrogen carbonate, calcium chloride dehydrate, potassium chloride, magnesium sulfate heptahydrate and n-glucose were purchased from Merck (Darmstadt, Germany). Nitrocellulose was from GE Healthcare (Upplands, Sweden).

Anti-phosphotyrosine antibodies (clone 4G10) were from Upstate Biotechnology (Lake Placid, NY, USA); anti-HSP90 (anti-GRP94) rabbit polyclonal (Cpn10) was from Abcam (Cambridge, UK); anti-DNAJB1 (anti-HSP40) rabbit polyclonal and goat anti-rabbit (immunoglobulin G, IgG) and rabbit anti-goat (IgG) horse-radish peroxidase (HRP) conjugates were from Calbiochem (La Jolla, CA, USA). Anti-HSPD1 (anti-HSP60) rabbit polyclonal, anti-HSPA1A (anti-HSP70) mouse monoclonal, anti-HSPA5 (anti-GRP78) goat polyclonal and goat polyclonal HRP-conjugated goat anti-mouse (IgG) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-HSFCA (anti-HSP90) mouse monoclonal and anti-HSPH1 (anti-HSP10) were from BD Biosciences (Mississauga, ON, Canada). Anti-TRAIL (anti-GRP94) rat monoclonal was from Neomarkers (Fremont, CA, USA). Goat anti-rat (IgG) HRP conjugate was from Oncogene Research Products (San Diego, CA, USA). Mouse anti-human CD59 was purchased from Soreotec, UK. All FITC-conjugated secondary antibodies were purchased from Sigma.

Preparation of human spermatozoa

Human semen samples were obtained from a panel of healthy normozoospermic volunteer donors assembled for the Reproductive Science Research Group at the University of Newcastle. After at least 48 h abstinence, semen samples were produced by masturbation and collected into sterile sample containers which were delivered to the laboratory within 1 h of ejaculation. Scientific use of these samples was approved by the University of Newcastle Human Ethics Committee.

Purification of human spermatozoa was achieved using a 44 and 88% discontinuous Percoll (GE Healthcare) centrifugation gradient. For this procedure, Percoll (90 ml) was supplemented with 10 ml of 10× Ham’s F10 solution, 370 μl sodium lactate syrup, 3 mg sodium pyruvate, 210 mg sodium hydrogen carbonate and 100 mg polyvinyl alcohol (PVA). This isotonic Percoll solution was diluted with HEPES-buffered Biggers, Whitten and Whittingham medium (BWW) supplemented with 1 mg/ml PVA, maintained at an osmolarity of 300 mOsm/kg (Biggers et al., 1971) in order to create the discontinuous gradient. Up to 3 ml semen was layered on top of each gradient and centrifuged at 500 × g for 30 min. Following centrifugation, the seminal plasma and Percoll were removed and discarded. Purified spermatozoa were recovered from the base of the 88% Percoll fraction and washed with medium BWW, pelleted by centrifugation at 500 × g for a further 15 min and finally resuspended at a concentration of 6 × 10^6 cells/ml.

Capacitation of human spermatozoa

Following dilution in BWW purified spermatozoa were incubated at 37°C under an atmosphere of 5% CO₂/95% air. Non-capacitated cells were incubated in BWW prepared without NaHCO₃ (BWW-HCO₃⁻). Capacitated cells were incubated in BWW prepared with CaCl₂ (BWW-Ca⁺²) but supplemented with 3 mM pentoxyfilline (pxt) and 5 mM dibutyryl cyclic adenosine monophosphate (dbcAMP). This treatment has been shown to induce optimal levels of tyrosine phosphorylation in human spermatozoa (Baker et al., 2004). Incubations were conducted for a period of 3 h, after which the percentage of motile cells was assessed and the sperm were prepared for the various treatments outlined below.

Collection and preparation of mouse spermatozoa

Experimental procedures involving animals in this study were performed in accordance with the University of Newcastle Animal Care and Ethics Committee (Ethics Number 748/0606). Inbred Swiss male mice, greater than 8 weeks of age, were obtained from the University of Newcastle’s Central Animal House. The mice were maintained under a lighting regime of 16 h light:8 h dark, at 21–22°C, with food and water available ad libitum. Prior to dissection, mice were euthanized via CO₂ asphyxiation.

Immediately after the mice had been euthanized, the epididymides were isolated and excess fat, mesentery and residual blood were carefully removed. The caudal region of the epididymus was isolated and immersed under pre-warmed water-saturated mineral oil. A small incision was made into the epididymal tubules and the sperm were gently teased out into a droplet of BWW media. The sperm were then incubated for 5–10 min at 37°C in an atmosphere of 5% CO₂/95% air to allow them to disperse into the media. Negative control incubations were conducted in media devoid of NaHCO₃, whereas positive control incubations were conducted in media devoid of CaCl₂ but supplemented with 1 mM ptx and 1 mM dbcAMP. Following isolation, cell concentration was determined and the cells diluted as required.

Preparation of human oocytes

Fresh oocytes, excess to requirements for in vitro fertilization (IVF) programmes at Sydney IVF (Sydney, Australia), were obtained after patient consent. Oocytes were stripped of cumulus cells by brief treatment with 80 IU/ml hyaluronidase (Sigma) followed by gentle pipetting through a 200 mm diameter pipette. Oocytes were then fixed in a high salt storage medium consisting of 1.5 M MgCl₂, 0.1% dextran, 0.01 mM HEPES buffer and 0.1% PVA and maintained at 4°C until use. Importantly, storage of oocytes in high salt medium has been demonstrated to retain the biological characteristics of the zona pellucida (Yanagimachi et al., 1979).

Human sperm–zona pellucida binding assay

Human spermatozoa were diluted to 5 × 10⁵ cell/ml and incubated under conditions that either would, or would not, induce capacitation. Non-capacitated spermatozoa were incubated in bicarbonate-free medium BWW, whereas capacitated spermatozoa were incubated in a medium lacking calcium but supplemented with 1 mM ptx, 1 mM dbcAMP and 1.7 mM strontium chloride that has previously been shown to induce maximal levels of zona binding in the mouse (Asquith et al., 2004). Following incubation, the spermatozoa were deposited under water-saturated mineral oil at 37°C, and 10–20 salt-stored human ova (Yanagimachi et al., 1979) were added to each sperm suspension and incubated for a further 30 min at 37°C in an atmosphere of 5% CO₂/95% air. Following co-incubation, the oocytes were washed three times by serial aspiration through a fine bore glass micropipette to remove any unbound or loosely adhered sperm. The oocytes were then incubated for 10 min in a droplet of BWW supplemented with 5 μg/ml of the DNA-specific fluorochrome 4',6-diamidino-2-phenylindole (DAPI). The oocytes were then washed in BWW and mounted on glass slides over 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 using a Zeiss Axioskop 2 microscope.

Identification of proteins

In order to determine the complement of molecular chaperone proteins present in human spermatozoa, the cells were lysed in 10 mM CHAPS (3-[3-cholamidopropyl] dimethylammonio)-1-propanesulfonate) (Research Organics, Cleveland, OH, USA) for 30 min on ice with regular vortexing. Following incubation, soluble protein extracts were subjected to proteomic analysis at the Australian Protein Analysis Facility.

The samples were transferred to Vivaspin 500 centrifugal filter units (5000 MWCO; Viva Science Ltd, Gloucestershire, UK), centrifuged at 12 000 × g for 15 min, and the pellets re-suspended in 30 mM ammonium bicarbonate. The samples were then reduced using dithiothreitol and alkylated with iodoacetamide before being digested with trypsin overnight at 37°C, dried and redissolved in reverse phase nanoLC MSMS sample loading solution (0.1% formic acid, 2% acetonitrile, 97.9% water). The samples were then injected onto Michrome Peptide Trap C18 pre-columns at 10 μl/min for concentration and desalted with 0.1% formic acid. The pre-column was then switched into line with the analytical column containing C18 RP silica (150 μm × 100 mm, Protocol C18 3 micro, Scientific Glass Engineering, Austin, TX). Peptides were eluted from the
column using a solvent gradient from H2O:CH3CN (90:10; +0.1% formic acid) to H2O:CH3CN (60:40; +0.1% formic acid) at 600 nL/min over a 120 min period. An Applied Biosystems QSTAR mass Spectrometer (BI, Foster City, CA, USA) on information dependent acquisition mode subjected the LC eluent to a positive ion nanoflow electrospray analysis, acquiring a TOFMS survey scan (m/z 370–2000, 1.0 s), with the four largest multi-charged ions (counts >50) in the survey scan sequentially subjected to MS/MS analysis. The LC/MS/MS data were searched using the Mascot (Matrix Science, London, UK) search engine using monoisotopic masses of tryptic peptides as inputs to search the NCBI non-redundant protein database. Peptide mass tolerance of ±200 ppm and a fragment mass tolerance of ±0.3 Da were considered.

**Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting**

Following incubation, cells were collected by gentle centrifugation (500 g for 3 min) and protein extracts were prepared by solubilizing the cells in a sodium dodecyl sulfate (SDS) Extraction Buffer (2% SDS, 10% sucrose in 0.1875 M Tris pH 6.8) supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany) for 5 min at 100°C. Insoluble constituents were removed by centrifugation at 10,000 g for 15 min. Protein quantification of the isolated supernatant was determined using a bicinchoninic acid Protein Assay Kit (Pierce, Rockford, IL, USA) according to the manufacturer’s instructions.

Equivalent amounts of protein (2 μg) were boiled in SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer (SDS Extraction Buffer supplemented with 2% mercaptoethanol and bromophenol blue) for 5 min and resolved on 10% polyacrylamide gels (Laemmli, 1970). The proteins were then electro-transferred onto nitrocellulose membranes under a constant current of 300 mA for 1 h (Towbin et al., 1979).

Membranes were blocked for 1 h at room temperature with 3% bovine serum albumin (BSA) (Research Organics) in Tris-buffered saline (TBS) pH 7.4 supplemented with 0.1% polyoxyethylene sorbitan monolaurate (Tween-20).

Membranes were rinsed in TBS and then probed with appropriate primary antibodies at 1:1000 dilution in 1% BSA, 0.1% Tween-20 in TBS for 2 h at room temperature. Following incubation, membranes were washed three times in TBS containing 0.01% Tween-20 (TBST) for 10 min. Membranes were then probed for 1 h with a 1:3000–1:5000 dilution of HRP-conjugated secondary antibody at room temperature. Following a further three washes in TBST, cross reactive proteins were visualized using an enhanced chemiluminescence kit (GE Healthcare) according to the manufacturer’s instructions.

**Immunodetection of chaperones on fixed spermatozoa**

Following incubation, spermatozoa were fixed in 4% paraformaldehyde, washed three times with phosphate-buffered saline (PBS), plated onto poly-L-lysine coated glass slides and air-dried. All subsequent incubations were performed in a humid chamber at 37°C. The cells were permeabilized with 0.2% Triton X-100 for 15 min, rinsed with PBS and blocked with 10% serum/3% BSA for 1 h. Slides were washed three times with PBS for 5 min and incubated in a 1:50 dilution of primary antibody at 4°C overnight. Slides were then subjected to 3 × 5 min washes with PBS and incubated in a 1:100 dilution of HRP-conjugated secondary antibody for 2 h at 37°C. Slides were again washed and mounted in 10% mowiol 4–88 (Calbiochem) with 30% glycerol in 0.2 M Tris (pH 8.5) with 2.5% 1,4-diazobicyclo[2.2.2]octane (DABCO). Cells were examined using either a Zeiss Axioplan 2 fluorescence microscope or an LSM510 laser scanning confocal microscope equipped with argon and helium/neon lasers.

**Immunodetection of chaperones on live sperm using Dynabeads®**

Magnetic beads coated with Protein G (Dynal Biotech ASA, Oslo, Norway) were washed three times in 0.1% BSA in PBS. This was followed by conjugation with primary antibody (1:50) for 3 h at room temperature with constant mixing. A control sample of beads was left non-conjugated and was incubated with PBS only. Beads were washed three times with BW2 media and added to capacitated spermatozoa (1:40) following pre-incubation. The sperm/bead suspension was incubated for 30 min at 37°C with constant mixing. Following incubation, an aliquot of the sperm/bead suspension was visualized by phase contrast microscopy. The percentage of cells bound to beads was determined by scoring 100 motile cells. It is important to note that the sperm suspension was viewed using both warm slides and coverslips.

**Immunodetection of chaperones on live sperm using fluorescence-activated cell sorter**

Following purification, spermatozoa were diluted to 1 × 106 cell/ml, and incubated in capacitation medium. The sperm suspension was then incubated with primary antibody at 1:100 dilution for 1 h. The cells were subsequently washed 2× with BW2 and incubated with FITC-conjugated secondary antibody at 1:500 for a further 40 min. Following additional washes with BW2, the cells were incubated with propidium iodide (20 μg/ml) and analyzed using a fluorescence-activated cell sorting (FACS) Calibur FACs (Becton Dickinson, Franklin Lakes, NJ, USA) with a FL4 530/30 nm band-pass filter, allowing the collection of 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 second. Data were analysed using the Cell Quest package.

**Statistics**

All experiments presented in this study were performed a minimum of three times. Graphical data presented represent mean data ± SEM. Statistical difference between group means were determined using an analysis of variance (ANOVA) or paired t-test. P-values of <0.05 were considered significant.

**Results**

**Sperm–zona pellucida binding and tyrosine phosphorylation**

In order to confirm the fundamental tenet that only capacitated cells possess the ability to engage in sperm–oocyte interaction, human spermatozoa were incubated under conditions that we have optimized for driving (BW2-Ca2+ + Sr2+ + 3 mM ptx + 5 mM dbcAMP) and suppressing (BW2-HCO3–) capacitation, respectively (Asquith et al., 2004). Following this initial incubation, we performed an in vitro sperm–zona binding assay to assess the functionality of the cells. Although we observed no difference in the percentage motility of sperm in either treatment, there was a clear distinction in their competence to adhere to the zona pellucida (Fig. 1A). As anticipated, capacitated sperm readily bound to the zona pellucida (35.5 ± 8.0 sperm/egg). In contrast, non-capacitated sperm showed only background levels of binding (4.8 ± 1.7 sperm/egg; Fig. 1B; P < 0.01).

**Tyrosine phosphorylation and zona binding**

Recent studies in the mouse have revealed that sperm–zona binding ability is causally related to the tyrosine phosphorylation status of these cells (Asquith et al., 2004). To investigate whether a similar relationship exists in the human, the expression of tyrosine phosphorylation by human spermatozoa was assessed in similar populations of cells to those used for the sperm–zona binding assay. As illustrated in Fig. 2, there is a clear distinction between the phosphotyrosine expression patterns of non-capacitated and capacitated human spermatozoa. Approximately 75% of the capacitated sperm population were characterized by intense phosphotyrosine labeling over the mid-piece and principle piece of the tail (complete flagellum). In contrast, non-capacitated cells showed only background staining (Fig. 2A and B). These data were supported by western blot analysis of the levels of tyrosine phosphorylation expression exhibited by both non-capacitated and capacitated human spermatozoa. As we have previously reported (Nixon et al., 2005), the tyrosine phosphorylation associated with capacitation involved a large number of target proteins, particularly those with molecular weights of >75 kDa. By contrast, non-capacitated were again characterized by weak background staining.

**Surface expression of tyrosine phosphorylation**

Although our results corroborate the relationship between phosphotyrosine expression and capacitation, the internal tail localization of phosphotyrosine residues fails to account for the enhanced zona
binding ability of capacitated human spermatozoa. However, in light of recent data demonstrating a capacitation-dependent expression of the phosphotyrosine residues on the outer surface of the sperm head in both murine and porcine sperm (Asquith et al., 2004; Piehler et al., 2006), we investigated whether a similar pattern of expression exists in human spermatozoa. For this purpose, we used an immuno-bead assay, the fidelity of which was confirmed using a sample of capacitated mouse spermatozoa, which revealed the anticipated binding of anti-phosphotyrosine beads to ~15% of the population (Asquith et al., 2004). However, neither non-capacitated nor capacitated human spermatozoa displayed detectable levels of surface phosphotyrosine residues (Fig. 3A).

This result was confirmed through the use of a more sensitive, FACS-based assay. Again, only a very small percentage of the

Figure 1: Analysis of the effects of capacitation on the ability of human spermatozoa to bind to the zona pellucida of homologous oocytes
Following incubation in media optimized to either drive or suppress capacitation, human spermatozoa were co-incubated with salt-stored human oocytes. (A) After stringent washing to remove loosely adherent spermatozoa, those remaining bound to the zona pellucida were stained with DAPI, washed in BWW and mounted on glass slides under coverslips and counted. (B) Representative fluorescence microscopy images of oocytes depicting the number of zona bound spermatozoa are shown. This experiment was replicated three times using a minimum of 12 oocytes per treatment. **P < 0.01 by paired t-test

Figure 2: Tyrosine phosphorylation of human spermatozoa
Purified human spermatozoa were incubated in capacitating or non-capacitating media for 3 h. (A) Following incubation the sperm were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 and stained with anti-phosphotyrosine (1:100). Non-capacitated cells revealed only background fluorescence, whereas capacitated cells were characterized by strong mid-piece and principle piece staining. (B) The cells were scored based on their staining pattern. This experiment was replicated three times scoring a minimum of 100 sperm per treatment. ***P < 0.001. (C) An aliquot of sperm from each population was also lyzed and prepared for immunoblotting with an anti-phosphotyrosine antibody. Following development, blots were stripped and reprobed with anti-α-tubulin as an internal control to ensure equivalent protein loading. Each of these experiments was repeated three times and a representative western blot is shown.
sperm population displayed detectable levels of surface phosphotyrosine expression (Fig. 3B). This result was not attributed to an inability to effectively label human sperm surface proteins, since >90% of the population were shown to express CD59, a known surface antigen used as an internal positive control.

Identification of chaperones in human sperm

In mouse spermatozoa, it has been proposed that the activation of sperm-surface chaperones by tyrosine phosphorylation during capacitation may trigger conformational changes facilitating the assembly of functional zona pellucida receptor complexes on the surface of these cells. By analogy, it is possible that similar mechanisms participate in the functional maturation of human spermatozoa, but that this process requires a different set of chaperones that operate independently of phosphorylation. In order to explore this possibility, we conducted a comprehensive proteomic analysis of the complement of chaperone proteins that are present in human spermatozoa. As anticipated, this study revealed the presence of a number of chaperones and their associated proteins (Table 1). Although DNAJB1, HSPD1, HSPA1A, HSPCA, HSPA5 and TRA1 have been identified in the sperm of humans and/or other mammalian species, HSPE1 and HSPA5 have not previously been described in these cells. Given the novelty of these findings, we first sought to confirm the presence of each of the chaperones in human sperm using immunoblotting and then to localize the respective antigens within these cells.

As illustrated in Fig. 4, cross reactive proteins corresponding to the appropriate molecular weight of each of the eight chaperones were detected in sperm cell lysates extracted from both non-capacitated and capacitated cells. Similarly, each of the chaperones were detected in fixed populations of human spermatozoa and the distinct staining patterns, described below and illustrated in Fig. 5, were not altered in response to capacitation (results not shown). Interestingly, each of the eight chaperones was characterized by a unique labeling pattern and few appeared to co-localize in human sperm cells. Furthermore, none were detected within the anterior region of the head, as would be expected of proteins involved in sperm–zona binding.

HSPE1 was localized to the mid-piece, principle piece and posterior head. DNAJB1 localized to the mid-piece and principle piece of the tail. HSPD1 localized predominantly to the mid-piece and faintly along the principle piece of sperm the tail. HSPA1A was also present along the entire length of the sperm tail but displayed a more uniform and intense, localization than that of HSPD1. Interestingly this chaperone was also detected within the equatorial region of the sperm head, as was HSPCA. Finally, TRA1 displayed perhaps the most surprising localization pattern, appearing to be restricted to a small spot at the base of the sperm head. Unfortunately, neither HSPA5 nor HSPH1 could be localized using the antibodies obtained for this study.

Surface expression of chaperones in human sperm

Given the unusual distribution of the chaperones of interest and our failure to co-localize these proteins within the head of fixed spermatozoa, we next sought to investigate their surface expression in live cells. For this purpose, we again employed the use of immunobead and FACS-based assays.

Although our previous studies of mouse spermatozoa have revealed that around 20–25% of capacitated cells bound to beads conjugated with anti-chaperone antibodies (anti-Hspd1 and anti-Tra1), under identical conditions human spermatozoa failed to bind beads conjugated with any of the eight chaperones of interest in the present study (Fig. 6). The validity of the assay for detection of human sperm antigens was confirmed using anti-CD59 conjugated beads, which consistently bound to over 90% of all sperm cells (Fig. 6). Furthermore, to alleviate the possibility that chaperone function was dependent upon the presence of calcium, it was found that the inclusion of calcium in the external media did not influence these results (data not shown). It thus appears that the chaperones under study are not superficially expressed on the human sperm surface. This conclusion was supported by the use of a more sensitive FACS assay which again revealed essentially no cells expressing surface chaperones (Fig. 7) in contrast to the abundant surface expression of CD59.

Discussion

The requirement for mammalian spermatozoa to undergo a period of residence within the female reproductive tract before acquiring the
that engage in little
dence suggests that spermatozoa are transcriptionally inactive cells
reports to the contrary (Gur and Breitbart, 2006), the balance of evi-
ability to engage in sperm–oocyte interaction has been recognized for
SDS Extraction Buffer. Cell extracts (2
Purified human spermatozoa were incubated in capacitating or non-
Figure 4:
Table 1: Chaperone proteins identified in human spermatozoa

<table>
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<tr>
<th>Abbreviation</th>
<th>Chaperone</th>
<th>Synonym</th>
<th>Accession number</th>
<th>Previously identified in sperm (species)</th>
<th>References</th>
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<td>HSPE1</td>
<td>Heat shock 10 kDa protein 1 (chaperonin 10)</td>
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<td>NP_002148</td>
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<td>DNAJB1</td>
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<td>NP_955472</td>
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<td>Kamaruddin et al. (2004)</td>
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<td>HSPA1A</td>
<td>Heat shock 70 kDa protein 1-like 2, 4, 6 and 8</td>
<td>HSP70</td>
<td>NP_005336</td>
<td>Yes (rodent, bovine)</td>
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<td>HSPCA</td>
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<td>HSPA5</td>
<td>Heat shock 70 kDa protein 5 (glucose-regulated protein 78 kDa)</td>
<td>GRP78</td>
<td>NP_005338</td>
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<td>TRA1</td>
<td>Tumor rejection antigen (gp96) 1 (endoplasm)</td>
<td>GRP94</td>
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Figure 4: Detection of chaperones of interest in human spermatozoa

Purified human spermatozoa were incubated in capacitating or non-
capacitating media for 3 h. Following incubation, sperm were solubilized in
SDS Extraction Buffer. Cell extracts (2 μg) were resolved by SDS–PAGE
and immunoblotted with anti-HSPE1, anti-DNAJB1, anti-HSPD1, anti-
HSPA1A, anti-HSPCA, anti-HSPH1, anti-HSPA5 or anti-TRA1 antibodies,
respectively. This experiment was replicated three times using pooled semen
samples and representative blots are depicted

ability to engage in sperm–oocyte interaction has been recognized for
more than half a century (Austin, 1951; Chang, 1951). However,
despite its physiological importance, the intricacies of this capacita-
process remain to be fully elucidated. Notwithstanding recent
reports to the contrary (Gur and Breitbart, 2006), the balance of evi-
dence suggests that spermatozoa are transcriptionally inactive cells
that engage in little de novo protein translation. Thus the functional
activation of these cells during capacitation must be underpinned by
post-translational modifications of existing proteins and remodeling
of the sperm surface architecture. In this context, one of the key un-
iversal associates of capacitation in mammalian sperm appears to be a
dramatic up-regulation of protein tyrosine phosphorylation (Visconti
et al., 1995a; 1995b) via an unusual protein kinase A (PKA)-mediated
pathway (Visconti et al., 1995a, 1997) involving SRC as an intermedi-
ate kinase (Baker et al., 2006).

Although the central role of protein tyrosine phosphorylation in
intracellular signal transduction cascades is well documented, its
importance in the control of sperm capacitation has only recently
been appreciated. Of particular interest is the finding that virtually
the entire population of mouse spermatozoa recovered from the
zona pellucida of homologous oocytes was tyrosine phosphorylated
compared with only 10–15% of the free swimming population
(Urner et al., 2001; Asquith et al., 2004). Furthermore, the inhibition
of tyrosine phosphorylation under these circumstances compromises
their ability to adhere to the zona (Asquith et al., 2004). Although
such studies invite speculation that phosphotyrosine residues are of
fundamental importance in gamete interaction, such a conclusion is
not supported by the inability of anti-phosphotyrosine antibodies to
compromise this interaction (Asquith et al., 2004). Instead, emerging
evidence suggests that tyrosine phosphorylation plays an indirect role
through the activation of target proteins including several members of
the molecular chaperone family. We contend that such proteins may in
turn facilitate the assembly of key recognition molecules into a func-
tional ZP receptor complex that ultimately becomes expressed on the
sperm surface. In this study, we have explored the validity of this
model for sperm–oocyte interaction in the human.

Consistent with our findings in the mouse (Asquith et al., 2004) and
those of Sakkas et al. (2003) and Liu et al. (2006), capacitation of
human spermatozoa and the associated increase in phosphotyrosine
expression are indeed positively associated with the ability of these
cells to interact with homologous oocytes. However, as recently
reported (Sakkas et al., 2003; Buffone et al., 2005; Liu et al., 2006),
phosphotyrosine expression appeared primarily restricted to the prin-
cipal piece of the flagellum of fixed human spermatozoa. In contrast,
to our own studies on mouse spermatozoa (Asquith et al., 2004) and
the results secured by Topfer-Petersons group with porcine gametes
(Piehler et al., 2006), we found no evidence for surface phosphotyros-
ine expression on live capacitated human spermatozoa using either
immunofluorescence, immunobead or highly sensitive flow FACS-
based assays. Similarly, although immunoblotting analysis confirmed
a marked increase in the tyrosine phosphorylation status of capacitated
sperm, the most notable bands were of ~85 and 105 kDa. These two
proteins are believed to correspond to A-kinase anchoring proteins
(AKAPs) that reside within the sperm flagellum and participate in the
acquisition of hyperactivated motility (Turner et al., 1999; Luconi et al., 2004). Although such changes may therefore account
for alterations in the motility patterns of capacitated sperm, they fail
to accommodate the enhanced sperm–zona pellucida binding ability
of such populations.

The molecular chaperones are a diverse group of proteins that are
characterized by their ability to recognize exposed hydrophobic
surfaces of newly synthesized or partially folded proteins and assist them to reach their functional conformation. In this capacity, molecular chaperones prevent improper or incorrect reactions that could otherwise result in protein misfolding and aggregation (Bukau and Horwich, 1998; Treweek et al., 2003). The archetype chaperones were originally identified by their increased expression following exposure of cells to environmental stresses such as heat shock and are thus referred to as ‘heat shock’ or ‘cell stress response’ proteins. However, it is becoming increasingly apparent that such proteins play important roles in a variety of essential cellular processes from aiding protein folding through to intracellular transport, membrane translocation and also protein degradation (Lund, 1995). As such, these proteins have been highly conserved during evolution and are widely expressed in virtually all eukaryotic and prokaryotic cells (Meinhardt et al., 1995).

Several somatic-and germ-cell specific molecular chaperones have been identified in the male germline and appear to be necessary for progression of the cell cycle during the initial stages of spermatogenesis (reviewed by Nixon et al., 2005). However, the present study represents the first attempt to define the repertoire of chaperone proteins that reside within mature human spermatozoa and the first to explore their functional significance to these cells.

Among the numerous proteins identified by our proteomic strategy were several classical chaperone and chaperone-associated proteins in addition to a number of non-archetype chaperone proteins (Table 1). The presence of such an array of chaperones was validated through immunoblotting experiments and thus raises the possibly that these proteins perform important functions in mature spermatozoa. By analogy with our evidence from the mouse, it is tempting to speculate that this role may, in part, be to facilitate the assembly of functional zona receptor complexes (Asquith et al., 2004). However, in the human, no surface expression of chaperones was observed and no surface expression of phosphotyrosine residues was observed; therefore, we conclude that no tyrosine phosphorylated chaperones are present on the human sperm surface. Although we acknowledge the possibility that these proteins may be constitutively active and thus do not require phosphorylation for their chaperoning activity, the subcellular localization of these proteins in human spermatozoa confirms that their functions must lie outside the realm of zona pellucida receptor assembly. In this context, it was also of interest that each of the eight chaperones examined displayed unique patterns of localization within human sperm despite the fact that proteins such as HSPD1

Figure 5: Localization of chaperones of interest in fixed human spermatozoa
Purified human spermatozoa were incubated in either capacitating or non-capacitating media for 3 h. They were then fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and stained with anti-HSPE1, anti-DNAJB1, anti-HSPD1, anti-HSPA1A, anti-HSPCA, anti-HSPH1, anti-HSPA5 or anti-TRA1 antibodies, respectively, followed by a FITC-conjugated secondary antibody. Negative controls were performed by incubating cells without primary antibody for each of the chaperone proteins investigated, these revealed no staining beyond background fluorescence (data not shown). This experiment was replicated three times using pooled semen samples and representative images are shown.

Figure 6: Immunodetection of chaperones on the surface of live human spermatozoa using an immunobead assay
Purified human spermatozoa were incubated in capacitating or non-capacitating media for 3 h. Presence of the chaperones on the surface of live spermatozoa was assessed with anti-HSPE1, anti-DNAJB1, anti-HSPD1, anti-HSPA1A, anti-HSPCA, anti-HSPH1, anti-HSPA5 or anti-TRA1 coated magnetic Dynabeads, respectively. A known sperm surface marker, CD59, was utilized as an internal control. (A) Representative images of live spermatozoa both unbound and bound to beads. (B) The percentage of motile spermatozoa expressing the chaperone proteins on their surface as assessed by immunobead assay. This experiment was repeated three times with a minimum of 100 motile cells scored for each experiment. CD59 antibodies were used as a positive control treatment and exhibited the anticipated high level of binding. ***P < 0.001

Human sperm chaperones
and HSPE1 (Xu et al., 1997) and DNAJB1 and HSPA1A (Terada et al., 2005) are known to form cooperative complexes and act synergistically to achieve productive protein folding in other cell types. At present, it remains unclear what role, if any, the numerous chaperone proteins we have identified play in modulating the functional competence of mature human spermatozoa. On the basis of our preliminary evidence, it is difficult to refute the possibility that their presence simply represents a carry over from spermatogenesis where proteins such as HSPD1 have been implicated in the mitochondrial protein import machinery of early germ cells (Paranko et al., 1996). Indeed, this notion is consistent with our localization of HSPD1 within the mid-piece of mature human sperm flagellum. Of more interest may be the fact that both HSPA1A and HSPCA were detected in the equatorial segment of the sperm head, a location compatible with a role in sperm–oocyte fusion. Interestingly, a similar pattern of HSPA1A immunoreactivity has also recently been reported in the equatorial segment of freshly ejaculated porcine spermatozoa (Spinaci et al., 2005). The fact that incubation of these cells with anti-HSPA1A antiserum impaired the fertilization of homologous zona-free oocytes provides compelling evidence for an involvement of HSPA1A in sperm–oocyte membrane fusion in this species (Spinaci et al., 2005). An inhibitory effect of anti-HSPA1A antibodies has also been observed in the bovine although in this species, the protein localizes to the acrosome of ejaculated cells and the inhibition seems to occur between sperm–zona binding and fusion of the sperm plasma membrane with the oolemma (Matwee et al., 2001). A putative role for HSPA1A during human fertilization has also been proposed on the basis that the presence of anti-HSPA1A antibodies in seminal plasma inhibits fertilization (Bohring and Krause, 2003), and the fact that reduced expression of HSPA1A in ejaculated sperm has been implicated in the pathogenesis of certain subtypes of male infertility (Cedenho et al., 2006). Nevertheless, a direct involvement of HSPA1A in human gamete interaction awaits further investigation. Taken together, these results indicate that although ejaculated human spermatozoa are endowed with a diverse array of molecular chaperones, it is unlikely that these proteins play an active role in the capacitation-associated remodeling of the sperm surface and zona binding. This is contrary to the model that we have proposed for mouse spermatozoa and suggests that alternative, chaperone-independent, mechanisms must underlie the creation of fertilization competent human spermatozoa. Ultimately, such differences may account for the species specificity of gamete interaction.

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