Identification of the Molecular Chaperone, Heat Shock Protein 1 (Chaperonin 10), in the Reproductive Tract and in Capacitating Spermatozoa in the Male Mouse

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ABSTRACT

Mammalian spermatozoa must undergo epididymal maturation in the male reproductive tract and capacitation in the female tract before acquiring the ability to fertilize an oocyte. Previous studies from our laboratory have demonstrated a causal relationship between capacitation-associated surface phosphotyrosine expression and the ability of mouse spermatozoa to recognize the oocyte and engage in sperm-zona pellucida interaction. Our previous analyses of the surface phosphoproteome of capacitated murine spermatozoa identified two molecular chaperones, heat shock protein (HSP) D1 and HSP90B1, with well-characterized roles in protein folding and the assemblage of multimeric protein complexes. The expression of these chaperones was restricted to the rostral aspect of the sperm head, in an ideal position to mediate sperm-zona pellucida interaction. Herein, we report the characterization of an additional chaperone in this location, HSPE1 (chaperonin 10; HSP10). This chaperone was identified using a coimmunoprecipitation strategy employing HSPD1 as bait. The putative interaction between HSPE1 and HSPD1 was supported by reciprocal immunoprecipitation and colocalization studies, which demonstrated the coordinated appearance of both proteins on the surface of the sperm head during capacitation. However, the surface exposure of the protein was lost upon induction of acrosomal exocytosis, as would be expected of a protein potentially involved in sperm-zona pellucida interaction. Collectively, these data invite speculation that a number of molecular chaperones are involved in modification of the sperm surface during capacitation to render these cells functionally competent to engage the process of fertilization.

INTRODUCTION

The development and maturation of spermatozoa is a complex, multifaceted process. Initially, spermatogenesis within the testes produces spermatozoa that, although structurally complete, are functionally deficient and incapable of progressive motility, zona pellucida recognition, or acrosomal exocytosis. Spermatozoa subsequently acquire these functional attributes after leaving the testes during their transit through the epididymal lumen. It has been well established that the epididymal maturation of spermatozoa is accompanied by significant changes in the composition and localization of sperm membrane proteins (reviewed in [1]). Even so, the exact mechanism by which spermatozoa gain their functional potential during epididymal maturation is yet to be elucidated.

After ejaculation, spermatozoa transit the female reproductive tract to the site of fertilization in the fallopian tubes. Prior to engaging in the cascade of events associated with fertilization, including successive penetration of the cumulus oophorus and zona pellucida and subsequent fusion with the oocyte (reviewed in [2]), spermatozoa must undergo a process of post-ejaculatory maturation, termed capacitation [3, 4]. One of the most important properties acquired by spermatozoa during capacitation is the ability to recognize the zona pellucida. This exquisitely specific cell-cell recognition event signals the initiation of fertilization, and yet its molecular basis is still poorly understood. From the oocyte’s perspective, ZP3 is acknowledged as the primary zona pellucida glycoprotein responsible for mediating mouse sperm-zona interaction [5–7]. In terms of the corresponding receptor on the surface of capacitated spermatozoa, a number of candidate proteins have been identified in previous studies including: SPAM1 (PH-20), ZP3R (SP56), B4GALT1 (β-1,4-galactosyltransferase), zonadhesin, zona receptor kinase, arylsulfatase A, and MAN2B2 (α-D-mannosidase) (reviewed in [8]). This range of putative ZP3 receptors has lead to the proposal that zona recognition is coordinated by the sequential action of a variety of sperm proteins, each performing a specific role [9, 10].

More recently, tyrosine phosphorylation of proteins in the rostral region of the sperm head has been shown to be important for zona recognition [11]. Two of the major tyrosine-phosphorylated proteins were identified as the molecular chaperones, heat shock protein (HSP) 1 (HSPD1; formerly HSP60) and HSP90, beta (Grp94), member 1 (HSP90B1; formerly endoplasm), both of which appear to be presented to the sperm surface during capacitation [11]. This has lead to the proposal that these chaperones may mediate the assembly of a protein receptor complex for the recognition of the zona pellucida [11]. Interestingly, this is not the first instance of chaperones acting in concert with other proteins on the surface of cells. For instance, HSP90z (HSP90AA1) has been shown to interact with matrix metalloproteinase 2 on the surface of fibrosarcoma cells and play an important role in promoting cancer invasiveness [12]. Furthermore, both HSP90 and HSP70...
form an integral part of the “activation complex” assembled in monocytic cell lines in response to bacterial lipopolysaccharide challenge [13]. Such findings highlight the emerging roles of chaperones as potential mediators of cell surface protein complex presentation and/or assembly.

To further our understanding of the role of molecular chaperones in the ability of mouse sperm to bind to the outer vestments of the oocyte, we have previously reported the patterns of HSPD1 and HSP90B1 expression in these cells throughout spermatogenesis and during their posttesticular development [11, 14]. As a continuation of these studies, we report here the characterization of an additional chaperone protein, HSPE1 (chaperonin 10; formerly known as HSP10), identified in capacitated mouse spermatozoa using an immunoprecipitation strategy with HSPD1 as the bait. Although eukaryotic HSPE1 has primarily been described as a mitochondrial chaperone [15–18], more recent reports suggest that it may also function in signal transduction, cell cycle regulation, nucleocytoplasmic transport, and metabolism [19]. This study extends the potential functions of HSPE1 by describing, for the first time, the progressive expression of this protein on the surface of murine spermatozoa during capacitation.

MATERIALS AND METHODS

Reagents

Unless otherwise stated, chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and were of molecular biology or research grade. Rabbit polyclonal anti-HSPE1 antibody (anti-Cnp10, ab13528) was obtained from Abcam (Cambridge, MA). Anti-rabbit immunoglobulin (Ig) G-HRP was from Upstate Biotechnology (Lake Placid, NY). Goat polyclonal anti-HSPD1 antibody (anti-HSP60 N-20), anti-goat IgG-fluorescein isothiocyanate (FITC), anti-rabbit IgG-FITC, and anti-goat IgG-tetramethylrhodamine isothiocyanate (TRITC) conjugates were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-ZP3R (clone 5F12) and anti-α-LePhos complex I were obtained from Biosieden International (Saco, ME) and Molecular Probes (Eugene, OR), respectively. Recombinant human HSPE1 was purchased from Abnova (Taipei City, Taiwan). Heps, penicillin, and streptomycin were obtained from Gibco (Paisley, UK). BSA was obtained from Research Organics (Cleveland, OH). Minicomplete protease inhibitor tablets (Roche (Mannheim, Germany). Nitrocellulose and percoll were from Amersham (Buckinghamshire, UK). Mowiol 4–88 was from Calbiochem (La Jolla, CA), paraformaldehyde was supplied by ProSciTech (Thuringowa, Australia), and protein G-coated Dynabeads were from Dynal (Oslo, Norway).

Animals

All experimental procedures were carried out with the approval of the University of Newcastle’s Animal Care and Ethics Committee (ACEC) and the Monash Medical Centre animal ethics committee. Inbred Swiss mice were obtained from a breeding colony held at the institute’s 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 killed via CO2 inhalation or cervical dislocation.

Collection and Preparation of Spermatozoa

Immediately after adult male mice (>8 wk old) were killed, 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 prewarmed, water-saturated mineral oil. Caudal spermatozoa were collected by back-flushing with water-saturated paraffin oil, after which the perfusate was deposited into a droplet of modified Biggers, Whitten, and Whittingham media (BBW, [20]) composed of 91.5 mM NaCl, 4.6 mM KCl, 1.7 mM CaCl2, 2H·O, 1.2 mM KH·PO4, 1.2 mM MgSO4·7H·O, 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 Heps buffer, and 3 mg/ml BSA, then allowed to disperse into the medium for 15 min. Where indicated, negative control (uncapacitated) incubations were conducted using medium prepared without NaHCO3, while positive control (capacitated) incubations were conducted in media supplemented with 1 mM pentoxifylline and 1 mM dibutylryl cyclic adenosine monophosphate. These treatments have been demonstrated to both suppress and promote sperm capacitation, respectively [11]. An osmolarity of 300 mOsm/kg was maintained.

Following collection, sperm concentration was determined and the cells diluted as required. Sperm were then assessed for motility and the uncapsacitated 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 spermatoza, the appropriate region of the epididymis was dissected out and placed in a 500-μl droplet of BBW 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 a discontinuous 25%/45%/65%/80% percoll gradient and centrifuged (1300 × g for 15 min). The 65/80 interface, consisting of >95% pure caput spermatozoa, was washed by gentle centrifugation (400 × g for 2 min) to remove excess percoll and then resuspended in fresh BBW medium and counted as described above. Similarly, testicular spermatozoa were prepared by decapsulating the isolated tests, making multiple incisions in the tissue with a razor blade, and allowing the cells to gently disperse into the medium with mild agitation. Although the purity of the spermatozoa isolated by this technique was not suitable for immunoblotting, it was appropriate for immunofluorescent labeling of HSPE1.

Coimmunoprecipitation Strategy

Coimmunoprecipitation (co-IP) was used to identify HSPD1-associated proteins in sperm lysates using the ProFound Mammalian Co-Immunoprecipitation kit (Pierce, Rockford, IL). A 50-μl aliquot of the supplied antibody-coupling gel slurry (AminoLink Plus gel) was washed three times with PBS followed by conjugation with 50 μg of primary antibody. The primary antibody was covalently linked to the coupling gel by the addition of sodium cyanoborohydride. Following a 4-h incubation at room temperature with constant agitation, unreacted sites on the coupling gel were quenched with 1 M Tris pH 7.4. The antibody-coupled gel was then washed twice with 1 M NaCl, followed by two washes with PBS, pH 7.4. The antibody-coupled gel was stored at 4°C in PBS containing 0.02% w/v sodium azide.

To generate lysates for co-IP, spermatozoa were capacitated as described above and cells were washed three times with protein-free media to remove any loosely associated proteins. Sperm viability and motility were assessed to ensure that neither parameter was compromised, and the cells were then resuspended in co-IP lysis buffer (M-PER, mammalian protein extraction reagent; Pierce) supplemented with a Complete Protease Inhibitor Cocktail tablet (Roche Applied Science, Indianapolis, IN) and lysed for 1 h at 4°C with constant agitation. Insoluble material was removed by centrifugation at 20000 × g for 10 min at 4°C.

For coexperiments, lysates were precleared against un conjugated gel slurry for 1 h prior to being diluted 1:1 with PBS and added to the prepared antibody-coupled gel. After incubation overnight at 4°C with constant agitation, unbound proteins were removed by centrifugation at 4000 × g for 1 min. The co-IP gel was washed five times with PBS before eluting the captured co-IP complexes in ImmuNoPure elution buffer at room temperature for 5 min. Similarly, control incubations were included where nonconjugated gel slurry was incubated with the sperm lysate. Eluates were methanol precipitated and stored at −20°C prior to analysis by SDS-PAGE or until being digested with trypsin in preparation for sequencing.

Proteomic Analysis ofCoprecipitated Proteins

Proteins communoprecipitated with HSPD1 were sequenced using an liquid chromatography tandem mass spectrometry (LC/MS/MS) interface at the Australian Proteome Analysis Facility. Briefly, precipitated samples were directly aliquoted into Vivaspin 500 centrifugal filter units (5000 MWCO; Viva Science Ltd, Gloucestershire, UK) and centrifuged at 12000 × g for 15 min. The pellets were resuspended in 50 mM ammonium bicarbonate before being reduced with dithiothreitol (DTT) and alkylated with iodoacetamide. The samples were then digested in 50 mM ammonium bicarbonate before being reduced with dithiothreitol (DTT) and alkylated with iodoacetamide. The samples were then digested in 50 mM ammonium bicarbonate. The samples were then digested in 50 mM ammonium bicarbonate. The samples were then digested in 50 mM ammonium bicarbonate. The samples were then digested in 50 mM ammonium bicarbonate.
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. The LC eluent was subjected to positive ion nanoflow electrospray analysis on an Applied Biosystems QSTAR mass spectrometer (ABI, Foster City, CA) in an information-dependent acquisition mode (IDA). In IDA mode, a time-of-flight mass spectrometry (TOF/MS) survey scan was acquired (m/z 370-2000, 1.0 s), with the four largest multiplied charged ions (counts > 50) in the survey scan sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 2 s (m/z 100-1600).

The LC/MS/MS data were searched through the MASCOT search engine using the National Center for Biotechnology Information (Bethesda, MD) non-redundant protein database. The following search parameters were used in all Mascot searches: *Mus musculus* taxonomy, maximum of one missed trypsin cleavage, cysteine carbamidomethylation, methionine oxidation, and a maximum 0.2-Da error tolerance in both the MS and MS/MS data. High-confidence positive identifications of HSPE1 was based on three matching peptides and the fact that significant peaks in the MS spectra were those used for the analysis.

**SDS-PAGE and Western Blotting**

Proteins were extracted in a modified SDS-PAGE sample buffer (2% w/v SDS, 10% v/v sucrose in 0.1875 M Tris, pH 6.8) with protease inhibitor tablets by incubation at 100°C for 5 min. Insoluble matter was removed by centrifugation at 20000 x g for 10 min and protein estimations were performed using the DC Protein Assay kit (Bio-Rad, Hercules, CA). Proteins were boiled in SDS-PAGE sample buffer (2% v/v mercaptoethanol, 2% w/v SDS, and 10% v/v glycerol in 0.1875 M Tris, pH 6.8, with bromophenol blue) and resolved by SDS-PAGE on polyacrylamide gels [21] followed by transfer onto nitrocellulose membranes [22]. Membranes were blocked with 3% w/v BSA in Tris-buffered saline (TBS; pH 7.4) for 1 h before being probed with 1:1000 dilutions of primary antibody in TBS containing 1% w/v BSA and 0.1% w/v polyoxyethylene sorbitan monolaurate (Tween-20; TBS-T) for 2 h at room temperature. Blots were washed three times in TBS-T followed by incubation with 1:1000 horseradish peroxidase-conjugated secondary antibody in 1% w/v BSA/TBS-T for 1 h. Following three washes in TBS-T, proteins were detected using an enhanced chemiluminescence kit (Amersham). Western blots were stripped in 100 mM mercaptoethanol, 2% w/v SDS, and 62.5 mM Tris (pH 6.7) at 60°C for 1 h, followed by several washes in TBS-T before reprobing.

**Tissue Immunofluorescence**

Tissue was collected from mouse testes and epididymides, fixed in formalin, embedded in paraffin, and cut into 5-μm sections. Following dewaxing and rehydration, antigen retrieval was performed by subjecting the slides to microwaves (500 W) 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 (w/v) normal goat serum supplemented with 3% w/v BSA and 0.1% v/v polyoxyethylene sorbitan mono-laurate ([22.2]-octane) and viewed using an LSM510 laser scanning confocal microscope equipped with argon and helium/neon lasers (Carl Zeiss Pty, Sydney, Australia). Excitation wavelengths of 488 and 545 nm and emission spectra of 500–530 and ≥560 nm were used for detection of FITC conjugates and propidium iodide, respectively.

**Immunolocalization of Chaperones on Fixed Spermatozoa**

Following incubation, spermatozoa were fixed in 4% v/v paraformaldehyde, washed three times with PBS, plated onto glass slides coated with 0.1% v/v poly-L-lysine, and air-dried. All subsequent incubations were performed at 37°C in a humid chamber, and all dilutions and washes were performed in PBS. Spermatozoa were permeabilized with 0.2% v/v Triton X-100 for 15 min, rinsed, and blocked in 10% v/v serum in 3% w/v BSA at 37°C for 1 h. Slides were rinsed (3 × 5 min) and incubated at 4°C overnight in 1:50 dilution of primary antibody. After three washes, cells were incubated with 1:100 FITC-conjugated secondary antibody at 37°C for 1 h, washed again, and mounted in antifade medium. Images were captured using a confocal microscope, as described above.

**Immunodetection of Chaperones on Live Sperm Using a Fluorescence-Activated Cell Sorter**

Following preparation, spermatozoa were diluted to 1 x 10⁶ cells/ml and incubated in either noncapacitating or capacitating medium. The sperm suspensions were then incubated with FITC-conjugated primary antibody for 10 min at 37°C. The cells were subsequently washed twice with BWW and incubated with FITC-conjugated secondary antibody at 1:200 for a further 10 min at 37°C. Following two additional washes with BWW, the cells were incubated with propidium iodide (20 mg/ml) and analyzed using a fluorescence-activated cell sorting (FACS) Calibur FACS (Becton Dickinson, Franklin Lakes, NJ) with an 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 analyzed using the CellQuest software (BD Biosciences, San Jose, CA).

**Acrosome Reaction**

Caual epididymal spermatozoa were capacitated as described above, followed by 15 min of incubation in 1.25 μM calcium ionophore A23187 as described previously [14]. Vehicle (dimethyl sulfoxide) controls were included. Samples were then diluted 1:10 in prewarmed hypo-osmotic swelling medium (25 mM sodium citrate, 7.5 mM fructose) and incubated at 37°C for 1 h. Spermatozoa were fixed and stained for HSPE1 using a TRITC-conjugated secondary antibody, as described above. The cells were then colabeled with FITC-conjugated *Arachis hypogaea* lectin (0.5 mg/ml in BWW) at room temperature for 15 min, washed, and mounted in antifade medium, as described above. At least 200 cells were scored on the basis of their viability, acrosomal status, and chaperone staining pattern.

**Sperm-Zona Pellucida Binding Assay**

Mouse oocytes were recovered from the oviduct of superovulated females as previously described [11]. Capacitated spermatozoa were incubated (30 min at 37°C) with either rabbit sera (control) or anti-HSPE1 antibodies (10 μg or 100 μg/ml) in BWW before being washed to remove unbound antibody. Washed spermatozoa (5 x 10⁶) were then coincubated with oocytes in a droplet of BWW under oil in 5% CO₂ for 30 min at 37°C. A separate treatment incorporating oocytes that had been preincubated with recombinant HSPE1 (5 μg/12 oocytes) was included in each experiment. Following incubation with spermatozoa, oocytes were washed by gentle pipetting through a fine-bore pipette in three changes of BWW to remove any loosely bound sperm. The number of sperm remaining bound to the zona pellucida was then recorded.

**Statistics**

Experiments were replicated with material collected from at least three different animals and the graphical data presented represent means ± SEM, the SEMs being calculated from the variance between samples. Statistical significance was determined using an ANOVA. The differences between group means were assessed using Fisher protected least-squares difference test.

**RESULTS**

**HSPE1 Coimmunoprecipitated with HSPD1 in Capacitated Mouse Spermatozoa**

A co-IP strategy was employed in order to identify proteins that associate with HSPD1 in capacitated mouse spermatozoa. For this purpose, soluble sperm lysates were incubated in the presence of polyclonal anti-HSPD1 serum covalently bound to agarose beads and associated proteins were resolved on 1D SDS-PAGE gels. Although some minor differences were observed between replicates, a number of interacting proteins were consistently detected using this approach (Fig. 1). The specificity of the co-IP strategy was demonstrated by the absence of these proteins in unconjugated bead controls (results not shown). Sequencing of the predominant proteins by LC/MS/MS analysis identified the HSPD1 cochaperone, HSPE1. The specificity of this putative interaction was confirmed by a reciprocal co-IP experiment with anti-HSPE1, which, as shown...
in Figure 1C, pulled down a protein of approximately 60 kDa that cross-reacted with anti-HSPD1 serum.

**HSPE1 Is Expressed in Spermatozoa from the Mouse Epididymis**

Given the novelty of the finding that HSPE1 is present in mouse spermatozoa, studies were undertaken to confirm this result and characterize the ontogeny of its expression during sperm development. For this purpose, soluble sperm lysates were prepared from populations of cells extracted from the caput, corpus, and cauda epididymis and isolated over percoll gradients to a purity that exceeded 95%. Western blot analysis of these samples revealed that a single, predominant, cross-reactive band of the appropriate size for HSPE1 was expressed in caput, corpus, caudal, and capacitated cauda spermatozoa (Fig. 2A). The specificity of this binding was confirmed by stripping the blot and reprobing with anti-HSPE1 antibodies. An immunoblot of the proteins was probed successively with anti-HSPE1 (HSPE1) and anti-HSPD1 (HSPD1). The proteins corresponding to HSPE1 and HSPD1 are indicated by arrowheads.

**HSPE1 Localizes to the Periacrosomal Region And Colocalizes with Mitochondria in the Cytoplasm of Precursor Germ Cells in the Testes**

Having confirmed the presence of HSPE1 in testicular tissue, we next sought to examine the localization pattern of the target protein in mouse testicular sections. This was achieved using laser confocal microscopy and indirect immunofluorescence. Sections were counterstained with propidium iodide, a nuclear stain, to assist in cell structure visualization and target protein localization. Anti-HSPE1 showed relatively weak, punctate labeling of the cytoplasm of spermatogonia (Fig. 3A, green) and, in the testis, this protein was indeed colocalized with mitochondria in precursor germ cells (Fig. 4, B–D). However, in round spermatids, particularly intense labeling was observed in a discrete crescent-shaped pattern characteristic of that displayed by the developing acrosomal vesicle (Fig. 3A and Fig. 4D, arrows). This is consistent with the fact that, in other seminiferous tubule sections, possessing more advanced stages of spermatogenesis, HSPE1 was also localized in the crescent-shaped acrosomal region of late-elongating spermatids (Fig. 3B, arrows). However, in these tissue sections, this protein was not detectable in fully differentiated spermatozoa (Fig. 3A, red arrow). The localization pattern of HSPE1 in round and elongating spermatids in mouse testes suggests that it is acquired by spermatozoa during spermatogenesis, and fulfils a role other than that associated with its traditional mitochondrial function. Importantly, the specificity of this HSPE1 labeling was confirmed by the fact that preabsorption of the antibody with recombinant HSPE1 protein eliminated this labeling (results not shown).

**HSPE1 Localizes to the Epididymal Epithelium and Dense Bodies in the Epididymal Lumen**

Localization of HSPE1 in mouse epididymal sections was achieved as described above for mouse testes sections.
Polyclonal anti-HSPE1 labeling within caput, corpus, and caudal epididymal tissue sections was consistently seen in a diffuse pattern throughout the principal epithelial cells (Fig. 5, A–C). Interestingly, this antibody also strongly labeled discrete structures within the lumen of the duct that we have previously termed “dense bodies” [14] (Fig. 5, A–C). Consistent with our previous reports, the dense bodies observed in the epididymal lumen were distinct from spermatozoa, as shown by their failure to colocalize with the propidium iodide-labeled sperm nuclei (Fig. 5, A–C, arrowheads). The pattern of HSPE1 labeling in the epididymal lumen also precluded localization to the sperm flagellum, including the mitochondrial gyres. Interestingly, these unique structures have also been shown to contain HSPD1 and HSP90B1 [14].

**HSPE1 Is Expressed in Spermatozoa Isolated from the Testis and Epididymis and Colocalizes with HSPD1 in Capacitated Spermatozoa**

In light of our inability to detect HSPE1 in spermatozoa within testicular or epididymal tissue sections, populations of spermatozoa were isolated from the testis and various sites along the epididymis. They were then fixed, immunostained with anti-HSPE1 polyclonal antibody, and the localization patterns viewed with laser confocal microscopy. This approach revealed distinct patterns of HSPE1 expression coinciding with different stages of sperm maturation. In testicular spermatozoa, anti-HSPE1 strongly labeled the acrosomal region of the sperm head, the midpiece, and cytoplasmic droplet, and was also detected, albeit more weakly, in the principal piece of the tail (Fig. 6, testis). A very similar HSPE1 labeling pattern was observed in sperm isolated from the caput epididymis (Fig. 6, caput), although the head labeling appeared to be restricted to a discrete crescent overlapping the acrosomal cap and posterior head, while the cytoplasmic droplet was still strongly labeled. During epididymal transit, the cytoplasmic droplet was lost and HSPE1 labeling of spermatozoa from the corpus epididymis was characterized by a similar crescent-shaped area of fluorescence over the acrosomal cap and strong labeling of the principal piece of the tail (Fig. 6, corpus). Finally, mature spermatozoa isolated from the caudal epididymis showed anti-HSPE1 labeling over the apical crescent of the head, weak midpiece labeling, and strong labeling of the principal piece (Fig. 6, cauda).

To gain further insight into the putative association between HSPE1 and HSPD1, the two chaperones were colocalized in capacitated mouse spermatozoa. As shown in Figure 7, both antigens localized to the apical domain overlying the acrosome, the site of sperm-ZP interaction. However, while HSPD1 was also localized to the midpiece of the tail, HSPE1 was predominantly localized in the principal piece of the tail. The co-IP of HSPE1 with HSPD1 and their colocalization over the apical acrosomal domain support a role in capacitation-
dependent acquisition of zona binding ability, as previously suggested for HSPD1 [11].

**HSPE1 Appears on the Surface of Spermatozoa During Capacitation**

Expression of HSPE1 over the apical domain of the sperm acrosome is suggestive of a role in the interaction between the sperm and the oocyte during fertilization. In order to confirm that HSPE1 is surface localized and, therefore, available to participate in this interaction, antigen localization studies were performed and surface labeling detected by flow cytometry. In these studies, nonviable cells were excluded from the analysis through the use of propidium iodide as a viability stain. In uncapacitated sperm populations, anti-HSPE1 was exposed on the surface of around 25% of the live cells (Fig. 8). However, after capacitation, there was a significant increase ($P < 0.001$) in sperm surface expression of HSPE1 (94 ± 1.2%) (Fig. 8). As anticipated, spermatozoa in the control population, prepared in the absence of primary antibody, displayed only background levels of fluorescence.

**Loss of Acrosome Correlates with Loss of HSPE1 from the Sperm Head**

The acrosomal localization of HSPE1 was further confirmed by analysis of its fate subsequent to loss of the apical sperm membrane through induction of acrosomal exocytosis. To perform these studies, mouse caudal sperm were capacitated and acrosomal exocytosis induced by addition of the calcium ionophore, A23187. Cells were then incubated in prewarmed hypo-osmotic swelling medium, allowing identification of the live sperm and, after fixation, they were successively immunostained with anti-HSPE1 and a TRITC-conjugated secondary antibody (Fig. 9, red labeling). The spermatozoa were then counterstained with FITC-conjugated *Arachis hypogaea*, a lectin that selectively stains acrosome-intact, but
not acrosome-reacted, sperm (Fig. 9, green labeling). Analysis of the merged images revealed that sperm stained with anti-HSPE1 were also acrosome intact (Fig. 9, A–D). In contrast, those sperm that had undergone acrosomal exocytosis failed to bind anti-HSPE1 (Fig. 9, E–H).

**HSPE1 Antibodies Inhibit Sperm-Zona Pellucida Interaction**

In order to begin to assess the functional significance of HSPE1 in relation to fertilization, capacitated spermatozoa were preincubated with anti-HSPE1 antibodies and subsequently examined for their ability to adhere to the zona pellucida of homologous mouse oocytes. As illustrated in Figure 10, anti-HSPE1 suppressed sperm-zona pellucida interaction in a significant dose-dependent manner without compromising either sperm viability or motility. At the highest concentrations of antibody used (100 μg), anti-HSPE1 reduced sperm binding to around 30% of that observed in the control populations. In contrast, preincubation of oocytes with recombinant HSPE1 failed to compromise sperm-zona pellucida interaction. From these data, we infer that HSPE1 does not, in itself, represent a receptor for the zona pellucida. Rather, it appears that the HSPE1 antibodies prevent sperm-zona interaction through mechanisms associated with steric hindrance of the cognate receptors or, alternatively, by modifying the sperm surface architecture by promoting the movement of antigens within the plasma membrane.

**DISCUSSION**

Mammalian sperm-oocyte interaction is preceded by capacitation, a critical phase of cellular maturation during which immature spermatozoa are transformed into functionally competent gametes. Although numerous correlates of capacitation have been established, the molecular mechanisms that underpin many aspects of this process remain poorly understood. Recent studies from a number of independent laboratories have drawn attention to the importance of capacitation-associated increases in tyrosine phosphorylation in the functional maturation of mammalian spermatozoa [24–27]. In the mouse, phosphotyrosine expression on the sperm surface is causally related to the acquisition of zona binding potential [11]. Studies from our laboratory have previously identified two of the major tyrosine-phosphorylated proteins as the molecular chaperones, HSPD1 and HSP90B1. In view of their recognized roles in facilitating protein folding and the assembly of multiple peptide subunits into mature protein complexes, these findings led us to hypothesize that, upon activation, these chaperones direct the assembly of a functional zona pellucida-receptor complex on the sperm surface (reviewed in [28]). In light of these data, we sought to further

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**FIG. 7.** Immunofluorescent colocalization of HSPE1 and HSPD1 in mouse spermatozoa. A) Sperm were isolated from the cauda epididymis, fixed, and successively stained with (B) anti-HSPE1 and a TRITC-conjugated secondary antibody (red) and then anti-HSPD1 followed by a FITC-conjugated secondary antibody (green). HSPE1 colocalized with HSPD1 in the periacrosomal region of sperm head (D), while only HSPE1 appeared in the principal piece (C), and only HSPD1 appeared in the midpiece (C). Bar = 10 μm.
our investigation of the biological role played by chaperones in fertilization by identifying proteins that associated with these molecules during capacitation. Herein, we describe the characterization of an additional chaperone protein, HSPE1, isolated from capacitating mouse spermatozoa using an HSPD1 co-IP strategy.

Our discovery of a putative molecular association between HSPD1 and HSPE1 is not without precedent. Classically, in Escherichia coli, the GroEL (HSPD1) and GroES (HSPE1) families of molecular chaperones interact to fulfill a well-characterized role in mediating the correct folding of a variety of protein substrates (reviewed in [29, 30]). GroEL forms a dual ringed tetradecamer structure, capped at either or both ends by a heptamer of GroES to form a symmetrical structure [31–34]. Although eukaryotic HSPD1 and HSPE1 also interact to form an oligomeric structure that participates in protein folding, this differs from that of the GroES and GroEL complex [35]. In eukaryotic cells, HSPE1 and HSPD1 were originally described as mitochondrial proteins. However, consistent with the findings of the present study, an emerging body of literature suggests these proteins are also present on the cell surface [36–42] and in the extracellular fluid [43, 44].

In the current study, anti-HSPE1 staining revealed two distinct patterns of labeling within testicular sections. In spermatogonia and spermatocytes, a punctate staining pattern in the cytoplasm probably reflected the mitochondrial localization of this chaperone, given the colocalization of HSPE1 with an antibody targeting complex 1 of the inner mitochondrial membrane (Fig. 4). However, as these precursor germ cells differentiated into haploid spermatids, this mitochondrial staining was lost and replaced by an intense crescent-shaped staining pattern corresponding to the developing acrosomal vesicle. From these data, we infer that HSPE1 may have a dual role in the development and/or function of both the mitochondria and acrosomal vesicle. In this context, it is
possible that HSPE1 is integral to the mitochondrial protein import and assembly machinery in early, mitotically active germ cells, as has been proposed for HSPD1, based on similar patterns of expression in the testes of rats, humans, monkeys, and mice [14, 45–47]. Similarly, the staining of the residual bodies with anti-HSPE1 may be accounted for by the fact that only a limited number of mitochondria are retained as sperm mature and the rest are expelled within these structures (reviewed in [48]). Although the precise role of HSPD1 and HSPE1 during spermatogenic development awaits further investigation, the importance of chaperone proteins in this process is highlighted by the demonstration that male mice lacking HSPA2 (formerly HSP70–2) are infertile (reviewed in [49]). Removal of HSPA2 causes the arrest of spermatogenesis during prophase I at the pachytene stage of development, and germ cells are lost by apoptosis.

In addition to their role in spermatogenesis, a number of chaperones have also been identified in posttesticular spermatozoa, including HSPA2, HSP90AA1, and HSPD1 [11, 14, 50–53], although their role in these cells remains less clear. Our studies of the ontogeny of HSPE1 expression during posttesticular sperm maturation revealed a similar pattern to that previously reported by us for both HSPD1 and HSP90B1 [14]. In purified populations of epididymal spermatozoa, HSPE1 was found to be predominantly localized to the apical (periacrosomal) region of the sperm head. Although this pattern was not altered at different stages of sperm development, the tail localization of HSPE1 appeared to undergo a dramatic reorganization from predominantly mid-piece in testicular spermatozoa to predominantly principal piece in cells from the cauda epididymis. This temporal pattern of HSPE1 redistribution is similar to that previously documented for a number of additional sperm proteins, including SPAM1 and ADAM2 [54]. However, in contrast to these proteins, we found no evidence to indicate that HSPE1 was processed during epididymal transit, and, thus, the mechanism responsible for its redistribution remains to be established.

Interestingly, HSPE1 antibodies consistently failed to label sperm within the lumen of testicular or epididymal tissue sections. They did, however, reveal diffuse labeling of the principal cells of the epididymal epithelium in addition to a large number of discrete entities within the lumen of the duct that appeared in the distal caput:proximal corpus region and beyond. Consistent with our previous account of these chaperone-laden dense bodies [14], they appeared to be closely apposed to the sperm heads, but failed to colocalize with these structures. Our previous ultrastructural analysis of dense bodies revealed that they are distinct from epidydymosomes, membrane-bound vesicles secreted from the epididymal epithelium in an apocrine manner (reviewed in [55]). However, there are only a limited number of independent studies in which similar epididymal structures have been described. Among these are reports of electron-dense, carbohydrate-rich aggregates that are apparently involved in the formation of sperm rosettes [56–58]. At present, it is unclear whether the dense bodies that we have identified are in fact the same or separate aggregations to those associated with rosette formation. Nevertheless, this seems likely, as they do possess a similar amorphous morphology and pattern of distribution within the epididymis.

In previous studies, we have speculated that the formation of dense bodies may be an effective way of simultaneously delivering a number of proteins to spermatozoa through the mediation of the chaperones that they contain (HSPD1, HSPE1, HSP90B1, and possibly others). This notion is consistent with emerging evidence that extracellular chaperones are not only able to bind and transport a myriad of “cargo” proteins, but that the interaction of these complexes at the surface of cells leads to the subsequent uptake of the chaperone-cargo protein complex (reviewed in [59]). This conclusion is also commensurate with the observation that the electron-dense material responsible for rosette formation contains CRISP1, a glycoprotein secreted by the corpus epididymis that is involved in sperm maturation [57]. Thus, the proposed ability of this material to embed the sperm head and bind several cells together may increase the efficiency of such transfer processes. However, the mechanism by which proteins are translocated from the dense bodies to either the surface or intracellular compartments of the sperm remains to be determined.

If HSPE1 participates in gamete interaction, as we have hypothesized for the chaperones HSPD1 and HSP90B1, then these proteins would be expected to colocalize on the apical surface of the head of live, capacitated spermatozoa. Using immunofluorescent staining of capacitated caudal spermatozoa, we have demonstrated that this was the case. Additional studies employing a flow cytometry assay not only confirmed the surface localization of the chaperones, but also revealed a dramatic increase in the level of surface exposure of HSPE1 during capacitation. In this context, HSPE1 was only observed on the surface of approximately 25% of uncapacitated cells, but was detected on virtually all capacitated spermatozoa. Although the superficial expression of both HSPD1 and HSP90B1 is also correlated with sperm capacitation [14], the level of exposure of these chaperones is much less than that
observed for HSPE1. Such findings not only implicate HSPE1 in the capacitation-dependent acquisition of zona binding potential [11, 60], but also indicate the possible use of this protein as a marker to monitor the capacitation status of spermatozoa. Further support for the functional significance of HSPE1 in relation to fertilization was advanced by the demonstration that anti-HSPE1 antibodies inhibited sperm-zona pellucida interaction in a concentration-dependent manner. Nevertheless, the possibility that HSPE1 serves as a receptor to directly mediate sperm-zona pellucida interaction has been discounted on the basis that recombinant HSPE1 protein failed to adhere to the zona pellucida and competitively inhibit sperm interaction. Rather, these data support the hypothesis that HSPE1 is localized within close proximity of the zona receptor, and may be involved in chaperone-mediated assembly of a zona-receptor complex, as we have previously proposed for its cochaperone, HSPD1 [11].

Although chaperones were originally thought to be restricted to organelles within the cell, recent reports are highlighting their role on the cell surface. These include cancer cells, blood cells, fibroblasts, hematopoietic stem cells, and neural progenitors [12, 41, 61–66]. Our evidence that the surface expression of HSPD1, HSPE1, and HSP90B1 is involved in acquisition of zona binding potential in mouse spermatozoa [11, 14] has highlighted another novel extracellular role for these important chaperone proteins. Further studies into the identity of the chaperone client proteins may yield important insights into the molecular basis of sperm maturation and fertilizing potential.

REFERENCES


