

The Chaperonin Containing TCP1 Complex (CCT/TRiC) Is Involved in Mediating Sperm-Oocyte Interaction[§]

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Background: Mammalian fertilization is initiated by sperm-zona pellucida (ZP) interactions.

Results: The chaperonin-containing TCP1 complex (CCT/TRiC) was identified on the surface of mouse spermatozoa and shown to have an indirect role in ZP adhesion.

Conclusion: CCT/TRiC mediates the presentation of ZP receptors.

Significance: These data provide an important insight into the molecular basis of sperm-ZP interactions.

Sperm-oocyte interactions are among the most remarkable processes in cell biology. These cellular recognition events are initiated by an exquisitely specific adhesion of free-swimming spermatozoa to the zona pellucida, an acellular matrix that surrounds the ovulated oocyte. Decades of research focusing on this interaction have led to the establishment of a widely held paradigm that the zona pellucida receptor is a single molecular entity that is constitutively expressed on the sperm cell surface. In contrast, we have employed the techniques of blue native-polyacrylamide gel electrophoresis, far Western blotting, and proximity ligation to secure the first direct evidence in support of a novel hypothesis that zona binding is mediated by multimeric sperm receptor complex(es). Furthermore, we show that one such multimeric association, comprising the chaperonin-containing TCP1 complex (CCT/TRiC) and a zona-binding protein, zona pellucida-binding protein 2, is present on the surface of capacitated spermatozoa and could account for the zona binding activity of these cells. Collectively, these data provide an important biochemical insight into the molecular basis of sperm-zona pellucida interaction and a plausible explanation for how spermatozoa gain their ability to fertilize.

Sperm-oocyte recognition not only initiates fertilization but also represents a key process in the etiology of male infertility and a strategic target for fertility regulation. When they are released from the testes, spermatozoa are completely lacking in any capacity to recognize the surface of the oocyte. They only acquire this competence as a consequence of a post-testicular maturation process, which proceeds in two major phases. The first of these phases occurs in the epididymis and bestows upon spermatozoa the potential to exhibit progressive motility and interact with the oocyte (1). However, it is not until these cells enter the female reproductive tract that this functional potential is expressed as the spermatozoa complete the second phase of maturation, known as capacitation (2, 3).

Because spermatozoa are transcriptionally inactive, their functional transformation during capacitation is reliant upon post-translational modifications and surface remodeling events. These processes are associated with the activation of complex, capacitation-associated signal transduction pathways, one of the most significant of which leads to a dramatic increase in protein tyrosine phosphorylation (4–6). Interestingly, these intracellular events also have a profound influence on the sperm surface architecture, rendering these cells competent to bind to the zona pellucida (ZP)² and to respond to this cell recognition event with the initiation of acrosomal exocytosis (7, 8).

The molecular mechanisms that direct sperm-ZP interactions remain controversial and are the subject of ongoing debate (9–13). The most widely accepted paradigm holds that spermatozoa interact in a relatively species-specific manner with O-linked carbohydrate ligands that furnish the ZP3 glycoprotein (14–23). Consistent with this notion, a number of candidate ZP3 receptors have been identified (8). However, it has become increasingly apparent that no single receptor candidate is uniquely responsible for mediating ZP adhesion (24, 25). Rather, the work of a number of independent laboratories has raised the intriguing prospect that individual receptor candidates may be presented to the ZP in the form a functional multimeric complex. Moreover, there is strong correlative evidence to suggest that the assembly of such a complex may be choreographed by the concerted action of a family of molecular chaperones that are themselves activated during capacitation (26–29).

Although such a model of sperm-ZP interaction would account for both the diversity of candidate receptors and the dependence of sperm-zona binding on capacitation (2, 3), at present there is no direct evidence that spermatozoa possess the proposed multimeric zona receptor complexes nor express them in a subcellular region compatible with zona binding. In

[§] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Tables S1 and S2 and Figs. S1–S5.

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² The abbreviations used are: ZP, zona pellucida; BN, blue native; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; MPC, multiprotein complex; ESI, electrospray ionization; CTB, cholera toxin B; CCT, cytosolic chaperonin containing TCP-1; TRiC, TCP1 ring complex; G_{M1}, monosialotetrahexosylganglioside.

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the studies described herein we have employed blue native-polyacrylamide gel electrophoresis (BN-PAGE) as a means of providing direct evidence for the presence and functional significance of multimeric sperm surface complexes. BN-PAGE is an electrophoretic technique for high-resolution separation of biologically active, native protein complexes. The isolation of such complexes is facilitated by solubilization of cellular membranes in a weak, nonionic detergent that maintains protein-protein interactions (30). Although originally developed for the analysis of the large multienzyme complexes comprising the mitochondrial electron transport chain (31, 32), BN-PAGE has since been successfully adapted for the isolation and functional characterization of protein complexes embedded within the plasma membrane of a variety of cell types (31, 33, 34). Utilizing this technique, we have been able to substantiate the presence of chaperone-laden protein complexes on the surface of capacitating mouse spermatozoa and demonstrate that a subset of these complexes display high affinity binding to the zona pellucida.

EXPERIMENTAL PROCEDURES

Reagents—Unless otherwise specified, chemicals and antibodies were purchased from Sigma and were of molecular biology or research grade. BSA and CHAPS detergent were purchased from Research Organics (Cleveland, OH). HEPES, penicillin, and streptomycin were obtained from Invitrogen (Paisley, UK). Protease inhibitor tablets were obtained from Roche Applied Science. Nitrocellulose membranes were from Amersham Biosciences (Buckinghamshire, UK). Coomassie Brilliant Blue G-250 was from Serva (Heidelberg, Germany). Anti-CCT2 (rat monoclonal, catalog number sc-58864), anti-CCT6A (goat polyclonal, catalog number sc-13897), anti-CCT8 (goat polyclonal, catalog number sc-13891), and anti-PRM2 (rabbit polyclonal, catalog number sc-30172) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CD59 (mouse monoclonal, catalog number ab9182) and anti-ZP3R (mouse monoclonal, catalog number ab17358) were purchased from Abcam (Cambridge, UK). Anti-ZPBP2 (mouse polyclonal, catalog number H00124625-B01) was purchased from Abnova (Taipei, Taiwan). Anti-OxPhos Complex IV subunit 1 (mouse monoclonal, catalog number 459600) was purchased from Invitrogen. Fluorescein isothiocyanate (FITC)-conjugated secondary antibodies and Alexa Fluor 594-conjugated *Arachis hypogaea* lectin were purchased from Sigma. Proximity ligation reagents were all acquired from Olink Bioscience (Uppsala, Sweden). Horseradish peroxidase (HRP)-conjugated streptavidin was purchased from Chemicon (Temecula, CA). Protein G Magnetic Beads were obtained from Millipore (Billerica, MA). 3,3'-Dithiobis(sulfosuccinimidyl propionate) was purchased from Pierce Biotechnology. Native PAGE Novex 4–16% Bis-Tris gels and Native Mark-unstained protein standards were bought from Invitrogen.

Preparation of Mouse Spermatozoa—All animal procedures described in this article received approval from our institutional animal ethics board. Spermatozoa were isolated from the caudae epididymides of euthanized adult Swiss mice by retrograde perfusion. Both negative control (noncapacitated) and positive control (capacitated) spermatozoa were prepared as

previously described (26). Spermatozoa were isolated from caput epididymides following gentle homogenization of the tubules in Biggers, Whitten, and Whittingham media (35). The sperm suspension was then overlaid above a 27% Percoll gradient and centrifuged at $600 \times g$ for 15 min at 37 °C (36).

Biotinylation of Sperm Surface Proteins—To investigate the surface orientation of the native protein complexes isolated in this study, spermatozoa were vectorially labeled with sulfo-NHS-LC-biotin (Pierce), a membrane impermeable derivative of biotin as previously described (36).

Blue Native-Polyacrylamide Gel Electrophoresis—Following incubation under either capacitating or noncapacitating conditions (26), suspensions of 2×10^6 sperm/ml were lightly pelleted ($300 \times g$ for 5 min) and resuspended in 2 volumes of native protein lysis buffer consisting of: 1% *n*-dodecyl- β -D-maltoside (a weak, nonionic detergent; adjusted to a final concentration below that of the critical micelle concentration; Sigma), 0.5% Coomassie Blue G250n (Serva), and a mixture of protease inhibitors (Roche Applied Science). The samples were gently mixed using a slow revolution vortex and then incubated at 4 °C on an orbital rotator for 30 min. Following incubation, the samples were again lightly vortexed, then centrifuged at $14,000 \times g$ at 4 °C for 15 min to remove cellular debris. The solubilized protein suspension was then dialyzed against Biggers, Whitten, and Whittingham media overnight at 4 °C to remove excess salts and detergent. In experiments designed to examine the ability of these native protein preparations to adhere to the zona pellucida, sperm were extracted using native protein lysis buffer prepared without Coomassie Blue G-250.

For the purpose of one-dimensional BN-PAGE, native protein lysates were loaded onto pre-cast BN-PAGE gels (Native PAGE Novex 4–16%, Bis-Tris; Invitrogen), and resolved using the Native PAGE cathode and anode buffer system (Invitrogen). The gels were then removed from the electrophoresis apparatus and stained with Coomassie Blue G-250. Where necessary, this was followed by silver staining to detect less abundant material. Alternatively, the gels were prepared for either Western blot analysis or two-dimensional BN-PAGE.

Two-dimensional BN-PAGE was conducted to separate native protein complexes into their individual components. Preparation for this analysis involved excising a single lane of a one-dimensional BN-PAGE gel and subjecting it to reduction and alkylation in 0.5% (w/v) DTT and 4% (w/v) iodoacetamide, respectively. The lane was then loaded above a 10% SDS-PAGE gel and secured in place with molten agarose. The second dimension gel was resolved at 100 V for 2 h as described (31). Gels were then removed from their cassette and either stained with colloidal Coomassie Blue G-250 or prepared for Western blotting.

Western Blot and Far Western Blot Analyses—Proteins resolved by either one- or two-dimensional BN-PAGE or SDS-PAGE were transferred onto nitrocellulose membranes using conventional Western blotting techniques. The primary antibody was diluted in TBS containing 1% (w/v) BSA and 0.1% (v/v) polyoxyethylenesorbitan monolaurate (Tween 20; TBS-T) (anti-CCT2, 1:1000; anti-CCT6A, 1:1000; anti-CCT8, 1:1000; anti-CD59, 1:4000; anti-COX, 1:1000; anti-PRM2, 1:2000; anti- α -tubulin, 1:4000; anti-ZP3R, 1:1000; and for anti-ZPBP2,

1:1000) and incubations were conducted for 1 h at room temperature. Labeled proteins were detected using an enhanced chemiluminescence (ECL) kit (Amersham Biosciences).

To identify native protein complexes with affinity for zona pellucidae, one-dimensional BN-PAGE gels were transferred to nitrocellulose membranes and prepared for far Western blotting with solubilized, biotin-labeled preparations of mouse zona pellucidae. These zona pellucida were prepared by incubation of oocytes (~200/experiment) in 1 mg/ml of sulfo-NHS-LC-biotin at 37 °C for 30 min. The biotin reaction was quenched by the addition of Tris (pH 7.4) to a final concentration of 1 mM. Oocytes were washed 3 times to remove unbound biotin and the zona pellucida were solubilized by incubation in acidified Hanks' balanced salt solution/polyvinyl alcohol (pH adjusted to 2.0 with 1 M HCl) for 15 min at 37 °C. Solubilized zona proteins were removed from insoluble oocyte material by aspiration with a fine bore micropipette, and then the pH was readjusted to 7.4 with 1 M NaOH. This preparation was then incubated with one-dimensional BN-PAGE Western blots overnight at 4 °C on an orbital rotator. Membranes were then washed 3 times in TBS-T before incubation with HRP-conjugated streptavidin (diluted 1:3000 in 1% (w/v) BSA/TBS-T). Labeled complexes were then detected using ECL as described above.

Immunolocalization of Proteins of Interest—Following isolation, spermatozoa were diluted to 1×10^6 cells/ml, and incubated in either noncapacitating or capacitating medium (26). The cells were then fixed in 1% paraformaldehyde, washed 3 times with 0.05 M glycine in phosphate-buffered saline (PBS), plated onto poly-L-lysine-coated glass slides, and allowed to settle overnight at 4 °C. Unless indicated all incubations were performed in a humid chamber at 37 °C. The cells were blocked with 10% serum, 3% BSA for 1 h. Slides were washed 3 times with PBS for 5 min and incubated in a 1:100 dilution of primary antibody at 4 °C overnight. Slides were then subjected to 3 \times 5-min washes with PBS and incubated in a 1:300 dilution of the appropriate FITC-conjugated secondary antibody for 1 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).

In Situ Proximity Ligation Assay—Sperm cells were prepared as described for the immunolocalization procedure above until the addition of the primary antibody step. At this point, two individual primary antibodies from different host species (mouse anti-ZPBP2 and anti- α -tubulin antibodies (1:100 dilution) and goat anti-CCT6A and anti-CCT8 (1:100 dilution)) were incubated with the cells simultaneously overnight at 4 °C, and were then washed 3 times with PBST. To detect primary antibodies with the *in situ* proximity ligation assay, the proximity ligation assay probes mouse PLUS and goat or rabbit MINUS (Olink Bioscience, Sweden) were added at a 1:5 dilution in antibody dilution buffer (Olink Bioscience, Sweden) for 60 min at 37 °C. After washing the coverslips 3 times with PBST, the probe was detected using *in situ* proximity ligation assay detection kit 594 (Olink Bioscience) according to the manufacturer's instructions. Immunolocalization on sperm cells was examined using either a Zeiss Axioplan 2 fluorescence microscope or an LSM510 laser scanning confocal microscope

equipped with argon and helium/neon lasers (Carl Zeiss Pty., Sydney, Australia).

To assess whether protein localization was influenced by the acrosomal status of spermatozoa, acrosomal exocytosis was induced by incubation of capacitated cells with 5 μ M calcium ionophore A23187 for 30 min as previously described (20). Following incubation, the cells were sequentially labeled with the appropriate primary and FITC-conjugated secondary antibody as indicated above. Spermatozoa were then dual labeled with *A. hypogaea* lectin conjugated to Alexa Fluor 594. The slides were then washed, mounted in antifade medium as described above, and assessed by fluorescence microscopy.

Flow Cytometry—The surface localization of proteins in live spermatozoa was assessed by flow cytometry following labeling of these cells with the appropriate primary and secondary antibodies as previously described. The spermatozoa were then counterstained for 15 min with propidium iodide (0.5 μ M) and after washing were analyzed on a fluorescence-activated cell sorting (FACS) Vantage flow cytometer (BD Biosciences). 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/s. Data were analyzed using the Cell Quest package.

Immunobead Detection of Proteins on Live Spermatozoa—To confirm the surface expression of the chaperonin proteins, 5 μ g of each chaperonin antibody was conjugated to protein G magnetic beads (Millipore) overnight at 4 °C. Controls included beads conjugated to an irrelevant, antibody (anti- α -tubulin) and a sample of nonconjugated beads incubated with PBS only. The antibody-bead complexes were washed twice in PBS and then incubated with populations of noncapacitated and capacitated spermatozoa. The sperm-bead suspensions were plated onto pre-warmed microscope slides and recorded using an Axiovert S100 phase-contrast microscope with a JVC digital CCD color video camera attached. Following a 15-min coincubation with beads, the percentage of live motile spermatozoa with bound beads was determined for each treatment.

Sperm-Zona Pellucida Binding Assay—To examine the physiological importance of the proteins identified by electrospray ionization (ESI)-MS in relationship to the sperm-zona pellucida interaction, capacitated and noncapacitated spermatozoa were prepared as described and incubated with the appropriate antibodies (diluted 1:100) for 30 min at 37 °C as described (26) in the presence of 10 to 12 cumulus-free mouse oocytes per experiment. At the end of this incubation period the eggs were removed, washed 3 times to remove loosely adherent spermatozoa and mounted in 5 μ l of warm Biggers, Whitten, and Whittingham media. The number of sperm bound to each zona pellucida was counted using a phase-contrast microscope.

Co-immunoprecipitation of Chaperonin Interacting Proteins—Approximately 60 μ l (per treatment) of protein G magnetic beads (Millipore) were washed 3 times in PBS. This was followed by conjugation with 5 μ g of either anti-CCT6A antibody or an irrelevant, isotype matched antibody (anti- β -tubulin) overnight at 4 °C with constant mixing. Following conjugation, the antibody-bead complexes were washed 2 times and then covalently cross-linked by incubation in 15 mM 3,3'-dithiobis(sulfosuccinimidyl propionate) (Pierce) for 2 h at 4 °C. The

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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 nonconjugated and was incubated with PBS only. Each of the conjugated bead preparations were then incubated with $\sim 100 \mu\text{g}$ of native sperm lysates (prepared as described above) that had been pre-cleared against unconjugated beads to limit nonspecific interactions. After an overnight incubation at 4 °C with constant mixing, the beads were washed 3 times prior to elution of bound proteins with 0.2 M glycine (pH 2.5) for 2 min at room temperature. The beads were finally boiled in SDS running buffer at 100 °C for 5 min. Precipitated proteins were resolved on 10% polyacrylamide gels and silver stained.

Mass Spectrometry Identification of Proteins of Interest—Proteins of interest were carefully excised and separation of tryptic peptide mixtures was achieved by nanoscale reversed phase high pressure liquid chromatography (HPLC), in combination with on-line ESI-MS. The mass spectrometric analysis was performed on an LTQ-linear ion trap system (Thermo Scientific, West Palm Beach, FL). Prior to ion trap analysis, an HPLC separation was performed using a nano-AQUITY system (Waters, Rydalmere, Australia), employing a linear gradient of 2–40% buffer B (100% ACN, 0.1% formic acid) over 60 min. The C18 column system consisted of a trapping column (5 μm bead, 180 μm inner diameter \times 20 mm) and a separation column (1.7 μm bead, 75 μm inner diameter \times 150 mm length). For online coupling, a nano-ion spray source was used, equipped with an ESI needle (10- μm silica tip). The needle voltage was 2.1 kV in positive ion mode. The scan cycle consisted of a survey scan (mass range 500–2000 atomic mass units) followed by MS/MS of the 4 most intense signals in the spectrum with an exclusion list for ion signals set to 25 s after one occurrence. For CID analysis, we used normalized collision energies set to 26 or 35; $q = 0.18$ with an activation time set to 30 ms and isolation width set to m/z 1.0. Analysis was performed in CID only mode as previously described (37).

Bioinformatics—The derived mass spectrometry datasets were converted to generic format (dta) files using the Bioworks Browser (version 3.3.1). These files were then searched against the mouse IPI data base (version 3.44 containing 55,078 proteins) using the Bioworks search algorithm, TurboSequestTM (version 3.3.1; Thermo-Finnigan). The species subset was set to *Mus musculus*, the number of allowed trypsin missed cleavages was set to 2.0. Carbamidomethylation of cysteine was set as a fixed modification, whereas oxidation of methionine and phosphorylation of serine, threonine, and tyrosine were set as differential modifications. The parent ion selection was set to 1.4 Da with fragment ion set to 0.7 Da. Peptides failing the preliminary filters set for the charge states +1 to +4, respectively, with x_{corr} values lower than 2.5, 2.9, 3.1, and 3.2 were automatically rejected. Those spectra meeting these criteria were manually inspected on a residue by residue basis to ensure accurate y - and b -ion detection with overlapping sequence coverage.

LTQ RAW files were also processed by the in house licensed Mascot data base search program (version 2.2; Matrix Science) utilizing the LCQ_DTA script to convert the RAW files into peak lists prior to MS/MS data base searching. Peak lists were searched against the SwissProt Data Base (version 51.6). The

taxonomy, missed cleavage sites, modifications, and mass tolerances were kept the same as the TurboSequest searches. Results were filtered to a confidence level of 0.001 and the peptides were manually inspected and validated.

Statistics—All experiments were replicated a minimum of 3 times with pooled sperm samples obtained from at least 3 mice. Graphical data are presented as mean \pm S.E., being calculated from the variance between samples. Statistical significance was determined using an analysis of variance.

RESULTS

Mouse Spermatozoa Express a Number of Surface-oriented Multimeric Protein Complexes—The separation of native multimeric protein complexes from mouse spermatozoa was achieved using BN-PAGE techniques optimized from the methodology originally described by Schagger and von Jagow (31). This system consistently resolved in excess of 10, high molecular mass (100 to 1100 kDa) entities, which we cautiously define as multiprotein complexes (MPCs), from populations of both caput and caudal epididymal spermatozoa (Fig. 1, A and B). Interestingly, the profile of MPCs showed several qualitative changes during epididymal maturation, with caput cells being characterized by a number of bands that appeared to be absent in the later stages of their development. However, the subsequent capacitation of these spermatozoa did not appear to be associated with further overt changes in the MPC profile (Fig. 1C).

To explore the possibility that the putative protein complexes reside on the surface of spermatozoa, a position compatible with a role in sperm-zona pellucida interaction, populations of capacitated sperm were surface labeled with a membrane-impermeable derivative of biotin. These cells were then lysed under native conditions, the proteins were resolved by BN-PAGE, transferred to nitrocellulose membranes, and affinity labeled with HRP-conjugated streptavidin. As depicted in Fig. 1D, this approach revealed at least eight prominent biotinylated protein bands ranging in molecular mass from 60 to >800 kDa. Importantly, a number of the putative protein complexes identified by one-dimensional BN-PAGE (Fig. 1C) appeared to co-migrate with those detected by biotinylation, suggesting that at least some of the components of these complexes are surface expressed. Support for this conclusion is also afforded by the specificity of surface biotinylation. In this context, representative proteins that are abundantly expressed in the cytosol (α -tubulin), mitochondria (Complex IV Subunit 1), or nucleus (protamine) were not detected among any of the biotinylated complexes (supplemental Fig. S1, A–E). To the best of our knowledge, these results represent the first reported examples of the isolation of native protein complexes from the surface of mouse spermatozoa.

Mouse Sperm MPCs Adhere to Solubilized Zona Pellucida—To investigate the functional significance of the putative sperm-protein complexes, their affinity for solubilized zona pellucida was assessed under conditions of high stringency using far Western blotting. This revealed at least two relatively large protein complexes, of ~ 820 and ~ 220 kDa (hereafter referred to as Complex I and Complex II, respectively) (Fig. 1E), that bound to the zona pellucida proteins. It was of considerable

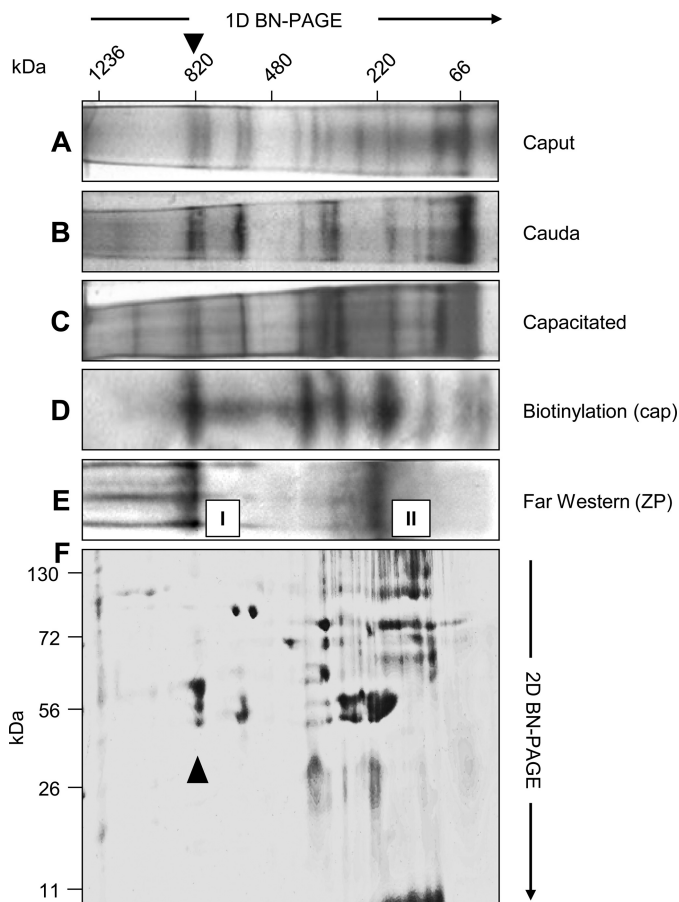


FIGURE 1. Separation and functional analysis of multiprotein complexes isolated from mouse spermatozoa. Native lysis prepared from (A) caput and (B and C) cauda (noncapacitated and capacitated) epididymal sperm were resolved by BN-PAGE and stained with Coomassie Blue G-250. The numbers on the top of panel A, correspond to the molecular mass (kDa) of BN-PAGE protein markers. D, capacitated spermatozoa were surface labeled with biotin prior to lysis of the cells under native conditions. The lysate was then prepared for BN-PAGE and affinity labeling with HRP-conjugated streptavidin. E, following separation of capacitated sperm proteins by BN-PAGE, the gel was transferred to a nitrocellulose membrane and incubated with solubilized, biotinylated zona pellucida recovered from ~200 oocytes. The blot was then washed thoroughly prior to detection of bound zona with HRP-conjugated streptavidin. Two predominant bands (I and II) were detected that possessed ZP affinity. F, a single lane of a one-dimensional BN-PAGE gel comprising capacitated sperm protein complexes was excised and individual proteins resolved on a 10% SDS-PAGE gel. These experiments were each replicated 3 times and representative gels and Western blots are shown.

interest that the migration of both of these complexes corresponded with bands that featured prominently among those displaying surface expression (Fig. 1D).

Identification and Characterization of the Individual Proteins That Comprise Sperm Protein Complex I (820 kDa)—Two-dimensional BN-PAGE was used to confirm that the putative MPCs recovered from spermatozoa were indeed composite structures comprising multiple proteins. As anticipated, this technique was effective in resolving the majority of the MPCs into a number of discrete proteins (Fig. 1F). It was of particular interest that the large, surface-expressed complex of ~820 kDa, Complex I, resolved into several individual proteins with molecular masses ranging from 55 to 65 kDa (Fig. 1F). Given that this complex was demonstrated to possess an affinity for the zona pellucida, these individual proteins were excised

and prepared for identification using an electrospray ionization mass spectrometry interface. This sequencing strategy identified all 8 members of the eukaryotic cytosolic chaperonin containing TCP-1 (CCT), also known as the TCP1 ring complex (TRiC) (*i.e.* CCT1–CCT8) (supplemental Table S1). In light of the novelty of this finding, studies were undertaken to confirm both the validity of our sequencing data and the putative surface orientation of this complex. For this purpose, commercial antibodies against three of the 8 CCT proteins (CCT2, CCT6A, and CCT8) were sourced and validated (supplemental Fig. S2) prior to use in immunoblotting and immunofluorescence analyses.

As demonstrated in Fig. 2, A–C, each of the three CCT proteins demonstrated cross-reactivity with a single band in one-dimensional BN-PAGE blots corresponding to Complex I. Following resolution of this complex by two-dimensional BN-PAGE, a single cross-reactive protein of the appropriate molecular mass of ~60 kDa was detected using anti-CCT2 and anti-CCT8 antibodies (Fig. 2, D and F). Anti-CCT6A generated similar results, however, in addition to the expected band of 58 kDa it also labeled a second protein of ~60 kDa (Fig. 2E). Our preliminary analysis of this additional protein indicates that it may originate from a post-translational modification of the parent protein during capacitation (supplemental Fig. S2). However, we are yet to explore the nature of this potential modification.

Having confirmed that the chaperonin proteins were constituents of the approximate 820-kDa multimeric zona-binding Complex I, we sought to localize the proteins on populations of fixed but nonpermeabilized spermatozoa (Fig. 3A). Three predominant labeling patterns: (i) mid and principal piece of the tail, (ii) periacrosomal region combined with the mid and principal piece of the tail, and (iii) periacrosomal region of the head alone, were observed in these cells (Fig. 3A). However, the percentage of cells in each category differed among the chaperonin subunits examined and was also influenced by the capacitation status of the sperm population. Interestingly in this regard, the portion of cells displaying periacrosomal labeling for the CCT2 and CCT6A proteins increased following the induction of capacitation (Fig. 3B). In contrast, CCT8 was consistently observed in the periacrosomal region of the sperm head, regardless of the capacitation status of the cell (Fig. 3B).

Given the resemblance between the spatial and temporal patterns of CCT labeling and that previously recorded for the membrane raft marker, G_{M1} ganglioside (28, 38), we conducted co-localization studies using the cholera toxin B subunit (CTB) to determine whether the CCT/TRiC complex resides within membrane rafts. As illustrated in Fig. 3C, the most substantive CTB/CCT co-localization was confined to the periacrosomal region of the head of capacitated sperm, the exact location where sperm-zona interaction occurs. The surface orientation of the CCT/TRiC complex was examined through the use of a flow cytometry assay, which demonstrated that the capacitation-associated increase in the intensity of head labeling was complemented by a significant increase in the number of spermatozoa expressing CCT2 and CCT6A on their surface after this event (Fig. 3D and supplemental Fig. S3, A–C). These findings were further confirmed through the application of an

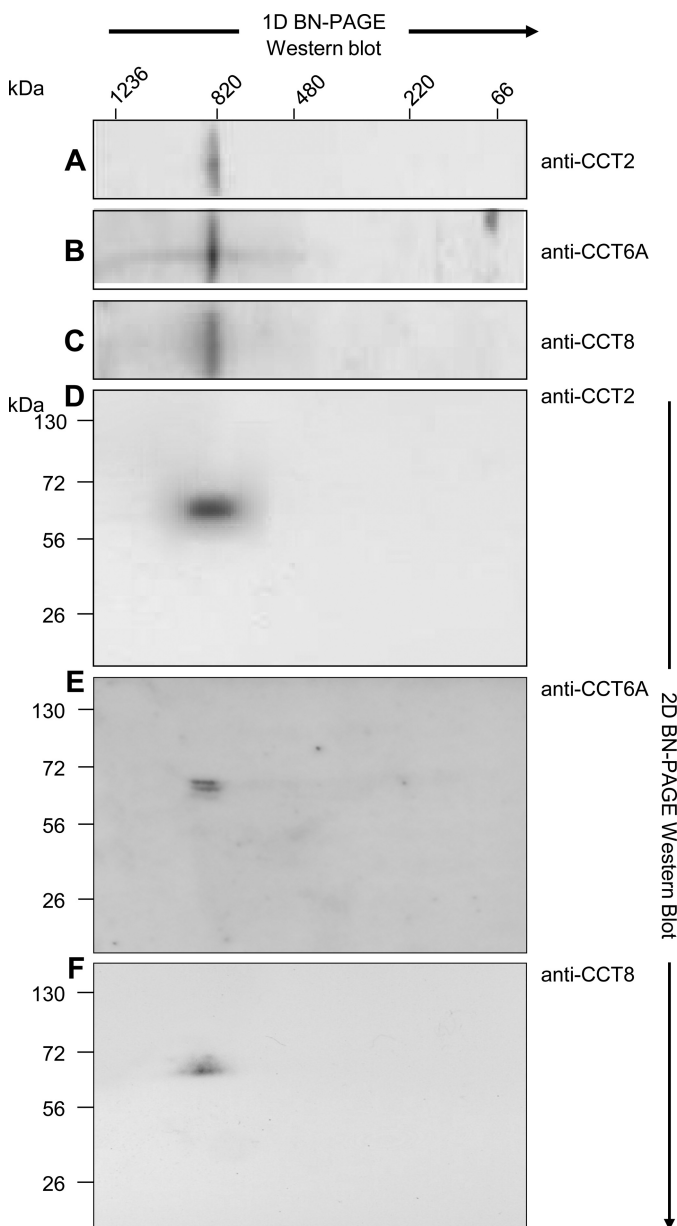


FIGURE 2. Western blot analysis of the composition of sperm membrane Complex I. A–C, native sperm lysates were resolved by one-dimensional BN-PAGE and transferred to nitrocellulose membranes. The membranes were then probed sequentially with: A, anti-CCT2; B, anti-CCT6A; or C, anti-CCT8 followed by an appropriate HRP-conjugated secondary antibody. D–F, two-dimensional BN-PAGE Western blots were probed with a similar panel of antibodies, anti-CCT2 (D), anti-CCT6A (E), and anti-CCT8 (F). Each experiment was replicated 3 times and representative Western blots are shown.

immunobead assay (24), the results of which demonstrated that a significantly higher percentage of capacitated spermatozoa bound to protein G beads conjugated with anti-CCT2 and anti-CCT6A antibodies compared with that of noncapacitated cells (supplemental Fig. S4B).

The CCT/TRiC Complex Participates Indirectly in Sperm-Zona Pellucida Interaction—On the basis of the evidence presented above, experiments were conducted to determine whether the CCT/TRiC complex participates either directly or indirectly in gamete interactions. For the former studies, capacitated spermatozoa were preincubated in antibodies directed against the subunits of the CCT/TRiC complex. Irrespective of

whether these antibodies were presented to sperm individually or as a mixture of 3 antibodies (*i.e.* anti-CCT2, anti-CCT6A, and anti-CCT8) they failed to disrupt significantly the efficacy of sperm-zona adhesion (Fig. 4A). From these results we infer that the CCT/TRiC complex does not participate directly in sperm-zona pellucida binding.

To determine whether the CCT/TRiC complex instead participates indirectly in zona adhesion, possibly through the presentation of zona receptors, we exploited the fact that ATP binding and hydrolysis stimulates the CCT/TRiC complex to release bound substrates (39, 40). Capacitating populations of spermatozoa were therefore incubated with varying concentrations of exogenous adenosine 5'-triphosphate magnesium salt (ATP; 20–2000 μ M) and examined for their zona binding affinity (Fig. 4B). Consistent with our hypothesis, ATP elicited a significant, dose-dependent inhibition of sperm-zona pellucida interactions (Fig. 4B). To control for the possibility that this inhibition was attributed to the exogenous ATP stimulating plasma membrane depolarization and acrosomal exocytosis (41), the integrity of the acrosome was examined in each of the treatment groups (supplemental Fig. S5). Importantly, under the conditions employed in the present study, ATP did not elicit an increase in acrosomal exocytosis with at least 75% of the cells remaining acrosome intact across all treatment groups. It is also noteworthy that, in contrast to ATP, the exposure of mouse spermatozoa to GTP, incorporated as a purine nucleoside control in this experiment, led to a significant increase in the number of sperm that bound to the zona pellucida (Fig. 4B).

As an additional strategy for confirming the physiological relevance of the CCT/TRiC complex, native lysates were prepared from capacitated spermatozoa and incubated with zona pellucida intact mouse oocytes. The oocytes were then subjected to stringent washing before being probed with anti-chaperonin antibodies. This assay demonstrated that chaperonin proteins were indeed present within a complex that possessed an affinity for the zona pellucida (Fig. 4C). The specificity of this interaction and the requirement for the native conformation of this complex was demonstrated by the fact that anti-chaperonin antibodies failed to label the zona pellucida of 2-cell embryos (Fig. 4D), which have lost their sperm-binding properties. Similarly, the anti-chaperonin antibodies also failed to label oocytes that were incubated in denatured sperm lysates (results not shown). Secondary antibody only controls showed modest staining of the oocyte but consistently failed to label the zona pellucida (Fig. 4E). Commensurate with the results of the previous study, chaperonin binding to the zona pellucida was eliminated if the native lysates were prepared from sperm that were pre-treated with 2 mM ATP (Fig. 4F).

The CCT/TRiC Complex Interacts with Putative Zona Pellucida-binding Proteins—The cumulative results presented above raise the possibility that the CCT/TRiC complex mediates the presentation of zona pellucida receptor(s) in capacitated spermatozoa. To test this possibility we employed a co-immunoprecipitation strategy to identify the cohort of potential receptors contributing to the zona-binding complexes following capacitation. As demonstrated in Fig. 5A, at least three predominant protein bands appeared to be precipitated with anti-CCT6A when incubated with native lysate and

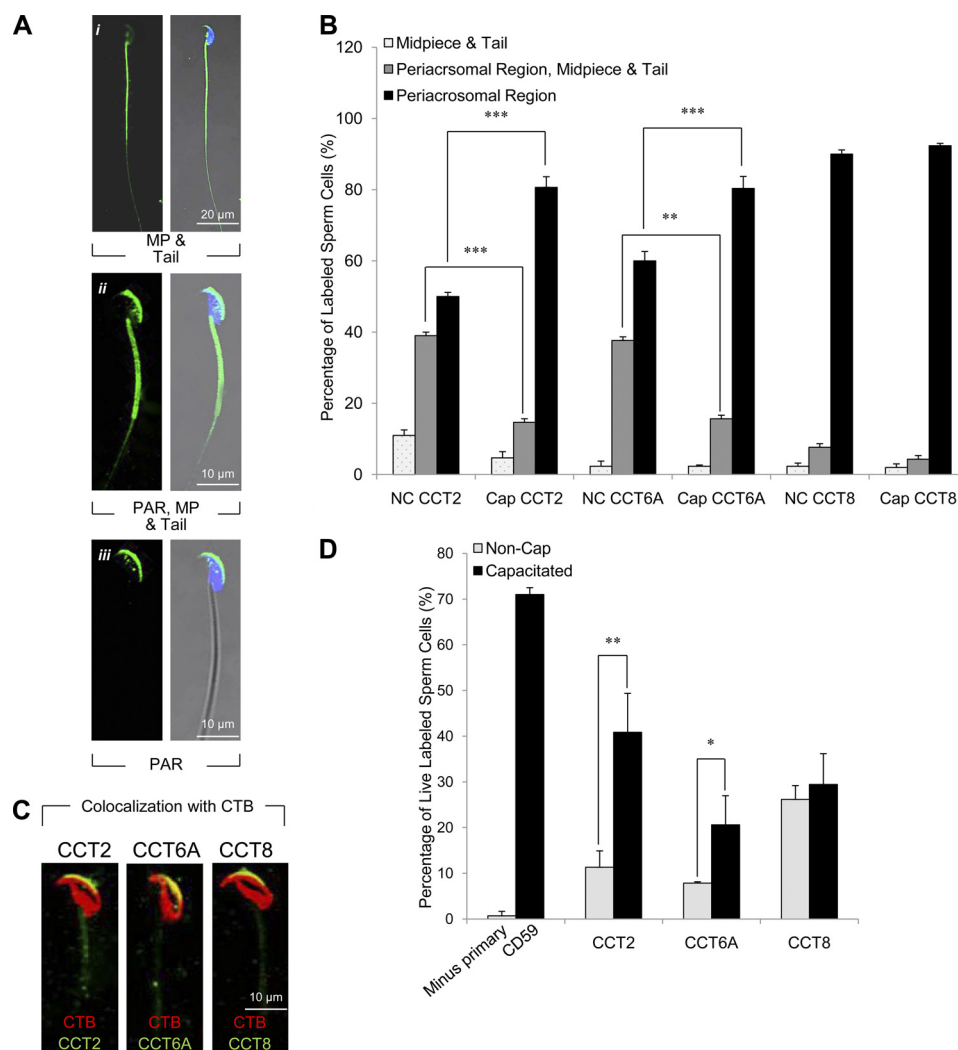


FIGURE 3. Immunolocalization of the subunits of the CCT/TRiC in mouse spermatozoa. Fixed populations of noncapacitated and capacitated epididymal spermatozoa were sequentially labeled with anti-CCT2, anti-CCT6A, or anti-CCT8 and the appropriate FITC-conjugated secondary antibody. *A*, representative images of the patterns of (i) midpiece and tail; (ii) periacrosomal region, midpiece, and tail; and (iii) periacrosomal region only labeling obtained for CCT2, CCT6A, and CCT8 are depicted. *B*, because the labeling patterns differed with each antibody and the capacitation status of the spermatozoa, a quantitative assessment was performed of the labeling patterns observed across a minimum of 300 cells. These data are expressed as the mean \pm S.E. from 3 replicates. **, $p < 0.01$, and ***, $p < 0.001$. *C*, the interaction between CCT subunits and CTB (a high affinity label of the membrane raft marker, G_{MI} gangliosides) was investigated using standard colocalization techniques. Merged images of spermatozoa dual labeled with antibodies against CCT subunits (green) and CTB (red), co-localization results in a yellow fluorescent signal. *D*, the expression of each of the chaperonin subunits (CCT2, CCT6A, and CCT8) was examined on the surface of live populations of noncapacitated and capacitated spermatozoa via flow cytometry. Importantly, no labeling was observed in the absence of primary antibody. In contrast, cells labeled with the anti-CD59 as a positive control revealed surface expression on more than 70% of the viable sperm population. This experiment was repeated 3 times with a minimum of 10,000 viable cells scored for each experiment; data are expressed as the mean \pm S.E. *, $p < 0.05$ and **, $p < 0.01$ compared with noncapacitated sample.

eluted under conditions of low pH. These bands did not appear to co-migrate with proteins precipitated with an alternative, isotype-matched antibody, nor were they detected in either the bead only or anti-CCT6A antibody only controls. The analysis of these bands by LC-MS (Fig. 5B) revealed the anticipated presence of members of the CCT/TRiC complex in addition to a number of putatively interacting molecular chaperones including HSPD1, a protein that has previously been implicated in the formation of multimeric zona-receptor complex (26) (Fig. 5B, II). The lower band (Fig. 5B, III) yielded one of the most interesting findings with the identification of zona pellucida-binding protein 2 (ZPBP2), one of several proteins implicated in sperm-zona pellucida interactions (42).

Confirmation of the Presence of ZPBP2 in a Zona Pellucida-Receptor Complex—Consistent with our sequencing data, antibodies directed against ZPBP2 labeled a single protein band that co-migrated with Complex I in one- and two-dimensional BN-PAGE gels (Fig. 6A). Furthermore, the use of ESI-MS confirmed that ZPBP2 peptides were indeed present within Complex I (supplemental Table S2). Interestingly, this sequencing strategy revealed the presence of two additional proteins with previously reported zona affinity, ZP3R (formerly SP56) (43) and ZPBP1 (formerly SP38) (42), that also putatively reside within Complex I (supplemental Table S2).

Immunolocalization of ZPBP2 demonstrated that this molecule resides within the acrosomal domain of acrosome intact (Fig. 6B), but not acrosome-reacted spermatozoa (results not

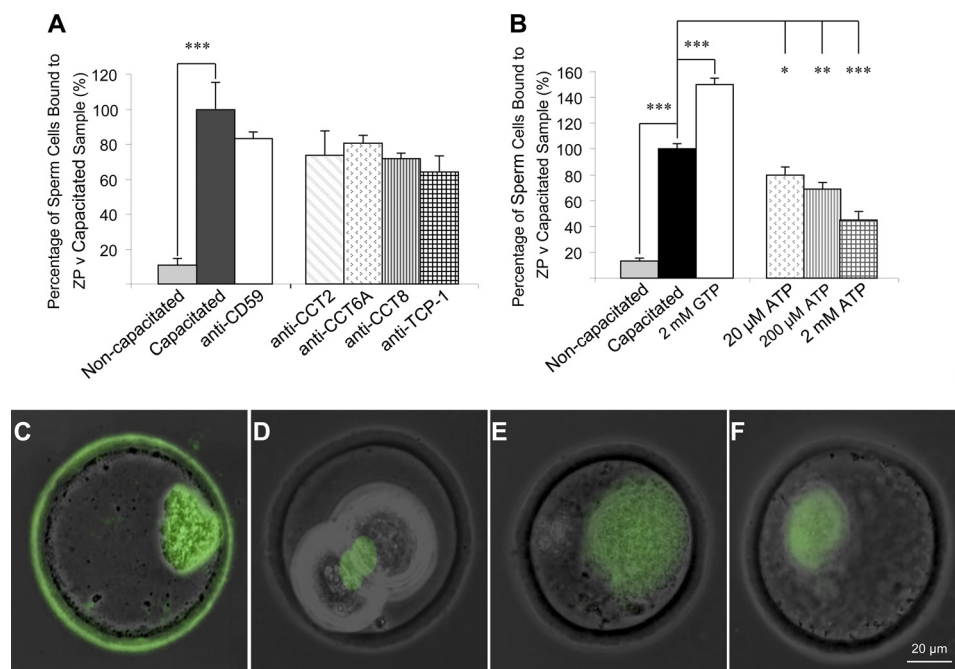


FIGURE 4. Investigation of the role of the CCT/TRiC in sperm-zona pellucida interaction. *A*, capacitated sperm were incubated with anti-CCT2, anti-CCT6A, and anti-CCT8 antibodies (diluted 1:100) individually or in the presence of a mixture of antibodies against the CCT/TRiC (anti-TCP-1; *i.e.* anti-CCT2, anti-CCT6A, and anti-CCT8 each diluted 1:100) for 30 min at 37 °C. The sperm suspensions were then washed and $\sim 5 \times 10^5$ cells were placed in a droplet containing 10–12 salt stored oocytes. The latter were then washed to remove loosely adherent spermatozoa and the number of cells remaining bound to each zona was scored and expressed as a percentage of the positive control (*i.e.* capacitated sperm sample). An antibody directed against the sperm surface marker CD59 (anti-CD59) was included as a control. This experiment was replicated 3 times and the data are expressed as the mean \pm S.E., as a percentage change from the capacitated control, $***, p < 0.001$. *B*, caudal epididymal spermatozoa were capacitated for 45 min prior to exposure to exogenous ATP (20–2000 μ M) or GTP (2000 μ M) for 15 min at 37 °C. The sperm were assessed for their affinity for the zona pellucida as described above. This experiment was replicated 3 times and the data are expressed as the mean \pm S.E. as a percentage change from the capacitated control, $*, p < 0.05$, $** , p < 0.01$, or $***, p < 0.001$. *C–F*, native lysates were prepared from capacitated spermatozoa as described and then incubated with zona intact oocytes. *C*, after stringent washing, the oocytes were probed with anti-CCT2 and FITC-conjugated secondary antibody. Controls consisted of *D*, 2-cell embryos (which have lost their sperm-binding properties) probed with anti-CCT2 and secondary antibody; *E*, oocytes incubated with secondary antibody only. *F*, in addition, oocytes were incubated with native lysate prepared from sperm pre-treated with 2 mM ATP and then probed as outlined above. This experiment was replicated 3 times with representative images of oocytes presented. Scale bar represents 20 μ m.

shown). *In situ* proximity ligation revealed a striking co-localization of ZPBP2 and CCT6A in the acrosomal region of the head of nonpermeabilized capacitated spermatozoa. In contrast, sperm cells coincubated with 2 mM ATP during capacitation did not show colocalization within the periacrosomal region (Fig. 6C).

As previously noted for CCT2 and CCT6A, the surface exposure of ZPBP2 increased significantly in cells in which capacitation was actively driven (Fig. 6D). Moreover, the anti-ZPBP2 antibodies were able to reduce sperm-ZP interactions (Fig. 6E) emphasizing the significance of this protein in the molecular mechanisms regulating sperm-oocyte recognition.

In view of these data, additional studies were undertaken to determine whether the presence of ZPBP2 accounts, at least in part, for the zona affinity displayed by the CCT/TRiC complex. For this purpose, native lysates were prepared from capacitated spermatozoa, incubated with zona pellucida intact mouse oocytes, and probed with anti-chaperonin antibodies as previously described (Fig. 4C). This assay revealed that preincubation of the lysates with anti-ZPBP2 antibodies led a marked reduction in the zona binding affinity of the CCT/TRiC complex (Fig. 7B). Because binding was not eliminated we conducted similar studies in the presence of antibodies against ZP3R, an additional zona adhesion candidate identified as a putative client protein of the CCT/TRiC complex (supplemen-

tal Table S2). The inclusion of these antibodies also reduced CCT/TRiC binding albeit to a lesser degree. Interestingly, however, the combined use of anti-ZPBP2 and anti-ZP3R antibodies virtually eliminated binding of the CCT/TRiC complex (Fig. 7D). As anticipated, an irrelevant antibody control (anti-CD59) had no effect on binding of the CCT/TRiC complex. Taken together these data highlight the importance of the CCT/TRiC complex and its client proteins in the mediation of sperm-zona pellucida adhesion.

DISCUSSION

Recent research into the mechanisms that underpin sperm-zona pellucida interaction have highlighted the potential importance of molecular chaperone proteins in this process (26, 29). Specifically, it has been suggested that a subset of molecular chaperones are responsible for the assembly of key recognition molecules into functional zona pellucida-receptor complexes that are expressed on the sperm surface during capacitation (26). This novel hypothesis draws on the well characterized roles of molecular chaperones in mediating the folding of polypeptides and their assembly into multisubunit structures. It also accounts for the myriad of candidate zona pellucida receptors that have been identified in the literature and the apparent redundancy evident from studies in which these individual candidates have been eliminated through the

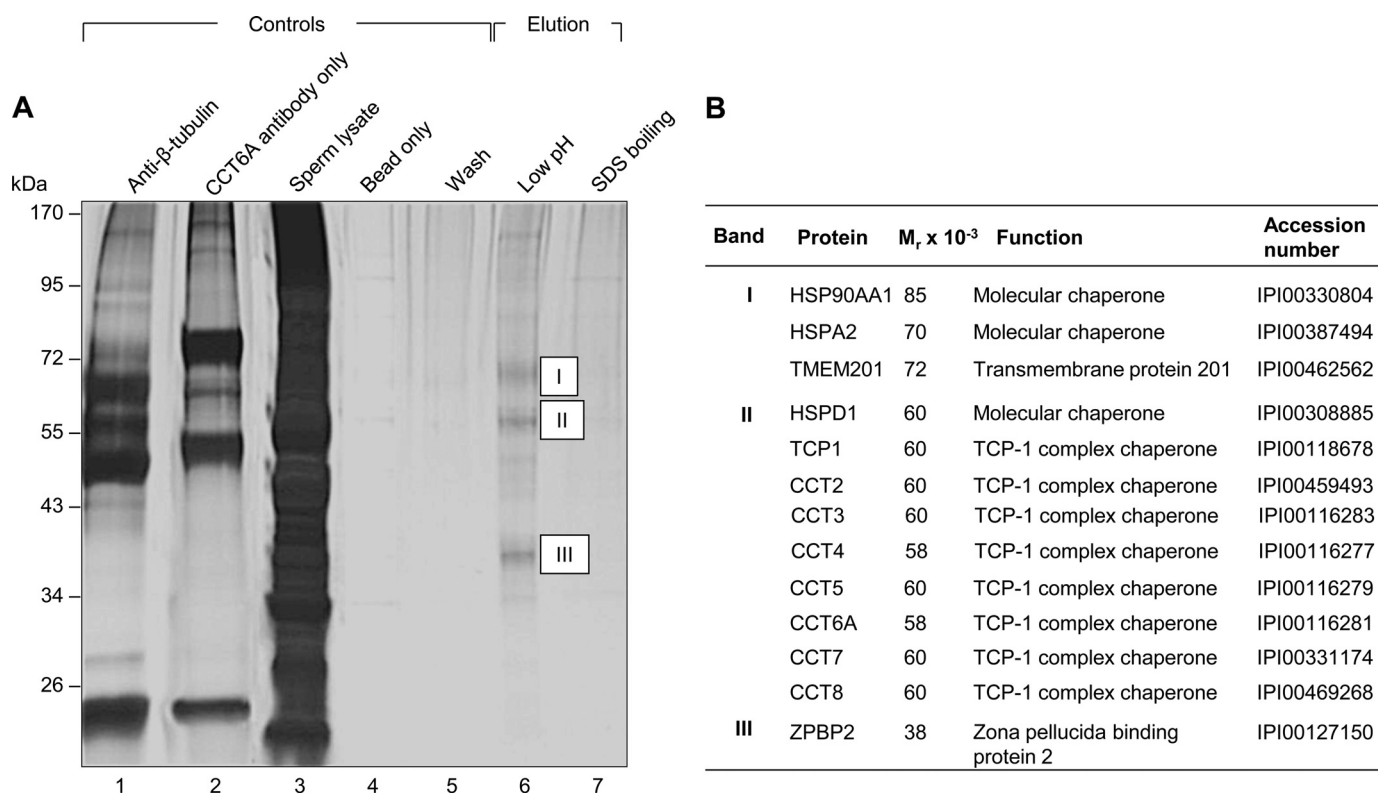


FIGURE 5. Co-immunoprecipitation of CCT/TRiC interacting proteins. A, native lysate prepared from capacitated spermatozoa was immunoprecipitated with anti-CCT6A (lanes 6 and 7). Experimental controls included native lysates precipitated with an irrelevant isotype-matched antibody (anti- β -tubulin, lane 1), the CCT6A antibody only (lane 2), whole sperm lysate (lane 3), and proteins recovered by boiling unconjugated beads following their incubation with native lysate (lane 4). In addition, the final bead wash (lane 5) and proteins recovered from beads were boiled in SDS buffer after the low pH elution (0.2 M glycine, pH 2.5) (lane 7). B, mass spectrometry data obtained from sequencing of proteins within bands I–III of panel A.

creation of knock-out models (25, 29, 44–50). However, to date, no studies have formally demonstrated the existence of chaperone containing multimeric protein complexes on the sperm surface.

To directly investigate whether spermatozoa express multimeric protein complexes, we employed the technique of BN-PAGE. This native gel system was selected as a separation platform because it represents a robust and versatile technique, suitable for the analysis of interacting proteins on both an analytical and preparative scale (30–32). This technique also affords greater resolution than gel filtration or sucrose density ultracentrifugation, and in contrast to immunoprecipitation and two-hybrid approaches, it allows the determination of the size, relative abundance, and subunit composition of multiprotein complexes. To the best of our knowledge, the studies described herein represent the first recorded use of BN-PAGE for the analysis of mouse sperm proteins and provide compelling evidence that sperm do indeed express a number of large multiprotein complexes. By coupling BN-PAGE with far Western immunoblot analyzes it was further demonstrated that the isolated complexes retain their biological activity. In this context, at least two of the high molecular weight sperm surface complexes demonstrated an affinity for solubilized zona pellucida. Proteomic analysis of one such complex identified the 8 unique subunits of the CCT/TRiC complex (*i.e.* CCT1–CCT8). The original TCP-1 polypeptide was identified as a highly expressed mouse testicular protein (51) encoded by a gene located within the t-complex on chromosome 17 (52). The

t-complex is of interest as it is known to harbor genes that influence mouse development and male, but not female, fertility (51, 53). For instance, it has been variously reported that t-specified differences exist in sperm antigenic properties (54, 55), metabolic levels (56, 57), and significantly, sperm-zona pellucida receptor activity (58).

Similar to other molecular chaperones, CCTs form a high molecular weight complex, with subunits arranged into two superimposed multimeric rings, each enclosing a central cavity that facilitates substrate binding (59–61). However, unlike other chaperone complexes that are generally assembled from one or two different types of subunits, the CCT/TRiC complex is a hetero-oligomeric complex comprising eight different subunits. The CCT subunits share only 22.5–36.2% amino acid sequence identity but possess several conserved motifs (62) that may contribute to their function. The presence of nonconserved regions, particularly within the putative CCT substrate-binding domains, suggests that each subunit may possess differing affinities for target sequences or structures within substrate proteins.

The CCT/TRiC complex is believed to function mainly as folding machinery for cytosolic proteins. Although little is known regarding the mechanisms that determine its substrate specificity, recent reports suggest that this may be influenced by interaction of the complex with other molecular chaperones, including HSPD1 (40, 63), a finding consistent with the results of the present study (Fig. 5B). Alternatively, it has also been suggested that the phosphorylation status of the CCT subunits

Sperm-Oocyte Interaction

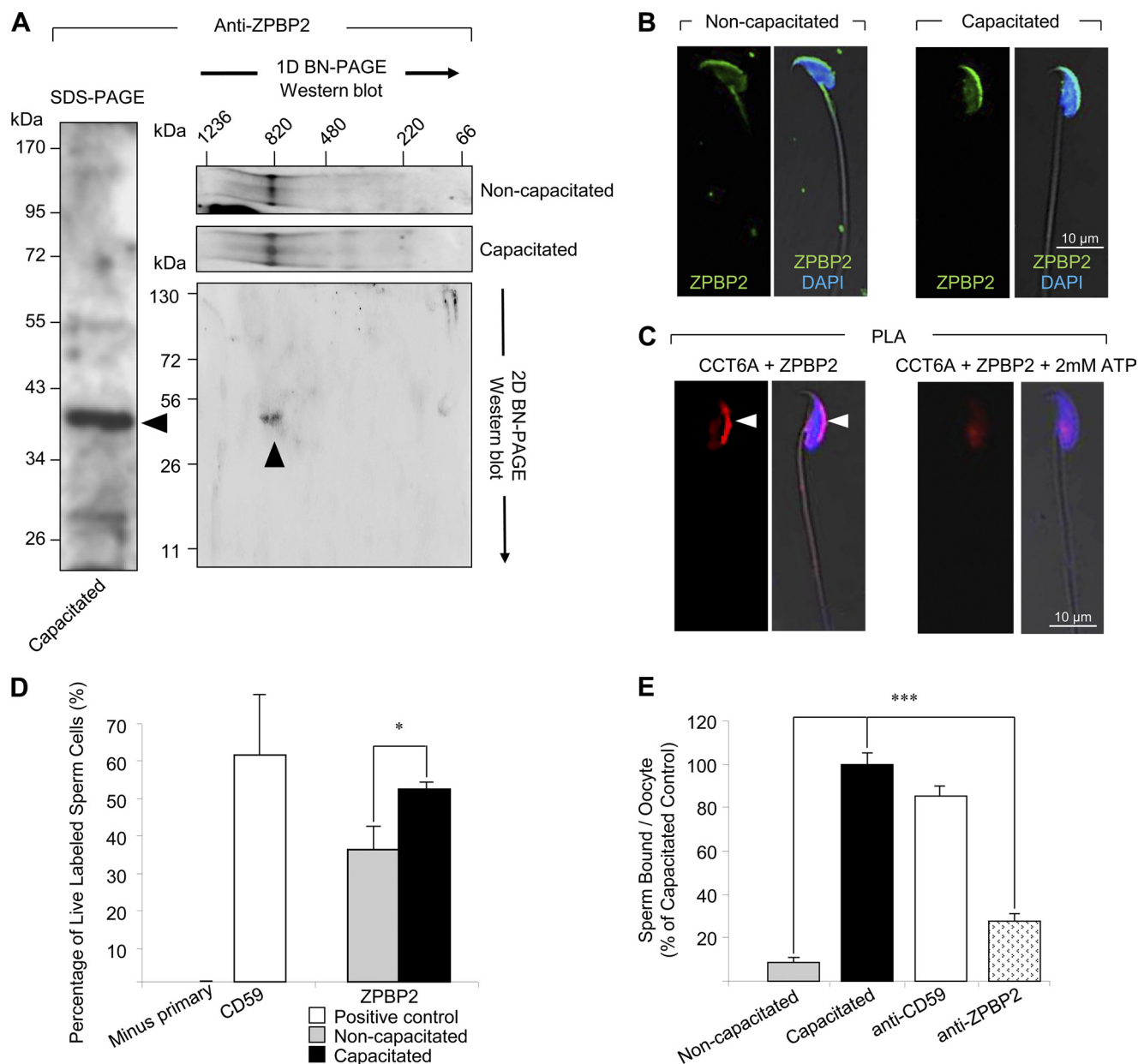


FIGURE 6. Characterization of CCT/TRiC-associated zona pellucida receptor protein ZBPB2. *A*, Western blots of SDS-PAGE, one- and two-dimensional BN-PAGE gels containing lysates prepared from noncapacitated and capacitated spermatozoa probed with anti-ZBPB2 antibodies. *B*, fixed populations of noncapacitated and capacitated spermatozoa were sequentially labeled with anti-ZBPB2 and an appropriate FITC-conjugated secondary antibody (green). They were then counterstained with DAPI (blue). *C*, detection of the interactions of CCT6A and ZBPB2 achieved using *in situ* proximity ligation assay in spermatozoa capacitated under either normal conditions or in the presence of 2 mM ATP. *D*, the expression of ZBPB2 was examined on the surface of live populations of noncapacitated and capacitated spermatozoa via flow cytometry analysis. The percentage of live spermatozoa expressing surface immunofluorescence specific for ZBPB2 was recorded and graphed. *E*, the role of ZBPB2 in zona pellucida binding was assessed by examining the ability of either anti-CD59 (control) or anti-ZBPB2 (diluted 1:100) to inhibit sperm binding to zona-intact oocytes. The number of spermatozoa bound to each zona was scored and expressed as a percentage of the positive control (*i.e.* capacitated sperm sample). Each experiment was replicated 3 times and representative Western blots and immunocytochemical pictures are shown. Graphical data are expressed as the mean \pm S.E., *, $p < 0.05$, and ***, $p < 0.001$. Arrowhead in *A*, indicates protein of appropriate molecular weight for ZBPB2. Arrowhead in *C*, indicates region of colocalization of CCT6A and ZBPB2.

may determine which specific proteins are accepted into the substrate binding pocket (64). This is of significance as our proteomic analyses indicated that a number of the identified CCT peptides displayed charge shifts consistent with those expected of phosphorylation (supplemental Table S1).

The major CCT/TRiC complex substrates identified to date appear to be the cytoskeletal proteins of tubulin and actin. However, the fact that these proteins show no significant amino acid sequence similarity and represent some of the most abun-

dant proteins within the cell (and are thus the easiest proteins to detect as substrates), raises the possibility that the CCT/TRiC complex may interact with additional protein substrates. Indeed, a number of studies have provided evidence that the CCT/TRiC complex is in fact relatively promiscuous, suggesting that ~5–10% of newly synthesized proteins flow through the CCT/TRiC complex (62, 65, 66).

The highest expression of the CCT/TRiC complex is observed within the mammalian testis (67) where it appears to

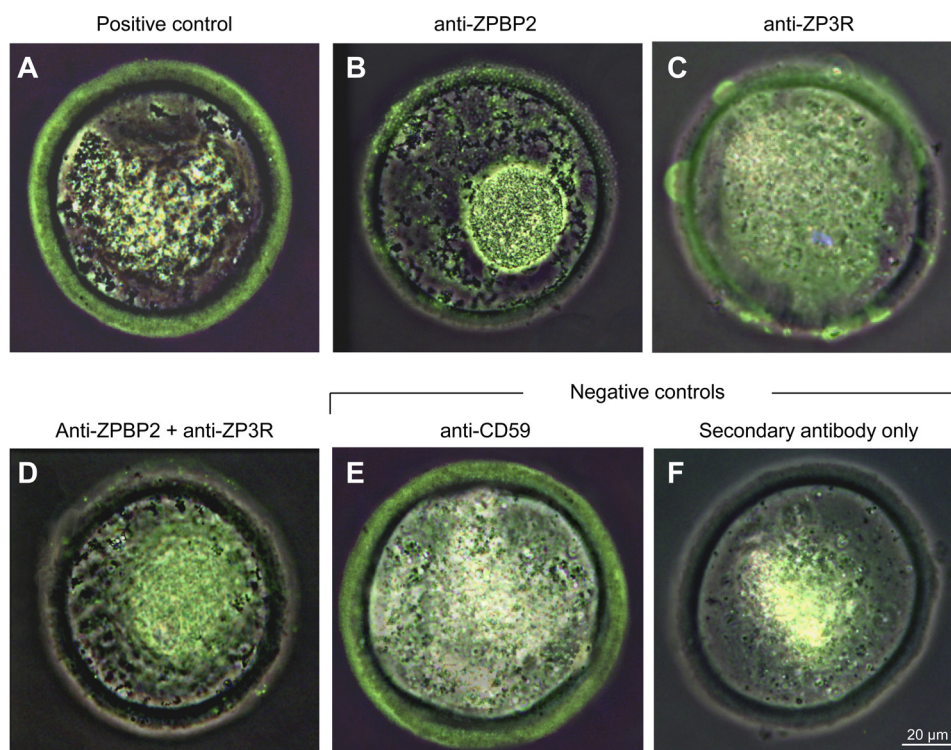


FIGURE 7. Investigation of the role of ZPBP2 in mediating the CCT/TRiC-zona pellucida interaction. *A*, native lysates were prepared from capacitated spermatozoa and then incubated with zona-intact oocytes (positive control). The oocytes were then sequentially probed with anti-CCT2 and FITC-conjugated secondary antibody. Alternatively, the native lysate was pre-treated with: *B*, anti-ZPBP2 antibodies; *C*, anti-ZP3R antibodies; or *D*, a mixture of these two antibodies prior to incubation with oocytes. Negative controls for this experiment consisted of: *E*, lysates pretreated with antibodies against CD59 (a sperm surface protein not involved in zona adhesion), and *F*, oocytes incubated with secondary antibody only. This experiment was replicated 3 times with representative images of oocytes presented. Scale bar represents 20 μm .

play a key role in the cytodifferentiation of spermatids, including nuclear shaping and reorganization of the cytoplasmic organelles, two processes that rely on extensive remodeling of the microtubule cytoskeleton (67). However, the presence and function of the CCT/TRiC complex has not previously been investigated in mature spermatozoa. Our finding that components of the CCT/TRiC complex are expressed on the surface of capacitated spermatozoa and comprise part of a complex that displays affinity for the zona pellucida therefore extends the known localization and potential functions of this intriguing multimeric structure. Interestingly, we documented marked changes in the subcellular localization of the different CCT subunits during capacitation, characterized by a striking increase in the surface labeling of at least two CCT subunits. At present it is not known whether such changes reflect the structural assembly or rearrangement of each subunit leading to the novel exposure of certain epitopes and the masking of others (68), or if this represents a genuine relocation of the CCT/TRiC complex as has been suggested for other molecular chaperones.

In addition to far Western blotting, the notion that the CCT/TRiC complex participates in zona pellucida interactions is supported by the demonstration that the native complex shows selective adherence to intact zona pellucida. Notwithstanding these data, it is considered unlikely that the CCT/TRiC complex serves as a primary receptor for zona proteins. Rather, our collective evidence suggests that it plays an intermediary role in the presentation of the appropriate adhesion molecules. Such a conclusion is consistent with the proposed role of alternative

chaperone proteins (26–29, 69). Calmegin, for instance, fulfills a critical role in spermatogenesis by ensuring the correct folding of nascent glycoproteins destined for the acrosomal matrix and the plasma membrane of mature spermatozoa. Targeted disruption of the calmegin gene compromises the delivery of several proteins to the sperm surface and results in a concomitant reduction in male fertility attributable to the loss of sperm-zona binding ability (29). Our model for chaperone-mediated sperm-zona pellucida interaction also aligns with the role of surface-expressed molecular chaperones in other cell types. For example, HSP90 has been shown to direct the presentation of matrix metalloproteinases on the surface of fibrosarcoma cells and thus play an important role in promoting cancer invasiveness (70).

Among the potential CCT/TRiC interacting proteins that could account for its zona affinity, we identified ZPBP2 as a compelling candidate. ZPBP2 is a paralog of the acrosomal matrix protein, zona pellucida-binding protein 1 (ZPBP1), which has been implicated in secondary sperm-zona pellucida interactions (42, 71, 72). However, in contrast to ZPBP1, which is distributed throughout the acrosome, ZPBP2 is characterized by a discrete localization along the rostral ridge of the acrosome (42). Furthermore, whereas male mice lacking ZPBP1 bear substantial morphological abnormalities arising as a result of incomplete acrosome compaction, males null for ZPBP2 display subfertility primarily associated with defects in zona pellucida interaction (42). It is also of interest that although preincubation of spermatozoa with an anti-ZPBP2 antibody

significantly suppressed sperm-zona binding, the inhibition was incomplete and did not reach the low levels typical of non-capacitated spermatozoa (Fig. 6E). Such incomplete inhibition is consistent with our hypothesis that the multimeric zona-binding complexes expressed on the sperm surface during capacitation contain more than one type of zona receptor. For example, both ZBP1 and ZP3R (SP56) were found in Complex I (supplemental Table S2) and additional receptors may well be present in Complex II. At present it is not known how ZBP2 or any of the other potential zona-binding proteins interact with the CCT/TRiC complex, nor the mechanism by which this protein is presented to zona ligands. It is of interest, however, that some acrosomal matrix proteins, including ZP3R, are released to the sperm surface during capacitation (73). Additional work is now required to determine whether this relocation is influenced by their interaction with the CCT/TRiC complex.

One particularly interesting finding was that the zona binding affinity of the CCT/TRiC complex is greatly reduced by the exposure of sperm to exogenous ATP, a result that suggests this treatment induces the release of ZBP2 and potentially other ZP-binding proteins. This interpretation is consistent with former accounts that ATP induces sequential conformational changes in the CCT/TRiC complex that facilitate the release of its bound substrates (39, 40, 74). Further compositional analysis of released CCT/TRiC substrates will aid in elucidating the full complement of sperm surface proteins with which the complex interacts.

In summary, these studies have demonstrated that capacitated spermatozoa contain chaperone-laden multiprotein complexes that display affinity for the zona pellucida and are presented at the sperm surface during capacitation. These observations encourage a complete reappraisal of the molecular mechanisms underpinning sperm-oocyte interaction; away from the single ZP3 sperm receptor model that has dominated thinking in this area for the past half-century and toward a novel construct involving the dynamic presentation of zona-binding complexes on the sperm surface during capacitation. This model will open up new avenues of research into areas such as male infertility, which frequently involve failures of sperm-zona recognition (75), and contraception, where the inhibition of this process is an important strategic aim (76).

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