Native human zona pellucida glycoproteins: purification and binding properties

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BACKGROUND: Fertilization starts with the binding of the spermatozoa to the zona pellucida (ZP) of the oocyte. Such binding is a carbohydrate-mediated event and consists of a series of tightly regulated events. Molecular interactions between spermatozoon and ZP in human are not well characterized due to limited availability of oocytes for research. Our current technology cannot generate recombinant human ZP (hZP) glycoproteins with native glycosylation. METHODS AND RESULTS: In this study, hZP glycoproteins, hZP2 (~120 kDa), hZP3 (~58 kDa) and hZP4 (~65 kDa) were purified from ZP (purity >88%) by immunoaffinity columns. The binding sites of the purified native hZP3 and hZP4 were localized to the acrosome region of the capacitated human spermatozoa, and were lost after acrosome reaction. Purified human hZP2 bound to this region only in acrosome-reacted spermatozoa. Differential binding of the three glycoproteins to the post-acrosomal region and the midpiece of the spermatozoa was observed. In addition, hZP3, but not hZP2 and hZP4, induced hyperactivation. The stimulatory activity was dependent partly on N-linked glycosylation of hZP3. CONCLUSIONS: This manuscript describes the biological activities of purified hZP glycoproteins from the native source for the first time.

Keywords: zona pellucida; spermatozoa; glycoprotein; antibody; acrosome

Introduction

Mammalian eggs are enveloped by an extracellular matrix, the zona pellucida (ZP) that serves as an important regulator of fertilization. The interaction between the spermatozoon and the ZP marks the first step in fertilization. The ZP mediates binding of the spermatozoon to the oocyte in a relatively species-specific manner, induces acrosome reaction in the ZP bound spermatozoa and blocks polyspermic fertilization (Yanagimachi, 1994; Wassarman et al., 2001). Most of our knowledge on spermatozoon-ZP interaction comes from studies in mice. The mouse ZP (mZP) consists of three major glycoproteins, mZP1, mZP2 and mZP3. mZP3 is responsible for the initial spermatozoon-ZP binding and induction of acrosome reaction (Bleil and Wassarman, 1980; Beebe et al., 1992; Wassarman, 2005). After acrosome reaction, mZP2 acts as a secondary sperm receptor, binds to the acrosome-reacted spermatozoon and facilitates the penetration of the ZP (Bleil et al., 1988). Dimers of mZP1 form bridges linking ZP filaments composed of a succession of mZP2 and mZP3 molecules (Greve and Wassarman, 1985). Further investigations using Zp1 null mice revealed that though mZP1 is not required for sperm binding or fertilization, it is required for the structural integrity of ZP to minimize precocious hatching and reduced fecundity (Rankin et al., 1999). In Zp2 null mice, a thin zona matrix in early follicles was observed that could not be sustained in pre-ovulatory follicles (Rankin et al., 2001). No 2-cell embryos were recovered after mating Zp2 null females with normal male mice, suggesting that mZP2 has a role during fertilization and early embryo development (Rankin et al., 2001). The Zp3 null mice have follicles with germinal vesicle intact oocytes but completely lack a ZP matrix and have a disorganized corona radiata (Liu et al., 1996; Rankin et al., 1996). The females of these mice are sterile and the developmental potential of their oocytes is highly compromised.

In contrast to mZP, human ZP (hZP) is composed of four glycoproteins designated as ZP1 [1-638 amino acids (aa)], ZP2 (1-745 aa), ZP3 (1-424 aa) and ZP4 (1-540 aa) (Lefie`vre et al., 2004; Conner et al., 2005). A critical appraisal of the biochemical properties and biological activities of the hZP glycoproteins has been hindered due to the paucity of availability of human eggs for research. To circumvent this difficulty, various investigators have employed recombinant hZP glycoproteins (rhZP) and investigated their binding characteristics to...
Table 1. Binding pattern of purified hZP glycoproteins on human spermatozoa.

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*A, acrosomal; E, equatorial; PA, post-Acrosomal; M, midpiece.

(Table I) and biological activities on spermatozoa (van Duin et al., 1994; Chapman et al., 1998; Tsubamoto et al., 1999; Bray et al., 2002; Chakravarty et al., 2005, 2008; Caballero-Campo et al., 2006). However, it is likely that the glycosylation of rhZP glycoproteins is different from that of the native counterparts. It is known that glycosylation has significant impact on the biological activity of ZP glycoproteins (Dell et al., 1999). However, it is likely that the glycosylation of rhZP glycoproteins is different from that of the native counterparts. It is known that glycosylation has significant impact on the biological activity of ZP glycoproteins (Dell et al., 1999; Topfer-Petersen, 1999; Wassarman et al., 2001).

Hyperactivation is a specialized motility pattern seen in spermatozoa at the fertilization site. It is characterized by a highly vigorous, non-progressive, random motion with high-curvature flagellar movement (Suarez and Ho, 2003). The proportion of hyperactivated spermatozoa is positively correlated with ZP binding and penetration (Yanagimachi, 1994; Stauss et al., 1995), ZP-induced acrosome reaction (Liu et al., 2007) and IVF (Wang et al., 1993). Solubilized hZP or rhZP glycoproteins induce hyperactivation (Caballero-Campo et al., 2006; Bastiaan and Franken, 2007). The action of purified native ZP glycoproteins on hyperactivation is not known.

There were three objectives in this report. First was to purify native hZP glycoproteins from heat solubilized isolated ZP using monoclonal antibody (MAb) based immunoaffinity columns. Second was to compare the binding characteristics of different hZP glycoproteins to the capacitated and acrosome-reacted spermatozoa. Third was to study the effect of hZP glycoproteins on hyperactivation.

**Materials and Methods**

**Semen samples**

The Ethics Committee of the University of Hong Kong approved the research protocol. Spermatozoa from normal semen (World Health Organization, 1999) were processed by Percoll (Pharmacia, Uppsala, Sweden) density gradient centrifugation (Chiu et al., 2003). Percoll-processed spermatozoa were capacitated in Earle’s balanced salt solution (EBSS; Flow Laboratories, Irvine, UK) supplemented with sodium pyruvate (0.033 mg/ml), penicillin-G (0.06 mg/ml), streptomycin sulfate (0.075 mg/ml) and 3% bovine serum albumin (BSA) at 37°C in an atmosphere of 5% CO₂ in air. EBSS was previously used in our assisted reproduction program resulting in a fertilization of >70%. The medium has been used for capacitation (e.g. Kirkman-Brown et al., 2004; Bedu-Addo et al., 2005). After overnight capacitation, the percentage of capacitated spermatozoa was 63.4 ± 5.5% (mean ± SEM) as determined by chlorotetracycline staining (Chiu et al., 2005). The capacitated spermatozoa were resuspended in EBSS containing 0.3% BSA (EBSS/BSA).

**MAbs against rhZP proteins**

To generate MAbs reactive with human zona proteins, baculovirus-expressed rhZP2, rhZP3 and rhZP4 were purified as described previously (Chakravarty et al., 2005). After due approval from the Institutional Animal Ethical Committee, male BALB/c mice (8–10-week-old, Small Experimental Animal Facility, National Institute of Immunology, New Delhi, India) were immunized i.c. with the respective purified recombinant protein (50 μg/animal) emulsified with complete Freund’s adjuvant (Difco Laboratories, Detroit, MI, USA). Animals received two booster injections i.p. at 4 week intervals of the same amount of the respective recombinant protein emulsified in incomplete Freund’s adjuvant (Difco Laboratories). Splenocytes from the animals showing highest antibody titres against the respective protein as determined by enzyme-linked immunosorbent assay (ELISA) were used to generate hybrid cell clones secreting MAb essentially as described previously (Govind et al., 2000). The specificity of MAbs for a given human zona protein was verified both in ELISA and Western blot employing all the three rhZPs (Bukovsky et al., in press). MAbs were purified by a HiTrap Protein-G Sepharose column (Pharmacia) attached to an AKTA system (Pharmacia). The bound MAbs were eluted with 0.1 M glycine–HCl pH 2.7. The concentrations of the purified MAbs were determined by a commercial protein assay kit (Bio-Rad, Hercules, CA, USA). The MAB MA-1615 generated against rhZP2 failed to react with rhZP3 and rhZP4 both in ELISA and Western blot (Bukovsky et al., in press) and was used to purify hZP2 from native source as described below. Similarly, MAB MA-1558, generated against rhZP3 and devoid of reactivity with hZP2 and hZP4, was used to purify hZP3 from...
native source (Bukovsky et al., in press). MAb MA-1660, reactive with rhZP4, was also used for characterization of purified glycoproteins in Western blot and other experiments.

**Immunoreactivity of MAbs with human oocyte**

Unfertilized human oocytes from our assisted reproduction program were incubated with MAbs against rhZP2 (MA-1615), rhZP3 (MA-1558) or rhZP4 proteins (MA-1660) at a concentration of 1 µg/ml in 60 µl of phosphate-buffered saline (PBS) containing 1% BSA for 1 h at 25°C. After extensive washing with PBS, the bound antibodies were detected by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (IgG) (Sigma, St Louis, USA) and were observed under a fluorescence microscope. Oocytes incubated with the secondary antibody alone or with unrelated mouse anti-goat IgG (Sigma) served as the negative controls.

**Effect of MAbs against rhZP proteins on the binding of human spermatozoa to ZP in a hemizona assay**

The hemizona binding assay was performed as described (Yao et al., 1996). Unfertilized oocytes from our assisted reproduction program were micro-bisected into two identical hemizonae. Each hemizona was pre-incubated with the anti-rhZP antibodies (0.1, 1 or 5 µg/ml) at 25°C for 1 h. The matching hemizona pre-incubated with PBS alone or unrelated mouse anti-goat IgG was used as the control. Subsequently, hemizona from the experimental and the control groups were incubated with 2×10⁵ spermatozoa/ml in a 20 µl droplet of EBSS/BSA under mineral oil for 3 h at 37°C in an atmosphere of 5% CO₂ in air. After incubation, the loosely bound spermatozoa were removed by several washings with EBSS/BSA and the numbers of tightly bound spermatozoa on the outer surface of the hemizonae were counted. The results are expressed as hemizona index (HZI) which is the ratio of the number of bound spermatozoa in the test droplet to that in the control droplet ×100.

**Purification of ZP glycoproteins from human oocytes**

Three thousand two hundred and eighty-four unfertilized human oocytes from the assisted reproduction program at Queen Mary Hospital, Hong Kong, were collected. The research protocol was approved by the Ethics Committee of the University of Hong Kong. Prior exposure to spermatozoon did not affect the sperm-binding ability of hZP (Liu et al., 1988; Franken et al., 1991; Gracyzkowski et al., 1998). hZP from unfertilized oocytes with or without spermatozoa penetration also had similar acrosome reaction inducing ability (Liu and Baker, 1996). The ZP were bisected and separated from the human oocytes using glass micropipette under a dissection microscope. The ZP were stored in a buffer containing 25 mM triethanolamine, 150 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 1% polyvinylpyrrolidone, pH 7.8, at −70°C before use. On the day of experimentation, they were thawed and heat-activated at 70°C for 30 min in 5 mM NaH₂PO₄ buffer pH 2.5 as described (Jungnickel et al., 2001). The preparation was centrifuged at 15 000g for 10 min at 4°C to remove debris, and the supernatant was collected.

Solubilized hZPs were incubated in 5 M guanidine hydrochloride and 1.5 M Tris (2-carboxyethyl) phosphate (TCEP) at pH 4.5 for 2.5 h. The mixture was desalted and diluted with a HiTrap™ Desalting Column (GE Healthcare, Piscataway, USA) at 4°C using 20 mM Tris—HCl containing 1% Nonidet P-40, 10 mM EDTA, 0.1 M NaCl, pH 6.0. Anti-rhZP2 and anti-rhZP3 MAbs were covalently coupled to Sepharose 4B according to the manufacturer’s instructions (Pharmacia). The resulting affinity columns were used to purify hZP2 and hZP3. The bound ZP glycoproteins were eluted with 0.1 M glycine, pH 2.6, neutralized, and renatured by dialysis overnight as described (McCoy and Wong, 1981) using micro-dialyzer (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA). The concentrations of the purified ZP glycoproteins were determined by the Bio-Rad Protein Assay kit (Bio-Rad). Their purities and identities were determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and mass spectrometry analysis. The identity of the purified hZP3 was further confirmed by Western blotting using a polyclonal antibody (1 µg/ml) against bovine ZP3 protein (Sumitro and Aulanniam, 2002), which cross-reacted with hZP3. The flow-through fraction after successive immunoaffinity chromatographies was collected. Adsorbent (Calbiochem, San Diego, CA, USA) was used to remove the detergent in the fraction, which was then subjected to dialysis. The fraction was designated as ZP1/ZP4 as it contained both hZP1 and hZP4.

**Mass spectrometry analysis of the purified hZP glycoproteins from native source**

The purified ZP glycoproteins were resolved in SDS–PAGE and visualized by silver staining. The protein bands corresponding to the ZPs were excised and digested in situ with trypsin (0.1 mg/ml in 25 mM NH₄HCO₃, pH 8.0). The digested peptides were recovered with MilliPore C18 ZipTips and dissolved in 60% acetonitrile–0.1% trifluoroacetic acid containing 0.2% 4-hydroxycinnamic acid matrix. The masses of the peptides were analyzed by a matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS/MS). The proteins were identified by matching to the public protein databases at the SwissProt (http://www.ebi.ac.uk/swissprot/) as described (Lee et al., 2004).

**Binding of purified ZP glycoproteins to spermatozoa**

Acrosome-reacted spermatozoa were prepared by incubating capacitated spermatozoa in EBSS/BSA containing 2.5 µM of ionophore A23187 for 30 min. The viability of spermatozoa as determined by Hoechst staining was similar before (82.0 ± 4.6%) and after (76.8 ± 4.6%) ionophore treatment. They were then washed, mildly fixed in 0.5% paraformaldehyde for 10 min at room temperature (Harrison and Vickers, 1990), smeared on slides and air-dried followed by the incubation with 1 pmol/20 µl native hZP glycoproteins at 4°C. After 24 h, the spermatozoa were washed, and further incubated successively with anti-rhZP glycoprotein antibodies (MA-1615, MA-1558 or MA-1660) and Cy3-conjugated goat anti-mouse IgG (Sigma). The slides were then washed, immersed for 1 min in ice-cold methanol for cell permeabilization and incubated with FITC labeled peanut (Pisum sativum) agglutinin (FITC-PSA; Sigma) for 9 min. The acrosomal binding of FITC-PSA and hZP glycoprotein immunoreactivity was examined under a fluorescence microscope. The experiment was repeated without ionophore treatment on mildly fixed capacitated spermatozoa. The binding patterns of the hZP glycoproteins were then determined.

**Deglycosylation of ZP glycoproteins**

N-linked glycans of ZP glycoproteins were removed selectively by incubation with 0.2 mU N-glycosidase-F at 37°C for 24 h according to the protocol of the Glycoprotein Deglycosylation Kit (Calbiochem). The enzyme was inactivated by boiling for 5 min. Removal of O-linked glycans was conducted by alkali hydrolysis (β-elimination) as described (Florman and Wassarman, 1985). In brief, ZP glycoproteins were incubated with 5 mM NaOH at 37°C for 24 h, the reaction was stopped by neutralization with acetic acid. Total deglycosylation was performed by the GlycoProfile IV Chemical Deglycosylation Kit (Sigma) using trifluoromethane sulfonic acid (TFMS, Florman and Wassarman, 1985; Dunbar et al., 2001).
according to the manufacturer’s protocol. Though chemical deglycosylation might cause partial protein destruction leading to loss of biological activity of the treated molecules (Sojar and Bahl, 1987), the method had been used successfully in studying the role of glycans on the biological activities of mouse (Florman and Wassarman, 1985) and porcine ZP glycoproteins (Paterson et al., 1992; Dunbar et al., 2001). The purity of the deglycosylated proteins was checked by SDS–PAGE after dialysis.

**Effect of native/deglycosylated ZP glycoproteins on hyperactivation of capacitated human spermatozoa**

The kinetics of action of hZP on sperm motility was determined by incubating spermatozoa with purified hZP glycoproteins at a concentration of 1 pmol/20 µl for 5, 15 and 120 min. As the amount of native hZPs used was insignificant when compared with the amount of BSA present in the culture medium, spermatozoa incubated in EBSS/BSA without native hZPs under similar conditions was used as control. Hobson Sperm Tracker System (Hobson Tracking Systems Ltd., Sheffield, UK) was used to study sperm motility parameters. The set-up parameters of the system and the procedures have been described elsewhere (Mortimer et al., 1998; Chiu et al., 2002). The Mortimer (1998) criteria for hyperactivation were used as follows: curvilinear velocity \( \geq 150 \text{ mm/s} \) and linearity \( \leq 50\% \) and amplitude of lateral head displacement \( \geq 7.0 \text{ µm} \).

**Statistical analysis**

All the data were expressed as mean and SEM. The data were analyzed by SigmaPlot 8.02 and SigmaStat 2.03 software (Jandel Scientific, San Rafael, USA). For all experiments, the non-parametric analysis of variance on rank test for multiple comparisons followed by Mann–Whitney \( U \)-test was used. A probability value <0.05 was considered to be statistically significant.

**Results**

**MAbs against rhZP proteins react with hZP and inhibit spermatozoa-ZP binding**

The three anti-rhZP MAbs tested bound specifically to intact ZP of human oocytes (Fig. 1). The oocytes incubated with the secondary antibody alone (data not shown) or unrelated mouse anti-goat IgG gave no fluorescent signals (Fig. 1). The MAab against rhZP3 (MA-1558) and rhZP4 (MA-1660) when tested at concentrations of 0.1, 1 and 10 µg/ml, significantly decreased the number of spermatozoa bound to the hemizona when compared with the control (\( P < 0.05 \)), resulting in a dose-dependent decrease in HZI (Fig. 2). The spermatozoa-ZP inhibitory activity of the MAab against rhZP2 was much weaker. Among the antibodies used, anti-rhZP3 antibody had the greatest inhibitory activity on sperm binding. At a concentration of 10 µg/ml, rhZP3 showed inhibition to the extent of 84.7 \( \pm 4.4\% \) of the sperm binding, whereas that of anti-rhZP2 and -rhZP4 antibodies inhibited 45.0 \( \pm 5.1\% \) and 70.7 \( \pm 2.7\% \) of the binding, respectively.

**Purification of hZP glycoproteins**

The affinity purified hZP2 and hZP3 showed a major band of \( \sim 120 \) (Fig. 3A, lane 3) and \( \sim 58 \) kDa (Fig. 3B, lane 3), respectively, in SDS–PAGE. The identities of these bands were confirmed to be that of hZP2 (NCBI Accession No: gi4508045) and hZP3 (NCBI Accession No: gi297791) by MALDI-TOF-MS/MS with a sequence coverage of 12% (MASCOT protein score: 86; nine peptide fragments) and 19% (MASCOT protein score: 211; four peptide fragments) as retrieved from ProteinProspector MS-Fit search.
respectively. Western blotting analysis using anti-bovine ZP3 antibody that cross-reacted with hZP3 revealed a major band of ~58 kDa in the purified hZP3 fraction and solubilized hZP, further supporting the identity of the purified hZP3 (data not shown). The purity of the isolated hZP2 and hZP3 were >88% and >92%, respectively, as determined by the relative proportion of the major band in SDS–PAGE. Their yields ranged from 13 to 17 ng/ZP (~0.13 pmol/ZP) for hZP2 and 4 to 10 ng/ZP (~0.11 pmol/ZP) for hZP3 without accounting for the loss during purification. The total protein recovered in the ZP1/ZP4 fraction ranged from 3 to 8 ng/ZP (~0.075 pmol/ZP). More than 93% of the protein in this fraction was hZP4 (NCBI Accession No: gi10863987) with identity confirmed by SDS–PAGE followed by MALDI-TOF-MS/MS (Sequence coverage: 10%; MASCOT protein score: 88; six peptide fragments) and Western blot analysis (Fig. 4, lane 3). The amount of hZP1 in the ZP1/ZP4 fraction was too low for characterization. Comparing the protein concentration in solubilized ZP with the total amount of purified hZP glycoproteins (ZP1+ZP2+ZP1/4) obtained, it was estimated that ~18% of the solubilized hZP glycoproteins were lost during purification.

Following N-glycosidase-F treatment, hZP2, hZP3 and hZP4 migrated as broad bands on SDS–PAGE, with their apparent molecular weights decreased by ~45 (Fig. 3A, lane 4), ~16 (Fig. 3B, lane 4) and ~12 kDa (Fig. 4, lane 4), respectively. Alkaline reduction removed O-linked oligosaccharides, and reduced the molecular mass of hZP2 by ~10 kDa (Fig. 3A, lane 5) and of ZP3 by ~5 kDa (Fig. 3B, lane 5) but had no apparent effect on that of hZP4 (Fig. 4, lane 5). Removal of both N- and O-linked oligosaccharides by TFMS treatment reduced the molecular size of the ZP glycoproteins to that of their polypeptide chain (hZP2: Fig. 3A, lane 6, ~65 kDa; hZP3: Fig. 3B, lane 6, ~40 kDa; hZP4: Fig. 4, lane 6, ~57 kDa).

**Binding of native hZP glycoproteins on spermatozoa**

Mildly fixed spermatozoa were used in this experiment to avoid the loss of hZP glycoprotein bound on the plasma membrane over the acrosomal region should ZP-induced acrosome reaction occur during treatment. The mildly fixed cell model had been used successfully to characterize mouse (Thaler and Cardullo, 1996) and porcine (Burkin and Miller, 2000) spermatozoa-ZP interaction. The hZP binding pattern needed longer (24 h) to develop in such conditions probably because of the reduced accessibility of the hZP binding sites after mild fixation and the reduced incubation temperature (4°C). Native hZP2 bound to 32.8 ± 1.6% of the capacitated spermatozoa. Strong hZP2 staining was demonstrated over the equatorial region, post-acrosomal region and midpiece of acrosome-intact spermatozoa (Fig. 5A). Ionophore treatment increased the percentage of acrosome-reacted spermatozoa...
from 7.0 ± 0.7% to 52.1 ± 3.2%. In acrosome-reacted spermatozoa, hZP2 bound to the acrosome region in addition to its binding over the post-acrosomal region, equatorial region and midpiece (Fig. 5A).

Native hZP3 bound to 51.2 ± 2.4% of the capacitated spermatozoa. The binding sites were on the acrosomal region, equatorial region and midpiece of acrosome-intact spermatozoa. After acrosome reaction, hZP3 binding sites were detected only in the midpiece (Fig. 5B). The entire head of acrosome-intact spermatozoa possessed hZP4 binding sites that disappeared after acrosome reaction (Fig. 5C). The percentage of capacitated spermatozoa with bound hZP4 was 49.5 ± 1.8%.

**Effect of hZP glycoproteins on hyperactivation**

Incubation of spermatozoa for 15 min in the presence of purified native hZP3 significantly increased the numbers of hyperactivated spermatozoa ($P < 0.05$), whereas similar treatment with either hZP2 or hZP1/4 produced no significant effect ($P > 0.05$) as compared with the respective controls (Fig. 6). Prolonging treatment of capacitated spermatozoa with hZP3 to 3 h did not further enhance hyperactivation of spermatozoa (data not shown). N-linked and complete deglycosylation, but not O-linked deglycosylation, diminished the hyperactivation-stimulatory effect of hZP3 (Fig. 6). On the contrary, N-linked and complete deglycosylation of hZP1/ZP4 increased the hyperactivation of the treated spermatozoa. hZP2 and its deglycosylation forms did not affect hyperactivation.

**Discussion**

Our knowledge on the functions of hZP glycoproteins is restricted due to their limited availability. To circumvent the limitation, several groups studied the biological activities of rhZP (Table I). Although the amino acid sequences of ZP glycoproteins are highly conserved in vertebrates, post-translational glycosylation is species-specific, and is crucial to spermatozoa-ZP interaction (Dell et al., 1999; Topfer-Petersen, 1999; Wassarman et al., 2001). The binding of mouse but not human spermatozoa to oocytes from mZP2- and mZP3-deficient transgenic mice expressing hZP2 and hZP3 (Rankin et al., 1998, 2003) is consistent with species-specific recognition of ZP mediated by the mouse-specific glycosylation irrespective of the protein core. Unfortunately, our current technology cannot generate recombinant proteins with proper glycosylation. Although rhZP3 expressed in mammalian cells is glycosylated, their carbohydrate moieties differ from those in native hZP3. These recombinant glycoproteins have variable capability in inducing acrosome reaction of capacitated human spermatozoa (van Duin et al., 1994; Dong et al., 2001; Bray et al., 2002; Caballero-Campo et al., 2006). Furthermore, porcine ZP proteins expressed in SF9 cells bind to bovine but not to porcine spermatozoa (Yonezawa...
et al., 2005). Therefore, hZP glycoproteins with native glycosylation were purified and used in this study.

Two strategies were employed to obtain a good yield of hZP glycoproteins. The first strategy was to use specific immuno-affinity chromatography for isolating hZP glycoproteins, effectively reducing loss during purification. In the present study, the three MAbs used bound to intact hZP and inhibited spermatozoa-hZP binding, supporting previous reports on the interference of antibodies against ZP components on sperm binding (Hinsch et al., 2003; Gupta et al., 2004). The antibodies may prevent spermatozoa-hZP interaction by direct binding to the sperm receptor or indirectly by preventing access of spermatozoa to their receptors via steric hindrance (Henderson et al., 1988). The specific MAbs used enabled the isolation of hZP glycoproteins of high purities (88–93%) after a single affinity chromatographic step.

The second strategy was to include procedures for efficient dissociation of the macromolecular structure of hZP. This was accomplished by utilizing (i) guanidine hydrochloride to disrupt non-covalent interactions between hZP glycoproteins; (ii) TCEP to reduce the intra- and probably inter-disulfide bonds between hZP glycoproteins at low pH (Han and Han, 1994; English et al., 2002); (iii) low-pH conditions to inhibit intra- and inter-hZP glycoprotein molecular interactions (Rankin et al., 1999; Hoodbhoy and Dean, 2004; Litscher and Wassarman, 2007; Litscher et al., 2008) during removal of guanidine hydrochloride and TCEP (English et al., 2002). Gradual dialysis was finally used to renature the isolated hZP glycoproteins. In our experience, the purities of the hZP glycoproteins prepared by this protocol were higher than with the protocol using heat-solubilized hZP directly for immunoaffinity purification possibly because heat solubilization alone cannot separate hZP glycoproteins completely from their supramolecular complexes.

On the basis of the electrophoretic mobility in denaturing SDS–PAGE, the molecular masses of native hZP2, hZP3 and hZP4 are ~120, ~58 and ~65 kDa, respectively. The values for hZP2 and hZP3 are similar to those in other reports (Moos et al., 1995; Gupta et al., 1998; Bauskin et al., 1999). N-linked glycosylation occupies ~37%, ~27% and ~18% of the molecular mass of hZP2, hZP3 and hZP4, respectively. The percentages of O-linked glycosylation are ~8% for hZP2 and ~9% for hZP3, whereas hZP4 seems to have no O-linked glycosylation.

To the best of our knowledge, this is the first report on the purification of native hZP4. The identities of the purified hZP glycoproteins were confirmed by Western blotting and MALDI-TOF-MS/MS. The flow-through fraction after hZP2 and hZP3 affinity chromatographies contained hZP4 and hZP1. In order not to lose hZP4 with further purification, the fraction was not fractionated to remove the contaminating hZP1. Gel-electrophoresis and Western blotting showed that hZP4 constituted >93% of the protein in the fraction, consistent with other reports that ZP1 had low abundance in ZP (Epifano et al., 1995; Lefèvre et al., 2004). It is likely that the binding of the fraction seen in the present study came from hZP4, as ZP1 does not appear to be required for spermatozoa-ZP interaction (Rankin et al., 1999).

An essential event in fertilization is spermatozoa-ZP binding (Yanagimachi, 1994; Wassarman et al., 2001). Although such binding has been studied extensively in the murine and porcine models (Nakano et al., 1996; Burkin and Miller, 2000; Wassarman, 2005), similar study using native hZP glycoproteins is not available. The present report demonstrates some differences in the binding properties between native hZP glycoproteins and their recombinant counterparts as reported in the literature (Table I).

Native hZP3 bound to the acrosomal region of acrosome-intact spermatozoa. The hZP3 binding sites were lost after acrosome reaction, consistent with the role of hZP3 as a primary sperm receptor in human (Wassarman et al., 2001). The acrosome region of human spermatozoa also binds rhZP3 from Escherichia coli and baculovirus (Chakravarty et al., 2008). However, native hZP3 binds to the midpiece, whereas rhZP3 does not (Table I). The binding of native hZP2 to the acrosome region of acrosome-reacted spermatozoa was in line with previous studies using rhZP2 (Tsubamoto et al., 1999; Chakravarty et al., 2008), and supported the role of hZP2 as a secondary binding site in human. Differences in the binding profiles between native and rhZP2 were observed. Escherichia coli- and baculovirus-expressed rhZP2 binds primarily to the equatorial segment of the acrosome-reacted spermatozoa (Chakravarty et al., 2008), whereas native hZP2 binds to the acrosome, post-acrosome and midpiece.

A new insight obtained in the present study is that the binding of hZP2 to the post-acrosomal region of acrosome-intact and -reacted spermatozoa, suggesting that hZP2 may have other functions as well. Similar observation has been reported in mouse (Kerr et al., 2002). The binding of hZP2 to acrosome-intact spermatozoa may ensure that spermatozoa remain bound to the hZP when the plasma membrane overlying the acrosome with its hZP3 binding sites is disintegrating during acrosome reaction. Another possible role of hZP2 binding may be to trigger calcium signaling, facilitating hZP3-induced acrosome reaction. It has been shown that solubilized hZP-induced acrosome reaction is associated with [Ca2+]i increase that spreads from the equatorial segment to the post-acrosomal region (Shirakawa and Miyazaki, 1999).

rhZP2 and pig ZP2 bind to the midpiece of acrosome-reacted spermatozoa (Tsubamoto et al., 1996, 1999). In this study, native hZP2 and hZP3 bind to the midpiece of both acrosome-intact and -reacted spermatozoa. The midpiece contains mitochondria producing energy for sperm motion. The binding of hZP2 and hZP3 to this region may be related to the stimulatory action of hZP3 on hyperactivation that enable spermatozoa to penetrate the hZP (Suarez and Ho, 2003). The observation is consistent with the hyperactivation inducing activity of solubilized hZP (Bastiaan and Franken, 2007) and the detection of hyperactivation-associated intracellular signaling molecules in the midpiece (Suarez and Ho, 2003). On the other hand, rhZP2, but not rhZP3, from insect cells induces hyperactivation-like motion of the treated spermatozoa (Caballero-Campo et al., 2006). The discrepancy in the hyperactivation-inducing activity between native and rhZP2 and rhZP3 could be due to their differences in glycosylation. In line with the importance of glycosylation in ZP, the
stimulatory effect of native hZP3 on hyperactivation was abolished after deglycosylation. A recent study demonstrated that a mechanosensory signal produced during sperm penetration through the zona matrix is necessary to trigger acrosome reaction (Baibakov et al., 2007). Together with the observation that hyperactivation is significantly correlated with ZP-induced acrosome reaction (Liu et al., 2007), the hyperactivation stimulatory effect of native hZP3 may indirectly promote the hZP-penetration induced acrosome reaction.

There is only one study examining the binding of native hZP4 to spermatozoa (Chakravarty et al., 2008): using heat-solubilized hZP and polyclonal anti-ZP4 antibody, the binding site of native hZP4 was shown only in the acrosomal region which disappeared after acrosome reaction. In addition to the acrosomal region, purified native hZP4 also bound to the post-acrosomal region in the present study. The discrepancy between the two studies on hZP4 binding sites may be due to the presence of other hZP glycoproteins in the solubilized ZP. Recent study suggests that mouse spermatozoa recognize the supramolecular structure of ZP (Hoodbhoy and Dean, 2004) containing zona filaments made of interspersed heterodimers of ZP glycoproteins (Litscher and Wassarman, 2007). Native mZP2 and mZP3 polymerize in non-denaturing conditions (Litscher et al., 2008). Thus, it is possible that solubilized hZP may contain heteropolymers of hZP glycoproteins that function differently from purified hZP glycoproteins.

In conclusion, we have provided the first report on the binding characteristics of the purified native hZP glycoproteins to the capacitated and acrosome-reacted spermatozoa. Differences in the binding properties between the native hZP and their reported rhZP were noted. The ability of hZP3 to enhance hyperactivation could be important for sperm penetration through the hZP.

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