In humans, zona pellucida glycoprotein-1 binds to spermatozoa and induces acrosomal exocytosis

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BACKGROUND: It has been suggested that the zona pellucida (ZP) may mediate species-specific fertilization. In human the ZP is composed of four glycoproteins: ZP1, ZP2, ZP3 and ZP4. In the present study, the expression profile of ZP1 in human oocytes and ovaries, and its role during fertilization, is presented.

METHODS: Human ZP1 (amino acid residues 26–551) was cloned and expressed in both non-glycosylated and glycosylated forms and its ability to bind to the capacitated human spermatozoa and to induce acrosomal exocytosis was studied. Monoclonal antibodies (MAbs), specific for human ZP1 and devoid of reactivity with ZP2, ZP3 and ZP4 were generated and used to localize native ZP1 in oocytes and ovarian tissues.

RESULTS: The MAbs generated against ZP1 recognized specifically the zona matrix of secondary and antral follicles, ovulated oocytes, atretic follicles and degenerating intravascular oocytes, but failed to react with the Fallopian tube, endometrium, ectocervix and kidney. Escherichia coli and baculovirus-expressed recombinant human ZP1 revealed bands of ~75 and ~85 kDa, respectively, in western blot. Lectin binding studies revealed the presence of both N- and O-linked glycosylation in baculovirus-expressed ZP1. Fluorescein isothiocyanate-labelled E. coli- and baculovirus-expressed recombinant ZP1 bound to the anterior head of capacitated spermatozoa, however, only baculovirus-expressed ZP1 induced acrosomal exocytosis in capacitated sperm suggesting the importance of glycosylation in mediating the acrosome reaction. The human ZP1-mediated acrosome reaction involved the activation of both T- and L-type voltage-operated calcium channels, but does not activate the G-coupled receptor pathway. Inhibition of protein kinase A and C significantly also reduced the ZP1-mediated induction of the acrosome reaction.

CONCLUSION: These studies revealed for the first time that in humans ZP1, in addition to ZP3 and ZP4, binds to capacitated spermatozoa and induces acrosomal exocytosis.

Key words: zona pellucida / sperm binding / acrosome reaction / fertilization / monoclonal antibodies

Introduction

Fertilization is an intricate process during which two haploid gametes, the oocyte and the spermatozoon unite to produce a genetically distinct individual. To accomplish this, initial binding of the spermatozoon to the oocyte takes place at the zona pellucida (ZP) matrix, an extra-cellular glycoprotein coat that surrounds all mammalian oocytes. The ZP matrix acts as an agonist for regulated exocytosis of the spermatozoon’s acrosomal vesicle and plays a crucial role in avoidance of polyspermy (Wassarman and Litscher, 2008). It also plays an important role in the protection of the preimplantation blastocyst. The ZP matrix of mouse is composed of three glycoproteins of the ZP family, ZP1, ZP2 and ZP3 (Bleil and Wassarman, 1980a). However, in humans, the ZP matrix is formed by four glycoproteins designated as ZP1, ZP2, ZP3 and ZP4 (Hughes and Barratt, 1999; Lefevre et al., 2004; Ganguly et al., 2008). The orthologue of the human Zp4 gene is present in the mouse genome as a pseudo gene.

The involvement and precise function of various glycoproteins that constitute the ZP matrix during fertilization is a topic of great interest. Although the number of ZP genes present in a given species is of...
interest in terms of evolution, the key question is whether this has functional relevance. Using the mouse model, the structural and functional significance of individual ZP glycoproteins has been extensively investigated (Bleil and Wassarman, 1980b; Greve and Wassarman, 1985; Bleil et al., 1988; Beebe et al., 1992). These investigations revealed that ZP3 binds to capacitated acrosome-intact spermatozoa and induces acrosomal exocytosis (Chakravarty et al., 2005; Caballero-Campo et al., 2006; Chakravarty et al., 2008; Chiu et al., 2008a; 2008b). However, in the report demonstrating the functional role of native human ZP4 (Chiu et al., 2008a), it should be noted that the ZP4 fragment purified from solubilized human ZP had ZP1 as a contaminant. Hence, the functional activity shown by the fragment may be due to the combined effect of both ZP1 and ZP4. Human ZP2 primarily binds to acrosome-reacted spermatozoa and fails to induce acrosomal exocytosis in capacitated human spermatozoa (Chakravarty et al., 2008; Chiu et al., 2008a; 2008b). Since the documentation of the existence of the true orthologue of mouse ZP1 in human oocytes (Hughes and Barratt, 1999; Lefevre et al., 2004), there has been little information on its expression profile in ovarian follicles and its role during fertilization in humans. Thus in the present manuscript, by employing ZP1-specific monoclonal anti-bodies (MABs), the expression of ZP1 in human ovaries has been investigated. Human ZP1 has been expressed for the first time in non-glycosylated as well as glycosylated forms and its role in binding to capacitated human spermatozoa and induction of acrosomal exocytosis during fertilization in humans has been examined.

Materials and Methods

Expression of human ZP1 in the Escherichia coli and baculovirus expression systems and its purification

For the expression of human ZP1 in the Escherichia coli and baculovirus expression systems, a synthetic gene (GenBank Accession number NM_207341) encoding human ZP1 glycoprotein till the furin cleavage site, i.e. 1–551 amino acid (aa) was obtained from GenScript Corporation (Piscataway, NJ, USA) in pUC57 vector. For its expression in E. coli, an internal cDNA fragment devoid of signal sequence and till the consensus furin cleavage site (aa residues 26–551), was PCR amplified from the pUC57 vector using forward 5′-CCCTGAGCGCCGCGTCGACGAAGGGACGCTGAC-3′ and reverse 5′-CGGAATTCTTAGGGTGCCGGTGCCTGAC-3′ primers with Xhol–EcoRI restriction sites, respectively, as described (Chakravarty et al., 2005). The PCR amplified fragment was cloned in pGEMT-Easy vector (Promega, Madison, WI, USA) following the manufacturer’s instructions.

The insert from one of the positive clones was excised by appropriate restriction and ligated downstream of the T7 promoter in prSET-A vector (Invitrogen Corporation, Carlsbad, CA, USA) in frame with a polyhistidine (His₆) tag at the N terminus for expression in E. coli. This construct was used to transform BL21(DE3)pLysS strain of E. coli, deficient in ompT and lon proteases, and one of the positive clones was used for the expression of ZP1 at the shake flask level in the presence of 1 mM isopropyl-β-d-thiogalactopyranoside for 2.5 h. The recombinant protein was purified by Ni-NTA affinity chromatography as described previously (Kaul et al., 1997; Chakravarty et al., 2005). The purified protein was extensively dialyzed against renaturation buffer [50 mM Tris–HCl pH 8.5, 1 mM ethylenediaminetetraacetic acid (Amresco, Solon, OH, USA), 0.1 mM reduced glutathione (Amresco), 0.01 mM oxidized glutathione (Amresco) and 10% sucrose (Sigma-Aldrich, Inc., St Louis, MO, USA)] for 96 h with six changes of the dialysis buffer with decreasing concentrations of urea (4, 3, 2, 1, 0.5 M and finally buffer without urea) to assist in removal of urea and refolding of the protein. The refolded protein was further dialyzed against 20 mM Tris (Sigma-Aldrich, Inc.) pH 7.4.

In addition, the cDNA encoding human ZP1 (26–551 aa) was also cloned into the baculovirus transfer vector pAcGP67-A (PharMingen, San Diego, CA, USA), under the control of late polyhedrin promoter and gp67, an insect secretory sequence. The forward and reverse primers, 5′-CGG AATTCGGCCCGGTGGCGTGCAAGCTTC3′ and 5′-GAAAGATCTTTAGT GGTGTGGGTGCGTGGTGGGGCTGCGGCTGCA3′ having the restriction sites EcoRI and BglII, respectively, were used. The sequence for a 6 x 48 bp tag was inserted in the reverse primer as the vector lacked a polyhistidine tag, PCR amplification, subsequent cloning of the PCR amplified product into the baculovirus transfer vector and generation of the recombinant baculovirus expressing the above recombinant protein was performed as described previously (Gahlay and Gupta, 2003). For large-scale purification of the recombinant protein, 50 × 10⁶ Spodoptera frugiperda (Sf21) insect cells growing in a suspension culture in Spinner bottles (Thermolyne; Barnstead International, Dubuque, IA, USA) were incubated with the recombinant virus at a multiplicity of infection of 3 at 42 rotations per minute (rpm) for 96 h, after which the cells were pelleted at 1000g for 15 min and recombinant proteins were purified using Ni-NTA resin (Gahlay and Gupta, 2003) and subsequently renatured as described above. Protein concentration was determined employing a bicinchoninic acid assay (Pierce, Rockford, IL, USA) using bovine serum albumin (BSA) as the standard. As the recombinant protein was secreted in very minute quantities in the supernatant, the functional assays were performed using the protein obtained from the cell pellet.

To characterize the recombinant ZP1 expressed either in E. coli or baculovirus expression systems, SDS–PAGE and western blot were performed using the respective cell pellets or purified proteins as described previously (Chakravarty et al., 2005). To detect the proteins, 1:100 dilutions of mouse polyclonal antibodies against ZP1 synthetic peptide (P3; Ganguly et al., 2008) or MABs generated as described below were used. The antibody bound to the respective recombinant protein was revealed by using horse-radish peroxidase-conjugated goat anti-mouse immunoglobulin (1:2000; Pierce). The glycosylation profile of the baculovirus-expressed human ZP1 was determined by a lectin-binding ELISA essentially as described previously (Bansal et al., 2009). The specificity of the lectin binding to the recombinant proteins was further confirmed by lectin blots (Chakravarty et al., 2005). E. coli-expressed recombinant human ZP1 was used as an internal control in the above lectin binding assays.
Generation of MAbS against synthetic peptide corresponding to human ZPI and their characterization

To obtain a handle for the detection of human ZPI, MAbs were generated against a 40 aa long synthetic peptide (P40; WDVKNKRDYGTHLS-QEQQVQASHLPCIVRTSKEACQQA), corresponding to aa residues 219–258 of human ZPI, obtained commercially from Peptron, Daejeon, South Korea. The purity of the synthetic peptide was checked by reverse phase high pressure liquid chromatography and mass spectrometric analysis. P40 was conjugated to diphtheria toxoid (DT; AXELL, Accurate Chemical and Scientific Corporation, Westbury, NY, USA) by using 0.1% glutaraldehyde (Grade II: Sigma-Aldrich, Inc.) as described previously (Ganguly et al., 2008). Murine MAbs were generated against P40-DT conjugate according to the standard protocol as described previously (Govind et al., 2000) after due approval from the Institutional Animal Ethical Committee.

The culture supernatant of the hybrid cell clones reactive with P40 were also evaluated for their reactivity in ELISA with recombinant human ZPI (generated as described in this manuscript) and previously generated recombinant human ZP2, ZP3 and ZP4 (Chakravarty et al., 2005). In addition, ascites fluid obtained by growing hybrid cell clones in Pristane (2,6,10,14-tetramethyl pentadecane, Sigma-Aldrich, Inc.) primed BALB/c mice was also tested at a 1:1000 dilution. The isotypes of the MAbs were determined by employing mouse MAb isotyping reagents (Sigma-Aldrich, Inc.) as described earlier (Govind et al., 2000). The specificity of the MAbs was further confirmed by immunoblots. E. coli-expressed recombinant human ZPI, ZP2, ZP3 and ZP4 proteins (2 μg/lane) were resolved on a 0.1% SDS–10% PAGE as described earlier (Chakravarty et al., 2005) and processed for western blotting using neat MAb culture supernatants (Bukovsky et al., 2008).

Localization of human ZPI in human oocytes and ovarian follicles

All the experiments using human oocytes, ovaries and other tissues were carried out after the due approval of and following the Institutional Human Ethical Committee Guidelines. Oocytes that had failed to fertilize in standard in vitro fertilization treatment due to severe male infertility factor were obtained from the Army Hospital, Research and Referral, New Delhi, India (Chakravarty et al., 2008). These oocytes were thawed, washed in phosphate buffered saline (PBS; 50 mM phosphate and 150 mM NaCl, pH 7.4) and incubated with neat culture supernatant of MAbs generated against human ZPI peptide or Sp2/O myeloma cells at room temperature (RT) for 1 h. Oocytes were washed thrice with PBS followed by incubation for 1 h with 1:800 dilution of goat anti-mouse immunoglobulins conjugated to fluorescein isothiocyanate (FITC; Pierce). Oocytes were washed four times with PBS and mounted in glycerol: PBS (9:1) and examined under a Nikon microscope (Nikon, Chiyoda-Ku, Tokyo, Japan). To check the specificity of the reactivity profile, MAbs (culture supernatant) were incubated with the P40 peptide (10 μg/ml) for 2 h at 37°C prior to the addition to the oocytes and then subsequently processed as described above.

In this study, samples from ovaries, oviduct and uterus (endometrium and ectocervix) which were hysterectomy specimens from six females, ages 26–38, and one kidney (autopsy specimen, age 40) were included. The study was approved by the Institutional Review Board, and a signed written consent was obtained. Tissues were frozen in optimal cutting temperature compound (Miles, Inc., Elkhart, IN, USA) and stored at −80°C until use. Cryosections (7 μm thickness) were fixed in acetone, treated with an optimized concentration of MAbs to ZPI (MA-1756 and MA-1757), processed for immunohistochemistry and the images were captured and compiled as described earlier (Bukovsky et al., 1995, 2008). For comparison, we also utilized MAbs to ZP2 (MA-1620), ZP3 (MA-1556) and ZP4 (MA-1660) reported previously (Bukovsky et al., 2008). CD3 MAb to T cells (Dako Corporation, Carpinteria, CA, USA) was utilized as a control.

Evaluation of binding of recombinant human ZPI to capacitated human spermatozoa

All experiments using human spermatozoa were carried out under informed consent and following the clearance from the Institutional Bio-safety and Ethical Committee. Both E. coli- and baculovirus-expressed recombinant human ZPI were conjugated with FITC and fluorescein/ recombinant protein molar ratio (F/P) determined as described previously (Chakravarty et al., 2008). Semen samples were collected from healthy donors, liquefied and motile sperm were separated and set up for capacitation as described previously (Chakravarty et al., 2005). To prepare acrosome-reacted sperm, capacitated sperm were incubated with calcium ionophore A23187 (Cal; 10 mM, Sigma-Aldrich, Inc.) for 20 min at 37°C and 5% CO2 in humidified air. Non-capacitated, capacitated and acrosome-reacted spermatozoa (5 × 106), pre-fixed for 10 min at RT with 0.5% paraformaldehyde, were incubated with varying concentrations (from 20 to 50 μg/ml) of FITC-labelled E. coli- or baculovirus-expressed recombinant ZPI alone or in the presence of a 5-fold molar excess of cold E. coli- or baculovirus-expressed human ZPI in a reaction volume of 50 μl at 37°C and 5% CO2 in humidified air for 60 min. Post-incubation, sperm were processed for simultaneous assessment of the status of acrosome by double labelling with 5 μg/ml tetramethylrhodamine isothiocyanate conjugated Pisum sativum agglutinin (TRITC–PSA; Vector Laboratories, Inc., Burlingame, CA, USA) and for binding of FITC-labelled human ZPI as described earlier (Chakravarty et al., 2008).

Induction of acrosome reaction by recombinant human ZPI in capacitated human spermatozoa

Capacitated sperm [1 × 106 in Biggers–Whitten–Whittingham (BWW)] (4% BSA) were incubated at 37°C with 5% CO2 in humidified air for varying times and with varying concentrations of recombinant human ZPI in a total reaction volume of 100 μl and processed as described earlier (Chakravarty et al., 2005). In addition, baculovirus-expressed recombinant ZPI at an optimized concentration of 1 μg/ml was pre-incubated for 30 min at RT with baculovirus-expressed ZP3 and/or ZP4 generated as described previously (Chakravarty et al., 2005) and the reaction mix evaluated for its ability to induce acrosome reaction. The effect of treatment with various recombinant proteins on sperm with respect to viability was assessed by eosin–nigrosin staining (Björndahl et al., 2003) and total motility as per the WHO guidelines (WHO, 1992). In order to account for the spontaneous induction of acrosome reaction, sperm were also incubated with BWW and 0.3% BSA alone. The sperm were stained with 5 μg/ml TRITC–PSA for 30 min at RT. Any spermatozoa that demonstrated complete loss of PSA staining in the acrosome or revealed staining at the equatorial region was classified as acrosome reacted. Sperm showing TRITC fluorescence in the acrosomal region of the head were classified as acrosome intact. All slides were read ‘blind’ with coded samples. Two hundred sperm were scored for every spot and the percentage of acrosome reaction was calculated by dividing the number of acrosome-reacted sperm by the total number of sperm counted and multiplying by 100.
Delineation of downstream signalling events associated with human ZP1-mediated induction of acrosomal exocytosis

To elucidate the downstream signalling mechanism of the acrosome reaction induced by functionally active recombinant human ZP1, capacitated spermatozoa were pre-treated with different pharmacological inhibitors at varying concentrations as reported previously (Chiu et al., 2008b; Bansal et al., 2009). These include: inhibitor of G protein: pertussis toxin (PTX); T-Type Ca²⁺ channel blocker (CCB); pimozide and amiloride; L-Type CCB: verapamil and nifedipine; GABA-A receptor antagonist: picrotoxin; extracellular calcium chelator: EGTA; inhibitor of protein kinase A (PKA): H89 and inhibitor of protein kinase C (PKC): chelerythrine for 10 min (except PTX, kept for 30 min incubation) at 37°C in humidified air prior to the addition of ZP1. All the above inhibitors were procured from Sigma-Aldrich, Inc. The values have been represented as percentage stimulation of acrosome reaction by normalizing to a maximum induction by Cal (10 μM) and a minimum induction by negative/vehicle controls for each individual experiment, employing the equation:

\[
\% \text{ stimulation of acrosome reaction} = \frac{\text{AR} - \text{negative control}}{\text{Cal} - \text{negative control}} \times 100
\]

where AR, percent induction of acrosome reaction by various inducers, negative control is the parallel vehicle control or spontaneous acrosome reaction and Cal is the percent acrosome reaction by incubation with Cal.

**Statistical analysis**

For experiments pertaining to binding and induction of acrosome reaction, the results are expressed as mean ± SEM of 3–4 different experiments using 2–3 male donors. The statistical analysis was done by comparing the means of the medium control (BWW and 0.3% BSA) and experimental sets or within two experimental groups by using one-way analysis of variance followed by Newman–Keuls multiple comparison test. The statistical analysis with respect to the effect of various pharmacological inhibitors on ZP1-mediated induction of acrosome reaction was performed by Student’s t-test. A P-value of < 0.05 was considered to be statistically significant.

**Results**

**Characteristics of E. coli- and baculovirus-expressed recombinant human ZP1**

Employing appropriate expression vectors, recombinant human ZPI was cloned and expressed in the E. coli as well as baculovirus expression systems. The E. coli-expressed recombinant human ZP1 was observed in the inclusion bodies. In the baculovirus expression system, immunoblot studies revealed the expression of ZP1 in both cell lysate and culture supernatant (Fig. 1A and B). However, recombinant ZP1 could be detected only in 10x concentrated culture supernatant. The expressed protein was purified from the respective cell pellets employing Ni-NTA affinity chromatography as described elsewhere (Kaul et al., 1997; Gahlay and Gupta, 2003) and refolded. SDS–PAGE and western blot analysis of the purified E. coli (Fig. 1F and G) and baculovirus (Fig. 1C–E) expressed recombinant human ZPI revealed a single band at ~75 and ~85 kDa, respectively. Analysis of the baculovirus-expressed recombinant ZP1 by lectin binding ELISA showed a high reactivity with Jacalin followed by concanavalin A, wheat germ agglutinin, Lens culinaris agglutinin and Ricinus communis agglutinin and weak reactivity with Erythrina cristagalli lectin and Vicia villosa agglutinin (Fig. 2a). E. coli-expressed recombinant ZP1 served as a negative control in these experiments as E. coli-expressed proteins being non-glycosylated do not show significant binding to any of the lectins under similar experimental conditions. The lectin blots, pertaining to the selected lectins exhibiting reactivity in ELISA with the recombinant proteins, reconfirmed the specificity of the above lectins to bind to recombinant baculovirus-expressed ZP1. Representative lectin blots of the E. coli- as well as baculovirus-expressed recombinant ZPI with ConA and Jacalin are shown in Fig. 2b and c. ConA recognizes N-linked (mannose α 1–3/1–6 residues) and Jacalin O-linked (α-O glycosides of Gal or GalNAc moieties) glycosylation.

**Human ZPI-specific MAbs and expression profile of ZPI in human oocytes and ovarian follicles**

In order to generate MAbs against human ZP1, BALB/cj mice, immunized with the P40 synthetic peptide corresponding to aa residues...
219–258 of human ZP1 conjugated with DT, were used to produce hybrid cell clones secreting MAbs as described in Materials and Methods. A panel of three MAbs was obtained against P40 peptide [MA-1756, MA-1757 and MA-1758; Table I]. All the MAbs showed strong reactivity with recombinant ZPI generated as described above and no cross-reactivity with E. coli- and baculovirus-expressed recombinant human ZP2, ZP3 and ZP4 (Chakravarty et al., 2005) in ELISA. The isotype analysis revealed MA-1756 to be of IgG1, and MA-1757 and MA-1758 to be of IgG2b isotypes (Table I). The reactivity of the MAbs was further confirmed in western blot showing specificity for ZP1. Representative immunoblot pertaining to MA-1756 is shown in Fig. 3a.

Immunofluorescence studies with human oocytes revealed distinct staining of the zona matrix by MA-1756 and MA-1757 (Fig. 3bB and D) though some degree of cytoplasmic staining was also observed. Oocytes incubated with neat culture supernatant of Sp2/O myeloma cells did not show any staining (Fig. 3bA). Prior incubation of MA-1757 with P40 peptide (10 μg/ml) abrogated its reactivity with ZP (Fig. 3bE). A significant reduction in the reactivity with ZP of MA-1756 previously incubated with P40 peptide was also observed (Fig. 3bC).

Analysis of the reactivity of the MAbs against ZPI with human ovarian cryosections by immunohistochemistry revealed that these failed to react with oocytes in healthy primordial and primary follicles (Fig. 4A) but strong staining was observed in oocytes of primordial/primary follicles undergoing atresia (Fig. 4B). Oocytes in secondary and antral follicles showed strong surface ZPI expression (Fig. 4C–E). Oocytes of antral follicles also showed strong surface ZP2 expression (Fig. 4F). Similar to ZPI, MAb against ZP3 exhibited reactivity with the ZP matrix of secondary follicles (Fig. 4G). The MAb against ZP4 showed strong surface staining of oocytes in primordial, primary and secondary follicles (Fig. 4H). No ZPI expression was observed in human Fallopian tube, kidney, endometrium and ectocervix samples (Fig. 4I–L).

Besides reactivity with follicular oocytes, all ZP MAbs also stained degenerating intravascular oocytes in either ovarian or extra ovarian venules. Figure 5 shows 5 cases (ages 28–38) with ZP1 immunoreactivity in degenerating oocytes in ovarian venules (Fig. 5A and B), ZP2 in ectocervical venule (Fig. 5C) and ZP3 and ZP4 in ovarian venules (Fig. 5D and E). CD3 antibody stained ovarian T cells (Fig. 5F) but not oocytes (Fig. 5G).

### Figure 2
Characterization of carbohydrate residues present in recombinant human ZP1. (a) Profile of lectins binding to E. coli- and baculovirus-expressed human ZP1 in ELISA: microtitration plates were coated with the E. coli- (grey bar) and baculovirus- (black bar) expressed recombinant ZPI (500 ng/well) and processed for the evaluation of binding to 21 different biotinylated lectins in an ELISA. Values are expressed as absorbance obtained with various lectins binding to the respective recombinant protein, after deducting the non-specific binding of the lectins to the uncoated wells. Each bar represents a mean of duplicate experiments. The lectins tested were GSL-I: Griffonia simplicifolia lectin I, SBA: soybean agglutinin, DSL: Datura stramonium lectin, PSA: Pisum sativum agglutinin, ConA: concanavalin A, ECL: Erythrina cristagalli lectin, LCA: Lens culinaris agglutinin, DBA: Dolichos biflorus agglutinin, LEL: Lycopersicon esculentum lectin, PHA-L: Phaseolus vulgaris leucoagglutinin, UEA-I: Ulex europaeus agglutinin I, STL: Solanum tuberosum lectin, PHA-E: Phaseolus vulgaris erythroagglutinin, RCA: Ricinus communis agglutinin, VVA: Vicia villosa agglutinin, SJA: Sophora japonica agglutinin, PNA: peanut agglutinin, S-WGA: succinylated wheat germ agglutinin, Jacalin, WGA: wheat germ agglutinin and GSL II: Griffonia simplicifolia lectin II. (b, c) Profiles of lectins binding to E. coli- and baculovirus-expressed ZPI1 in lectin blots: the recombinant proteins (2 μg/lane) were resolved on SDS–PAGE and processed for lectin blots with 20 μg/ml each of Con A (b) and Jacalin (c). Lanes are represented as M: molecular weight markers; lane 1 represents E. coli-expressed ZPI1 and lane 2 baculovirus-expressed ZPI1.

### Binding characteristics of recombinant human ZPI with human spermatozoa
To obtain a handle for direct detection of recombinant ZPI bound to human spermatozoa in in vitro sperm binding assays, the Ni-NTA affinity purified E. coli- and baculovirus-expressed recombinant ZPI were conjugated to FITC as described in Materials and Methods. The molar F/P ratios of the FITC-conjugated recombinant proteins were found to be 1.5 for E. coli-expressed ZPI and 1.2 for baculovirus-expressed ZPI. These FITC-labelled recombinant proteins were analysed for binding to spermatozoa and the acrosomal status of the spermatozoa was also simultaneously assessed by double labelling with TRITC–PSA (Vector Laboratories). Varying concentrations of the FITC-labelled recombinant proteins were used for the binding experiments. In case of studying the binding profile of recombinant ZPI, the results obtained by using 50 μg of protein/ml (Table II and Fig. 6) have been included and in the specificity studies, results obtained by using 20 μg of protein/ml (Fig. 7) have been shown. FITC-conjugated Fetuin bound to 3.95–4.00% of capacitated spermatozoa while the...
positive controls, FITC-labelled E. coli- and baculovirus-expressed ZP3 bound to 24.08 ± 3.15 and 26.08 ± 2.75% and ZP4 bound to 15.96 ± 1.47 and 17.85 ± 2.87% of capacitated spermatozoa, respectively. The baculovirus-expressed recombinant human ZPI showed binding to 16.50 ± 1.50% of capacitated acrosome-intact sperm and to 14.40 ± 1.09% acrosome-reacted sperm (Table II). Interestingly, E. coli-expressed FITC-labelled recombinant human ZP1 also showed similar binding profile to the capacitated and acrosome-reacted human sperm as observed with FITC-labelled baculovirus-expressed recombinant human ZP1 (Table II and Fig. 6). In contrast to capacitated sperm, E. coli-expressed ZP1 showed binding to 6.00 ± 0.69% and baculovirus-expressed ZP1 to 5.10 ± 0.54% of non-capacitated sperm (data not shown) and binding was predominantly localized to the equatorial segment. Analysis of

Table I  Characterization of MAbs generated against P40 synthetic peptide corresponding to human ZP1.

<table>
<thead>
<tr>
<th>MAbs*</th>
<th>Antibody isotypes</th>
<th>Absorbance at 492 nm with P40 peptide</th>
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<tbody>
<tr>
<td></td>
<td>E. coli-expressed</td>
<td>Baculovirus-expressed</td>
</tr>
<tr>
<td></td>
<td>ZP1</td>
<td>ZP2</td>
</tr>
<tr>
<td>MA-1756</td>
<td>IgG1</td>
<td>3.00</td>
</tr>
<tr>
<td>MA-1757</td>
<td>IgG2b</td>
<td>3.00</td>
</tr>
<tr>
<td>MA-1758</td>
<td>IgG2b</td>
<td>3.00</td>
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*Neat culture supernatant of the respective MAbs was tested in ELISA.

Figure 3  Characterization of the MAbs generated against P40 peptide corresponding to human ZP1. (a) Specificity of MAbs generated against synthetic peptide corresponding to ZP1 in western blot: various E. coli-expressed recombinant human zona proteins (2 μg/lane) were resolved on SDS–PAGE and processed for immunoblot with neat culture supernatant of MA-1756. Lanes are represented as M: molecular weight markers; lane 1–4: E. coli-expressed recombinant human ZP1, ZP2, ZP3 and ZP4, respectively. (b) Reactivity of MAbs with human oocytes: human oocytes were incubated with the neat culture supernatant of MA-1756 and MA-1757 as well as the culture supernatants pre-incubated with the P40 peptide (10 μg/ml) and processed for their reactivity with native ZP. Representative immunofluorescence patterns are shown. The top panel shows representative pictures taken under phase contrast and the bottom panel represents immunofluorescence of the same oocyte. (A) Neat culture supernatant of Sp2/O myeloma cells, (B and D) oocytes incubated with neat culture supernatants of MA-1756 and MA-1757, respectively; (C and E) oocytes treated with culture supernatant of MA-1756 and MA-1757 pre-incubated with 10 μg/ml of P40 peptide, respectively. The scale bar represents 36 μm.
binding profile of the capacitated acrosome-intact human sperm revealed that ~70% sperm showed binding of ZP1 to the acrosomal cap and ~30% in the equatorial region (Fig. 6). Recombinant protein failed to show any binding to the acrosomal cap of the acrosome-reacted human spermatozoa and primarily showed binding to the equatorial region. In addition, a few spermatozoa also showed binding of FITC-labelled recombinant ZPI to either the post-acrosomal region or midpiece of capacitated and acrosome-reacted spermatozoa.

The binding of FITC-labelled E. coli-expressed ZPI1 to capacitated spermatozoa was reduced from 21.5 to 4.0% when co-incubated with a 5-fold molar excess of unlabelled E. coli-expressed ZPI1 (P < 0.02) and to 7.5% (P < 0.01) when co-incubated with baculovirus-expressed unlabelled ZPI1 (Fig. 7A). Similarly, the binding of baculovirus-expressed ZPI1 to capacitated sperm was also

Table II  Binding characteristics of FITC-labelled recombinant human ZPI1 with human spermatozoa.

<table>
<thead>
<tr>
<th>Test protein*</th>
<th>Percent binding to spermatozoa</th>
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<tr>
<td></td>
<td>Capacitated (acrosome-intact)</td>
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<tr>
<td>Baculovirus-expressed ZP proteins</td>
<td></td>
</tr>
<tr>
<td>Fetuin</td>
<td>3.95 ± 2.12</td>
</tr>
<tr>
<td>ZP3</td>
<td>26.08 ± 2.75</td>
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<tr>
<td>ZP4</td>
<td>17.85 ± 2.87</td>
</tr>
<tr>
<td>ZPI</td>
<td>16.50 ± 1.50</td>
</tr>
<tr>
<td>E. coli-expressed ZP proteins</td>
<td></td>
</tr>
<tr>
<td>Fetuin</td>
<td>4.00 ± 3.52</td>
</tr>
<tr>
<td>ZP3</td>
<td>24.08 ± 3.15</td>
</tr>
<tr>
<td>ZP4</td>
<td>15.96 ± 1.47</td>
</tr>
<tr>
<td>ZPI</td>
<td>17.40 ± 1.09</td>
</tr>
</tbody>
</table>

*All the recombinant proteins were used at a concentration of 50 μg/ml (2.5 μg/reaction).
significantly inhibited in the presence of baculovirus as well as E. coli-expressed unlabelled ZP1 (Fig. 7b).

Baculovirus-expressed recombinant human ZP1 induces acrosomal exocytosis in capacitated human spermatozoa

Initially, the culture supernatants obtained from Sf21 cells infected with recombinant virus and wild-type AcNPV were harvested and 20 µl of 10× concentrated supernatants were used to study induction of acrosome reaction as described in Materials and Methods. A significant increase in acrosome reaction was observed in the presence of the culture supernatant from Sf21 cells infected with recombinant virus when compared with wild-type AcNPV (33.1 ± 1.3 versus 17.4 ± 1.9; P = 0.0332). For subsequent characterization and delineation of downstream signalling components involved in ZP1-mediated induction of acrosome reaction, cell-pellet purified recombinant ZP1 was employed. Incubation of the capacitated human sperm with either E. coli- or baculovirus-expressed recombinant human ZP1 did not result in any significant decrease in the viability of the sperm (data not shown). Subsequent to incubation with recombinant ZP1, no significant increase in the percentage of non-motile sperm was observed when compared with medium/vehicle control. The total motility of sperm after capacitation and treatment with recombinant ZP1 ranged from 80 to 92% in different experiments. A dose–response effect in the induction of acrosome reaction was observed when varying concentrations of baculovirus-expressed human ZP1 were incubated with capacitated spermatozoa. A significant increase in acrosomal exocytosis even at 1 µg/ml when compared with the medium control (Table III, P = 0.0041) was found to occur. No further increase in acrosomal exocytosis was observed if the concentration of ZP1 was increased beyond 10 µg/ml (Table III). Hence, all subsequent studies using the human ZP1 protein were performed using 2 µg/ml of the protein. Time kinetic studies with baculovirus-expressed recombinant ZP1 revealed that the induction of acrosome reaction could be seen as early as 10 min after exposure of the capacitated sperm to the above recombinant protein (Supplementary Fig. S1). The highest level of induction of acrosome reaction was observed at 60 min. E. coli-expressed recombinant human ZP1 failed to induce any significant increase in the induction of acrosomal exocytosis (Table III, P = 0.7301). CaI, a chemical agonist of acrosomal exocytosis.

Figure 6 Binding profile of recombinant human ZP1 with human spermatozoa. Capacitated (left panel) or acrosome-reacted sperm (right panel; 5 × 10⁶/50 µl) were incubated with 2.5 µg of FITC conjugated E. coli-expressed recombinant ZP1 (A–C) and baculovirus-expressed ZP1 (D–F). The acrosomal status was determined by labelling the sperm with PSA–TRITC. The images were captured using Eclipse 80i fluorescence microscope (Nikon). In each panel, the subpanels are represented as (a) phase contrast; (b) PSA–TRITC fluorescence; (c) FITC-ZP protein fluorescence and (d) overlap of fluorescent frames. The scale bar represents 2.5 µm.
(used as a positive control) and baculovirus-expressed recombinant human ZP3 also showed a significant increase in the percentage of sperm undergoing acrosome reaction. However, Fetuin and baculovirus-expressed ZP2 used as internal controls, failed to induce any significant increase in acrosomal exocytosis when compared with medium control (Table III). Incubation of capacitated human sperm with a combination of baculovirus-expressed recombinant ZP1 and ZP3, ZP1 and ZP4 or ZP1, ZP3 and ZP4 resulted in an increase in induction of acrosome reaction when compared with ZP1 alone, which was, however, statistically insignificant (Table III).

**Downstream signalling events associated with ZP1-mediated induction of acrosomal exocytosis**

Different pharmacological inhibitors at varying concentrations were used to reveal the downstream signalling pathway mediated by baculovirus-expressed ZP1 to bring about acrosomal exocytosis. The typical results obtained at a given concentration with various inhibitors are shown in Table IV and Fig. 8. Induction of acrosomal exocytosis mediated by ZP1 required extracellular Ca\(^{2+}\) as prior incubation of capacitated sperm in BWW medium with EGTA before exposing to recombinant protein led to a significant decrease in percent stimulation of acrosome reaction \((P < 0.0001; \text{Table IV})\). In contrast to baculovirus-expressed recombinant human ZP3, induction of acrosomal exocytosis by ZP1 is independent of activation of G\(_\text{i}\) pathway since no significant decrease in ZP1-mediated percent stimulation of acrosome reaction was observed \((P > 0.05)\) after incubation with PFX pre-treated capacitated human spermatozoa (Table IV). Also, unlike in the case of ZP3-mediated acrosomal exocytosis, GABA-A receptor antagonist, picrotoxin did not inhibit the induction of acrosomal exocytosis mediated by ZP1 suggesting that the GABA-A receptor-associated Cl\(^{-}\) channel might not be involved in the process (Table IV). Inhibition of acrosomal exocytosis by inhibitors of both T- and L-type Ca\(^{2+}\) channels suggests that ZP1-mediated induction of acrosome reaction involves activation of both Ca\(^{2+}\) channels (Fig. 8a). Activation of PKA and PKC by recombinant human ZP1 is also crucial as treatment of capacitated sperm with the respective inhibitors led to a significant decrease in stimulation of the acrosome reaction (Fig. 8b).

**Discussion**

The critical appraisal of the role of ZP glycoproteins during fertilization in humans has been hampered due to their non-availability in highly purified form from native source. In humans, ZP1 is documented as being a low abundance protein when compared with ZP2, ZP3 and ZP4 in the ZP matrix (Lefievre et al., 2004). Hence, there is an inherent scarcity of ZP1 combined with the paucity of human oocytes for research purpose, to elucidate the functional attributes of human ZP1. Thus for this study, ZP1 has been expressed as a polyhistidine-tagged fusion protein in *E. coli* as well as in baculovirus expression systems. Western blot analysis of the lysate of SF21 cells infected with the recombinant virus expressing ZP1 revealed a lower molecular weight band in addition to the full-length protein, which may represent either a premature translational product or a degradation product and is absent from the Ni-NTA affinity purified protein (Fig. 1). SDS-PAGE and western blot analyses of the purified
Youssef et al. revealed the presence of mannose binding sites, which have been implicated to play a crucial role in ZP1 as revealed by high ConA binding. The presence of mannose is also the case with the baculovirus-expressed recombinant human ZP1 in human ovaries is available. The MAbs reported in the present manuscript, which are devoid of reactivity in ELISA and western blot with human ZP2, ZP3 and ZP4 reacted with the ZP matrix of secondary and antral follicles and failed to react with the ZP of primordial and primary follicles. These studies revealed the distribution of ZP1 throughout the width of ZP matrix in both ovarian cross-sections as well as ovulated oocytes. At this stage it is not clear, how ZP1 is incorporated into the ZP matrix which is preformed at the primary follicle stage. One of the possible explanations may be that when oocytes enter the growth phase and the ZP matrix starts growing, the epitope(s) recognized by ZP1 MAbs become accessible only in the secondary and antral follicles. This notion is supported by the observations that these MAbs recognize ZP1 in primordial/primary follicles undergoing atresia. By employing specific MAbs, the expression of ZP2 and ZP3 in growing and antral follicles and their respective absence in primordial and primary follicles has been shown (Fig. 4; Bukovsky et al., 2008). In another study, expression of ZP3 has been documented in granulosa cells and primordial follicles in human ovaries (Grootenhuis et al., 1996). However, non-reactivity of the MAbs used in these studies with other zona proteins was not established. The MAbs against ZP4 reacted with ZP matrix of primordial, growing and antral follicles (Fig. 4). High resolution scanning

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent induction of acrosome reaction* (Mean ± SEM)</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (BWW + 0.3% BSA)</td>
<td>7.9 ± 2.6</td>
<td></td>
</tr>
<tr>
<td>Calcium ionophore (10 μM)</td>
<td>56.7 ± 4.9</td>
<td>*P = 0.0001&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fetuin (10 μg/ml)</td>
<td>6.8 ± 2.9</td>
<td>*P = 0.9620&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Baculovirus-expressed ZP3 (10 μg/ml)</td>
<td>32.4 ± 3.4</td>
<td>*P = 0.0139&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Baculovirus-expressed ZP2 (10 μg/ml)</td>
<td>15.3 ± 4.7</td>
<td>*P = 0.1037&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>E. coli-expressed ZP1 (10 μg/ml)</td>
<td>8.4 ± 2.2</td>
<td>*P = 0.7301&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Baculovirus-expressed ZP1 0.5 μg/ml</td>
<td>11.3 ± 1.5</td>
<td>*P = 0.6064&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>1 μg/ml</td>
<td>24.1 ± 3.9</td>
<td>*P = 0.0041&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2 μg/ml</td>
<td>26.6 ± 4.2</td>
<td>*P = 0.0007&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>5 μg/ml</td>
<td>25.5 ± 3.8</td>
<td>*P = 0.0013&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>10 μg/ml</td>
<td>30.0 ± 3.3</td>
<td>*P = 0.0004&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Experiment II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (BWW + 0.3% BSA)</td>
<td>8.7 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>Calcium ionophore (10 μM)</td>
<td>55.2 ± 1.9</td>
<td>*P = 0.0010&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Baculovirus-expressed ZP1 (1 μg/ml)</td>
<td>25.1 ± 0.8</td>
<td>*P = 0.0002&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Baculovirus-expressed ZP3 (1 μg/ml)</td>
<td>28.4 ± 0.5</td>
<td>*P = 0.0001&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Baculovirus-expressed ZP4 (1 μg/ml)</td>
<td>26.2 ± 0.7</td>
<td>*P = 0.0010&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Baculovirus-expressed ZP1 + ZP3 (1 μg/ml each)</td>
<td>28.4 ± 1.7</td>
<td>*P = 0.0005&lt;sup&gt;b&lt;/sup&gt;; *P = 0.1490&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Baculovirus-expressed ZP1 + ZP4 (1 μg/ml each)</td>
<td>27.2 ± 1.6</td>
<td>*P = 0.0004&lt;sup&gt;b&lt;/sup&gt;; *P = 0.3147&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Baculovirus-expressed ZP1 + ZP3 + ZP4 (1 μg/ml each)</td>
<td>30.2 ± 1.9</td>
<td>*P = 0.0005&lt;sup&gt;b&lt;/sup&gt;; *P = 0.0692&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Percent induction of acrosomal exocytosis was calculated by dividing the number of acrosome-reacted sperm by total number of sperm counted and multiplying by 100. Values represent mean ± SEM of at least four independent experiments.

<sup>b</sup>Statistical significance with respect to the medium control was calculated by one-way ANOVA followed by Newman–Keuls multiple comparison test.

<sup>c</sup>Statistical significance with respect to baculovirus-expressed ZP1 was calculated by Student’s t-test.

<sup>d</sup>Values are statistically significant.

The understanding of the expression of zona proteins as a function of folliculogenesis and their subsequent assembly in the ZP matrix in humans is very limited. To date, no information about the expression of ZPI in human ovaries is available. The MAbs reported in the present manuscript, which are devoid of reactivity in ELISA and western blot with human ZP2, ZP3 and ZP4 reacted with the ZP matrix of secondary and antral follicles and failed to react with the ZP of primordial and primary follicles. These studies revealed the distribution of ZP1 throughout the width of ZP matrix in both ovarian cross-sections as well as ovulated oocytes. At this stage it is not clear, how ZP1 is incorporated into the ZP matrix which is preformed at the primary follicle stage. One of the possible explanations may be that when oocytes enter the growth phase and the ZP matrix starts growing, the epitope(s) recognized by ZP1 MAbs become accessible only in the secondary and antral follicles. This notion is supported by the observations that these MAbs recognize ZP1 in primordial/primary follicles undergoing atresia. By employing specific MAbs, the expression of ZP2 and ZP3 in growing and antral follicles and their respective absence in primordial and primary follicles has been shown (Fig. 4; Bukovsky et al., 2008). In another study, expression of ZP3 has been documented in granulosa cells and primordial follicles in human ovaries (Grootenhuis et al., 1996). However, non-reactivity of the MAbs used in these studies with other zona proteins was not established. The MAbs against ZP4 reacted with ZP matrix of primordial, growing and antral follicles (Fig. 4). High resolution scanning
electron microscopy studies with mouse as well as human oocytes show that ZP appears as a delicate meshwork of thin interconnected filaments in a regular alternating pattern of wide and tight meshes/pores (Familiari et al., 2006). These pores appear larger at the outer surface of the zona than the inner surface. In mouse, using immunola-belling techniques, it has been shown that Golgi apparatus, secretory granules and a complex structure called vesicular aggregates are involved in processing of the three zona glycoproteins before their secretion to form the ZP matrix (El-Mestrah et al., 2002). These inves-tigators revealed asymmetrical spatial distribution of the three zona glycoproteins in the ZP matrix at various stages of follicular development. The evolving picture of biogenesis of the mouse ZP is that only ZP2 and ZP3 are required to form a ZP matrix that functions bio-logically and that the two proteins are co-expressed during oocyte maturation. The evolving picture of biogenesis of the mouse ZP is that only ZP2 and ZP3 are required to form a ZP matrix that functions bio-logically and that the two proteins are co-expressed during oocyte maturation. McLeskey et al. (1998). However, baculovirus-expressed human ZP2 failed to induce significant increase in the induction of acrosome reaction in spite of having high-mannose residues. This suggests that the polypeptide backbone of human ZP1 per se may be sufficient to act as a docking site for sperm on the ZP matrix. These results are corroborated by similar findings wherein the polypeptide backbone of bonnet monkey as well as human ZP3 and ZP4 have been independently shown to be sufficient to bind to homologous spermatozoa (Govind et al., 2001; Gahlay et al., 2002; Chakravarty et al., 2008). Our understanding of the molecular basis of the primary sperm binding to ZP is still elusive. It has been proposed that multiple low-affinity bonds between spermatozoa and the ZP may be sufficient for primary binding (Castle, 2002). The avidity of these low-affinity interactions may exceed the strength of a single high-affinity bond and may help to tether a spermatozoon to the ZP (Castle, 2002). On the basis of extensive studies done in mouse, two models have been proposed for sperm–oocyte binding (Clark and Dell, 2006). One of the models proposes that the primary binding of sperm to ZP is predominated by protein–protein interaction, which may explain binding of the E. coli-expressed ZP1 to spermatozoa. The second model suggests that protein–carbohydrate interaction is responsible for 75–80% of the sperm binding to the ZP and remaining sperm bind by protein–protein interactions (Clark and Dell, 2006).

Recent studies have shown that quail ZP1 and dimeric chicken ZP1 are capable of inducing the acrosome reaction (Okumura et al., 2004; Sasanami et al., 2007). However, no information pertaining to the ability of ZP1 to induce acrosomal exocytosis in homologous sperm is available in any mammalian species. The results pertaining to the induction of acrosome reaction show that ZP1 may also have a functional role in acrosomal exocytosis in humans. In the present study, induction of acrosome reaction by baculovirus-expressed recombinant ZP1 but not by E. coli-expressed recombinant ZP1 (Table III), reiterate that glycosylation of zona proteins is essential for the induction of acrosome reaction (Chakravarty et al., 2005, 2008). The recombinant ZP1 protein employed in the present study has been shown to have high mannose sugar moieties added to the polypeptide backbone (Fig. 2). These findings correlate well with earlier observations, where mannose has been implicated to play an important role in the sperm receptor activity (Mori et al., 1989; Cornwall et al., 1991; McLeskey et al., 1998). However, baculovirus-expressed human ZP2 failed to induce significant increase in the induction of acrosome reaction in spite of having high-mannose residues. This suggests that the polypeptide backbone of ZP1 may be playing an important role in appropriate disposition of carbohydrates and hence enables

### Table IV Role of extracellular calcium, Gt protein and GABA-A receptor-associated Cl− channels in ZP1-mediated induction of acrosomal exocytosis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent stimulation of acrosome reaction of acrosome reaction (mean ± SEM)</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZP1 (2 μg/ml)</td>
<td>31.3 ± 0.64</td>
<td>P &lt; 0.0001*</td>
</tr>
<tr>
<td>ZP1 (2 μg/ml)</td>
<td>10.1 ± 0.53</td>
<td>P = 0.2041</td>
</tr>
<tr>
<td>ZP1 (2 μg/ml)</td>
<td>30.4 ± 0.32</td>
<td>P = 0.5029</td>
</tr>
<tr>
<td>ZP1 (2 μg/ml)</td>
<td>28.6 ± 3.98</td>
<td>Statistical significanceb</td>
</tr>
</tbody>
</table>

*Percent stimulation of acrosome reaction has been calculated as described in Materials and Methods.

bStatistical significance (P-values) with respect to the recombinant protein-treated sperm.

Values are statistically significant.
Role of VOCCs and protein kinases in ZP1-mediated induction of acrosomal exocytosis. To study the effect of blocking of L-type VOCC, capacitated sperm (1 x 10⁷/100 μl) were pre-incubated for 10 min with 10 μM verapamil and 10 μM of nifedipine and for T-type VOCC with 10 μM pimozide and 100 μM amiloride (a). Similarly, capacitated sperm were also pre-treated for 10 min, with 3 μM chelerythrine (PKC inhibitor) and 20 μM H89 (PKA inhibitor) to study the effect of protein kinase inhibitors (b). These pre-treated sperm were incubated with 0.2 μg of ZP1 in a reaction volume of 100 μl for 60 min, and subsequently analysed for acrosomal status by PSA-TRITC staining. Negative controls comprised BWW medium or solvent used for dissolving inhibitors. Induction of acrosome reaction by 10 μM of calcium ionophore was taken as maximum value. Values are expressed as percent stimulation of acrosome reaction and represent mean ± SEM of three different experiments using semen samples from at least two different male donors. *P < 0.05 when compared with ZP1-mediated acrosome reaction without prior treatment with inhibitors.

Figure 8 Role of VOCCs and protein kinases in ZP1-mediated induction of acrosomal exocytosis. To study the effect of blocking of L-type VOCC, capacitated sperm (1 x 10⁷/100 μl) were pre-incubated for 10 min with 10 μM verapamil and 10 μM of nifedipine and for T-type VOCC with 10 μM pimozide and 100 μM amiloride (a). Similarly, capacitated sperm were also pre-treated for 10 min, with 3 μM chelerythrine (PKC inhibitor) and 20 μM H89 (PKA inhibitor) to study the effect of protein kinase inhibitors (b). These pre-treated sperm were incubated with 0.2 μg of ZP1 in a reaction volume of 100 μl for 60 min, and subsequently analysed for acrosomal status by PSA-TRITC staining. Negative controls comprised BWW medium or solvent used for dissolving inhibitors. Induction of acrosome reaction by 10 μM of calcium ionophore was taken as maximum value. Values are expressed as percent stimulation of acrosome reaction and represent mean ± SEM of three different experiments using semen samples from at least two different male donors. *P < 0.05 when compared with ZP1-mediated acrosome reaction without prior treatment with inhibitors.
Studies from various species such as chicken, pig, rabbit and bonnet monkey have earlier suggested that more than one zona protein is involved in binding to capacitated spermatozoa and induction of acrosome reaction (Prasad et al., 1996; Yurewicz et al., 1998; Govind et al., 2001; Okumura et al., 2004). Hence, to conclude, these studies suggest a probable model wherein in humans, ZP1, ZP4 and ZP3 are involved in binding of capacitated spermatozoa to the ZP matrix and subsequent induction of acrosome reaction, whereas in mice, ZP3 is primarily responsible for binding of capacitated spermatozoa to the ZP matrix and induction of acrosome reaction. In a recent study, the N- and C-terminal fragments of human ZP3 have been expressed using baculovirus expression system. Though both fragments bind to the capacitated spermatozoa, only the C-terminal fragment (214–348 aa) induces acrosomal exocytosis (Bansal et al., 2009). Taking cue from this study, efforts are under way to delineate the domain(s) of ZP1 that is responsible for acrosomal exocytosis and preliminary findings suggest that the ‘ZP domain’ module might be responsible for the same (Anasua Ganguly, unpublished observations). It is imperative to pursue further studies either by site-directed mutagenesis or expression of deletion constructs of ZP1 to delineate the motifs responsible for the binding to spermatozoa and induction of acrosomal exocytosis enabling unearthing of comprehensive information on the molecular mechanisms involved in human fertilization.

**Authors’ roles**

The unfertilized human oocytes were kindly provided by R.K.S. A.B. performed the localization studies employing human ovarian sections and helped in writing and reviewing the manuscript. S.K.G. and A.G. participated in the study design, execution, analysis, manuscript drafting and critical discussion. P.B. and B.B. helped in performing additional sperm binding and induction of acrosome reaction experiments.

**Supplementary data**

Supplementary data are available at http://humrep.oxfordjournals.org/.

**Acknowledgements**

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Gautier L, Dean J. ZP2 and ZP3 traffic independently within oocytes prior to assembly into the extra cellular zona pellucida. Mol Cell Biol 2006;26:7991–7998.


