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Characterization of human zona pellucida glycoproteins

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The human egg may only be fertilized by one spermatozoon to prevent polyploidy. In most mammals, the primary block to polyspermy occurs at the zona pellucida (ZP). Little is known of the human ZP and the changes occurring following fertilization to prevent polyploidy. Using antibodies directed against synthetic peptides predicted from the human ZP2 and ZP3 cDNA, we identified ZP3 as a 53–60 kDa glycoprotein and ZP2 as a 90–110 kDa glycoprotein in prophase-I oocytes. Characterization of the ZP from metaphase II arrested eggs (inseminated–unfertilized and fertilized–uncleaved), shows no visible modification of ZP3, but demonstrates that ZP2 undergoes limited proteolysis in the amino terminal domain, to a 60–73 kDa species, denoted $ZP2_p$, which remains linked to the proteolysed fragments by intramolecular disulphide bonds. A lack of ZP2 proteolytic activity in acrosomal supernatants is consistent with an oocyte origin for the protease. The ZP2-specific protease may be released during cortical granule exocytosis which occurs during meiotic maturation and following sperm–egg fusion as part of the block to polyspermy. Since mouse ZP2 acts as a secondary sperm receptor, it is possible that intact ZP2 binds a secondary egg binding protein, whereas cleaved ZP2 does not, suggesting a possible mechanism for the block to polyspermy.

Key words: glycosylation/human zona pellucida/proteolysis/ZP2/ZP3

Introduction

The zona pellucida (ZP) is a transparent, porous, glycoprotein coat that surrounds mammalian eggs. The ZP is formed in the early stages of ovarian follicular development and plays an important role in fertilization and early development. It contains the species-specific receptors for spermatozoa and can induce the acrosome reaction. The ZP is involved in establishing the ZP block to polyspermy as well as serving to protect the cleaving embryos as they traverse the female reproductive tract (Wassarman, 1988; Snell and White, 1996).

Much of what is known about the ZP in mammalian fertilization has been obtained from studies on the mouse. The mouse ZP (mZP) is composed of three glycoproteins: ZP1, ZP2 and ZP3 (Bleil and Wassarman, 1980a). Fertilization begins when a capacitated mouse spermatozoon binds the ZP, activating the acrosome reaction. ZP3 acts as the primary sperm receptor, mediating both initial binding of the spermatozoon to the egg and activation of the acrosome reaction (Bleil and Wassarman, 1980b, 1983). Following induction of the acrosome reaction, ZP2 acts as the secondary sperm receptor, binding acrosome-reacted spermatozoa and facilitating penetration of the ZP for fusion with the egg plasma membrane (Bleil and Wassarman, 1986). Following sperm–egg fusion, the egg releases its cortical granule contents into the ZP, modifying the zona to prevent any further sperm binding and penetration. Both ZP2 and ZP3 are modified by the zona reaction: ZP2 undergoes limited proteolysis (Bleil *et al*., 1981; Moller and Wassarman, 1989) and ZP3 loses both sperm receptor activity and ability to induce the acrosome reaction (Wassarman, 1988).

Human homologues of the mZP1, mZP2 and mZP3 cDNA have been isolated (hZP3, Chamberlain and Dean, 1990; hZP2, Liang and Dean, 1993; hZP1, Harris *et al*., 1994) and recombinant hZP3 expressed in chinese hamster ovary cells has been shown to activate the acrosome reaction (van Duin *et al*., 1994). However, very little is known of the native human ZP (hZP) proteins. Sodium dodecy sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of solubilized ZP demonstrates three ZP proteins, 90–110 kDa (ZP1), 64–78 kDa (ZP2) and 57–73 kDa (ZP3), with the 90–110 kDa protein disappearing following fertilization (Shabinowitz and O'Rand, 1988a,b; Bercegeay *et al*., 1995).

In this study we compare the ZP from prophase I (Pro I) oocytes and metaphase II (Met II) arrested human eggs which were inseminated–unfertilized and fertilized–uncleaved [from failed in-vitro fertilization (IVF) preparations]. ZP2- and ZP3-specific antibodies were used to examine the changes in the ZP which may be responsible for establishing the ZP block to polyspermy.

Materials and methods

Immune reagents

Rabbit polyclonal antisera against ZP2 and ZP3 were obtained using synthetic peptides coupled to keyhole limpet haemocyanin (KLH) as immunogen. Coupling to KLH was carried out using *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester (Pierce Chemical Co., Rockford, USA) essentially according to manufacturer's instructions. For the anti-ZP2 serum, synthetic peptides corresponding to amino acid residues 424–440 (CGTRYKFEDDKVVYENE) and 535–545 (NRDDPNIKLVLDDC) (Liang and Dean, 1993) were co-injected, and for the anti-ZP3 serum the synthetic peptide corresponding to residues 327–341 (CGTPSHSRRQPHVMS) (Chamberlain and Dean, 1990) were used. The ZP3 peptide has already been shown to generate antibodies capable of recognizing the native protein (Mahi-Brown and Moran, 1995). Specificity was demonstrated by immunoprecipitation analysis of heat-solubilized iodinated ZP.

Collection of oocytes

Human ZP were obtained from post-mortem-derived Pro I oocytes and Met II arrested eggs obtained from follicular aspirates from patients participating in the IVF–embryo transfer programme at Tygerberg Hospital, Cape Town as previously described (Franken *et al*., 1991a, 1996). All prescribed legal and ethical procedures concerning the Human Tissue Act have been fulfilled throughout the study. Pools of Met II arrested eggs, induced by exogenous gonadotrophins, had been subjected to IVF by either regular insemination or intracytoplasmic sperm injection. Pools of eggs which were inseminated but failed to fertilize or which fertilized but failed to undergo cleavage were used with the patients' consent and represent materials which would normally have been discarded. These eggs have been collectively termed 'failed IVF' throughout. Post-mortem-derived oocytes are termed Pro I oocytes throughout. All eggs were stored in 1.5 mol/l $MgCl_2$, 0.1% polyvinylpyrrolidone, 40 mmol/l HEPES, pH 7.2 at 4°C for up to several weeks prior to use. ZP retain their biological activity and function following storage in salt solution for at least 1 month (Franken *et al*., 1991b; Kruger *et al*., 1991).

Isolation and radiolabelling of ZP

Zonae were mechanically isolated from oocytes using glass micropipettes under a stereomicroscope (Franken *et al*., 1996). ZP were cleaned of cumulus–corona cells as described (Franken *et al*., 1996) and either heat-solubilized at 70°C for 90 min in distilled water adjusted to pH 9 with Na_2CO_3 (Dunbar *et al*., 1980) or acid-solubilized in 5 mmol/l NaH_2PO_4, pH 2.5 and then neutralized (Bleil and Wassarman, 1980; Franken *et al*., 1996). Solubilized ZP were iodinated using 0.1 mCi $Na^{125}I$ (Amersham) and iodobeads (Pierce Chemical Co.) essentially according to manufacturer's instructions.

Immunoprecipitation and gel electrophoresis

Iodinated heat-solubilized ZP were diluted in 50 mmol/l HEPES, pH 7.4, 1% Triton X-100, 5 mmol/l EDTA, 0.1% bovine serum albumin and various protease inhibitors (2 mmol/l phenylmethylsulphonyl fluoride, 5 μg/ml leupeptin, 0.3 U/ml aprotinin). For non-reducing SDS–PAGE analysis, 50 mM iodoacetamide was included. Immunoprecipitation was carried out essentially as described (Bauskin *et al*., 1991). Antisera were used at dilutions of 1:75 for anti-ZP3 serum and 1:100 for anti-ZP2 serum. Immunoprecipitated proteins were analysed on polyacrylamide gels in the presence of SDS either with reduction (Laemmli, 1970) or without (Olson *et al*., 1988). Experiments intended for parallel analysis by both non-reducing and reducing SDS–PAGE were treated as previously described (Bauskin *et al*., 1991). Both reducing and non-reducing gels were 7.5%.

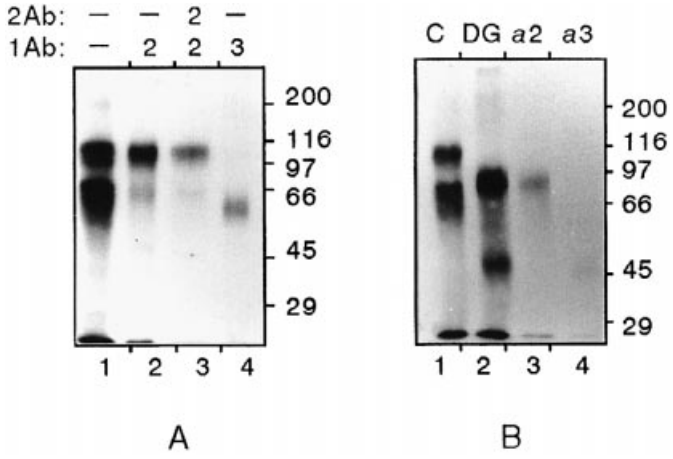

Figure 1. Immunoprecipitation analysis of zona pellucida (ZP)2 and ZP3 from immature prophase I oocytes. (**A**) Iodinated heat-solubilized ZP (lane 1) was immunoprecipitated with anti-ZP2 (lane 2) and anti-ZP3 (lane 4). Immunoprecipitated ZP2 was eluted and reprecipitated with anti-ZP2 (lane 3). (**B**) Iodinated heat-solubilized ZP (lane 1) was digested with *N*-glycosidase F (lane 2) and immunoprecipitated with anti-ZP2 (lane 3) and anti-ZP3 (lane 4). Ab = antibody; C = untreated ZP; DG = deglycosylated ZP.

Sequential immunoprecipitation analysis was carried out as described (Bauskin *et al*., 1991).

Glycosylation analysis

For endoglycosidase analysis, either heat solubilized iodinated ZP or immunoprecipitated proteins eluted from antibody coated protein A Sepharose beads were treated with *N*-glycosidase F, endoglycosidase H, *O*-glycosidase and neuraminidase (Boerhinger Mannheim, Mannheim, Germany) essentially according to manufacturer's instructions.

Preparation of acrosomal extract

Semen was provided by normal donors and motile spermatozoa were selected by the swim-up procedure as previously described (Franken *et al*., 1994). Acrosomal exocytosis of washed spermatozoa (300×10^6) was induced by A23187 ionophore (Sigma, St Louis, MO, USA) and Ca^{2+} as described (Cross *et al*., 1986). Confirmation of acrosomal exocytosis was obtained by staining with fluorescein isothiocyanate-conjugated *Pisum sativum* agglutinin (Sigma) before and after incubation with ionophore and calcium, (Cross *et al*., 1986). Acrosomal supernatant was collected after centifugation of spermatozoa (400 *g*, 5 min) for incubation with Pro I ZP. Pro I ZP were incubated with acrosomal supernatant for 2 h at 37°C prior to solubilization and immunoprecipitation analysis with anti-ZP2-specific serum.

Results

Characterization of the ZP proteins from immature prophase I oocytes

Reducing SDS–PAGE analysis of iodinated heat-solubilized ZP isolated from Pro I oocytes demonstrated two major bands at 95–110 and 57–73 kDa (Figure 1A). Immunoprecipitation analysis with anti-ZP3 serum raised against a synthetic ZP3 peptide (residues 327–341; Chamberlain and Dean, 1990) demonstrates ZP3 as a 53–60 kDa component of the lower major band (lane 4). Antiserum raised against two ZP2 synthetic peptides predicted from the ZP2 cDNA sequence

(residues 424–440 and 535–545; Liang and Dean, 1993) precipitates the upper major 105–110 kDa band (lane 2). There also appears to be a minor reactivity of the serum with a 60–73 kDa component of the lower major band (lane 2). As solubilization by heating may not completely solubilize the ZP and result in the formation of supramolecular complexes, sequential immunoprecipitation analysis was performed. Iodinated heat-solubilized ZP was immunoprecipitated with anti-ZP2 serum, the precipitated proteins eluted with reducing SDS–PAGE sample buffer, then reprecipitated with anti-ZP2 serum. Both the major upper 105–110 kDa band as well as the minor 60–73 kDa component reprecipitated (lane 3), suggesting that there are two forms of ZP2 which may arise from post-translational modification (see below).

The sizes of ZP2 and ZP3 are larger than that predicted by their cDNA sequence, most likely due to glycosylation. To confirm this, heat-solubilized ZP were deglycosylated with *N*-glycosidase F to remove all *N*-linked oligosaccharides, which accounts for most of the carbohydrate content of the mammalian ZP proteins studied to date. Anti-ZP2 serum precipitates the 80–85 kDa polypeptide (Figure 1B, lane 3) while anti-ZP3 precipitates the 47 kDa polypeptide (lane 4), both being slightly larger than that predicted by their cDNA sequences after signal peptide cleavage (ZP2, 78 kDa; Liang and Dean, 1993); ZP3, 44 kDa (Chamberlain and Dean, 1990) due to the presence of *O*-linked oligosaccharides (see Figure 5). In the deglycosylated ZP preparation (lane 2), a minor band at ~60 kDa may represent the human ZPB/ZP1 (Harris *et al*., 1994), which is homologous to the porcine *ZP3*α and rabbit *rc55* genes (Schwoebel *et al*., 1991; Yurewicz *et al*., 1993).

ZP2 is proteolytically cleaved in ZP from failed IVF

In most mammals studied to date, the primary block to polyspermy occurs at the ZP following fusion of the sperm and egg and exocytosis of the contents of the cortical granules into the perivitilline space. The exudate, containing hydrolases (proteases and glycosidases), acts on the ZP (zona reaction) causing it to lose the ability to bind spermatozoa and to be penetrated by spermatozoa previously bound to the zona (Wassarman, 1988). To examine any modifications in the ZP which may arise following insemination, ZP were isolated from a pool of Met II arrested eggs (induced by exogenous gonadotrophins), which have been subjected to IVF by either regular insemination or intracytoplasmic sperm injection. Pools of eggs which were inseminated–unfertilized and fertilized–uncleaved were used. These ZP were heat-solubilized, iodinated, immunoprecipitated with anti-ZP3 and anti-ZP2 serum and analysed by reducing SDS–PAGE (Figure 2). Anti-ZP3 precipitated a 53–60 kDa band (lane 1), similar to that from Pro I oocytes (see Figure 1A), indicating no visible difference in ZP3 following insemination. This is consistent with previous reports which indicate that hZP3 is indistinguishable in SDS–PAGE before and after fertilization (Shabinowitz and O'Rand, 1988a,b), and similar to mZP3 (Wassarman, 1980). However, anti-ZP2 precipitated a major band at 60–73 kDa and a more minor band at 105–110 kDa. Confirmation that both bands were ZP2 was demonstrated by sequential immunoprecipitation analysis of ZP2. Primary immunoprecipitates of ZP2 were eluted with reducing SDS–PAGE sample buffer, diluted and reprecipitated with anti-ZP2 serum (lane 2). Both the 105–110 kDa and 60–73 kDa bands reprecipitated. This indicates that ZP2 (105–110 kDa) undergoes limited proteolysis to ZP2 (60–73 kDa) (denoted $ZP2_p$) during meiotic maturation/sperm–egg fusion, most likely due to cortical granule exocytosis.

It has been shown in the mouse that ZP2 undergoes limited proteolysis (from 120 to 90 kDa, denoted $ZP2_f$) following fertilization, as part of the block to polyspermy. The peptides

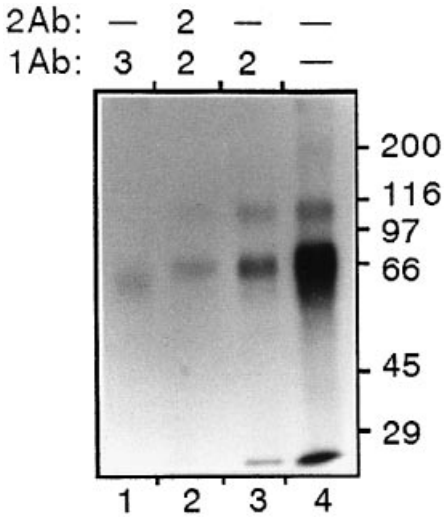

Figure 2. Immunoprecipitation analysis of zona pellucida (ZP)2 and ZP3 from failed in-vitro fertilization. Iodinated heat-solubilized ZP (lane 4) was immunoprecipitated with anti-ZP2 (lane 3) and anti-ZP3 (lane 1). Immunoprecipitated ZP2 was eluted and reprecipitated with anti-ZP2 (lane 2). Ab = antibody.

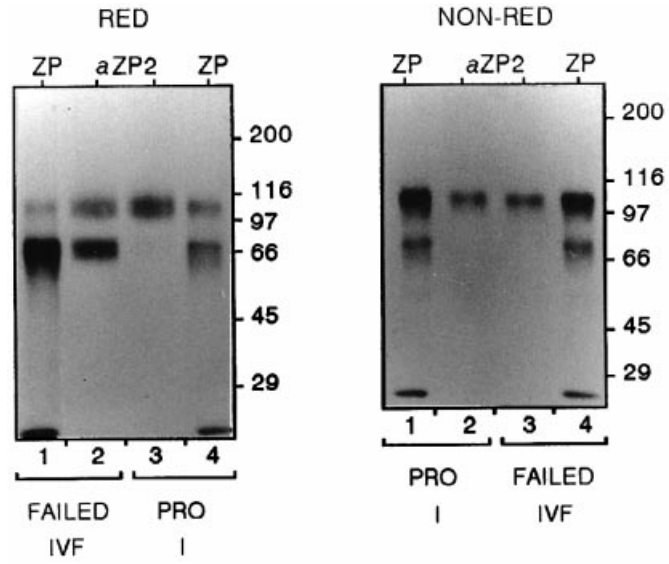

Figure 3. The zona pellucida (ZP)2 peptides generated by proteolysis are covalently linked by intramolecular disulphide bonds. Heat-solubilized iodinated ZP (lanes 1,4) was immunoprecipitated with anti-ZP2 (lanes 2,3) and analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis under non-reducing (Non-red) and reducing (red) conditions. red: lanes 1 and 2, failed in-vitro fertilization (IVF); lanes 3 and 4, prophase (Pro) I oocytes. Non-red: lanes 1 and 2, Pro I oocytes; lanes 3 and 4, failed IVF.

generated following proteolysis remain covalently linked to $ZP2_f$ by intramolecular disulphide bonds (Bleil *et al*., 1981). To investigate whether this is occurring here, ZP2 was immunoprecipitated from both immature Pro I and failed IVF ZP preparations and subjected to parallel analysis by SDS–PAGE under both reducing and non-reducing conditions (Figure 3). For parallel analysis the immunoprecipitated proteins were first eluted with non-reducing SDS–PAGE sample buffer and half were incubated with mercaptoethanol prior to reducing SDS–PAGE. Under non-reducing conditions, ZP2 migrates at 105–110 kDa in both preparations. However, under reducing conditions ZP2 migrates as two bands, 105–110 and 60–73 kDa in the failed IVF preparation, but mainly as 105–110 kDa in the immature Pro I ZP. A minor fraction of $ZP2_p$ can also be observed in Pro I ZP. This indicates that the peptides generated by limited proteolysis remain linked by intramolecular disulphide bonds. No large peptide fragment to account for the size difference between ZP2 and $ZP2_p$ was observed in reducing SDS–PAGE of solubilized ZP. It is possible that ZP2 is proteolytically cleaved into fragments that cannot be iodinated or are iodinated but too small to be observed.

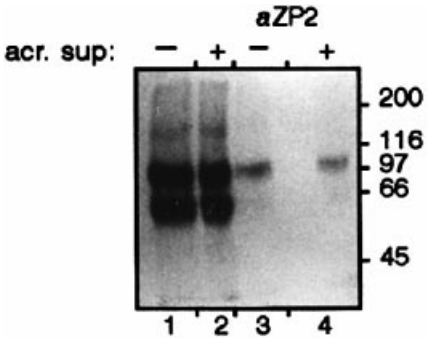

Figure 4. The zona pellucida (ZP)2-specific protease is not released during the acrosome reaction. Iodinated solubilized prophase (Pro) I ZP (lanes 1 and 2) were incubated with (+) and without (–) supernatant from acrosome-reacted sperm, ZP2 immunoprecipitated with anti-ZP2 serum (*a* ZP2) (lanes 3 and 4), and analysed by reducing sodium dodecyl sulphate–polyacrylamide gel electrophoresis and autoradiography. acr. sup: = acrosomal supernatant.

The ZP2-specific protease is not released during acrosomal exocytosis

Sequential immunoprecipitation analysis of $ZP2_p$ from several pools of immature Pro I oocytes demonstrated the presence of a minor population of $ZP2_p$ (see Figure 1A), probably due to contamination with meiotically maturing oocytes. The occurrence of any artefactual induction of $ZP2_p$, due to the release of the protease during ZP isolation, has not been considered as all eggs were stored in salt solution (1.5 mol/l $MgCl_2$) prior to ZP isolation, which renders both eggs and enzymes non-viable. To examine the possibility that the ZP2-specific protease is also released by the sperm during the acrosome reaction, Pro I ZP were incubated with supernatants from acrosome-reacted spermatozoa (acrosomal exocytosis was induced with A23187 ionophore and calcium). Immunoprecipitation analysis of ZP2 following incubation did not show any increase in $ZP2_p$ (Figure 4). In addition ZP2 from Pro 1 hemizonas incubated with and without spermatozoa (long enough to activate the acrosome reaction) was not induced to undergo further proteolysis (results not shown). This correlates with an origin of the ZP2-specific protease in the oocyte. The protease would most likely be released during cortical granule exocytosis which occurs spontaneously during meiotic maturation and following sperm–egg fusion as part of the block to polyspermy.

Glycosylation analysis of ZP2 and ZP3 from immature Pro I ZP and failed IVF

As various glycosidases have been demonstrated in mouse cortical granules (Miller *et al*., 1993), possible glycosylation differences in ZP2 and ZP3 from Pro I and failed IVF preparations were investigated (Figure 5). Iodinated heat-solubilized ZP were immunoprecipitated with anti-ZP2 and the eluted proteins subjected to digestion with various endo-

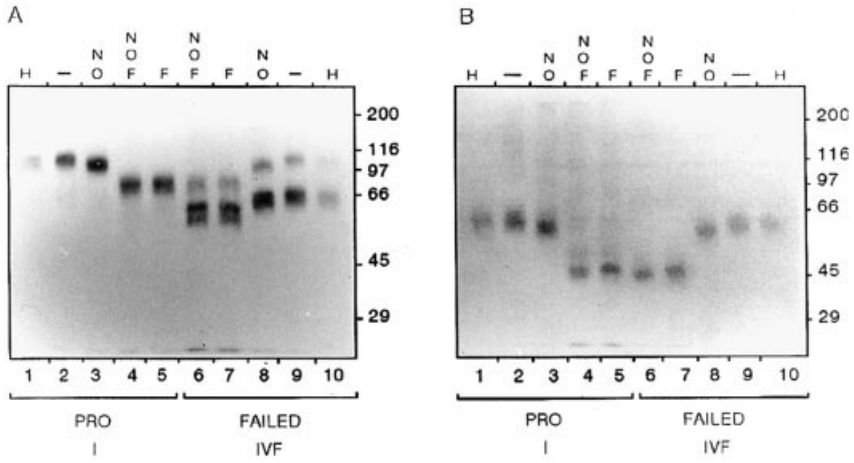

Figure 5. Glycosylation analysis of zona pellucida (ZP)2 and ZP3 from immature prophase (Pro) I oocytes and failed in-vitro fertilization (IVF). ZP2 (**A**) and ZP3 (**B**) from heat-solubilized iodinated zona were immunoprecipitated with anti-ZP2 and anti-ZP3 serum, eluted and incubated with endo H lanes (H; lanes 1 and 10), without enzyme (lanes 2 and 9), *O*-glycosidase and neuraminidase (N O; lanes 3 and 8), *N*-glycosidase F (F; lanes 5 and 7), *N*-glycosidase F, *O*-glycosidase and neuraminidase (N O F; lanes 4 and 6). Lanes 1–5, Pro I oocytes; lanes 6–10, failed IVF.

and exoglycosidases (Figure 5A). Both ZP2 and $ZP2_p$ were relatively insensitive to endo H (lanes 1, 10), indicating no high mannose *N*-linked oligosaccharides. Removal of most *O*-linked oligosaccharides with *O*-glycosidase and neuraminidase reduced ZP2 by ~5 kDa and $ZP2_p$ by only a fraction (lanes 3, 8). Removal of all *N*-linked oligosaccharides with *N*-glycosidase F (lanes 4 and 7), similar to the removal of both *O*- and *N*-linked oligosaccharides (lanes 5 and 6) demonstrated a 55/58 kDa doublet arising from $ZP2_p$ in failed IVF preparations. Following removal of all *N*-linked oligosaccharides, ZP2 is reduced by 25–30 kDa whereas $ZP2_p$ is reduced by only 10–15 kDa. This would indicate that the cleavage site(s) lie(s) in the amino terminal half of the protein which contains all the potential sites for *N*-linked oligosaccharides (Liang and Dean, 1993). No other visible differences in $ZP2_p$ were observed in the failed IVF.

Similar glycosylation analyses of ZP3 from Pro I and failed IVF preparations (Figure 5B) revealed that both preparations were relatively insensitive to endo H, indicating the absence of high mannose *N*-linked oligosaccharides (lanes 1 and 10). Removal of most *O*-linked oligosaccharides with *O*-glycosidase and neuraminidase reduced ZP3 by ~5 kDa in both preparations (lanes 3 and 8). Treatment with neuraminidase alone, which removes all terminal sialic (acid) residues, resulted in a minor reduction in size, but not as much as when used in combination with *O*-glycosidase (data not shown). Removal of all *N*-linked oligosaccharides with *N*-glycosidase F (lanes 4 and 7), similar to the removal of both *O*- and *N*-linked oligosaccharides (lanes 5 and 6) reduced the molecular weight by 10–15 kDa in both preparations.

Discussion

Studies here demonstrate that in human Pro I zonae, ZP2 and ZP3 are glycoproteins with both *O*- and *N*-linked oligosaccharides. Immunoprecipitation of solubilized Pro I zonae with anti-ZP3 serum shows that ZP3 as a 53–60 kDa glycoprotein. This is similar to other findings (Rankin *et al*., 1998), who have shown that immunoblot analysis of zonae from non-viable human eggs, with anti-ZP3 monoclonal antibodies, detects a 64 kDa (average) protein. Recombinant hZP3 has been shown to activate the acrosome reaction, suggesting a role as the primary sperm receptor (van Duin *et al*., 1994; Brewis *et al*., 1996; Whitmarsh *et al*., 1996), similar to mZP3 (Wassarman, 1988). Primary binding of spermatozoa to the egg is thought to involve receptors for mannosyl, *N*-acetylglucosaminyl and possibly fucosyl residues of ZP3 (Miranda *et al*., 1997; Benoff, 1998). Capacitated human spermatozoa express a surface plasma membrane receptor for mannose ligands and it is thought that this receptor may also bind ligands containing fucose and *N*-acetylglucosamine in a lectin-like manner (reviewed by Benoff, 1998). Pretreatment of spermatozoa with D-mannose but not with *N*-acetylglucosamine has been shown to block binding to the human zona, indicating that mannose is the more important of the two in regulating the sperm–egg interaction (Mori *et al*., 1989). Here endoglycosidase analysis shows that ZP3 appears to have only complex and no high mannose *N*-linked oligosaccharide moieties, suggesting that both core and terminal sugars within an oligosaccharide chain play a role in contributing to the tight binding between spermatozoa and the zona.

Following sperm–egg fusion, exocytosis of the cortical granules and subsequent release of proteases and glycosidases results in modification of the ZP which prevents further binding/penetration of spermatozoa on the ZP as part of the block to polyspermy. However, examination of any possible modifications to ZP3 in failed IVF reveals no visible changes. No visible glycosylation differences are apparent in ZP3, suggesting that any changes would involve mainly terminal sugars. This is similar to the mouse where no visible changes are observed in ZP3 following fertilization. This is not surprising in the mouse as specific terminal *N*-acetylglucosamine residues (Miller *et al*., 1992, 1993) as well as terminal galactose residues on *O*-linked oligosaccharides (Florman and Wassarman, 1983; Bleil and Wassarman, 1988) have been implicated in the sperm receptor activity of mZP3.

Immunoprecipitation of ZP2 in Pro I zonae with anti-ZP2 demonstrates ZP2 to be a 105–110 kDa glycoprotein. Examination of ZP2 in failed IVF reveals that ZP2 undergoes limited proteolysis in the amino terminal domain from 105–110 to 60–73 kDa. The peptides generated by proteolysis remain linked by intramolecular disulphide bonds. Previous studies on the hZP (Shabinowitz and O'Rand, 1988a,b; Bercegeay *et al*., 1995) reported that ZP1 and ZP2 co-migrate at 92–110 kDa and are only seen as individual species (90–110 kDa, ZP1; and 64–78 kDa, ZP2) following reduction. However, these studies were carried out on solubilized ZP and their identities were assigned based solely on their migration rates in SDS–PAGE. Here it has been demonstrated, using antibodies directed against ZP2 synthetic peptides, that both the 90–110 and 64–78 kDa species are ZP2, the latter arising by limited proteolysis of the former. It has been observed (Shabinowitz and O'Rand 1988a,b) that a dramatic reduction of the 90–110 kDa species in fertilized eggs occurs with a concomitant increase in the 64–78 kDa species, indicating almost complete conversion to $ZP2_p$ following fertilization. In addition, it has been observed (Moos *et al*., 1995) that hZP2 (~100 kDa) could only be observed under non-reducing conditions following fertilization. ZP1 has recently been characterized as ~60 kDa (Gupta *et al*., 1998) and most likely co-migrates with $ZP2_p$.

Similar limited proteolysis of both mouse and hamster ZP2 has been demonstrated following fertilization and artificial activation of eggs. This has been attributed to the release of a ZP2-specific protease during cortical granule exocytosis as part of the block to polyspermy (Bleil *et al*., 1981; Moller and Wassarman, 1989; Moller *et al*., 1990). Indeed a protein with an apparent molecular weight between 21 and 34 kDa has been isolated from mouse cortical granule exudates, which cleaves mZP2 *in vitro*, but not mZP1 or mZP3 (Moller *et al*., 1990). In addition, the occurrence of spontaneous cortical granule release and the appearance of proteolytically cleaved mZP2 have been shown to occur during and after meiotic maturation of mouse oocytes (Ducibella *et al*., 1990; Kalab *et al*., 1993; Okada *et al*., 1993). It has been suggested to play a role in conditioning the ZP prior to the ZP reaction (Okada

et al., 1993). A similar occurrence of a fertilization-independent release of cortical granules has been demonstrated by electron microscopy studies of maturing human oocytes (Rosseau *et al*., 1977). In addition, a recent study suggests that disappearance of the 90 kDa protein (ZP2) correlates with cortical granule loss during meiotic maturation of human oocytes (Ducibella *et al*., 1995). From the results described here, the human ZP2-specific protease most likely originates in the oocyte. There is a minor fraction of the 60–73 kDa band in immature Pro I oocytes which have not been exposed to spermatozoa. While this may be due to a possible cross-reaction of the antibodies with ZP1 (the ZP2 peptide 424–440, used in generating the antisera, has five identical amino acids to ZP1), this band disappears under non-reducing conditions and therefore more likely represents a minor portion of $ZP2_p$ due to contamination of the pool with meiotically maturing oocytes. Furthermore, acrosomal supernatants from spermatozoa induced to undergo the acrosome reaction did not induce limited proteolysis of ZP2, correlating with origin of the protease in the oocyte. $ZP2_p$, the major form of ZP2 in failed IVF, could then be due to cortical granule exocytosis which may occur either spontaneously during meiotic maturation and/or as part of the initial events in fertilization as described in the mouse.

Cortical granule release in maturing human oocytes has been demonstrated as early as Met I (Rosseau *et al*., 1977) and has been shown to increase zona resistance to sperm penetration when compared with less mature oocytes (Tesarik *et al*., 1988). It has been postulated that the zona resistance to sperm penetration during maturation of the human oocyte is counteracted by factors secreted by the cumulus cells, particularly during Met I to Met II transition (Tesarik, 1992). Interestingly in this respect, human follicular fluid has been shown to inhibit the conversion of mZP2 to $ZP2_f$ during in-vitro maturation of mouse oocytes (Kalab *et al*., 1993), suggesting that human eggs possess a ZP2-specific protease(s) with a substrate specificity similar to that of the mouse enzyme.

N-glycosidase analysis of ZP2 and $ZP2_p$ suggests two proteolytic cleavage sites among the *N*-linked sites in the amino terminal domain of $ZP2_p$. If hZP2 functions as a secondary sperm receptor similar to mouse ZP2 (Bleil and Wassarman, 1986), this would suggest a role for the amino terminal domain in the interaction with acrosome-reacted spermatozoa. Evidence that the amino terminus of ZP2 may play a role in the interaction with a secondary receptor may be obtained by analogy to the porcine model. Porcine ZP1 (92 kDa) is proteolytically cleaved at the amino terminus to give ZP2 (69 kDa) and ZP4 (23 kDa), the latter two being only visible under reducing conditions (Hedrick and Wardrip, 1987). In fact recent peptide mapping and cDNA cloning suggest the 90 kDa protein is actually ZP2 (Harris *et al*., 1994; Hassegawa *et al*., 1994). Interestingly, antisera to pZP4, which is homologous to the amino terminal domain of hZP2 (Harris *et al*., 1994; Hassegawa *et al*., 1994), blocked binding and penetration of human spermatozoa into the hZP, while antisera against pZP2 (69 kDa), which is analagous to $hZP2_p$, had no inhibitory effect on the human sperm–ZP interaction (Koyama *et al*., 1991). If the binding of hZP2 to a secondary sperm receptor involves a lectin-like mechanism, it is tempting to speculate that the proteolytic cleavages which occur in amongst the carbohydrate moieties on $ZP2_p$ interfere with this interaction by not providing the high local concentrations of complementary sugar in the correct conformation necessary for binding.

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