Characterization of a novel ZP3-independent sperm-binding ligand that facilitates sperm adhesion to the egg coat

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Summary

During mammalian fertilization, sperm adhere to the extracellular coat of the egg, or zona pellucida, in a species-specific manner. In mouse, evidence suggests that sperm recognize and bind to specific oligosaccharide ligands within the zona pellucida glycoprotein, ZP3, via β1,4-galactosyltransferase I (GalT I), a lectin-like receptor on the sperm surface. Although in vitro experiments using isolated gametes lend support to this model, recent in vivo studies of genetically altered mice question whether ZP3 and/or GalT I are solely responsible for sperm-egg binding. In this regard, sperm from GalT I-null mice bind poorly to ZP3 and fail to undergo a zona-induced acrosome reaction; however, they still bind to the ovulated egg coat in vitro.

In this report, we characterize a novel ZP3- and GalT I-independent mechanism for sperm adhesion to the egg coat. Results show that the ovulated zona pellucida contains at least two distinct ligands for sperm binding: a ZP3-independent ligand that is peripherally associated with the egg coat and facilitates gamete adhesion; and a ZP3-dependent ligand that is present in the insoluble zona matrix and is recognized by sperm GalT I to facilitate acrosomal exocytosis. The ZP3-independent ligand is not a result of contamination by egg cortical granules, nor is it the mouse homolog of oviduct-specific glycoprotein. It behaves as a 250 kDa, WGA-reactive glycoprotein with a basic isoelectric point, distinguishing it from the acidic glycoproteins that form the insoluble matrix of the egg coat. When eluted from isoelectric focusing gels, the acidic matrix glycoproteins possess sperm-binding activity for wild-type sperm, but not for GalT I-null sperm, whereas the basic glycoprotein retains sperm-binding activity for both wild-type and GalT I-null sperm. Thus, GalT I-null sperm are able to resolve gamete recognition into at least two distinct binding events, leading to the characterization of a novel, peripherally associated, sperm-binding ligand on the ovulated zona pellucida.

Key words: Fertilization, OGP, Sperm, Zona pellucida, ZP3, Mouse

Introduction

Gamete interaction is an obligatory event during mammalian fertilization when sperm bind to the egg in a species-specific manner. The most widely discussed models suggest that a lectin-like receptor on the sperm surface recognizes a specific carbohydrate ligand on the egg coat. The sperm receptor subsequently activates intracellular signaling cascades that culminate in exocytosis of the acrosomal vesicle from the sperm head. The released acrosomal contents enable sperm to penetrate the egg coat, fuse with the egg plasma membrane and fertilize the egg (Talbot et al., 2003).

Much of our current understanding of mammalian gamete recognition comes from studies in mouse. This is due, in large part, to the ability to perform quantitative in vitro assays of sperm-egg binding along with biochemical analysis of mouse gametes, as well as the ability to manipulate the mouse genome. Pioneering studies by Wassarman and colleagues suggest that in mouse, sperm bind to a specific class of oligosaccharides on ZP3 (also referred to as ZPC) (Spargo and Hope, 2003), one of the three glycoproteins that constitute the extracellular coat of the mouse egg, or zona pellucida. This was demonstrated by the ability of purified soluble ZP3, as well as its oligosaccharide chains, to competitively inhibit sperm-egg binding in vitro (Bleil and Wassarman, 1980a; Florman and Wassarman, 1985). Binding of ZP3 leads to sperm activation of both pertussis toxin (PTx)-sensitive heterotrimeric G-proteins as well as voltage-independent and -dependent cation channels that result in elevated pH_i and $Ca^{2+}{}_i$, thus triggering acrosomal exocytosis (Arnoult et al., 1996; Endo et al., 1987; Endo et al., 1988; O'Toole et al., 2000).

The sperm receptor for ZP3 oligosaccharides has been more difficult to identify, but most evidence is consistent with sperm surface β1,4-galactosyltransferase I (GalT I) performing this function. GalT I specifically binds to the same class of ZP3 oligosaccharides that possess sperm-binding activity, and removing or masking the GalT I binding site on these oligosaccharides removes their sperm-binding activity (Miller et al., 1992). The cytoplasmic domain of GalT I binds, directly or indirectly, to heterotrimeric G proteins that are activated following ZP3-induced aggregation of GalT I (Gong et al., 1995). In support of this, ectopic expression of GalT I on *Xenopus* oocytes results in ZP3-specific binding and G-protein activation, and mutagenesis of the GalT I cytoplasmic domain prevents ZP3-dependent G-protein activation (Shi et al., 2001). Transgenic sperm that overexpress GalT I bind more ZP3 than do normal sperm, have accelerated G-protein activation and undergo precocious acrosome reactions (Youakim et al., 1994). By contrast, sperm in which surface GalT I has been eliminated by homologous recombination, but which maintain normal intracellular galactosylation, no longer bind ZP3 in vitro or undergo zona-induced acrosomal exocytosis (Lu and Shur, 1997).

Despite these, and many other observations, the apparent role of ZP3 and GalT I in sperm-egg binding has recently been thrown into question. For example, GalT I-null sperm still retain the ability to bind to the ovulated egg coat, despite their inability to bind ZP3 in solution or undergo zona-induced acrosome reactions (Lu and Shur, 1997). This binding enables GalT I-null sperm to penetrate the zona pellucida matrix, presumably via spontaneous acrosome reactions, and fertilize eggs (although at only 7% the efficiency of wild-type sperm when assayed in vitro). Similarly, well-defined oligosaccharides that are not predicted to be substrates for GalT I competitively inhibit sperm-egg binding (Amari et al., 2001; Bendahmane et al., 2001; Johnston et al., 1998; Loeser and Tulsiani, 1999). Even though these inhibitory oligosaccharides do not appear to be present within the zona pellucida (Aviles et al., 1999; Aviles et al., 2000b), their ability to inhibit binding speaks against a role for GalT I in initial gamete adhesion. Finally, evidence that ZP3 is not the only sperm-binding ligand in the egg coat comes from the observation that mouse sperm still bind to eggs in which the mouse ZP3 polypeptide has been replaced by human ZP3 (Rankin et al., 1998; Rankin et al., 2003). Some of this confusion probably results from the fact that the biological activity of putative gamete receptors is usually assayed by competitive inhibition of sperm binding to the coats of ovulated eggs, whereas the source of competitive ligands for these experiments is frequently the ovarian zona pellucida. At any rate, all of these observations suggest that sperm binding to the zona pellucida may involve multiple receptor-ligand interactions of which ZP3 binding to GalT I may be one component.

In this report, we test the hypothesis that gamete adhesion involves a ZP3- and GalT I-independent receptor-ligand interaction. We have detected and characterized a novel ligand for sperm that is present in the ovulated zona pellucida, but not in the ovarian zona pellucida. This ligand activity does not result from contamination by egg cortical granules or by ZP3. In addition, this ligand is shown to be distinct from the mouse ortholog of oviduct-specific glycoprotein (OGP), which has been implicated in sperm-egg adhesion in hamster (Boatman and Magnoni, 1995). Lectin depletion studies and lectin-blotting of two-dimensional polyacrylamide gels indicate that the ovulated zona pellucida contains a relatively basic, high molecular weight, WGA-reactive glycoprotein that is not present in the ovarian zona pellucida. This protein, when eluted from isoelectric focusing gels, possesses sperm-binding activity for both wild-type and GalT I-null sperm, whereas ZP3 possesses sperm-binding activity for only wild-type sperm. These results suggest that the WGA-reactive, basic glycoprotein within the ovulated zona pellucida functions as a ZP3- and OGP-independent ligand that facilitates gamete adhesion.

Materials and methods

Preparation of zona glycoproteins

Ovarian zona glycoproteins were purified from CD-1 female mice (Charles River) as described (Bleil and Wassarman, 1986). Ovulated zona glycoproteins were purified from 8-week-old CD-1 superovulated females. Cumulus-oophorous masses were collected in modified Krebs-Ringer buffer (dmKRBT) and transferred to 0.2% hyaluronidase. Cumulus-free eggs were collected with a glass pipette twice the diameter of the egg and washed through three drops of either phosphate-buffered saline (PBS)/0.1% polyvinylpyrrolidone (PVP) or Tyrode's solution (Hogan et al., 1994). The zona pellucida was solubilized by one of three methods: heating the eggs to 65°C; replacement of the buffer with Tyrode's (pH 2.5); or replacement of the buffer with 0.04 N HCl (subsequently neutralized with NaOH). The biological activities of the solutions were equal under all preparation conditions. Zona glycoprotein preparations were stored at –80°C until the day of assay. The peripheral and matrix fractions of the ovulated zona pellucida were prepared by triturating the washed eggs 15 times in the same drop with a glass pipette of diameter equal to that of the egg. The wash solution was collected and called the 'peripheral fraction'. The washed zona pellucida surrounding the eggs was then solubilized as above and called the 'matrix fraction'.

Sperm-egg binding assay

Ovulated eggs were collected from superovulated CD-1 females and cleaned of cumulus cells as described. Two-cell embryos were collected into dmKRBT from the oviducts of superovulated females that were mated 15 hours earlier. The caudae epididymides of strain-matched wild-type and long isoform GalT I-null sperm (Lu and Shur, 1997) were dissected into dmKRBT and shredded. The epididymides were incubated for 15 minutes at 37°C to release the sperm, which were collected after filtration (3-35/27 Nitex, Sefar America, Kansas City, MO). The sperm suspension was centrifuged at 66 ***g*** for 5 minutes at 24°C and resuspended in fresh medium containing 10 µg/ml pertussis toxin (CalBiochem, San Diego, CA), to prevent sperm from undergoing zona-induced acrosome reactions that would artificially reduce the number of sperm bound to the egg. Sperm were capacitated at 37°C for 1 hour and diluted to a final concentration of 4×10^6 motile sperm/ml. Forty-thousand sperm were incubated in 50 µl drops of dmKRBT containing 30-40 ovulated eggs and 5-10 embryos (as a control for non-specific binding) for 30 minutes at 37°C. The incubation solution contained either the purified zona glycoproteins or an equal volume of buffer as control. Eggs and embryos were washed through sequential drops of dmKRBT until one to three sperm remained bound to the embryos. The gametes were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) and the number of sperm bound to each egg and embryo was counted at 200× magnification using phase-contrast optics. The average number of sperm bound/embryo was subtracted from the average number of sperm bound/ovulated egg. The average of triplicate drops for each time point was determined and normalized so that the number of sperm bound in the control is equal to 100%. The data presented are the average of at least three experiments (±s.e.m.).

Cortical reaction assay

The amount of contaminating cortical granule material in the zona pellucida preparations (50 egg equivalents) was measured by assaying for *N*-acetylglucosaminidase (GlcNAc'ase) as described (Miller et al., 1993). The reaction product was determined fluorometrically using a Perkin Elmer LS50B instrument (Beaconsfield, UK) at an excitation wavelength of 380 nm, an emission wavelength of 460 nm and a slit width of 2.5 nm. Fluorescence produced by the substrate solution alone was subtracted as background from the readings of the zona pellucida solutions. To determine the maximum amount of cortical granule material released, eggs were incubated with 10 µM A23187 for 30 minutes at 37°C prior to preparation of each zona pellucida fraction. Aliquots of the solutions assayed for GlcNAc'ase activity were subsequently tested for biological activity in the sperm-egg binding assay at a concentration of three zona equivalents/µl.

Anti-mouse ZP3 immunoblot

Twenty-five, 50, 100 and 200 egg equivalents of the peripheral and matrix fractions of ovulated zona glycoproteins were solubilized in reducing sample buffer and fractionated by gel electrophoresis. The proteins were transferred to PVDF (Millipore) and blocked with 5% milk, 0.05% Tween 20, 1×PBS. The blots were incubated with a 1:1000 dilution of IE-10, a rat monoclonal antibody against mouse ZP3 residues 336-342 (East et al., 1985), and subsequently in a 1:1000 dilution of sheep anti-rat IgG-HRP (Amersham). The blots were washed and the signal developed by chemiluminescence (ECL-Plus, Amersham). The blots shown are representative of two experiments.

Fecundity of GalT-null males crossed with OGP-null females

Twelve-week-old GalT I-null males were caged with at least three 6-week-old *Ogp*$^{+/+}$ or *Ogp*$^{-/-}$ females (Araki et al., 2003) for a period of 3 months. The average litter size resulting from these matings was calculated (±s.d.).

Lectin depletion of biological activity

WGA-agarose or BS-I agarose beads [100 μl (50% slurry)] (Vector Laboratories, Burlingame, CA) were pelleted at 1000 ***g*** for 5 seconds, washed 10 times in 1×PBS containing 0.1 mM $CaCl_2$ and resuspended in 50 μl wash solution. This slurry (20 μl) was blocked in 100 μl 0.1% PVP in wash solution at 4°C for 2 hours. The beads were subsequently resuspended in 1000 egg equivalents of the matrix or peripheral fraction prepared in 0.1% PVP/PBS and incubated at 4°C for 2 hours. As controls, 1000 egg equivalents of each solution were incubated in parallel in the absence of the lectin-agarose beads, or alternatively, lectin-agarose beads were incubated with buffer rather than with zona glycoproteins. Following incubation, the beads were pelleted at 1000 ***g*** for 5 seconds and the supernatant removed. Five-hundred egg equivalents of the depleted solution or the undepleted control were assayed for biological activity in the sperm-egg binding assay.

Lectin blot of zona glycoproteins

One-thousand egg equivalents of the peripheral fraction were solubilized in reducing sample buffer and fractionated by gel electrophoresis. The proteins were transferred to PVDF and subsequently blocked in 1% BSA, 0.1% Tween-20, 0.9%NaCl, 50 mM Tris•Cl, pH 7.4. To detect glycoproteins, the membranes were incubated with 1 μg/ml of biotinylated-WGA or BS-I (Sigma). The membranes were washed and subsequently probed with a 1:50,000 dilution of streptavidin-HRP (Zymed, S. San Francisco, CA). After washing, the signal was developed by chemiluminescence.

Two-dimensional polyacrylamide gel electrophoresis of zona glycoproteins and characterization by lectin blot

Five-hundred egg equivalents of ovarian (2.5 μg) and ovulated zona glycoproteins were precipitated by mixing with eight volumes of ice-cold acetone and incubating overnight at –20°C. The proteins were pelleted by centrifugation at 3000 ***g*** for 15 minutes at 4°C. After draining the acetone, the proteins were dried briefly at room temperature and then solubilized in IEF sample buffer [9.5 M recrystallized urea, 2% deionized NP-40, 5% β-mercaptoethanol (BioRad), 1.6% Servalyt 5-7 and 0.4% Servalyt 3-10 isodalt (Crescent Chemicals)] for 30 minutes at 24°C. Prior to loading the protein solution, urea-acrylamide tube gels (dimensions=5.5×0.1 cm) were cast according to the manufacturer's directions (BioRad, Hercules, CA). The pH gradient was established by electrophoresing gels in 10 mM NaOH and 10 mM H_3PO_4 for 10 minutes at 200 V, 15 minutes at 300 V and 15 minutes at 500 V. The buffers were replaced and the sample was loaded directly onto the surface of the gel and overlaid with 9 M recrystallized urea, 0.8% Servalyt 5-7, 0.2% Servalt 3-10, isodalt and 0.05% Bromophenol Blue. The proteins were electrophoresed at 500 V for 10 minutes and 750 V for 3.5 hours (until equilibrium). The gels were stored in SDS-equilibration buffer (62.5 mM Tris•Cl, pH 6.8, 2.3% SDS, 8% glycerol, 0.05% Bromophenol Blue) at –80°C. The IEF gels were warmed to 24°C and equilibrated for 30 minutes with gentle agitation. The gels were transferred into the well of a 4-12% gradient polyacrylamide gel (Jule, Milford, CT) and covered with agarose solution (1% low M_r agarose, 0.1% SDS, 125 mM Tris•Cl, pH 6.8). The proteins were fractionated by electrophoresis, transferred to PVDF, and then probed with biotinylated WGA as described.

Purification of the ZP3-independent ligand from IEF gels

Ovulated zona glycoproteins (5000 egg equivalents) were fractionated by IEF as described above. After electrophoresis, the tube gel was sliced into 2 mm pieces and transferred into siliconized 1.5 ml tubes. The gel pieces were incubated in 200 μl of 50 mM NH_4HCO_3, pH 7.6 containing 1 μg/ml fatty acid-free BSA for 12 hours at 4°C. This was repeated two additional times, after which the three wash solutions were combined and incubated with 0.12 g BioBeads (BioRad, Hercules, CA) for 15 minutes at 24°C. The solutions were concentrated to 100 μl and dialyzed against 50 mM NH_4HCO_3 for 1 hour at 24°C. The solutions were dried and the proteins washed twice in double-distilled H_2O before being solubilized in dmKRBT and tested in the sperm-egg binding assay.

Results

Zonae pellucidae isolated from ovarian and ovulated oocytes contain distinct biological activities

ZP3 purified from either the ovulated or ovarian zona pellucida competitively inhibits sperm-egg binding and induces the acrosome reaction in wild-type sperm (Bleil and Wassarman, 1980a; Bleil and Wassarman, 1986). Although GalT I-null sperm do not bind ZP3 (Lu and Shur, 1997), they still bind to the zona pellucida of ovulated eggs. Therefore, we determined whether the zona pellucida contains any other ligand activity that may be recognized by GalT I-null sperm. The presence of sperm-binding activity was assayed by the ability of solubilized zona glycoproteins to competitively inhibit sperm-egg binding, as originally used for the identification of ZP3 (Bleil and Wassarman, 1980a). Initially, wild-type or GalT I-null sperm were incubated with ovulated eggs in the presence of soluble ovarian zona glycoproteins (Fig. 1A). Consistent with published results, ovarian zona glycoproteins inhibited the binding of wild-type sperm to ovulated eggs in a concentration-dependent manner. By contrast, ovarian zona glycoproteins failed to competitively inhibit GalT I-null sperm binding to ovulated eggs. This result is consistent with previous evidence demonstrating that GalT I-null sperm do not bind ZP3 and indicates the absence of any other binding ligand in the ovarian zona pellucida for GalT I-null sperm.

Nevertheless, GalT I-null sperm still bind to ovulated eggs, which predicts that the ovulated, but not the ovarian, zona pellucida contains a ligand for GalT I-null sperm. To test this hypothesis, wild-type or GalT I-null sperm were incubated with ovulated eggs in the presence of solubilized ovulated zona glycoproteins (Fig. 1B). Unlike that seen with ovarian zona glycoproteins, zona glycoproteins from ovulated oocytes inhibited both wild-type and GalT I-null sperm binding, and did so in a concentration-dependent manner. Thus, as predicted, the ovulated zona pellucida contains a ligand to which both wild-type and GalT I-null sperm bind and which is absent from the ovarian zona pellucida.

The lack of ligand activity for GalT I-null sperm in ovarian

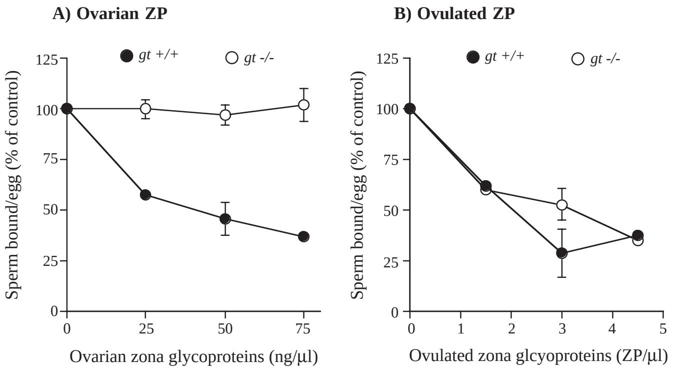


Fig. 1. Ovulated, but not ovarian, zona pellucida (ZP) glycoproteins contain a ligand that competitively inhibits GalT I-null sperm binding to ovulated eggs. (A) Inhibition of wild-type and GalT I-null sperm binding to ovulated eggs with 0-75 ng/µl solubilized ovarian ZP. (B) Inhibition of wild-type and GalT I-null binding with 0-5 zonae equivalents solubilized ovulated ZP. Each point represents the mean±s.e.m.; n=3 experiments. In a single experiment, the value for each data point represents the average of three determinations (for a total of nine assays per data point).

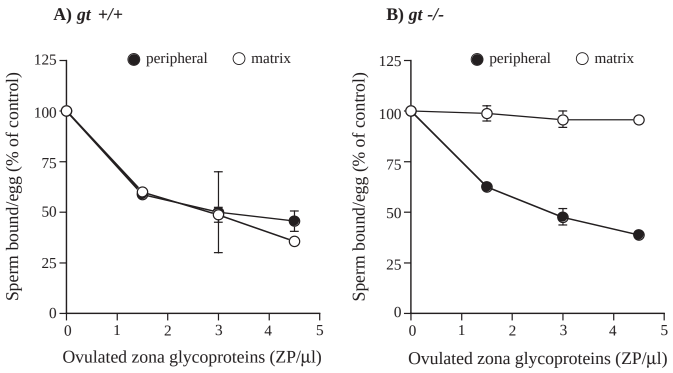


Fig. 2. Wild-type and GalT I-null sperm binding to ovulated eggs is competitively inhibited by a ligand peripherally associated with the ovulated zona pellucida (ZP). (A) Inhibition of wild-type sperm binding by glycoproteins solubilized from either the matrix fraction or peripheral fraction. (B) Inhibition of GalT I-null sperm binding by the matrix and peripheral fractions. Each point represents the mean±s.e.m.; n=3 experiments. In a single experiment, the value for each data point represents the average of three determinations (for a total of nine assays per data point).

zona glycoproteins predicts that GalT I-null sperm should be incapable of binding directly to the intact zona pellucida of ovarian oocytes. Zona-intact oocytes were collected from antral follicles, and their follicular cells removed following prolonged hyaluronidase digestion. GalT I-null sperm bound to zona-intact antral oocytes at 50% the level of binding to ovulated oocytes (11±1 sperm/ovarian oocyte versus 21±2 sperm/ovulated oocyte). However, we suspect that the 50% binding seen towards antral oocytes is an artifact of preparation, as the prolonged digestion with hyaluronidase required to remove follicular cells increased the binding of GalT I-null sperm to both ovarian and ovulated eggs (data not shown). To test more directly for the presence of ligand activity towards GalT I-null sperm, the zonae pellucidae of antral oocytes were solubilized and assayed in competitive sperm-egg binding assays; no inhibition of GalT I-null sperm binding occurred, whereas the solubilized zona glycoproteins readily inhibited wild-type sperm-egg binding (similar to that in Fig. 1). Collectively, these results suggest that GalT I-null sperm recognize a ligand present primarily, if not exclusively, on ovulated oocytes, whereas wild-type sperm recognize ligands in both the ovarian and ovulated egg coat.

Although GalT I-null sperm are unable to bind ZP3 or undergo ZP3-induced acrosome reactions, a small number of sperm (i.e. 7% of wild-type) are able to penetrate the zona pellucida in vitro (Lu and Shur, 1997). This low level of zona penetration is thought to account for the fertility of GalT I-null males in vivo, which may result from spontaneous acrosome reactions or other ‘non-physiological’ inducers of acrosomal exocytosis. In any event, we tested whether the sperm-binding ligand present in the ovulated zona could induce acrosomal exocytosis in GalT I-null sperm. This was shown not to be the case; ovulated zona glycoproteins failed to induce acrosomal exocytosis in GalT I-null sperm but, as expected, did induce acrosome reactions in wild-type sperm, presumably owing to the presence of ZP3 (data not shown).

The ovulated zona pellucida contains a peripherally associated ligand for sperm binding

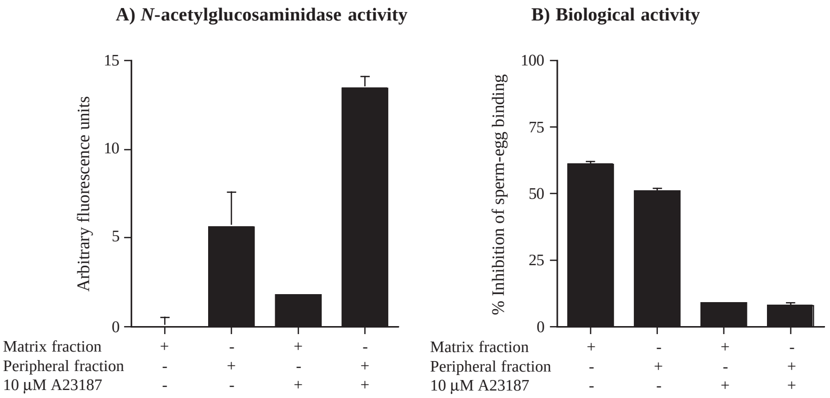
At the time of ovulation, epithelial cells lining the oviduct are actively secreting glycoproteins into the lumen where fertilization occurs. Some of these components have been postulated to play a role in maintenance of the oocyte, gamete interaction and development of the embryo (Buhi et al., 2000). We therefore tested whether the sperm-binding ligand in the ovulated zona pellucida resulted from addition to the zona pellucida during transit into the oviduct. Cumulus cell-free ovulated eggs were stringently washed to remove any material loosely associated with the zona pellucida, as described in the Materials and methods, and the wash solution was collected as the ‘peripheral fraction’. The remaining intact washed zona pellucida was solubilized as the ‘matrix fraction’. Both the peripheral and matrix fractions were tested for ligand activity in the competitive sperm-egg binding assay.

As expected, the matrix fraction inhibited wild-type sperm binding to eggs in a concentration-dependent manner (Fig. 2A), but had no activity against GalT I-null sperm (Fig. 2B). Thus, the matrix fraction recapitulates the activity of the ovarian zona glycoproteins, indicating that it contains ZP3 but not the ligand to which GalT I-null sperm bind. By contrast, the peripheral fraction inhibited the binding of both wild-type and GalT I-null sperm to eggs in a concentration-dependent manner. This demonstrates that the ovulated zona pellucida contains two distinct ligand activities: ZP3 associated with the insoluble matrix and a ZP3-independent component that is peripherally associated with the zona pellucida. The fact that the ZP3-independent ligand can be removed from the zona pellucida by stringent washing suggests it results from addition to the egg coat upon entry into the oviduct, and not from covalent modification of the zona matrix.

The ligand in the peripheral fraction is not a contaminant from the egg or from ZP3

To eliminate the possibility that the ligand activity in the

Fig. 3. The ligand in the peripheral fraction of the ovulated zona pellucida (ZP) is not a contaminant of the cortical granules. (A) *N*-acetylglucosaminidase activity of fractions of the ovulated ZP (three ZP equivalents/μl) prepared from eggs treated with 10 μM A23187 to induce the cortical reaction or DMSO, as control. (B) Biological activity of the ovulated ZP fractions (three ZP equivalents/μl) prior to and after treatment of the eggs with 10 μM A23187. Each bar represents the mean±s.d.; *n*=2 experiments. In a single experiment, the value for each data point represents the average of three determinations (for a total of six assays per data point). The peripheral fraction contains variable amounts of GlcNAc'ase, but this contribution does not account for its biological activity.



peripheral fraction is due to contamination by egg-released material during preparation, we assayed the individual zona pellucida fractions, as well as intact eggs and isolated zona, for *N*-acetylglucosaminidase (GlcNAc'ase) activity. This enzyme is highly concentrated in cortical granules and serves as a marker for the release of cortical granule contents (Miller et al., 1993).

No GlcNAc'ase activity above background was detected in the intact ovulated zona pellucida or in the solubilized matrix fractions (five different preparations assayed) (data not shown). This result argues against the possibility that the ZP3-independent ligand results from egg-derived material, as the intact ovulated zona pellucida contains both ZP3 and ZP3-independent ligands (Fig. 1).

However, preparing the peripheral fraction resulted in variable levels of GlcNAc'ase activity, ranging from 5-16% of the total enzyme activity detectable in eggs (1.7±0.6 to 5.2±0.5 fluorescence units relative to 32.2±5.4 fluorescent units in ovulated eggs). Thus, it remained a formal possibility that this contamination was responsible for the sperm-binding activity in the peripheral fraction. To test this possibility, we maximized the amount of potential egg-released material by treating eggs with the calcium ionophore A23187 to induce the cortical reaction, and the peripheral fraction was collected and assayed for GlcNAc'ase activity and sperm-binding activity.

As expected, treatment with A23187 increased the GlcNAc'ase activity of both the matrix and peripheral fractions (Fig. 3A) and of the intact ovulated zona pellucida (data not shown). However, A23187 treatment reduced the ability of the peripheral and matrix fractions to inhibit wild-type sperm binding, relative to solutions prepared from DMSO-treated eggs (Fig. 3B). This result indicates that egg-released material is not the source of the ligand in the peripheral fraction and, in fact, increasing the amount of contaminating egg material decreases the biological activity of the ligand, probably as a result of proteases and glycosidases released from cortical granules.

Previous studies have demonstrated that GalT I-null sperm do not bind soluble ZP3 (Lu and Shur, 1997). Nevertheless, we felt it important to eliminate the possibility that ZP3 may be present in the peripheral fraction and contribute to its sperm-binding activity. We took two approaches to this problem. First, we tested the ability of anti-ZP3 antibodies to inhibit the binding of either wild-type and/or GalT I-null sperm to ovulated eggs. As expected, the anti-ZP3 monoclonal antibody (East et al., 1985) prevented wild-type sperm, but not GalT I-null sperm, from binding to ovulated eggs. The control IgG did not affect binding of either wild-type or GalT I-null sperm (Fig. 4A). In agreement with previous results, these data suggest that GalT I-null sperm binding to the ovulated zona pellucida is independent of ZP3 (Lu and Shur, 1997). Second, we confirmed that ZP3 is not released from the zona matrix during the preparation of the peripheral fraction by probing both fractions with anti-ZP3 antibody (Fig. 4B). The antibody reacted strongly with as few as 25 egg equivalents of protein in the matrix fraction. By contrast, the antibody failed to react

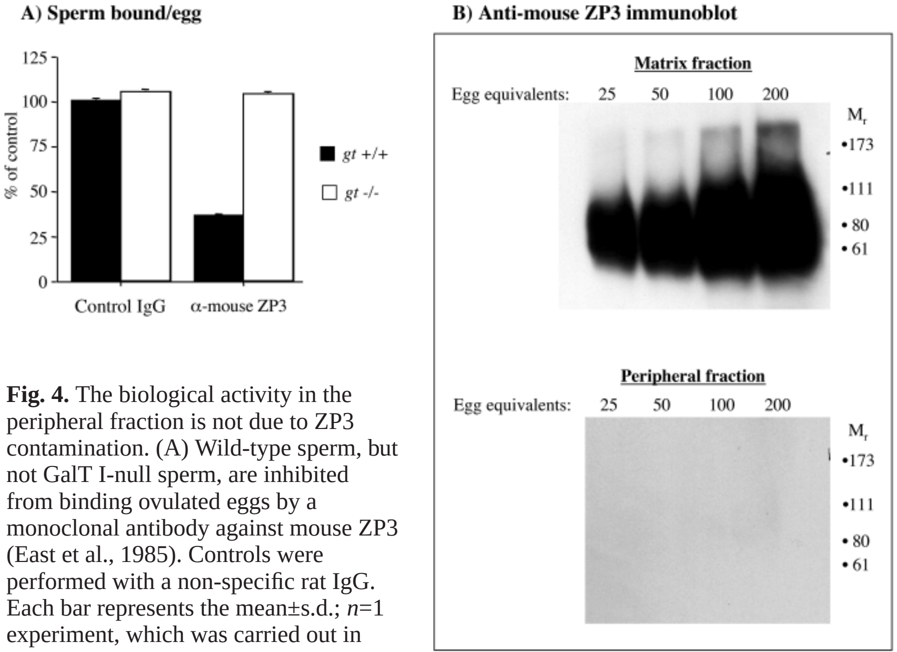


Fig. 4. The biological activity in the peripheral fraction is not due to ZP3 contamination. (A) Wild-type sperm, but not GalT I-null sperm, are inhibited from binding ovulated eggs by a monoclonal antibody against mouse ZP3 (East et al., 1985). Controls were performed with a non-specific rat IgG. Each bar represents the mean±s.d.; *n*=1 experiment, which was carried out in triplicate. (B) Immunoblot of matrix and peripheral fraction proteins from ovulated zonae pellucidae with a rat monoclonal antibody against mouse ZP3. Molecular weight markers are shown.

Table 1. GalT I-null males fertilize *Ogp*$^{-/-}$ females normally, and their sperm bind to *Ogp*$^{-/-}$ eggs at normal levels

(A) In vivo fertility*

Female genotype	Litter size (after mating with GalT I-null male)
Ogp$^{+/+}$	6 (±5); *n*=3
Ogp$^{-/-}$	8 (±2); *n*=5

(B) In vitro sperm-egg binding[†]

	Number of GalT I-null sperm/egg		
Female genotype	10^4 sperm/ml (*n*=2)	10^5 sperm/ml (*n*=1)	10^6 sperm/ml (*n*=1)
Ogp$^{+/+}$	4 (±1)	17 (±1)	34 (±1)
Ogp$^{-/-}$	2 (±0)	17 (±2)	32 (±0)

*Twelve-week-old GalT I-null males were mated with at least three 6-week-old females for a period of 3 months. The litter sizes (±s.d.) resulting from wild-type (*Ogp*$^{+/+}$), heterozygous (not shown), and OGP-null (*Ogp*$^{-/-}$) females were similar in size to one another. *n*, number of litters sired by one (*Ogp*$^{+/+}$) or two (*Ogp*$^{-/-}$) GalT I-null males.

[†] The binding of sperm from GalT I-null males to oocytes from either wild-type (*Ogp*$^{+/+}$), heterozygous (not shown), or OGP-null (*Ogp*$^{-/-}$) females was determined as described in the Materials and methods. Three different concentrations of sperm were assayed, from one or two males, each of which was conducted in triplicate. GalT I-null sperm bound similarly to oocytes from all three genotypes (sperm bound/oocyte±s.e.m.).

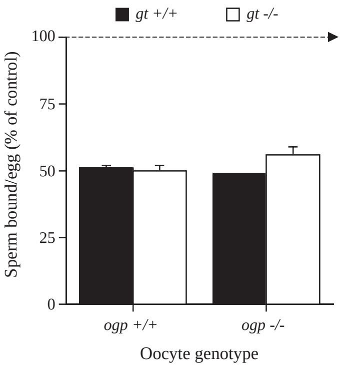


Fig. 5. Oviduct-secreted glycoprotein (OGP) is not the peripherally associated, ZP3-independent ligand. Inhibition of wild-type and GalT I-null sperm binding to eggs by ovulated zona glycoproteins (three ZP/µl) prepared from wild-type and OGP-null females (Araki et al., 2003). Each bar represents the mean±s.d.; *n*=1 experiment, which was carried out in triplicate. Wild-type and GalT I-null sperm are equally inhibited from binding to ovulated eggs by zona glycoproteins isolated from wild-type and OGP-null females. The broken line indicates the number of sperm bound in the control (buffer).

with any protein in the peripheral fraction, although 200 egg equivalents of this solution has the same amount of total protein as 25 egg equivalents of the matrix fraction (data not shown). Taken together, results in Figs 3 and 4 indicate that the peripheral fraction contains a sperm-binding ligand that is not due to contamination by egg-derived material or by ZP3.

The ZP3-independent ligand is not oviduct-secreted glycoprotein (OGP)

The most extensively studied of the oviduct-derived glycoproteins is oviduct-secreted glycoprotein (OGP), which, in hamster, is secreted at the time of ovulation, adheres to the zona pellucida and may promote zona-binding and penetration (Boatman and Magnoni, 1995; Kan et al., 1990; Robitaille et al., 1988; St-Jacques et al., 1992). We therefore tested directly whether OGP functions as the ZP3-independent ligand identified here. We reasoned that GalT I-null males should be unable to fertilize OGP-null females and unable to bind OGP-null oocytes if, in fact, OGP is the ZP3-independent ligand. However, when GalT I-null males were bred with wild-type or OGP-null females, the average litter sizes resulting from these matings were similar to one another (Table 1A), indicating that GalT I-null sperm are equally capable of fertilizing wild-type or OGP-null eggs in vivo. Similarly, at three different sperm concentrations, equivalent numbers of GalT I-null sperm bound to ovulated eggs isolated from wild-type or OGP-null females (Table 1B). To confirm that OGP does not function as the ZP3-independent ligand, soluble zona glycoproteins were prepared from ovulated eggs isolated from wild-type or OGP-null females, and tested for sperm-binding activity (Fig. 5). Similar to that shown in Fig. 1, zona glycoproteins from both wild-type and OGP-null females inhibited the binding of both wild-type and GalT I-null sperm to ovulated eggs. This indicates that ovulated zona glycoproteins from OGP-null females still contain the ZP3-independent ligand. Taken together, these experiments demonstrate that the sperm-binding ligand in the ovulated zona pellucida is neither ZP3 nor OGP, and is, therefore, a previously uncharacterized component of the ovulated egg coat.

The ZP3- and OGP-independent ligand is a high molecular weight, WGA-reactive molecule

We predicted that the peripherally associated ligand is a secreted molecule, and therefore glycosylated, and asked if its biological activity could be removed from the peripheral fraction by binding to lectin-agarose beads. The matrix and peripheral fractions were incubated with either wheat germ agglutinin (WGA) agarose or *Bandeiraea simplicifolia I* (BSI) agarose, lectins that bind *N*-acetylglucosamine and sialic acid residues, or galactosyl α(1-3) galactose disaccharides, respectively. The lectin beads and their bound glycoproteins were removed, and the ligand activity in the depleted supernatants was determined using the sperm-egg binding assay (Fig. 6A). WGA was able to deplete sperm-binding activity in both the matrix and peripheral fractions, as assayed by higher numbers of sperm binding to the zona pellucida in the presence of the WGA-depleted fractions, relative to non-depleted fractions. By contrast, BSI did not deplete either fraction of biological activity. Supernatants from mock-incubated controls, containing buffer rather than zona glycoproteins, had no effect on sperm-zona binding (data not shown), demonstrating that the effects were due to lectin-specific depletion of zona glycoproteins, rather than to any lectin that may have leached into the supernatant.

To determine if the ability of WGA to deplete biological activity from the peripheral fraction correlated with any molecular species, the glycoproteins in the peripheral fraction were examined by lectin-blotting (Fig. 6B). WGA reacted strongly with a high molecular weight species in the peripheral fraction, whereas BSI failed to react with any components in this fraction. Thus, biological activity in the peripheral fraction correlates with a WGA-reactive, high molecular weight glycoprotein.

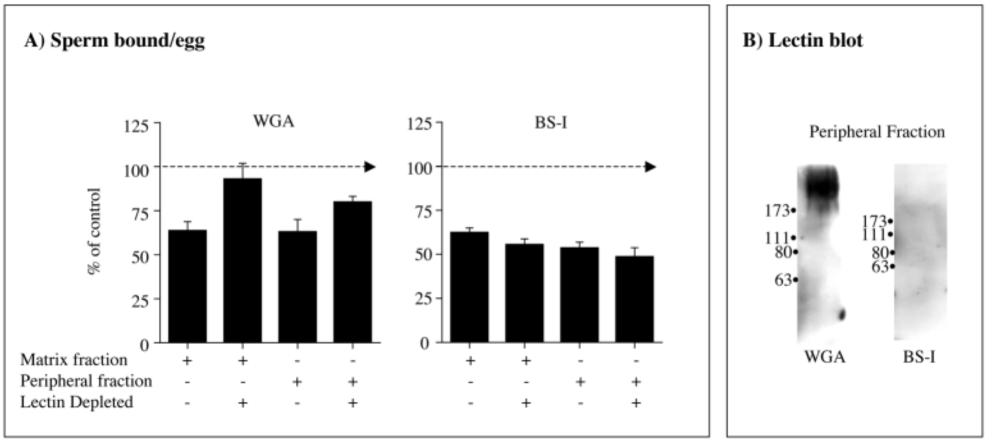


Fig. 6. The peripheral fraction contains a WGA-reactive high molecular weight glycoprotein that correlates with the biological activity of the ligand. (A) Biological activity of matrix and peripheral fractions before and after depletion with WGA-agarose or BS-I agarose. WGA depletes ligand activity, assayed against wild-type sperm, from both the matrix and peripheral fractions, whereas BS-I fails to deplete activity from either fraction. Each bar represents the mean±s.e.m. of triplicate assays from one experiment. The broken line indicates the number of sperm bound in the control (buffer). (B) WGA and BSI lectin blots of peripheral fraction glycoproteins. WGA reacts with high molecular weight glycoproteins in the peripheral fraction, whereas BSI does not react with any glycoprotein species. Each blot is representative of at least two experiments. Molecular weight markers are shown.

Multiple attempts to purify the biological activity from the peripheral fraction proved unsuccessful because of limiting amounts of protein (estimated at ~1 ng per egg equivalent) and variable amounts of biological activity, which is probably the result of cortical granule damage during preparation. Therefore, we returned to the original source of the peripherally associated ligand, the ovulated zona pellucida, and compared the migration of WGA-reactive proteins in ovarian and ovulated zonae by two-dimensional SDS-PAGE (Fig. 7). As expected, both the ovarian and ovulated zonae pellucidae consist of heavily glycosylated, relatively acidic proteins that migrate with a pattern consistent with ZP1, ZP2 and ZP3 (Bleil and Wassarman, 1980b). Furthermore, the ovulated zona pellucida contains a WGA-reactive, relatively basic protein of ~250 kDa that is absent from the ovarian zona pellucida. We determined that this glycoprotein originated from the zona pellucida and was not a contaminant of the hyaluronidase or buffers used to prepare the zona glycoproteins (data not shown). The molecular weight of this protein is comparable with the WGA-reactive protein detected in the peripheral fraction by one-dimensional SDS-PAGE, although the conditions of the electrophoresis and the molecular weight standards differed between the two experiments.

The ability of WGA to deplete biological activity from the peripheral fraction is consistent with the ~250 kDa, basic protein being the ZP3- and OGP-independent ligand. We tested this possibility more directly by isolating proteins resolved by isoelectric focusing (IEF) and assaying them for ligand activity in the sperm-egg binding assay (Fig. 8). IEF gels were divided into four fractions ranging from the most acidic region of the gel containing the matrix glycoproteins (fraction 1) to the most basic region of the gel containing the high molecular weight, WGA-reactive species (fraction 4). Proteins were eluted from each gel fraction, dialyzed, resuspended in dmKRBT and assayed for ligand activity. As control, pieces of a blank IEF gel were subjected to the same procedure. Wild-type sperm were competitively inhibited from binding to ovulated eggs by both the acidic (1) and basic (4) fractions, whereas GalT I-null sperm binding was inhibited only by the basic fraction (4). When the amount of starting ovulated zonae was doubled, the eluate from fraction 4 produced 69% inhibition of sperm-egg binding. Thus, the acidic region of the gel containing ZP3 recapitulates the behavior of ovarian zona glycoproteins, in that it competitively inhibits wild-type, but not GalT I-null, sperm binding to eggs. However, the basic region of the IEF gel, where the WGA-reactive, high molecular weight protein resolves, contains a ligand to which both wild-type and GalT I-null sperm bind, thus recapitulating the characteristics of the intact ovulated zona pellucida and the peripheral fraction.

Discussion

The molecular mechanisms underlying gamete recognition are not yet fully understood. Until recently, evidence suggested

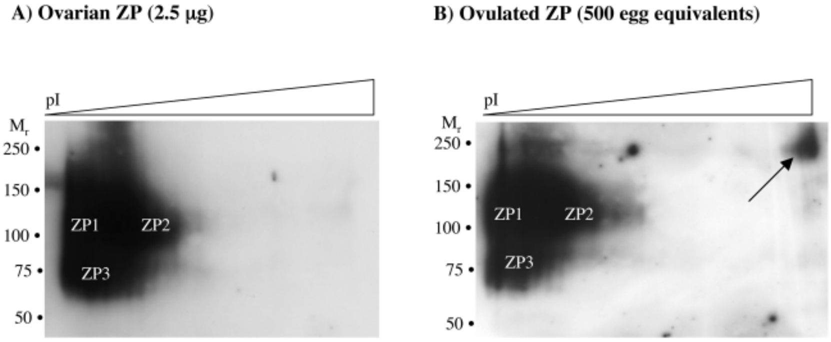


Fig. 7. Ovulated, but not ovarian, zona pellucida (ZP) glycoproteins contain a basic, WGA-reactive, 250 kDa glycoprotein. Ovarian (A) and 500 zonae equivalents of ovulated (B) zona proteins (2.5 µg) were separated by two-dimensional polyacrylamide gel electrophoresis, transferred to PVDF and visualized by staining with biotinylated-WGA. This blot is representative of five experiments. The arrow indicates a 250 kDa, basic, WGA-reactive protein that is present in ovulated ZP, but not ovarian ZP. The pH gradient, the theoretical location of the matrix proteins [according to Bleil and Wassarman (Bleil and Wassarman, 1980b)], and molecular weight markers are shown.

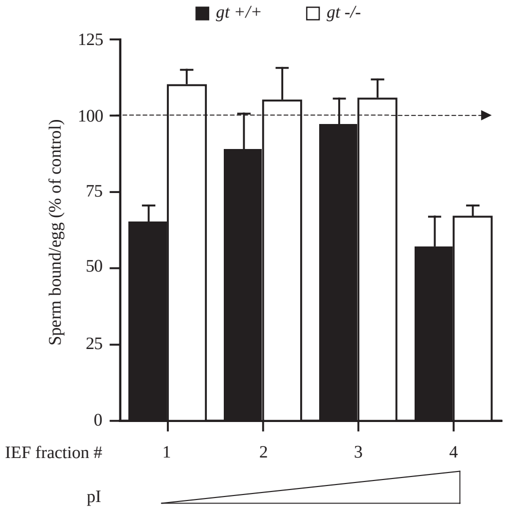


Fig. 8. Proteins isolated from the basic region of an IEF gel containing ovulated zona pellucida (ZP) glycoproteins inhibit wild-type and GalT I-null sperm binding to ovulated eggs. Proteins were isolated from acidic (fraction 1), neutral (fractions 2 and 3) and basic (fraction 4) regions of an IEF gel containing 5000 ovulated ZP and tested for biological activity in the sperm-egg binding assay. The acidic fraction (containing ZP3) inhibits wild-type, but not GalT I-null, sperm from binding to ovulated eggs. By contrast, the basic fraction (containing the basic ligand) prevents both wild-type and GalT I-null sperm from binding to ovulated eggs. Doubling the amount of starting material lead to 69% inhibition of sperm-egg binding by fraction 4. Each bar represents the mean±s.e.m.; *n*=4 experiments. In a single experiment, the value for each bar represents the average of three determinations (for a total of 12 assays per bar). The broken line indicates the number of sperm bound in the control, i.e. material obtained from a blank IEF gel treated identically to gels containing zona glycoproteins.

that gamete recognition in mouse is mediated by a single egg coat glycoprotein (ZP3) that is recognized by a specific sperm receptor, with most evidence implicating GalT I as, at least one of, the ZP3 receptors. In this study, data are presented suggesting that gamete recognition is more complex than a single receptor-ligand interaction, and can be resolved into at least two distinct binding events: a ZP3- and GalT I-independent interaction responsible for gamete adhesion, and a ZP3- and GalT I-dependent interaction that facilitates acrosomal exocytosis. As sperm are able to bind to ovarian eggs, ZP3 may support some degree of sperm adhesion as well, possibly via GalT I. However, sperm normally fertilize ovulated rather than ovarian eggs, and therefore must encounter the ZP3-independent binding activity under normal physiological conditions.

The two distinct sperm-binding activities can be attributed to two distinct sperm-binding ligands present in the ovulated egg coat: a ligand in the insoluble zona matrix and a peripherally associated ligand that can be removed by extensive washing. The matrix fraction can account for the behavior of ovarian zona glycoproteins in that it inhibits wild-type sperm-egg binding, but has no effect on GalT I-null sperm. By contrast, the peripheral fraction inhibits both wild-type and GalT I-null sperm binding. These results strongly suggest that the matrix fraction contains ZP3 and the peripheral fraction contains a ZP3-independent component. Furthermore, as wild-type sperm are sensitive to both the peripheral and matrix fractions, this indicates that the ZP3-independent ligand is physiologically relevant to wild-type sperm-egg binding, and not a peculiarity of the GalT I-null sperm phenotype.

The ligand activity in the peripheral fraction is not a byproduct of cortical granule secretions released during preparation or a result of to residual ZP3, nor is it a function of mouse oviduct-specific glycoprotein (OGP). This is consistent with recent data indicating that OGP-null females are fertile (Araki et al., 2003). Initial characterization suggests that the ligand is a relatively basic, WGA-reactive, high molecular weight (~250 kDa) glycoprotein that can be readily distinguished from the insoluble matrix glycoproteins by two-dimensional SDS-PAGE. Preliminary estimates indicate that the peripheral fraction contains ~1.7 ng protein/egg, whereas the insoluble matrix fraction contains ~5.1 ng protein/egg. Although the peripheral fraction probably contains proteins in addition to the ZP3-independent ligand, this suggests that the ligand is present at levels not grossly different from the individual zona matrix glycoproteins, which have both structural as well as sperm-binding capacities.

We expected that the matrix and peripheral fractions would be less efficacious in inhibiting wild-type sperm binding than the ‘unfractionated’ ovulated zona pellucida. Similarly, one would predict that wild-type sperm would continue to bind to the zona pellucida even in the presence of soluble ZP3, owing to the presence of the ZP3-independent ligand. However, we, and others, have observed that sperm-egg binding is effectively blocked when only one competitive ligand is present, e.g. ZP3 or the ZP3-independent ligand. The mechanism underlying this is unclear; blocking the binding site of one receptor may sterically interfere with the ability of the other receptor to recognize its ligand or may alter its affinity for ligand. Evidence for this comes from the observation that GalT I-null sperm bind to ovulated eggs in higher numbers than do wild-type sperm (Lu and Shur, 1997), suggesting that the affinity of the GalT I-independent receptor may be modulated by the presence of GalT I. This model cannot be definitively tested until the ligand and the receptor have been purified in sufficient quantities to perform binding analyses. It is noteworthy, however, that cellular interactions known to be mediated by multiple receptor-ligand pairs, such as the concerted action of selectins and integrins in mediating lymphocyte interactions with vascular endothelium, are readily inhibited by low molecular weight competitors of only one of the relevant receptor-ligand pairs (Bevilacqua, 1993; Lasky, 1992; Stoolman and Rosen, 1983).

There are several reasons that may explain why the ZP3-independent ligand has not been identified previously. First, the removal of GalT I from sperm by homologous recombination allowed us to eliminate, for the first time, the contribution of ZP3, and thereby reveal a novel, previously undetected binding activity. Second, the traditional procedure of extensively washing ovulated eggs before solubilizing their zona pellucida removes most of the peripherally associated sperm-binding ligand, as shown here by the ability to remove ligand activity by extensive washing. Finally, the majority of experiments characterizing the function of zona glycoproteins have used homogenized ovaries as a source of zona glycoproteins, which lacks the ZP3-independent ligand.

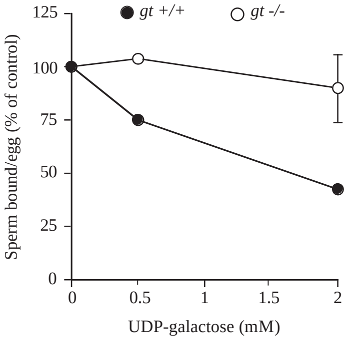


Fig. 9. Binding of GalT I-null sperm to the egg is independent of other members of the GalT family. UDP-galactose inhibits wild-type sperm, but not GalT I-null sperm, from binding to ovulated eggs. Each data point represents the mean±s.d.; *n*=2 experiments. In a single experiment, the value for each data point represents the average of three determinations (for a total of six assays per data point). UDP-glucose had no effect on the binding of either sperm genotype to eggs (data not shown).

The sperm receptor for the ZP3-independent ligand is of great interest. We tested the possibility that other members of the GalT family may function in this capacity, as GalT I is now known to be one of six enzymes in the β1,4-galactosyltransferase family (Almeida et al., 1999; Lo et al., 1998), some of which have been reported to be expressed in testis (Almeida et al., 1997; Sato et al., 1998). This possibility was shown to be unlikely, as the addition of UDP-galactose readily inhibited wild-type sperm-egg binding, as previously demonstrated (Lopez et al., 1985), whereas it had no effect on GalT I-null sperm-egg binding (Fig. 9). As UDP-galactose is able to force the catalytic dissociation of any putative galactosyltransferase from its galactosylated product, this result suggests that no other members of the GalT family function during the binding of GalT I-null sperm to the zona pellucida. However, any of the other sperm components recently implicated in ZP3-independent sperm-egg binding, such as SED1 or arylsulfatase A, could function as the receptor for this novel, ZP3-independent ligand (Ensslin and Shur, 2003; Tantibhedhyangkul et al., 2002; White et al., 2000).

As discussed here, several lines of evidence imply that the classical ‘one receptor-one ligand’ model of gamete interaction may be inaccurate. There is additional confusion in the literature regarding the composition of the zona pellucida and the structure of the biologically active glycosides. In this regard, at least four different monosaccharide residues have been implicated as being critical for initial sperm binding (Amari et al., 2001; Bendahmane et al., 2001; Johnston et al., 1998; Loeser and Tulsiani, 1999). Many of these observations result from the ability of defined glycoconjugates to competitively inhibit sperm-egg binding, but their interpretation is questioned by two findings. First, the sugar composition of the zona pellucida is heterogeneous (Aviles et al., 2000a). Immunocytochemical analysis at the ultrastructural level reveals that some sugars are confined to the inner regions of the zona pellucida, whereas other sugars are dispersed uniformly throughout the zona. Thus, it is impossible to know whether the inhibitory sugar being assayed is available in the zona pellucida to the sperm at initial binding or during later aspects of zona penetration. Furthermore, some of the most potent oligosaccharide inhibitors of sperm-egg binding do not appear to have analogous structures in the zona pellucida, and thus are unlikely to account for sperm binding activity in the intact zona (Aviles et al., 1999; Aviles et al., 2000b).

Finally, and perhaps most significantly, is that during fertilization sperm bind to the zona pellucida of ovulated oocytes, and not to the ovarian egg coat. In fact, oviductal glycoprotein secretions are known to permeate the zona pellucida, and, at least in hamster, there is evidence to suggest that the ovulated zona pellucida has biological activities that are distinctly different from those in the ovarian zona pellucida (Boatman and Magnoni, 1995; Kan et al., 1990; Robitaille et al., 1988; St-Jacques et al., 1992). All of these observations necessitate a re-examination of the simple premise that sperm-egg binding involves a single receptor-ligand interaction. In this regard, the fact that GalT I-null sperm fail to undergo acrosomal exocytosis even though they bind to the ovulated zona pellucida, clearly resolves gamete interaction into at least two distinct components, a ZP3-independent adhesive event and a ZP3-GalT I-dependent induction of acrosomal exocytosis.

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