Mouse oviduct-specific glycoprotein is an egg-associated ZP3-independent sperm-adhesion ligand

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Summary

Mouse sperm-egg binding requires a multiplicity of receptor-ligand interactions, including an oviduct-derived, high molecular weight, wheat germ agglutinin (WGA)-binding glycoprotein that associates with the egg coat at ovulation. Herein, we report the purification and identification of this sperm-binding ligand. WGA-binding, high molecular weight glycoproteins isolated from hormonally primed mouse oviduct lysates competitively inhibit sperm-egg binding in vitro. Within this heterogeneous glycoprotein preparation, a distinct 220 kDa protein selectively binds to sperm surfaces, and was identified by sequence analysis as oviduct-specific glycoprotein (OGP). The sperm-binding activity of OGP was confirmed by the loss of sperm-binding following immunodepletion of OGP from oviduct lysates, and by the ability of both immunoprecipitated OGP and natively purified OGP to competitively inhibit sperm-egg binding. As expected, OGP is expressed by the secretory cells of the fimбриae and infundibulum; however, in contrast to previous reports, OGP is also associated with both the zona pellucida and the perivitelline space of mouse oocytes. Western blot analysis and lectin affinity chromatography demonstrate that whereas the bulk of OGP remains soluble in the ampullar fluid, distinct glycoforms associate with the cumulus matrix, zona pellucida and perivitelline space. The sperm-binding activity of OGP is carbohydrate-dependent and restricted to a relatively minor peanut agglutinin (PNA)-binding glycoform that preferentially associates with the sperm surface, zona pellucida and perivitelline space, relative to other more abundant glycoforms. Finally, pretreatment of two-cell embryos, which do not normally bind sperm, with PNA-binding OGP stimulates sperm binding.

Key words: Sperm-egg binding, Fertilization, Oviduct-specific glycoprotein

Introduction

Successful fertilization requires numerous, specific interactions between the sperm and egg (Wassarman et al., 2001; Lyng and Shur, 2007). When sperm arrive at the ovulated oocyte, they first encounter the cumulus cells, which surround and nurse the egg during oogenesis. After traversing the cumulus layer, the sperm binds to the egg coat, or zona pellucida (ZP). Sperm binding to the ZP stimulates acrosomal exocytosis, which releases proteins that stabilize sperm adhesion to the ZP as well as degradative enzymes that enable the bound sperm to penetrate the ZP and reach the perivitelline space where it encounters the egg plasma membrane (Wassarman et al., 2001; Primakoff and Myles, 2002; Shur et al., 2004).

The mechanisms underlying sperm-ZP recognition and binding are highly contested (Clark and Dell, 2006; Williams et al., 2007). At the center of the debate is whether sperm binding is carbohydrate-mediated, in which case sperm are thought to bind specific carbohydrate structures on the ZP, and many studies support this interpretation (Florman and Wassarman, 1985; Miller et al., 1992; Shur, 2008). Alternatively, it has been argued that sperm recognize the overall supramolecular structure of the ZP rather than specific carbohydrate determinants (Hoodbhoy and Dean, 2004).

Pioneering studies by Wassarman and colleagues demonstrated that the murine ZP is composed of three glycoproteins, only one of which, ZP3, specifically binds to capacitated sperm and competitively inhibits sperm-egg binding in vitro, thus demonstrating its role as a sperm-binding ligand on the egg coat (Bleil and Wassarman, 1980). The sperm-binding activity of ZP3 has been attributed to specific glycan chains because deglycosylated ZP3 does not possess sperm-binding activity (Bleil and Wassarman, 1988; Florman and Wassarman, 1985). A wide range of sperm surface components have been implicated as receptors for the ZP, but only one, β-1,4-galactosyltransferase 1 (B4galT1, also known as GalT1), has been shown to selectively bind ZP3 glycans and trigger acrosomal exocytosis (Miller et al., 1992; Shi et al., 2001; Shur, 2008). Evidence consistent with sperm GalT1 functioning as a ZP3 receptor includes: (i) expression of GalT1 on Xenopus oocytes leads to selective ZP3 binding and GalT1-dependent signal transduction; (ii) overexpression of GalT1 on mouse sperm results in increased binding of soluble ZP3; whereas (iii) GalT1-null sperm do not bind ZP3 at significant levels and fail to undergo ZP-induced acrosomal exocytosis (Lu and Shur, 1997; Youakim et al., 1994; Shi et al., 2001). However, despite the low level of binding to ZP3, GalT1-null sperm still bind to the intact ZP suggesting that ovulated egg coats contain additional sperm-binding ligands in addition to ZP3.

Comparison of the egg coats from ovarian and ovulated oocytes revealed the presence of a ZP3-independent sperm-binding ligand that associates with the egg coat at ovulation (Rodeheffer and Shur, 2004). Characterization of this ZP3-independent ligand showed it to be a high molecular weight, basic glycoprotein whose sperm-binding activity could be depleted by wheat germ agglutinin
can be removed by stringent washing, and ZP3, a structural sperm-binding ligand: a peripherally associated glycoprotein that is capable of inhibiting sperm-egg binding.

Previous studies suggested that the ovulated egg coat contains two not GS1-reactive, basic, high molecular weight glycoproteins (OGP). Purification of native OGP and specific immunodepletion studies validate OGP as a sperm-binding ligand capable of competitively inhibiting sperm-ZP interactions. In contrast to earlier reports (Kapur and Johnson, 1986), we show here that OGP is associated with the ZP of the ovulated oocyte, as well as within the perivitelline space. Importantly, fractionation of native OGP through sequential chromatography on immobilized plant lectins reveals distinct glycoforms that show specific localizations and biological activities. In this regard, sperm-binding activity is specifically attributed to a peanut agglutinin (PNA)-binding glycoform that localizes to the ZP and perivitelline space, whereas the other glycoforms remain in the ampullar fluid and fail to interact significantly with sperm or eggs. Finally, the relationship of OGP to the previously characterized ZP3-independent ligand is addressed through use of GalT1-null sperm-binding assays.

Results

Hormonally primed oviduct lysates contain WGA-reactive, but not GS1-reactive, basic, high molecular weight glycoproteins capable of inhibiting sperm-egg binding

Previous studies suggested that the ovulated egg coat contains two sperm-binding ligands: a peripherally associated glycoprotein that can be removed by stringent washing, and ZP3, a structural component of the egg coat. The peripherally associated, ZP3-independent sperm-binding ligand behaves as a high molecular weight, basic glycoprotein that is bound by WGA lectins, but not by GS1 lectins (Rodeheffer and Shur, 2004). Because the ligand activity is specifically detected after ovulation, we predicted that the oviduct would be a rich source of the ligand. Consistent with this, 2D SDS-PAGE fractionation of oviduct lysates collected from superovulated (i.e. hormonally primed) females demonstrates the presence of WGA-binding, GS1-nonbinding, high molecular weight species with basic pI values (data not shown). Therefore, oviduct lysates were resolved by 1D SDS-PAGE and the WGA-binding species identified by lectin blot at 150-350 kDa, 100-150 kDa and 50-75 kDa (Fig. 1A). Corresponding areas of the gel were excised and assayed for sperm-binding activity as indicated in the Materials and Methods. Additionally, a WGA-nonbinding region (37-50 kDa) was prepared in parallel to control for nonspecific effects due to SDS-PAGE and sample preparation. Pre-incubation of wild-type sperm with 4 µg protein from the high molecular weight region (150-350 kDa) specifically inhibited sperm binding to cumulus-free oocytes (Fig. 1B), whereas none of the other WGA-binding or nonbinding material had any effect on sperm-binding activity.

Because a number of oviduct glycoproteins show hormone-dependent expression, we examined the possibility that the ZP3-independent ligand activity is hormonally regulated (Buhi et al., 2000; Buhi, 2002). High molecular weight (150-350 kDa) glycoproteins from hormonally primed and nonstimulated oviducts were obtained by identical methods and 4 µg of each were assayed for sperm-binding activity. Samples prepared from hormonally primed oviducts had more than twice the activity as samples prepared from nonstimulated, randomly cycling females (Fig. 1C). These results indicate that the sperm-binding activity present within the 150-350 kDa range is not a result of nonspecific effects from residual SDS or the purification protocol, and is upregulated during hormonal stimulation and ovulation.

![Fig. 1. High molecular weight proteins (150-350 kDa) from hormonally primed oviductal lysates demonstrate specific inhibition of sperm-ZP binding.](image-url)
Hormonally primed oviductal glycoproteins residing in the 200-250 kDa region exhibit similar capabilities and lectin characteristics to those of the ZP3-independent ligand. To further resolve the biologically active species in the high molecular weight range, the 150-350 kDa region was divided into four equal segments: 150-200 kDa, 200-250 kDa, 250-300 kDa and 300-350 kDa, each of which was extracted and assayed for sperm-binding activity. Of these four smaller molecular weight ranges, the strongest bioactivity was found in the 200-250 kDa fraction (Fig. 2A). The remaining regions showed minimal activity (data not shown). To determine whether this bioactivity possessed similar lectin characteristics as the previously identified ZP3-independent ligand, the 200-250 kDa fraction was depleted by WGA or GS1 agarose beads; bioactivity was eliminated by WGA-agarose depletion, but not by GS1-agarose depletion (Fig. 2A). This was similar to the results obtained for the ZP3-independent ligand.

The lectin binding and narrowed molecular weight range were used to further enrich the bioactive ZP3-independent ligand for subsequent identification. Hormonally primed oviduct lysates were enriched by GS1 depletion followed by WGA precipitation, and resolved by SDS-PAGE. The 200-250 kDa range was excised and prepared for measurement of sperm-binding activity. As expected, the enriched sample, designated LE (lectin-enriched) 200-250 kDa, is represented by a major Coomassie-stained polypeptide of ~220 kDa that is bound by WGA, but not GS1 (Fig. 2B). The LE 200-250 kDa species showed strong bioactivity against both wild-type and GalT1-null sperm at a concentration of 20 μg/ml (Fig. 2C), suggesting a ZP3/GalT1-independent interaction. Inhibition was dose-dependent, with a linear range of inhibition between 4 and 16 μg/ml (Fig. 2D).

The 220 kDa glycoprotein demonstrates a sperm-specific interaction. The ability of the LE 200-250 kDa species to competitively inhibit sperm-ZP binding implies that the bioactive species is binding to the sperm, occupying ZP recognition sites, and preventing those sites from interacting with the intact ZP. To explore whether there was a direct interaction between sperm and any species within the bioactive fractions, we used a pull-down assay with sperm. As shown in Fig. 3, a single ~220 kDa protein is extracted from the sperm surface following pre-incubation with hormonally primed 150-350 kDa, despite the presence of numerous proteins in the starting material. Identical results were obtained using hormonally primed 200-250 kDa and LE 200-250 kDa fractions as the starting material (data not shown).

The ZP3-independent ligand is identified as an OSG. The 220 kDa band, visualized by Pierce Imperial Protein Stain, was excised from a 1D SDS-PAGE of the LE 200-250 kDa fraction, and subjected to nano-electrospray ionization mass spectrometry (nanoESI-MS) by the Emory Microchemical Core Facility. Peptide analysis showed multiple sequence matches to human cytokeratin-1 (16 sequences, score: 1077), mouse myosin-11 (20 sequences, score: 1039) and mouse OGP (16 sequences, score: 702) (Fig. 4A). None of the other potential candidate proteins were represented by more than 1-2 peptide sequences, other than the laminin B and C chains, which were represented by five to six peptide sequences. Cytokeratin was eliminated as a candidate because it was of human origin. Myosin-11 was presumably derived from the smooth muscle of the oviduct and was also eliminated as a candidate because myosin-free fractions retained bioactivity. OGP remained a potential candidate.
Sperm-binding activity of OGP glycoforms

candidate because its presence within the LE 200-250 kDa fraction was validated by western blot analysis (data not shown).

To confirm that OGP is the bioactive species, OGP was immunodepleted from the LE 200-250 kDa fraction and bioactivity assessed (Fig. 4B). Consistent with previous results, the LE 200-250 kDa fraction exhibited high bioactivity, inhibiting wild-type sperm-egg binding by more than 80%. Mock depletions with control IgG beads showed a slight decrease in binding that was probably due to nonspecific protein loss, as judged by OGP western blot analysis. By contrast, depletion with anti-OGP beads resulted in undetectable levels of OGP by western blot and a coincident loss of bioactivity. Furthermore, material recovered from the anti-OGP immunodepletion was able to competitively inhibit sperm-egg binding, whereas parallel incubations with material removed from control beads showed negligible activity (Fig. 4B).

Native OGP competitively inhibits sperm-ZP binding
OGP was purified under native conditions to further test whether it functions as a ZP3-independent sperm-binding ligand. Native OGP was enriched by size separation, ion exchange and lectin affinity chromatography as described in the Materials and Methods. At each step, OGP-positive fractions were identified by western blot analysis and pooled (Fig. 5A-C). The nonbound fraction was collected and determined not to contain OGP by western blot (data not shown). The Superose-, MONO-Q- and WGA-enriched material behaved as one predominant silver-stained polypeptide of ~220 kDa and showed strong reactivity with anti-OGP antibodies (Fig. 5D). Although the oviduct preparations are believed to be free of contaminating oocytes, the presence of any contaminating ZP3 was ruled out by western blot analysis (Fig. 5D).

Native OGP was assayed for sperm-binding activity in competitive sperm-ZP binding assays. As expected, native OGP (20 μg/ml) competitively inhibited wild-type sperm-ZP binding by nearly 90%; however, identical concentrations inhibited GalT1-null sperm binding by only ~35% (Fig. 6A). Although OGP showed dose-dependent inhibition of sperm-ZP binding for both sperm genotypes (Fig. 6B and data not shown), the decreased bioactivity against GalT1-null sperm suggests that the loss of GalT1 might somehow influence the affinity of OGP binding to the sperm surface. In either event, bioactivity towards both wild-type and GalT1-null sperm could be removed by anti-OGP depletion (Fig. 6A).

Fig. 3. Sperm pull-down analysis reveals a single sperm-interacting protein of approximately 220 kDa present in the hormonally primed (HP) 150-350 kDa fraction. Protein stain and streptavidin-blotted biotinylated high molecular weight (150-350 kDa) proteins isolated from hormonally primed oviducts. Following incubation of sperm with the biotinylated proteins, sperm were pelleted, washed and extracted with either NaCl or detergent, which releases a distinct 220 kDa band from sperm, despite the large number of proteins present in the starting material. Molecular weight markers are indicated to the left.

Sperm pull-down analysis

Fig. 4. NanoESI sequence analysis of the 220 kDa band and immunodepletion studies confirms that OGP is the bioactive protein. (A) NanoESI mass spectrometric analysis of the 220 kDa band identifies 16 peptide sequences that exist within the polypeptide sequence of mouse OGP. Matches are indicated by the boxed sequences. (B) Quantitative analysis of biological activity, i.e. competitive inhibition of sperm-ZP binding, of the LE 200-250 kDa fraction before and after depletion with OGP or control antibodies, as well as of the recovered immunoprecipitated material. Each bar represents the mean ± s.e.m. of four assays, each conducted in triplicate. The relative amount of OGP in each fraction is illustrated by the accompanying western blot, which is representative of two assays. Representative oocytes are shown following each assay condition.
OGP expression and localization in superovulated oviducts

Because earlier results indicated that the sperm-binding activity of the 150-350 kDa oviduct polypeptides showed hormone-dependent activity, we examined the expression of OGP before and after pregnant mare’s serum (PMS) and human chorionic gonadotrophin (hCG) injection. For comparison, nonstimulated oviducts and ovarian tissue were collected. Because hormonal priming results in increased vascularization within the oviduct, OGP expression was normalized to ‘oviduct equivalents’ rather than to protein concentration. Western blot analysis shows a 2.2-fold increase in the 220 kDa OGP isoform following PMS stimulation and remained consistently elevated during hCG exposure (Fig. 7A).

OGP was localized in hormonally primed oviduct sections by indirect immunofluorescence. OGP immunoreactivity was observed in the fimbriae, infundibulum and ampulla, whereas the isthmus showed only background staining (Fig. 7B). Distinct OGP-reactive cells occur within the fimbriae and infundibulum, which are reminiscent of secretory, or ‘peg’, cells (Oliphant et al., 1984). Strong immunoreactivity is also observed in the ampulla, where the surface of the lumen appears coated by OGP. It is unclear whether such a coating exists, or if this reflects fixation of soluble OGP to the luminal surface. Regardless, OGP is clearly present within the secretory cells of the fimbriae and infundibulum, where it is presumably secreted and associates with the newly ovulated oocyte, as well as in the ampulla where fertilization occurs.

OGP shows distinct localizations within the cumulus-oocyte complex

Whereas the preceding results indicate specific localizations within the oviduct epithelium, we sought to determine whether OGP shows any distinct distribution within the cumulus-oocyte complex by using fractionation procedures coupled with western blotting, as well as by indirect immunofluorescence. The ampullar contents were collected as indicated in the Materials and Methods and fractionated as diagrammed in Fig. 8A.

Western blot analysis of the SDS-PAGE-resolved fractions illustrates the presence of two distinct pools of OGP: a freely soluble pool present in the ampullar fluid that can be removed from the cumulus-oocyte complex by washing, and a second pool associated with the cumulus-oocyte complex that is resistant to washing (Fig. 8B). Removal of cumulus cells by hyaluronidase treatment releases a portion of this cumulus-oocyte associated pool, and the remainder is released upon heat solubilization of the ZP. The cumulus cells themselves appear to be OGP-negative, as are the ZP-free oocytes. The OGP pool recovered following solubilization of the ZP could be associated with the ZP directly, as reported for other systems (O’Day-Bowman et al., 2002; McCauley et al., 2003), where it could function in sperm binding; or it could be released from the perivitelline space, where it might interact with sperm that have successfully penetrated through the ZP matrix. Consequently, we assessed OGP distribution within ovulated oocytes and two-cell embryos by indirect immunofluorescence. Contrary to our initial expectations, OGP showed minimal localization to the ZP but strong
localization to the perivitelline space (Fig. 8C). An earlier study reported the localization of an unidentified WGA-binding, 215-kDa glycoprotein in mouse oocytes (Kapur and Johnson, 1985) that we speculate might be OGP.

The presence of OGP in the ‘cumulus removal supernatant’ raised the possibility that OGP might be stripped from the ZP by hyaluronidase treatment and/or fixation methods. We therefore examined OGP distribution on oocytes following nonenzymatic (i.e. mechanical) removal of the cumulus cells by repetitively pipetting complexes through small pore pipettes. Strong OGP immunoreactivity on the ZP was observed following mechanical removal of cumulus cells. Confocal image analysis of mechanically treated oocytes shows clear surface staining with decreasing reactivity towards the interior regions of the ZP (Fig. 8C). By contrast, confocal imaging of hyaluronidase-treated oocytes shows greatly reduced OGP staining on the ZP surface, with punctate reactivity that is suggestive of a previously intact coating. Similar punctate OGP reactivity is also observed on the ZP of two-cell embryos.

The distinct OGP distributions correlate with specific OGP glycoforms
2D gel electrophoresis of secreted porcine oviductal proteins demonstrates high molecular weight proteins that range in pI from acidic to basic (Buhi et al., 2000). These protein species, although not identified, are assumed to be the porcine homolog of OGP. Consequently, we asked whether mouse OGP also exists as distinct isoforms (or glycoforms) and whether any show restricted distributions. Similar to the results reported for porcine proteins, mouse OGP isoforms ranging from acidic to basic were observed within the ampullar fluid. However, the number of isoforms decreased in the cumulus-oocyte-associated OGP pool, and reduced to a single, basic OGP species associated with the ZP (Fig. 9A).

To determine whether any of the specific OGP distributions and/or isoforms are associated with distinct OGP glycoforms, or differential OGP glycosylation, the various fractions were sequentially analyzed by chromatography on immobilized GS1, concanavalin A (Con A), Ricinus communis agglutinin 1 (RCA1), PNA and WGA (Fig. 9B). (These lectins recognize α-galactose, α-mannose, β-galactose, galactose-β1,3-N-acetylglactosamine [Gal-
GalNAc], and N-acetylglucosamine/sialic acid residues, respectively.) The soluble fractions of OGP derived from the ampullar fluid and washes possess significant levels of GS1-, ConA- and RCA1-binding glycoforms and minimal levels of a PNA-binding glycoform. This is distinct from the OGP binding pattern of the ‘cumulus removal supernatant’ and ZP-associated fraction, which show a single GS1-binding and a single PNA-binding glycoform, respectively. All OGP glycoforms could be accounted for by the four lectin columns (i.e. GS1, Con A, RCA1 and PNA) because there was no residual OGP binding to WGA-agarose, which binds to all OGP forms.

**Enrichment of distinct OGP glycoforms reveals glycoform-specific bioactivity and gamete interaction**

The realization that OGP distribution is correlated with distinct OGP glycoforms raised the possibility that the sperm-binding activity characterized in this report is actually associated with only a subset of the OGP glycoforms. Sequential GS1, Con A, RCA1 and PNA affinity chromatography of native OGP purified from oviduct lysates produced an array of OGP-reactive glycoforms similar to that observed in ampullar exudates, i.e. a significant amount of OGP was bound to GS1-, Con A- and RCA1-agarose columns, with a lesser amount bound to PNA-agarose (Fig. 10A).

Although it might appear ideal to normalize the bioactivity of the distinct OGP glycoforms to their protein concentration, this was not practical due to the small amount of recoverable PNA-binding OGP. Instead, bioactivity was normalized to relative OGP concentration, as determined by western blotting. Following elution from each lectin column, the OGP glycoforms were concentrated to equal volumes, resolved by SDS-PAGE and western blotting, and the OGP-reactive band intensities quantified using spot densitometer software. Only the Con-A- and PNA-binding glycoforms showed any sperm-binding activity, i.e. competitive inhibition of sperm-ZP binding (Fig. 10B). Normalized to OGP levels, the PNA-binding glycoform was 2.5-fold more bioactive than the Con-A-binding glycoform. The remaining two glycoforms, GS1- and RCA1-binding, did not competitively inhibit sperm-ZP binding, despite the present of significant amounts of OGP (Fig. 10B).
to OGP-treated two-cell embryos. This prediction was verified, as sperm nor express OGP on their ZP) would induce sperm binding PNA-binding glycoform to two-cell embryos (which no longer bind OGP in sperm-ZP binding. We predicted that the addition of the ZP of hyaluronidase-treated oocytes.

binding to the acrosomal cap of capacitated sperm, as well as to the equatorial segment suggests a role for Con-A-binding OGP other than during initial sperm-egg binding. In marked contrast to all other glycoforms, the Con-A-binding glycoform, which modestly inhibited sperm-ZP binding, shows a strong interaction with the equatorial region of the sperm head, but no interaction with the ZP. Localization to the equatorial segment suggests a role for Con-A-binding OGP other than during initial sperm-egg binding. In marked contrast to all other glycoforms, the PNA-binding glycoform demonstrates distinct binding to the acrosomal cap of capacitated sperm, as well as to the ZP of hyaluronidase-treated oocytes.

Overall, these results support a specific role for PNA-binding OGP in sperm-ZP binding. We predicted that the addition of the PNA-binding glycoform to two-cell embryos (which no longer bind sperm nor express OGP on their ZP) would induce sperm binding to OGP-treated two-cell embryos. This prediction was verified, as shown in Fig. 11, because PNA-binding OGP was found to bind to the ZP of two-cell embryos coincident with an increase in sperm binding relative to control two-cell embryos.

Bioactivity of the PNA-reactive OGP glycoform is carbohydrate-dependent

The ability of the LE 200-250 kDa OGP-enriched fraction to competitively inhibit sperm-ZP binding following excision from SDS-polyacrylamide gels suggests that the bioactivity is not dependent on protein tertiary structure. We therefore directly tested whether PNA-binding OGP inhibited sperm-egg binding in a carbohydrate-dependent manner by using heat denaturation and enzymatic deglycosylation (using a glycosidase cocktail that recognizes both N- and O-glycans). We reasoned that if bioactivity is dependent on OGP carbohydrate structures, then heat denaturation would have no affect on sperm-egg binding, but deglycosylation would ablate bioactivity. As before, the limited amount of the PNA glycoform precluded the ability to demonstrate efficient deglycosylation before and after enzymatic treatment. Consequently, validation of the glycosidase digestion was completed on a surrogate glycoprotein, bovine serum fetuin, which has both N- and O-glycans. Treatment of fetuin with the glycosidase cocktail led to the expected shift in electrophoretic migration, reflecting the deglycosylated polypeptide and demonstrating the effectiveness of the treatment procedure (Fig. 12A).

Heat denaturation did not eliminate the bioactivity of the PNA-binding glycoform, and in fact, it produced a slight increase (~10%) in activity compared with the native glycoform. Deglycosylation, however, significantly reduced the bioactivity of the PNA-binding glycoform to near background levels. This loss of bioactivity is not due to the presence of the denatured glycosidases because the glycosidase control did not affect the number of sperm bound per egg (Fig. 12B).

Discussion

Results presented here identify a specific OGP glycoform as a sperm-binding ligand in the mouse. The glycoform is not bound by sequential GS1, Con A and RCA1 columns, but is sequenced by a subsequent PNA-column. Functional analysis, as defined by an ability to competitively inhibit sperm-egg binding in vitro, revealed that the 200-250 kDa range of proteins isolated from hormonally primed oviductal lysates contains sperm-binding activity that is reminiscent of a previously identified ZP3-independent ligand (Rodeheffer and Shur, 2004). The biological activity is associated with a 220 kDa, WGA-binding glycoprotein that specifically binds to sperm surfaces, and was identified as OGP by sequence analysis. Natively purified OGP inhibits sperm-egg binding, and bioactivity can be removed from oviduct lysates by specific OGP immunodepletion.

Composed of two major domains, a catalytically inactive chitinase domain and a C-terminal O-glycosylation domain, OGP is expressed in response to estrogen stimulation in the oviduct of numerous mammals, including pig (Buhi et al., 1990), hamster (Robitaille et al., 1988), baboon (Boice et al., 1990), bovine (Abe et al., 1995), mice (Kapur and Johnson, 1985) and humans (Verhage et al., 1988). Co-incubation of OGP or media conditioned with OGP (i.e. oviductal fluid) with sperm, oocytes or embryos promotes fertilization and enhances early embryo development in numerous model systems (Killian, 2004). It is generally accepted that OGP is hormonally regulated and secreted from non-ciliated “peg” cells of the oviduct epithelium (Oliphant et al., 1984). Differences in the
expression of OGP within distinct regions of various mammalian oviducts have been validated by immunohistochemistry and in situ hybridization (Gandolfi et al., 1991; Kapur and Johnson, 1988), and it has been hypothesized that the region-specific expression is suggestive of location-specific function (Buhi, 2002). Our western blot and immunohistochemical data supports the notion of hormonal stimulation and regulated expression in the mouse oviduct.

OGP has been suggested to function during several different aspects of fertilization in a wide range of species (Araki and Yoshida-Komiya, 1998). Pretreatment of sperm with OGP has been shown to increase sperm motility, capacitation and the ability to fertilize in bovine (Abe et al., 1995; King et al., 1994; Martus et al., 1998); increase sperm viability, ZP penetration and block polyspermy in porcine (McCauley et al., 2003); and increase sperm-ZP binding and penetration in hamsters and humans (Boatman and Magnoni, 1995; O’Day-Bowman et al., 2002). Pretreatment of ovarian eggs with OGP increases sperm-ZP binding, penetration and overall fertilization in porcine and bovine (Martus et al., 1998; McCauley et al., 2003). Collectively, these findings suggest that OGP might play a role in initial sperm-ZP binding in most model systems, with the exception being mouse. Immunolocalization studies by Kapur and Johnson that utilized antibodies against an oviduct-associated glycoprotein, GP 215 (which we speculate is OGP), showed that this protein is not associated with the ZP, but is localized in the perivitelline space (Kapur and Johnson, 1985; Kapur and Johnson, 1986). Furthermore, female mice bearing targeted deletions in OGP remain fertile, and OGP-null eggs bind sperm in vitro (Araki et al., 2003). Although there is some indication that sperm binding to OGP-null eggs in vitro might be compromised when assaying sperm numbers closer to those in vivo (Carey Rodeheffer, Emory University School of Medicine, Atlanta, GA and B.S., unpublished data), it has been assumed that OGP does not have a role in mouse sperm-ZP binding, or a role in mouse fertilization. Our demonstration that OGP is present on the surface of the ZP, and that a selective subpopulation of OGP (a minor glycoform bound by PNA) interacts directly with gametes and...
Furthermore, OGP might present sugar structures that are known and peripherally associated (i.e. OGP) glycoproteins of the egg coat. O-glycans as sperm-binding epitopes on both structural (i.e. ZP3) activity. Collectively, these results focus attention on PNA-reactive exist within the O-glycans of ZP3, which have sperm-binding (Sendai et al., 1995). Similar PNA-reactive structures presumably linked glycans, of which mouse OGP contains 24 predicted sites the PNA-reactive epitope, Gal-GalNAc, traditionally occurs on O- glycoform significantly reduced bioactivity. Although we cannot deglycosylation, but not heat denaturation, of the bioactive OGP derived from the associated carbohydrate structures. Indeed, OGP possessed sperm-binding activity suggests that its function is minor OGP glycoforms. The fact that only a specific glycoform of OGP product, which might obscure the functional assessment of these results highlight the fact not all OGP possesses the same functionality.

To our knowledge, this is the first evidence showing that OGP functions as a sperm-binding ligand, indicates a need to re-examine the role of OGP in mouse fertilization using more rigorous in vitro and in vivo experimental protocols.

In this regard, it is noteworthy that our results indicate that OGP is removed by hyaluronidase treatment, a procedure routinely used in the preparation of oocytes for sperm-ZP binding assays. This might also explain why earlier studies failed to detect GP 215, presumably OGP, on hyaluronidase-treated oocytes (Kapur and Johnson, 1986). Furthermore, immunolocalization of GP 215 required reduced fixation protocols because traditional methods were detrimental to the ZP matrix, raising the possibility that GP 215 (OGP) peripherally associated with the ZP surface was lost during these fixation procedures (Kapur and Johnson, 1986). In any event, these studies emphasize the need for cautious interpretation of results derived from traditional in vitro binding assays using hyaluronidase-treated oocytes.

Several lines of evidence indicate that the sperm-binding activity of OGP is restricted to a minor PNA-binding glycoform that shows specific binding to sperm and oocytes. Most of the OGP in the ampulla is not associated with the cumulus-oocyte complex. However, the OGP that is associated with the cumulus-oocyte complex is specifically bound, because it could not be dissociated with repeated washing. Furthermore, numerous OGP isoforms exist with regards to pI and lectin binding; however, only a single basic, PNA-binding OGP associates with the ZP, binds to the acrosomal cap of the sperm head, and inhibits sperm-egg binding. Overall, these results highlight the fact not all OGP possesses the same functionality.

To our knowledge, this is the first evidence showing that OGP function is dependent on individual iso- or glycoforms. Previous studies have analyzed OGP function using the total, unfractionated OGP product, which might obscure the functional assessment of minor OGP glycoforms. The fact that only a specific glycoform of OGP possessed sperm-binding activity suggests that its function is derived from the associated carbohydrate structures. Indeed, deglycosylation, but not heat denaturation, of the bioactive OGP glycoform significantly reduced bioactivity. Although we cannot make any conclusions about the functional glycan ligand on OGP, the PNA-reactive epitope, Gal-GalNAc, traditionally occurs on O-linked glycans, of which mouse OGP contains 24 predicted sites (Sendai et al., 1995). Similar PNA-reactive structures presumably exist within the O-glycans of ZP3, which have sperm-binding activity. Collectively, these results focus attention on PNA-reactive O-glycans as sperm-binding epitopes on both structural (i.e. ZP3) and peripherally associated (i.e. OGP) glycoproteins of the egg coat. Furthermore, OGP might present sugar structures that are known to inhibit sperm-ZP binding in vitro, but which are not found on ZP3 (Johnston et al., 1998; Aviles et al., 2000). Such possibilities require additional study, but do provide an attractive opportunity to reconcile previous results.

The assignment of distinct biological activities, e.g. sperm-ZP binding, to specific OGP glycoforms raises interesting questions regarding the nature and derivation of the various glycoforms. It appears likely that they result from variable glycosylation, as the predicted molecular weight of the mouse OGP polypeptide is 76 kDa and the predicted pI is 9.19 (http://ca.expasy.org). Although the different glycoforms might reflect cell-type specific glycosyltransferase activity within the oviductal epithelium, it seems more likely that they result from heterogeneity of glycosylation within a given cell. One carbohydrate modification that might contribute to the generation of various isoforms is sialic acid, because neuraminidase treatment of bovine OGP collapses the diverse isoforms to pI 9.3 and exposes strong PNA reactivity (Satoh et al., 1995). In this light, the minor PNA-reactive glycoform
might reflect naturally occurring Gal-GalNAc epitopes or, possibly, be created by a neuraminidase activity derived from sperm and/or the cumulus-oocyte complex. Accordingly, glycoforms that do not bind sperm could become sperm-reactive through enzymatic processing. Similarly, the PNA-binding OGP within the perivitelline space might be processed to a Con-A-reactive form by cortical granule glycosidases, which would bind excess acrosome-reactive sperm in the perivitelline space to prevent their contribution to polyspermy, as has been suggested for OGP in other species (McCauley et al., 2003).

The original identification of a ZP3-independent ligand on ovulated mouse oocytes was facilitated by the development of GalT1-null mice; sperm from these mice show greatly reduced binding to soluble ZP3 yet retain binding to the intact ZP (Lu and Shur, 1997; Rodeheffer and Shur, 2004). Thus, studies of the GalT1-null mouse dissected sperm-egg binding into at least two distinct steps: a GalT1/ZP3-independent adhesion followed by a GalT1/ZP3-dependent binding that facilitates acrosomal exocytosis. Characterization of the ZP3-independent ligand recognized by GalT1-null and wild-type sperm raised the possibility that it could be OGP. However, OGP was eliminated as a candidate on the basis of the ability of GalT1-null sperm to bind OGP-null eggs. Nevertheless, these studies did not address the sperm-binding activity of OGP itself. In this regard, it is of interest that in the present study, denatured OGP inhibited the binding of both wild-type and GalT1-null sperm with similar efficacy, but native OGP showed reduced bioactivity against GalT1-null sperm. Because the sperm-binding activity of OGP appears to lie within its glycan chains, it is likely that denaturing the polypeptide backbone would relax the conformational specificity that restricts glycan presentation to its receptor. In this context, denatured OGP would present the glycan epitopes with limited specificity; similar to the reduced affinity seen when glycosyltransferase substrates are removed from their native polypeptide backbone (Baranski et al., 1990). Furthermore, the reduced activity of native OGP towards GalT1-null sperm suggests that the loss of GalT1 influences OGP binding to sperm, not unlike the reduced binding of ZP3 to GalT1-null sperm (Lu and Shur, 1997).

These issues are of interest in the light of the suggestion that the egg-binding machinery (EBM) is organized into lipid rafts on the sperm plasma membrane. Several studies have reported alterations in lipid raft composition during sperm capacitation that are thought to be a prerequisite for sperm binding to the egg coat (Cross, 2004; Bou Khalil et al., 2006). GalT1 has been shown to localize to lipid rafts in somatic cells (Hathaway et al., 2003), although it is still unclear whether GalT1 is present within lipid rafts on sperm as well. If so, then the loss of GalT1 might disrupt or alter the presentation of the EBM components within the lipid raft, leading to reduced affinity for egg coat ligands, including ZP3 and OGP. In any event, the results presented here indicate that OGP is secreted as a mixture of distinct glycoforms, one of which has specific affinity for the sperm surface and for ZP, and facilitates sperm-ZP adhesion.

Materials and Methods

Sperm-egg binding assay

All reagents were purchased from Sigma (St Louis, MO) unless otherwise noted. Eight-week old CD-1 female mice (Charles River, Wilmington, MA) were superovulated by hormone injection using 7.5 IU of PMS and hCG, 48 hours apart. Cumulus-oocyte masses were collected from the oviducts of superovulated females. The masses were transferred into 0.2% hyaluronidase in 1× phosphate-buffered saline, pH 7.4 (PBS). Cumulus-free eggs were then washed through three drops of modified Krebs-Ringer bicarbonate medium (mKRB) (Rodeheffer and Shur, 2004) via a glass pipette that was approximately twice the diameter of the egg. Two-cell embryos were collected into mKRB (but not washed) from the oviducts of superovulated CD-1 females that were mated 15 hours earlier. The caudal epididymides of CD-1 males or GalT1-null males were dissected into mKRB and shredded. The epididymides were incubated at atmospheric CO2 at 37°C for 15 minutes to release the sperm, which were collected after filtration (Nitiex; Sefar America; Kansas City, MO). The sperm were further capacitated for 45 minutes and the number of sperm determined. Some 40,000 sperm were then co-incubated with 25-35 ovulated cumulus-free eggs and three to five two-cell embryos (as a control for nonspecific binding) in 50 μl drops of mKRB for 30 minutes at 37°C. Eggs and embryos were washed through sequential drops of mKRB until ~1 sperm remained bound to the two-cell embryos. The gametes were fixed in 4% paraformaldehyde in PBS. The number of sperm bound to each egg and two-cell embryo was counted using phase-contrast optics. The average number of sperm bound per two-cell embryo (nonspecific binding) was subtracted from the average number of sperm bound per egg. The data presented are the average of at least three experiments ± s.e.m., each of which contained triplicate droplets for each experimental parameter, unless otherwise noted.

Western and lectin blot analysis of oviduct lysates

Eight-week old CD-1 female mice were superovulated as above and sacrificed at 16 hours post hCG injection. Oviducts were dissected from the ovary and uterus into ice-cold 500 μl PBS. Oviducts were homogenized, and the insoluble debris was removed by centrifugation for 1 hour at 16,060 g. Oviducts and ovaries from six un.injected, random cycling, females were also obtained. Samples were denatured with 2× loading buffer (0.125 M Tris, 2% glycerol, 2% SDS, 0.5% β-mercaptoethanol, 20 mM DTT) at 95°C for 3 minutes. Lysates were fractionated on a 7.5% Criterion SDS-PAGE gel (Bio-Rad Laboratories, Hercules, CA). Proteins were transferred to PVDF membrane (Millipore, Billerica, MA) and blocked in 1% BSA, TBS-T (0.1% Tween 20, 0.8% NaCl, 0.002% KCl, 25 mM Tris pH 7.4). Membranes were incubated with a 1:2000 dilution of goat anti-OGP polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and subsequently with 1:2000 dilution of a 1:2000 dilution of goat anti-OGP polyclonal antibody (Santa Cruz Biotechnology). Membranes were incubated with 1 μg/ml of immobilized lectin. Blots were washed and subsequently probed with a 1:50,000 dilution of streptavidin-HRP (Zymed, South San Francisco, CA). After washing, the chemiluminescence signal was assayed (GE Healthcare, Fairfield, CT) and band density was quantified using spot densitometer software (Alpha Innotech, San Leandro, CA).

ZP3-independent ligand and OGP purification

Twenty-four 8-week old CD-1 female mice were superovulated as above and sacrificed at 16 hours post hCG injection. Oviducts were placed in 1 ml ice-cold lectin affinity buffer (10 mM phosphate buffer, 150 mM NaCl, 0.25 mM CaCl2, pH 7.4). Oviducts were homogenized, and the insoluble debris was removed by centrifugation. For denaturing purification, oviduct lysates were denatured with 2× loading buffer and fractionated on a 5.0% Criterion SDS-PAGE gel. Electrophoresis conditions were optimized so that the separation between the 250 kDa and 150 kDa molecular weight markers was significant enough to properly select various ranges. Excised gel pieces were lyophilized and eluted in a Bio-Rad Elutrap according to the manufacturer’s instructions. Electroluted proteins were collected and dialyzed three times in a 10,000 MWCO Slide-A-Lyzer (Pierce Biotechnology; Rockford, IL) against 500 ml 8 M urea, 10 mM phosphate buffer, pH 7.4 at 4°C, followed by three times against 10 mM phosphate buffer.

For native purification, lysates from 48 superovulated oviducts were fractionated on a Pharmacia fast protein liquid chromatography (FPLC) system. Lysates were applied to a Superose 6 size separation column (GE Healthcare) at 0.3 ml/minute in 50 mM HEPES, 50 mM NaCl, ph 7.4. Fractions were collected in 0.5 ml volumes and assayed for OGP by western blot analysis. OGP-positive fractions were pooled and applied to a MONO-Q ion exchange column (GE Healthcare). After sufficient washing, bound proteins were eluted with a 0.05-1 M NaCl gradient in 50 mM HEPES, pH 7.4. OGP-positive fractions were pooled and concentrated. The sample was resuspended in 5 ml lectin affinity buffer and separated on various lectin columns (10 ml columns, run at 0.5 ml/minute) as indicated. Eluted proteins were concentrated and the free sugar was removed by dialysis against PBS. After dialysis for both denaturing and native purification, samples were concentrated to 30-50 μl. An aliquot of the fluid that passed through the concentrator was collected and served as a dialysis control in sperm-egg binding assays. Concentrated samples and dialysis controls were assayed for protein concentration using RC DC Protein Assay (Bio-Rad Laboratories). For lectin depletion or enrichment, oviduct lysates or native OGP fractions, in lectin affinity buffer, were added to 20 ml GS1-, Con-A-, RCA1-, PNA- or WGA-agarose columns (E.Y. Laboratories; San Mateo, CA). Depending on the desired purification, columns were used in a variety of sequential configurations. After sufficient washing of the columns with lectin affinity buffer, bound proteins were eluted with 0.5 M methylbiose, (GS1), 0.2 M D-mannose mannose (Con A), 0.1 M lactose (RCA), 0.05 M N-acetylglucosamine (PNA), or 1M N-acetylgalactosamine (WGA). Bound proteins were collected, dialyzed against PBS to remove free sugar or salt, concentrated by ion concentrators (Pierce Biotechnology), and resolved by SDS-PAGE or assayed for biological activity.
Gamete interaction assays
Sperm pull-down assay
Distinct bioactive fractions were biotinylated at a 20:1 molar ratio using Pierce EZ-Link Sulfo-NHS-LC-CL-biotin (Pierce Biotechnology). After dialysis to remove free biotin, individual fractions were incubated with 2,000,000 capacitated wild-type sperm at 12 μg/ml in mKRB. After 30 minutes incubation at 37°C, the sperm were collected by centrifugation, the supernatant was discarded and the sperm pellet washed several times in mKRB or PBS. After the final wash, the sperm were resuspended and all associated proteins extracted by either 1% Triton X-100, 1 M NaCl, or heat denaturation (70°C for 1 hour). The sperm were pelleted by centrifugation, and the extracted proteins within the supernatant were collected and prepared for 1D SDS-PAGE and western blot analysis. The washed oviductal fractions were further processed for 2D SDS-PAGE analysis, and samples were dialyzed into 50 mM HEPES, pH 7.4 overnight, acetone precipitated, and resolubilized in first-dimension buffer as per the manufacturer’s instructions (Bio-Rad Laboratories). Samples were run on Bio-Rad IEPG-3-10 nonlinear strips (11 cm) using the Bio-Rad Protein IEF Cell system. The second-dimension was run on Criterion 10% IPG-1 well gels (Bio-Rad Laboratories). Leetin affinity chromatography was performed as described above.

Deglycosylation of PNA-binding OGP
A preparation of the PNA-binding OGP glycoform was separated into three identical aliquots. The first aliquot served as an internal control for bioactivity. The second aliquot was heat-denatured for 10 minutes at 65°C. The final aliquot, as well as the control glycoprotein bovine fetuin, were deglycosylated using the E-DEGLY kit, as per the manufacturer’s instructions (Sigma), which enzymatically removes N- and O-glycans. After deglycosylation, the glycosidases were inactivated by heat denaturation. To control for any affect of the denatured glycosidases, a control sample containing glycosidases but lacking OGP was heat-denatured. All samples were assayed for sperm-binding activity.

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