Characterization of a Mouse ZP3-Derived Glycopeptide, gp55, That Exhibits Sperm Receptor and Acrosome Reaction-Inducing Activity *in Vitro*

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ABSTRACT: During fertilization, free-swimming mouse sperm bind to mZP3 (∼83 000 M_r), one of three zona pellucida glycoproteins, and once bound undergo the acrosome reaction, a type of cellular exocytosis [Wassarman, P. M., & Litscher, E. S. (1995) *Curr. Top. Dev. Biol. 30*, 1−19]. Sperm recognize and bind to specific serine/threonine-linked oligosaccharides located at the mZP3 combining site for sperm. Here, we examined certain characteristics of gp55, a ∼55 000 M_r glycopeptide derived from the carboxy-terminal half of mZP3 polypeptide to which sperm bind [Rosiere, T. K., & Wassarman, P. M. (1992) *Dev. Biol. 154*, 309−317]. gp55 is heterogeneous with respect to M_r (∼47 000−62 000 M_r) and has a relatively low p*I* (∼4.3−4.5) compared to the polypeptide portion of the glycopeptide (p*I* ∼6.5). gp55 inhibits binding of sperm to eggs (i.e., exhibits sperm receptor activity) and induces sperm to undergo the acrosome reaction *in vitro* at about the same concentrations required for intact mZP3 (∼50−200 nM). Each of three different size-fractions of gp55, separated by SDS−PAGE, also exhibits bioactivity *in vitro*. Removal of asparagine-linked (N-linked) oligosaccharides from gp55, by extensive digestion with *N*-glycanase, reduces its M_r to ∼21 000 and increases its p*I* to ∼5.3, but does not significantly affect its ability to inhibit binding of sperm to eggs or to induce sperm to undergo the acrosome reaction. Similarly, digestion of gp55 with either endo-β-galactosidase or neuraminidase alters its M_r and/or p*I*, but does not significantly affect either of its bioactivities. These observations are consistent with the proposal that neither N-linked oligosaccharides nor sialic acid is an essential element of the mZP3 combining site for sperm. They also indicate that a relatively small mZP3 glycopeptide is able to induce sperm to undergo the acrosome reaction (i.e., cellular exocytosis) *in vitro*.

Mammalian eggs are surrounded by a thick, transparent extracellular coat, called the zona pellucida (ZP;[1] Gwatkin, 1977; Dietl, 1989; Dunbar & O'Rand, 1991; Yanagimachi, 1994). Generally, the ZP is composed of three glycoproteins, called ZP1−3, that form a loose meshwork of interconnected filaments (Greve & Wassarman, 1985; Wassarman, 1988, 1993; Wassarman & Mortillo, 1991). In mice, hamsters, and many other mammals, including human beings, free-swimming sperm recognize and bind to ZP3 to initiate the fertilization process (Bleil & Wassarman, 1980; Wassarman, 1990, 1993; Wassarman & Litscher, 1995).

Mouse ZP3 (mZP3; ∼83 000 M_r) is an acidic glycoprotein (p*I* ∼4.5) that consists of a ∼44 000 M_r polypeptide (p*I* ∼6.5), three or four complex-type, asparagine-linked (N-linked) oligosaccharides, and an undetermined number of serine/threonine-linked (O-linked) oligosaccharides (Salzmann *et al*., 1983; Wassarman, 1988, 1993). Purified mZP3 inhibits binding of sperm to eggs and induces sperm to undergo the acrosome reaction *in vitro* (Bleil & Wassarman, 1980, 1983; Wassarman, 1990). Acrosome-intact sperm apparently recognize and bind to specific O-linked oligosaccharides located at the mZP3 combining site for sperm (Florman & Wassarman, 1985; Bleil & Wassarman, 1988; Kinloch *et al*., 1995; Wassarman, 1989, 1990, 1992; Litscher & Wassarman, 1993).

Previously, we reported that digestion of purified mZP3 by either papain or V8 protease produced an ∼55 000 M_r glycopeptide that was derived from the carboxy-terminal half of the polypeptide and inhibited binding of sperm to eggs *in vitro* (Rosiere & Wassarman, 1992). Here, following digestion of mZP3 by papain, we purified the glycopeptide, called gp55, in order to assess the potential role of N-linked oligosaccharides, sialic acid, and other factors in its bioactivities. In addition, we asked whether this portion of mZP3 was sufficient to induce sperm to undergo the acrosome reaction *in vitro*. A preliminary report of these results has appeared (Litscher & Wassarman, 1994).

MATERIALS AND METHODS

Enzymes and Chemicals. Immobilized papain (EC 3.4.22.2; Pierce), *N*-glycanase (N-GLYase; *Flavobacterium meningosepticum*; EC 3.5.1.52 and 3.2.2.18; Genzyme), endo-β-galactosidase (EBGase; *Bacteroides fragilis*; EC 3.2.1.103; Oxford GlycoSystems), and neuraminidase (NEURase; *Arthrobacter ureafaciens*; EC 3.2.1.18; Oxford GlycoSystems) were purchased from commercial sources. Ampholytes (Bio-Lyte 3/10 and 4/6; Bio-Rad), molecular weight standards (Bio-Rad), and carrier-free $Na^{125}I$ (Amersham) also were purchased from commercial sources.

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[1] Abbreviations: ZP, zona pellucida; PBS, phosphate-buffered saline; PVP, polyvinylpyrrolidone; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; N-linked, asparagine-linked; O-linked, serine/threonine-linked; N-GLYase, peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase; EBGase, endo-β-galactosidase; NEURase, neuraminidase.

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Collection and Culture of Gametes and Embryos. Sperm, ovulated eggs, and fertilized eggs were obtained from randomly bred, Swiss albino mice (CD-1; Charles River Breeding Labs) and cultured *in vitro*, essentially as previously described (Bleil & Wassarman, 1980; Florman & Wassarman, 1985; Moller *et al*., 1990).

Purification of Radiolabeled gp55. Production of ^{125}I-labeled gp55 and purification of the glycopeptide by HPLC were carried out essentially as previously described (Rosiere & Wassarman, 1992). mZP3 was purified by HPLC from ZP that were isolated by Percoll (Sigma) gradient centrifugation of ovarian homogenates (Bleil & Wassarman, 1986; Bleil *et al*., 1988). Purified mZP3 was radioiodinated in 0.2 M sodium phosphate (pH 6.8)/0.1% SDS in the presence of carrier-free Na^{125}I (specific activity, 14−16 mCi/μg; sufficient to convert all five mZP3 tyrosine residues to either monoiodo- or diiodotyrosine) and mixed with unlabeled mZP3 (1:10 labeled:unlabeled ratio). The mixture was digested with immobilized papain (250 μg/mL gel), at an enzyme-to-mZP3 ratio of 1:2 by weight, in 50 mM Hepes (pH 6.1)/10 mM EDTA/20 mM cysteine/0.1% SDS, at 37 °C for 2 h, with vortexing for a few seconds every 20 min. Protease was separated from mZP3 by centrifugation and gel-filtration (Bio-Gel P-2) into 0.2 sodium phosphate (pH 6.6)/0.1% SDS, followed by HPLC fractionation (Bio-Sil SEC-250 column, 300 × 7.8 mm; flow rate, 0.1 mL/min). Aliquots were analyzed in a gamma counter, to estimate recovery, and then examined by SDS−PAGE and autoradiography. Fractions containing gp55 were pooled and dialyzed extensively, first against 8 M urea and then against distilled water.

High-Resolution Two-Dimensional Electrophoresis. ^{125}I-gp55 and partially deglycosylated ^{125}I-gp55 were subjected to isoelectric focusing and SDS−PAGE by using a Mini Protean II-2D cell according to procedures described in the supplier's (Bio-Rad) guide [procedures based on O'Farrell (1975)]. Gels were stained with silver to visualize internal standards (Bio-Rad) and subjected to autoradiography to identify the positions of gp55 and glycosidase-treated gp55.

Fractionation of gp55 into Molecular Weight Classes. Purified ^{125}I-gp55 was subjected to SDS−PAGE followed by autoradiography of the wet gel. The region of gel containing ^{125}I-gp55 was excised and sliced into three fractions based on molecular weight (high, ∼60 000 M_r; medium, ∼55 000 M_r; low, ∼49 000 M_r), and each fraction was electroeluted from the gel slice (Centrilutor, Centricon-30; Amicon) and dialyzed extensively, first against 8 M urea and then against distilled water.

Treatment of gp55 with Glycosidases. Digestions of ^{125}I-gp55 with glycosidases were carried out at 37 °C for 48 h. Typically, ∼1 μg of radiolabeled gp55 was incubated with enzyme in a total volume of 10 μL (2 μL of enzyme plus 8 μL of buffer): 0.5 unit of N-GLYase in 20 mM sodium phosphate (pH 7.5); 8 milliunits of EBGase in 50 mM sodium acetate (pH 5.8)/250 μg/mL BSA/1 mM NaCl; 40 milliunits of NEURase in 0.1 M sodium acetate (pH 5.0). Digestions were terminated by addition of 40 μL of distilled water and boiling the sample for 5 min, followed by extensive dialysis against distilled water.

Bioactivity Assays. gp55 and its derivatives were tested for their ability to bind to sperm and, thereby, prevent sperm from binding to ovulated eggs *in vitro* ("competition assay"), essentially according to the procedure described previously (Bleil & Wassarman, 1980; Florman & Wassarman, 1985; Moller *et al*., 1990; Litscher *et al*., 1995). The concentration of gp55 used in the experiments was estimated by gamma-counting and autoradiography of gels. Briefly, papain digests of ^{125}I-mZP3 were subjected to one-dimensional SDS−PAGE followed by autoradiography, and the cpm associated with the entire digest as well as the cpm associated with gp55 were determined. Such measurements permitted an estimate to be made of the amount of gp55 present (25−30% of total digest). Capacitated sperm (10 μL) were incubated with gp55, or derivatives of gp55 (dissolved in 2 μL of water and 8 μL of M199-M), in a 20 μL drop, under oil, at 37 °C for 15 min. Control experiments included incubating sperm in the presence of intact mZP3, BSA, or distilled water under identical conditions. Following the 15 min incubation, 12 ovulated eggs and 2 two-cell embryos were added to the drop, and the incubation was continued an additional 40−45 min. Eggs and embryos were then washed by mouth-pipetting, fixed in 2% formaldehyde/PBS/PVP (pH 7.2) and M199-M (1:1), and the number of sperm bound per egg was determined by light microscopy.

gp55 and its derivatives also were tested for their ability to induce sperm to undergo the acrosome reaction *in vitro*, essentially according to the procedure described previously (Moller *et al*., 1990; Litscher *et al*., 1995). Briefly, sperm collected from the 20 μL drops described above ("competition assay") were fixed and stained with Coomassie blue, and the status of the acrosome was assessed by light microscopy.

RESULTS

Experimental Rationale. Previously, we reported that a ∼55 000 M_r glycopeptide, excised from the carboxy-terminal half of mZP3 by either papain or V8 protease, inhibited binding of sperm to eggs ("sperm receptor activity") *in vitro* (Rosiere & Wassarman, 1992). However, we did not determine whether or not the glycopeptide, like intact mZP3, could induce sperm to undergo the acrosome reaction subsequent to binding. Furthermore, we did not characterize the glycopeptide with respect to the potential role of N-linked oligosaccharides and/or other moieties in its bioactivities. Experiments that address these issues are presented here. It should be noted that all molecular weights reported here should be considered as "apparent molecular weights," since it is well known that glycoproteins and glycopeptides migrate anomalously on SDS−PAGE due, in part, to a low charge-to-mass ratio for the molecules (Leach *et al*., 1980).

Production and Electrophoretic Analysis of gp55. Papain digests of purified, radiolabeled mZP3 were fractionated by HPLC on a size-exclusion column, and aliquots of fractions were analyzed by SDS−PAGE and autoradiography, as described under Materials and Methods. As seen in Figure 1, an mZP3 glycopeptide with an average M_r of ∼55 000 (gp55) was a major digestion product. In addition to gp55, the digests contained mZP3 glycopeptides with average M_rs of ∼26 000, ∼13 000 and <10 000 that were derived from gp55, as previously described (Rosiere & Wassarman, 1992). HPLC fractions containing gp55 were pooled and dialyzed extensively prior to use. High-resolution two-dimensional gel electrophoretic analysis of purified gp55 revealed two major isoforms (p*I* ∼4.3 and ∼4.5) having M_rs that range from ∼47 000 to ∼62 000 (Figure 1). Thus, like intact mZP3 (Wassarman, 1988), gp55 is very heterogeneous with respect to both its charge and its molecular weight.

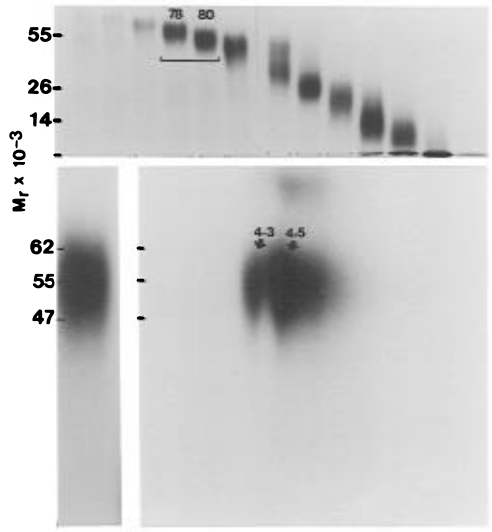

FIGURE 1: Purification and electrophoretic analysis of gp55. (Top) Autoradiograph obtained following SDS−PAGE of radiolabeled mZP3 digested with papain and subjected to HPLC gel filtration, as described under Materials and Methods. Approximately 100 fractions were collected, and all even-numbered fractions between 70 and 100 were subjected to SDS−PAGE and autoradiography. Even-numbered HPLC fractions between fractions 72 and 96 are shown here. HPLC fractions 78, 79, and 80, containing gp55, were pooled and used in the experiments described here. (Bottom, left) Autoradiograph obtained following one-dimensional SDS−PAGE analysis of purified gp55. Note that the molecular weight of gp55 extends from about 47 000 to 62 000. (Bottom, right) Autoradiograph obtained following high-resolution two-dimensional gel electrophoresis of purified gp55. Note the relatively low p*I*s (4.3 and 4.5) for gp55. Gels were calibrated with prestained molecular weight standards ranging either from 14 300 to 100 000 M_r (one-dimensional gels) or from 17 500 to 76 000 M_r (two-dimensional gels).

Bioactivity Assays of gp55. Purified gp55 was tested for bioactivity using two different assays (Figure 2). Previously, it was shown that, like mZP3, gp55 at relatively low concentrations inhibits binding of sperm to ovulated eggs in an *in vitro* competition assay (Rosiere & Wassarman, 1992), and that result was confirmed here (Figure 2). gp55 exhibited an ID_{50} of ~200 nM in this assay (60 ± 19% inhibition of sperm binding at a concentration of 10 ng/μL; $n = 3$), a value similar to that obtained with intact mZP3 (~120−200 nM; Wassarman, 1988) and gp55 (~120 nM; Rosiere & Wassarman, 1992) previously. In addition, gp55 was tested for its ability to induce sperm to undergo the acrosome reaction *in vitro* and was found to be virtually as effective as mZP3 in this assay (Figure 2). At a concentration of about 10 ng/μL, gp55 induced 49 ± 4% of sperm to undergo the acrosome reaction (background, 19 ± 2%; $n = 3$). Therefore, gp55 possesses both of the bioactivities exhibited by intact mZP3 *in vitro* and is active at about the same concentrations.

Bioactivity Assays of gp55 Fractionated by Molecular Weight. As shown above, gp55 is very heterogeneous with respect to molecular weight (~47 000−62 000 M_r). To determine whether all size-classes of gp55 are able to inhibit binding of sperm to eggs *in vitro*, the purified glycopeptide was subjected to SDS−PAGE, the portion of gel containing gp55 was cut into three equally sized gel pieces, and glycopeptides were eluted from each gel piece. When the three purified fractions were reexamined by SDS−PAGE, they displayed average M_rs of ~60 000, ~55 000, and ~49 000, respectively (Figure 3). Each fraction was then tested for bioactivity in the competition assay. As seen in Figure 3, each of the three fractions was about as effective as unfractionated gp55 at inhibiting binding of sperm to eggs *in vitro*. For example, at a concentration of 5 ng/μL (~100 nM), all three M_r fractions of gp55 inhibited binding of sperm to eggs by about 44% ($n = 3$).

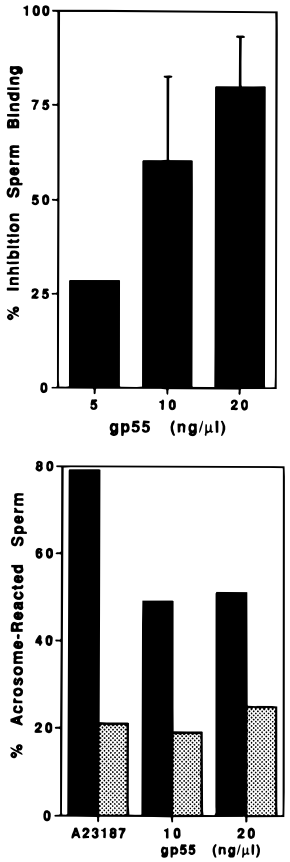

FIGURE 2: Effect of gp55 on binding of sperm to eggs and induction of the acrosome reaction *in vitro*. (Top) Inhibition of sperm binding to eggs as a function of gp55 concentration. Sperm were incubated in the presence of gp55, eggs were added, and binding of sperm to eggs was determined, as described under Materials and Methods. Each bar represents the mean of either two (5 ng/μL) or three (10 and 20 ng/μL) individual experiments, with error bars indicating the standard deviation. (Bottom) Percent acrosome-reacted sperm in the presence of either ionophore A23187 (10 μM) or gp55 (solid bars). Also shown are the levels of acrosome-reacted sperm in samples incubated without either A23187 or gp55 (background; stippled bars). Each bar represents the mean of either two (10 ng/μL) or three (A23187 and 20 ng/μL) individual experiments (error bars are not shown, but were less than 10% of the mean).

Electrophoretic Analysis and Bioactivity Assays of gp55 Treated with Glycosidases. (i) N-Glycanase (N-GLYase). Purified gp55 was digested by N-GLYase, an endoglycosidase that catalyzes the hydrolysis of N-linked oligosaccharides (high-mannose, complex, and hybrid) at the β-aspartylglycosylamine bond between the innermost GlcNAc and the Asn residue (converting Asn to Asp; Tarentino *et al.*, 1985; Chu, 1986). Removal of N-linked oligosaccharides from gp55 by N-GLYase reduced its average M_r to ~21 000 and increased the p*I* of both major isoforms to ~5.3 (Figure 4), but did not significantly affect its ability to inhibit binding of sperm to eggs or to induce the acrosome reaction (Figure 5). Therefore, N-linked oligosaccharides present on gp55 apparently are not essential for its bioactivities.

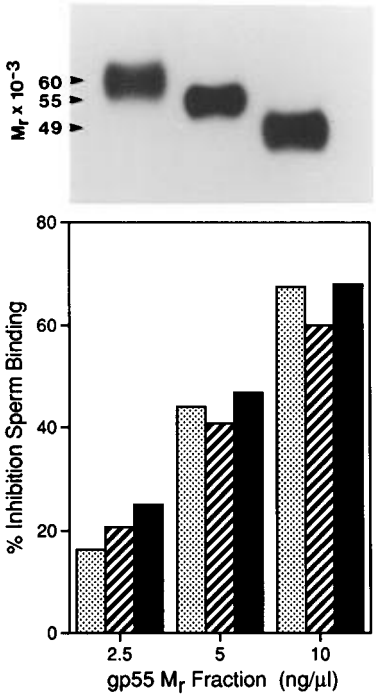

FIGURE 3: Effect of gp55 size-classes on binding of sperm to eggs. (Top) Autoradiograph of the three molecular weight fractions of gp55. Gels were calibrated with prestained molecular weight standards ranging from 14 300 to 200 000 M_r. (Bottom) Inhibition of sperm binding to eggs by three size-classes of gp55 at three different concentrations (2.5, 5, and 10 ng/μL). Sperm were incubated in the presence of gp55, eggs were added, and binding of sperm to eggs were determined, as described under Materials and Methods. Shown are the effects of high (~60 000 M_r; stippled bar), middle (55 000 M_r; striped bar), and low (~49 000 M_r; solid bar) molecular weight fractions of gp55 on sperm binding. Each bar represents the mean of either two (10 ng/μL) or three (2.5 and 5 ng/μL) individual experiments (error bars are not shown, but were less than 11% of the mean).

(ii) Endo-β-galactosidase (EBGase). Purified gp55 was digested by EBGase, an endoglycosidase that cleaves the 1−4-linked β-Gal in the linear sequence R-GlcNAcβ1−3Galβ1−4Glc/GlcNAc, provided that the Gal is not branched (Scudder *et al*., 1987). Removal of lactosaminoglycan units from gp55 by EBGase reduced its average M_r to ~40 000 and increased the p*I* of some molecules to 5.6 (Figure 4), but did not significantly affect its ability to inhibit binding of sperm to eggs or to induce the acrosome reaction (Figure 5). In this context, it should be noted that gp55 digested first with N-GLYase and then with EBGase resembled gp55 treated with N-GLYase alone; i.e., its average M_r was reduced to ~21 000, and its p*I* was increased to ~5.3 (see above). These observations suggest that the lactosaminoglycan units released from gp55 by EBGase were associated with N-linked oligosaccharides and were not essential for the glycopeptide's bioactivities.

(iii) Neuraminidase (NEURase). Purified gp55 was digested by a NEURase specific for a variety of nonreducing terminal sialic acids in α2−3/6/8 linkages to various monosaccharides (Uchida *et al*., 1977). Removal of sialic acid from gp55 by NEURase did not have a significant effect on its average M_r, but did alter the pattern of isoforms observed on isoelectric focusing gels, increasing its p*I* from ~4.3−4.5 to ~5.6−6.0 (Figure 4). This large change in p*I* suggests that much, but not all, of the acidic nature of gp55 is attributable to sialic acid residues that terminate its

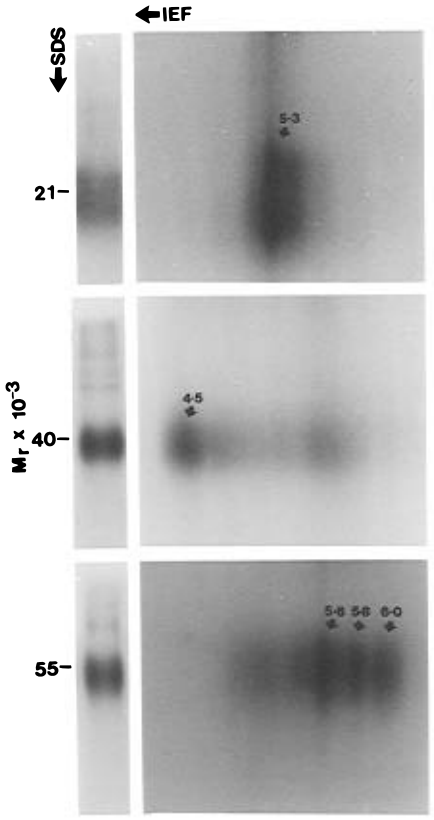

FIGURE 4: Gel electrophoretic analyses of gp55 following digestion by various glycosidases. Shown in each case is an autoradiograph obtained following either one-dimensional SDS−PAGE (left) or high-resolution two-dimensional gel electrophoresis (right) of glycosidase-treated gp55. Digestions were performed as described under Materials and Methods. (Top) *N*-Glycanase digest. (Middle) Endo-β-galactosidase digest. (Bottom) Neuraminidase digest. Shown are the molecular weights and p*I*s for glycosidase-treated gp55. Gels were calibrated with prestained molecular weight standards as described in the legend to Figure 1. IEF, isoelectric focusing.

oligosaccharides. Despite the relatively large change in its p*I*, gp55 digested by NEURase inhibited binding of sperm to eggs and induced sperm to undergo the acrosome reaction *in vitro* (Figure 5). Therefore, sialic acid does not appear to be essential for bioactivity.

DISCUSSION

gp55, a major product when mZP3 is digested by any of a number of proteases, including papain and V8 protease, is derived from the carboxy-terminal half of the polypeptide (Rosiere & Wassarman, 1992). It has been proposed that this specificity for digestion is attributable to the presence of a so-called "hinge" region, as in immunoglobulins (Braden & Tooze, 1991), located from amino acid residues 219 to 260 of mZP3 (Wassarman & Litscher, 1995). This region presumably serves as a flexible link between two different mZP3 polypeptide domains: one domain that possesses the combining-site for sperm (carboxy-terminal) and a second that, perhaps, participates in ZP filament assembly (amino-terminal). The latter would involve interactions between mZP3 and mZP2, a 120 000 M_r ZP glycoprotein, to form the heterodimers that polymerize into ZP filaments during oocyte growth (Greve & Wassarman, 1985; Wassarman & Mortillo, 1991).

gp55 binds to sperm and prevents them from binding to ovulated eggs *in vitro* (Rosiere & Wassarman, 1992), a result

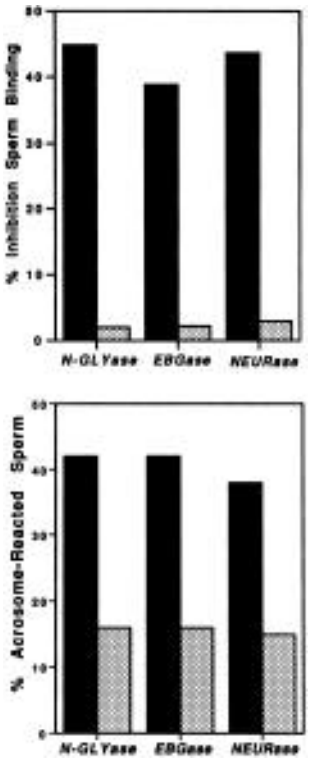

FIGURE 5: Effect of glycosidase-treated gp55 on binding of sperm to eggs and induction of the acrosome reaction *in vitro*. (Top) Inhibition of sperm binding to eggs by glycosidase-treated gp55 (10 ng/μL) ($n = 2$; solid bars). Sperm were incubated in the presence of glycosidase-treated gp55, eggs were added, and binding of sperm to eggs was determined, as described under Materials and Methods. Also shown is the extent of inhibition of sperm binding by eggs exposed to samples lacking gp55 ($n = 2$; stippled bars). (Bottom) Percent acrosome-reacted sperm in the presence of glycosidase-treated gp55 (10 ng/μL) ($n = 2$; solid bars). Also shown are the levels of acrosome-reacted sperm after incubation in the presence of samples lacking gp55 ($n = 2$; stippled bars).

confirmed here (Figure 2). gp55 possesses the combining site for sperm, located in a region of polypeptide that contains five serine residues (Kinloch *et al*., 1995; Wassarman & Litscher, 1995). Like intact mZP3, the glycopeptide is a very effective inhibitor of sperm binding, acting at nanomolar concentrations (~50−200 nM). This is somewhat surprising since oligosaccharides isolated from mZP3, as well as synthetic oligosaccharides of defined structures, must be present at much higher concentrations (~10 μM) to inhibit sperm binding (Florman & Wassarman, 1985; Litscher *et al*., 1995). It can be assumed that any constraints placed on oligosaccharide structure by mZP3 polypeptide are retained in gp55. In addition, it is clear from experiments carried out with gp55 size-fractions that each fraction recognizes and binds to sperm (Figure 3).

Although small mZP3 glycopeptides (~1500−6000 M_r) inhibit binding of sperm to eggs *in vitro*, they do not induce sperm to undergo the acrosome reaction (Florman *et al*., 1984; Wassarman, 1990). In a similar vein, mZP3 O-linked oligosaccharides (Florman & Wassarman, 1985; Wassarman, 1990), as well as related synthetic oligosaccharides of defined structures (Litscher *et al*., 1995), inhibit binding of sperm to eggs, but do not induce the acrosome reaction. In this context, there is some evidence to suggest that small mZP3 glycopeptides (<30 000 M_r) fail to induce the acrosome reaction, following binding to sperm, due to their inability to aggregate complementary mZP3-binding proteins in the sperm-head plasma membrane (Leyton & Saling, 1989). On the other hand, results presented here indicate that a larger glycopeptide, representing about one-third of the mZP3 polypeptide (~170 amino acids), can induce the acrosome reaction. This is consistent with our previous observation that, when the average size of mZP3 glycopeptides in Pronase digests falls below ~45 000 M_r, the glycopeptides are no longer able to induce sperm to undergo the acrosome reaction (Wassarman *et al*., 1985).

N-Linked oligosaccharides have been implicated in the biosynthesis, transport, and functions of many glycoproteins (Varki, 1993). gp55 has five potential N-linked glycosylation sites (N-205, N-251, N-282, N-305, and N-308; secreted mZP3 lacking signal-sequence), all of which have the consensus sequence N-X-S/T, where X can be any amino acid (Kornfeld & Kornfeld, 1985). Some structural studies have been carried out on mZP2 and mZP3 N-linked oligosaccharides (Noguchi & Nakano, 1993). They were found to be mainly fucosylated tri- and tetra-antennary complex-type oligosaccharides (no high-mannose or hybrid types detected), and similar N-linked oligosaccharides were found on mZP2 and mZP3. Here, digestion with *N*-glycanase reduced the M_r of gp55 from ~55 000 to ~21 000, and increased the p*I* from 4.3−4.5 to about 5.3, suggesting that the glycopeptide carries multiple N-linked oligosaccharides. Digestion of gp55 with EBGase reduced its M_r to ~40 000, and the lactosaminoglycan units removed apparently were present on N-linked oligosaccharides. However, results of experiments in which the ~21 000 M_r (N-GLYase treatment) and ~40 000 M_r (EBGase treatment) species were used strongly suggest that N-linked oligosaccharides are not involved in either the sperm receptor or the acrosome reaction-inducing activity of gp55 (Figure 5). This is consistent with results of previous experiments using intact mZP3 (Florman & Wassarman, 1985; Wassarman, 1989; Miller *et al*., 1992).

Sialic acid is frequently found at the nonreducing end of oligosaccharides present on glycoproteins and, in several instances, serves as an essential ligand in carbohydrate-mediated adhesion (Varki, 1992; Roth, 1993; Pilatee *et al*., 1993; Powell *et al*., 1993; Kelm *et al*., 1994). For example, sialylation of GlyCAM-1 is required for L-selectin binding (Rosen *et al*., 1989; Imai *et al*., 1991; Berg *et al*., 1992; Foxall *et al*., 1992). Digestion of GlyCAM-1 with neuraminidase from either *Arthrobacter ureafaciens* or Newcastle Disease virus (selective for sialic acid α2−3 linkages; Paulson *et al*., 1979) significantly decreases binding activity (Imai *et al*., 1992). On the other hand, we found that, while much of the acidic nature of gp55 is attributable to sialic acid (Figure 4; p*I* increased by as much as 1.7 units), desialylation of gp55 does not significantly affect its bioactivity (Figure 5). Therefore, sialic acid does not appear to be directly involved in binding of gp55 to sperm.

Overall, these results provide further support for the involvement of O-linked, rather than N-linked, oligosaccharides in mZP3 function (i.e., binding to sperm and induction of the acrosome reaction). gp55 is virtually as effective as intact mZP3 in preventing the binding of sperm to eggs and inducing the acrosome reaction. Therefore, gp55 should be suitable for structural studies of the sperm receptor's combining site for sperm, as well as for mechanistic studies of sperm−egg interaction and induction of the acrosome reaction.

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