Murine Sperm-Zona Binding, A Fucosyl Residue Is Required for a High Affinity Sperm-binding Ligand

A SECOND SITE ON SPERM BINDS A NONFUCOSYLATED, β-GALACTOSYL-CAPPED OLIGOSACCHARIDE*

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An essential initial step in murine fertilization is the binding of acrosome-intact sperm to specific O-linked oligosaccharides on zona pellucida glycoprotein 3. While there is agreement on the primary role of O-linked glycans in this process, there is a lack of consensus on both the terminal monosaccharide(s) required for a functional sperm binding site and the corresponding protein on the sperm cell surface that recognizes this ligand. Much current debate centers on an essential role for either a terminal N-acetylgalactosaminyl or, alternatively, a terminal α-galactosyl residue. To gain insight into the terminal saccharides required to form a functional sperm-binding ligand, dose-response curves were generated for a series of related tri- and tetrasaccharides to evaluate their relative effectiveness to competitively inhibit the in vitro binding of murine sperm to zona pellucida-enclosed eggs. A GlcNAc-capped trisaccharide, GlcNAcβ1,4GlcNAcβ1,4GlcNAc, was inactive (1–72 µM range). In contrast, a β4-galactosyl-capped trisaccharide (Galβ1,4GlcNAcβ1,4GlcNAc) and an α3-galactosyl-capped trisaccharide (Galα1,3Galβ1,4GlcNAc) inhibited sperm-zona binding with low or moderate affinity (ED50 = 42 µM and 5.3 µM, respectively). The addition of an α3-fucosyl residue to each of these two competitive inhibitors, forming Galβ1,4[Fucα1,3]GlcNAcβ1,4GlcNAc or Galα1,3Galβ1,4[Fucα1,3]GlcNAc, resulted in ligands with 85- and 12-fold higher affinities for sperm, respectively (ED50 = 500 and 430 nM). Thus, the presence of a fucosyl residue appears to be obligatory for an oligosaccharide to bind sperm with high affinity. Last, mixing experiments with pairs of competitive inhibitors suggest that murine sperm-zona binding is mediated by two independent oligosaccharide-binding sites on sperm. The first (apparently high affinity) site binds both the α3-galactosyl-capped trisaccharide and the two fucosylated tetrasaccharides. The second (apparently low affinity) site binds a nonfucosylated β4-galactosyl-capped trisaccharide.

The initial event in mammalian fertilization is the binding of a sperm to the zona pellucida (ZP),1 the extracellular glycoprotein matrix surrounding the egg. In the mouse, capacitated, acrosome-intact sperm initially bind to zona pellucida glycoprotein 3 (ZP3), one of three glycoproteins comprising the ZP (1, 2). The sperm binding activity of ZP3 is localized to a subset of O-linked oligosaccharides on ZP3 with an estimated molecular mass of about 3.9 kDa (3–5). While there is general agreement that the nonreducing terminal monosaccharides are critical for binding (4, 5), there is a lack of agreement on the identity of the terminal monosaccharide(s) required for a functional sperm-binding ligand and the corresponding binding site(s) on the sperm surface.

Two different models of murine sperm-ZP binding have been proposed that address the basic requirements for both a functional sperm-binding ligand and for the corresponding sperm surface ZP-binding protein. The first model posits that sperm surface β1,4-galactosyltransferase (β4GT) binds its acceptor sugar substrate, an N-acetylgalactosaminyl residue (GlcNAc), located at the nonreducing terminus of an O-linked oligosaccharide on ZP3 (5, 6). In support of this model, β4GT has been localized immunocytochemically with a polyclonal antiserum to the sperm plasma membrane that overlies the acrosome (6). Biochemical and immunological probes that can potentially interact with cell surface β4GT block the ability of sperm to bind zona-intact eggs in vitro (6). The activity of ZP3 in a competitive sperm-ZP binding assay was reduced either by enzymatic removal or β4-galactosylation of terminal GlcNAc residues (5). In conflict with this model are observations from two laboratories that independently report the generation of mice in which the β4GT gene is inactivated by homologous recombination (7, 8). The null mice survive to term, a significant percentage survive to maturity, and the males are fertile.

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1 The abbreviations used are: ZP, zona pellucida; ZP3, zona pellucida glycoprotein 3; β4GT, β4-galactosyltransferase; GNAc, N-acetylglucosamine; α1,4Fucα, α-fucosyltransferase; β1,4GT, β1,4-galactosyltransferase; UDP-GlcNAc, UDP-N-acetylglucosamine; α-Galβ1,3Galβ1,4GlcNAc, mesotetraosylglycan; α-Galβ1,3Galβ1,4GlcNAc, mesotetraosylglycan.

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The second model postulates that a sperm surface protein, sp56, binds nonreducing terminal α-galactosyl residues on ZP3 (4, 9–12). This model is supported by the observation that enzymatic removal of terminal α-galactosyl residues eliminated the inhibitory activity of ZP3 in the competitive sperm-ZP binding assay (4). Tetraantennary, α3-galactosyl-capped oligosaccharides have also been shown to inhibit sperm-ZP binding (12). Additionally, it has been demonstrated that sp56 binds galactose residues and competitively inhibits murine sperm-ZP binding in vitro (10, 11). Also consistent with this model is the observation that murine α1,3-galactosyltransferase, the candidate enzyme for the addition of terminal α-galactosyl residues on O-linked oligosaccharides on ZP3, is expressed in female but not male germ cells (13). In potential conflict with this model, however, is the observation that inactivation of the α1,3-galactosyltransferase gene by homologous recombination does not affect the fertility of female mice (14). Additionally, recent evidence indicates that sp56 is located primarily in the acrosomal contents and not on the plasma membrane (15). It has been postulated, however, that dynamic pores in the plasma membrane of acrosome-intact murine sperm may allow sp56 to bind ZP3 (15).

A determination of whether either or both of these models of sperm-ZP binding are correct has been hampered by the limited amounts of oligosaccharides that can be obtained from murine ZP3 for thorough structural and functional analyses and by the potential structural complexity of these oligosaccharides. To circumvent these limitations, we have used an in vitro sperm-ZP binding assay to generate dose-response curves for a series of tri- and tetrasaccharides with different nonreducing ends that these two current models for sperm-ZP binding predict might compete for ZP-binding sites on sperm. Precedent for this approach is provided by the demonstration that tri- and tetraantennary α- and β-galactosyl-capped oligosaccharides inhibit sperm-ZP binding (12) and by the extensive analysis of potential ligands for the family of cell surface receptors, the selectins, which mediate the binding of lymphocytes to the vascular endothelium (16). In this study, we show that a small oligosaccharide with a nonreducing terminal GlcNAc, which is a substrate for β4GT in an in vitro enzymatic assay, is not a competitive inhibitor of sperm-ZP binding. We also demonstrate that a terminal α3-galactosyl residue is not sufficient for an oligosaccharide to be a high affinity competitive inhibitor of sperm-ZP binding. Rather, an α3-fucosyl residue in the context of the LewisX trisaccharide, Galβ1,4[Fucα1,3]GlcNAc, is required to create a high affinity ligand for a ZP-binding site on murine sperm. Additionally, we present evidence that murine sperm-ZP binding is potentially mediated, in part, by a second, orphaned amounts of oligosaccharides that can be obtained from bovine α1,3-galactosyltransferase and UDP-[14C]Gal (0.23 Ci/mol), and the product was purified and analyzed as described previously (17–19). The mass of product was determined as described above. Two additional preparations of αG-βG-GN were purchased from V-Labs (Covington, LA).

**Oligosaccharide 5**—Galα1,3Galβ1,4(Fucα1,3)GlcNAc (αG-βG-[F]-GN) was synthesized by incubation of αG-βG-GN with purified human milk α1,3-fucosyltransferase and GDP-[3H]fucose (0.10 Ci/mol). The product was purified and characterized as described previously (17).

**1H NMR Spectroscopy**

Products obtained from the enzyme-assisted synthesis of each oligosaccharide were identified using 400-MHz 1H NMR spectra, which were recorded on a Bruker MSL-400 spectrometer (Department of Physics, Vrije Universiteit, Amsterdam) as described (20).

**Collection of Mouse Gametes and Embryos**

Eggs and sperm were collected from CD-1 mice and prepared as described by Bleil and Wassarman (1). Prior to use in the competitive sperm-ZP binding assay, sperm were capacitated by incubation for about 1 h in medium 199 supplemented with 2 mg/ml BSA and 30 μg/ml sodium pyruvate (M199-M). ZP were isolated from oocytes in Whitten’s medium (21) plus 3 mg/ml BSA (WM-BSA) and acid-solubilized as described by Bleil and Wassarman (2). Two-cell embryos were obtained by culturing fertilized eggs from mated females for 18 h in WM-BSA.

**Pipettes**

Micropipettes were fire-pulled from 4-mm internal diameter borosilicate glass tubing. A bore diameter of 228 μm was obtained by inserting a 0.009-inch diameter piano wire (Small Parts Inc.) down the pipette until it stopped; a flat break was made at that point. Pipettes having a tapered length of 3–4 cm were fired-polished, and the bore diameter was remeasured to ensure that the bore diameters were not reduced.

**The Competitive In Vitro Sperm-ZP Binding Assay**

This assay was performed essentially as described (1). Briefly, the test oligosaccharide was incubated at 37 °C in 95% air, 5% CO2 for 30 min with 30,000 sperm in a total volume of 25 μl of M199-M under oil, and then at least 10 eggs and three two-cell embryos were added in 5 μl of medium. Sperm, eggs, and embryos were incubated for an additional 15 min. Published results demonstrate that the sperm that bind zona-enclosed eggs during this 15-min incubation do not undergo the acrosome reaction (22). Sperm-egg and sperm-embryo complexes were then serially transferred through 40-μl drops of media until 2–5 sperm remained bound to the embryos. For each experiment, the cultures were pipetted an equal number of times with the same pipette. Cells were immediately fixed with an equal volume of 1.0% formaldehyde, 0.4% polyvinylpyrrolidone-40 in HEPES (pH 7.4) saline, transferred to a glass slide, and examined by phase contrast microscopy. Bound sperm were enumerated using (× 40; N.A. = 0.70) phase contrast objective. Sperm on the ZP overlying the upper 40% of an egg or a two-cell embryo were counted as the focal plane was moved down through the egg or embryo. This region of the ZP was examined because all sperm on this but not on lower regions could be counted rapidly and accurately. The number of bound sperm/egg was defined by subtracting the average number of sperm remaining on the embryos from the average number of sperm on the eggs. The accuracy of this method of counting sperm on eggs or embryos was verified by two methods. First, for a subset of samples, the number of sperm on the total surface of the ZP surrounding eggs and embryos was also determined. These numbers were consistently 2.5 times greater than when sperm on the upper 40% of the zona were enumerated. Second, selected samples were recounted independently by a second investigator, and similar results were obtained. In the experiments described, 69 ± 6 (mean ± S.E.) sperm bound to the entire surface of the eggs in the absence of any added competitor. Data are expressed as percentage of inhibition of sperm-ZP binding, where numbers of bound sperm in the absence of competitor equaled 0% inhibition.

**Demonstration That the Oligosaccharides Used as Competitors Did Not Trigger the Acrosome Reaction**

This study identifies small oligosaccharides of defined structure that inhibit the in vitro binding of acrosome intact sperm to the zona pellucida and correlates the structures of these oligosaccharides with their biological activities in the competitive sperm-ZP binding assay. Since the primary receptor of murine spermatozoa for the ZP resides on the...
plasma membrane overlying the acrosome (1, 2), a prerequisite for this analysis is that the test oligosaccharides themselves do not cause the sperm to undergo the acrosome reaction and, thus, lose their cell surface receptors for the ZP. To verify that the oligosaccharides do not trigger the acrosome reaction, 1 × 10^6 capacitated sperm were incubated for 45 min in a total volume of 50 µl of medium supplemented with 18 µM oligosaccharide or 10 µM concentration of the calcium ionophore (A23187), the positive control for this procedure. Sperm used as negative controls were incubated in medium to which was added the 2.8 µl of vehicle (water: 2 × M199M; 1:1). Sperm were incubated for 45 min with or without oligosaccharides, because in the competitive sperm-ZP binding assay, sperm were incubated with oligosaccharide competitors for a total duration of 45 min. Following the incubation, sperm were fixed in 4% formaldehyde in phosphate-buffered saline, and acrosomes were stained with 1% Coomassie Brilliant Blue G. A minimum of 200 sperm/sample were analyzed as acrosome-intact or as acrosome-reacted and data were expressed as percentage of acrosome-reacted sperm.

**Dose-response Analysis of Individual Oligosaccharides in the Competitive Sperm-ZP Binding Assay: Experimental Design**

To compare the abilities of oligosaccharides to inhibit sperm-ZP binding, each oligosaccharide was titrated against sperm and eggs in the competitive sperm-ZP binding assay. Depending on the oligosaccharide, the concentrations tested ranged from 0.25 to 144 µM. The ED_{50} and maximal percentage of inhibition for each oligosaccharide were calculated from the corresponding dose-response curve (see "Analysis of Data"). The ED_{50} is defined as the concentration of an oligosaccharide producing half-maximal inhibition of sperm-ZP binding.

Results from the competitive sperm-ZP binding assay are described by the probabilistic model of cell binding of Cozen-Roberts et al. (23). This model predicts that the probability that a sperm will bind a ZP-enclosed egg is a function of the incubation time of sperm with eggs, the number of unoccupied receptors on the sperm, and the rates of association and dissociation of sperm surface binding sites from specific ligands. In addition, this model predicts that saturation of this receptor with the appropriate free oligosaccharide will inhibit sperm-ZP binding by 100% if a single sperm surface receptor mediates this binding. However, if sperm-ZP binding is mediated by multiple zona-binding sites, each binding a different oligosaccharide, saturation of one of these receptors will reduce sperm-ZP binding by less than 100%.

**Additive Effects of Paired Oligosaccharides: Experimental Design**

Three experiments were conducted to test the hypothesis that different oligosaccharides bind independent ZP-binding sites on sperm. Each experiment consisted of four experimental groups.

**Group 1—**Sperm were incubated in a saturating concentration of either αG-βG-GN or αG-βG-[F]-GN. The saturating concentration was defined as the dose-response curve above which there was no significant increase in percentage of inhibition.

**Group 2—**Sperm were preincubated in twice the concentration of αG-βG-GN or αG-βG-[F]-GN used in group 1. We anticipated that there would be no significant difference in the results obtained between groups 1 and 2, confirming that for a particular experiment, the concentration of oligosaccharide used in group 1 was at apparent saturation.

**Group 3—**Sperm were preincubated in a saturating concentration of αG-βG-[F]-GN or a concentration of βG-βG-GN calculated from the competitive sperm-ZP binding assay to reduce sperm-ZP binding by 50%.

**Group 4—**Sperm were preincubated with the saturating concentration of the oligosaccharide used in group 1 plus the concentration of the second oligosaccharide used in group 3.

Last, to establish the numbers of sperm/egg at 0% inhibition, sperm were preincubated in the absence of any oligosaccharide. Two oligosaccharides were defined as binding independent sites on sperm if the percentage of inhibition with the oligosaccharide pair was greater than the percentage of inhibition with each oligosaccharide alone.

**Analysis of Data**

Dose response curves for individual oligosaccharides in the competitive sperm-ZP binding assay were fit to a rectangular hyperbola, and regression analysis was performed using the program Sigma Plot; the ED_{50} and percentage of maximal inhibition were calculated from this regression analysis. The ED_{50} values provided a measure of the relative inhibitory activities of the oligosaccharides in the competitive sperm-ZP binding assay. Two-way analysis of variance was used to compare the effects of incubating sperm with one or two different oligosaccharides. The latter analysis was conducted using the statistical package, SAS. Statistically significant differences were defined as p ≤ 0.05.

**RESULTS**

**Structural Identification of the Synthetic Oligosaccharides βG-βG-GN, βG-[F]-βG-GN, αG-βG-GN, and αG-βG-[F]-GN**

αG-βG-GN and αG-βG-[F]-GN were synthesized and characterized as described previously (17). Their chemical shift values are included in Table I to allow comparison with βG-βG-GN and βG-[F]-βG-GN.

The presence of the terminal β4-linked Gal residue in βG-βG-GN is confirmed in the 1H NMR spectrum by the Gal H-1 and H-4 signals at δ 4.465 and δ 3.925, respectively. These values are comparable with those of the Gal residue in the Galβ1,4GlcNAc element (17).

In the 1H NMR spectrum of βG-[F]-βG-GN, the presence of the α3-linked Fuc residue is reflected in the Fuc H-1, H-5, and CH3 structural reporter group signals, which have similar values as those of the Fuc residue in αG-βG-[F]-GN (see Table I). In addition, the α3-linkage of the Fuc residue to the internal GlcNAc-2 is deduced from the upfield shifts of the GlcNAc-2 NAc (Δδ −0.011) and βGal H-1 (Δδ −0.022) signals compared with βG-βG-GN. Similar upfield shifts are observed when comparing the same signals in αG-βG-GN and αG-βG-[F]-GN.

**Validation of the Competitive Sperm-ZP Binding Assay**

Initially, we validated the competitive sperm-ZP binding assay using acid-solubilized total ZP as the competitor. Preincubation of sperm with one, two, or four solubilized ZP/µl for 30 min inhibited sperm-ZP binding in a dose-dependent manner (data not shown). In agreement with Bleil and Wassarman (1), four ZP/µl inhibited sperm-ZP binding by approximately 90% relative to the negative control in which no solubilized ZP was added. By this criterion, results from our experiments are comparable with those from other laboratories. Second, we established in blind trials that incubation of sperm under these assay conditions with a 9 or 72 µM concentration of each oligosaccharide did not alter either sperm motility or viability. Third, we determined in 33 independent experiments that three different preparations of αG-βG-GN, at 9 µM, reproducibly inhibited

### Table I

| Reporter group | Residue | Chemical shift |
|----------------|--------|----------------|
| H-1            | GlcNAc-1 | 5.191 |
|                | GlcNAc-2 | 6.415 |
|                | GlcNAc-2(b) | 4.619 |
|                | Fuc | 4.833 |
|                | Fuc | 4.833 |
|                | NaC | 2.039 |
|                | GlcNAc-2 | 2.065 |
| CH3            | Fuc | 1.174 |

αG-βG-GN and αG-βG-[F]-GN were synthesized and characterized as described previously (17). Their chemical shift values are included in Table I to allow comparison with βG-βG-GN and βG-[F]-βG-GN.
50–60% of sperm-ZP binding. In three additional experiments where the positive control fell outside this range, the data were excluded from the statistical analysis. The data for the positive control are shown as the bar graph to the right of the dose-response curves in Fig. 1. Finally, it was demonstrated that incubation of capacitated sperm for 45 min with a 18 μM concentration of each oligosaccharide did not cause the sperm to undergo the acrosome reaction (Table II). In contrast, this concentration of βG-βG-GN, αG-βG-GN, αG-βG-[F]-GN, and βG-[F]-βG-GN inhibited sperm-ZP binding (see Fig. 1). Taken together, these data demonstrate that the competitive sperm-zona binding assay measures the abilities of specific oligosaccharides to inhibit the binding of acrosome-intact murine spermatozoa to the zona pellucida.

Fig. 1. Dose-response analysis of a series of short oligosaccharides with different nonreducing ends in the competitive sperm-ZP binding assay. A, βG-βG-GN was tested at 1, 9, 18, and 36 μM; B, βG-βG-GN was tested at 1, 9, 18, 36, and 72 μM; C, αG-βG-GN was tested at 1, 5, 9, 18, and 36 μM; D, αG-βG-[F]-GN was tested at 0.25, 0.5, 0.75, 1, and 9 μM (inset, 1, 5, 9, 18, and 36 μM); E, βG-[F]-βG-GN was tested at 0.25, 0.5, 0.75, 1, and 9 μM. Data (mean ± S.E. for 4–6 experiments) are expressed as percentage of inhibition relative to negative controls (no competitor). These panels were drawn by connecting the mean value of percentage of inhibition for each oligosaccharide concentration. Results for the internal positive control (9 μM αG-βG-GN) are shown by the bar graph in appropriate panels.
10^6 capacitated sperm were incubated for 45 min in 50 µl of M199M supplemented with 18 µM of an oligosaccharide, with vehicle (1.4 µl of water plus 1.4 µl of 2 × M199M) or with 10 µM A23187, and sperm were then fixed in buffered 4% formaldehyde. Following staining of the acrosomes with Coomassie Blue G, a minimum of 200 sperm/sample were analyzed for the presence or absence of an acrosome. Sperm without an acrosome are defined as acrosome-reacted. Results shown in this table are from four independent experiments.

**Table II**
The oligosaccharides tested do not cause murine spermatozoa to undergo the acrosome reaction.

| Test substance | Acrosome-reacted sperm (mean ± S.E.) |
|----------------|--------------------------------------|
| αGalβ1,4GalNAcβ1,4GlcNAc | 14.6 ± 2.2 |
| Galβ1,4GlcNAcβ1,4GlcNAc | 11.6 ± 2.7 |
| Galα1,3Galβ1,4GlcNAc | 10.5 ± 2.8 |
| Galβ1,3Galβ1,4[Fucα1,3]GlcNAc | 12.9 ± 4.3 |
| Galβ1,4[Fucα1,3]GlcNAcβ1,4GlcNAc | 15.1 ± 5.9 |
| Vehicle | 10.7 ± 4.8 |
| A23187 | 67.5 ± 8.2 |

**Dose-response Analysis of Individual Oligosaccharides in the Competitive Sperm-ZP Binding Assay**

The Trisaccharide βG-βGN-βG-βGN Does Not Inhibit Sperm-ZP Binding—If sperm-zona binding is mediated by a sperm surface β1,4-galactosyltransferase and its acceptor sugar substrate, then an oligosaccharide with a nonreducing terminal GlcNAc residue would be anticipated to inhibit sperm-ZP binding. To test this prediction, sperm were preincubated with 1, 9, 18, or 36 µM βG-βG-βGN-βG-βGN, which is an appropriate substrate for β1,4-galactosyltransferase. In four replicate experiments, no competition was observed (Fig. 1A). Competition was also not observed when sperm were incubated with 72 µM βG-βGN-βG-βGN (data not shown).

Substitution of the Terminal GlcNAc with a β4-galactosyl Residue Results in a Trisaccharide That Inhibits Sperm-ZP Binding—Bleil and Wassarman (4) reported that enzymatic removal of α-galactosyl residues reduced ZP3’s ability to compete for ZP-binding sites on sperm. Removal of an α-galactosyl residue would be anticipated to yield an O-linked oligosaccharide with Galβ1,4GlcNAc-R at its terminus. Shur and colleagues (6) have reported that β4-galactosylation of ZP3 also reduced the sperm binding activity of ZP3. This galactosylation would also have produced O-linked oligosaccharides which terminated in Galβ1,4GlcNAc-R. Therefore, we anticipated that βG-βGN-βGN would not inhibit sperm-ZP binding. However, as shown in Fig. 1B, this was not the case. Significant inhibition (14%) was observed with 9 µM βG-βGN-βG-βGN; inhibition increased to 47% at 72 µM, the highest concentration tested.

While this concentration was not saturating, regression analysis indicated that βG-βGN-βGN would produce an estimated 74% maximal inhibition and an ED50 of 42 µM. This ED50 value, coupled with the fact that this site was difficult to saturate, indicates that βG-βGN-βG-βGN is a low affinity competitive inhibitor for ZP-binding sites on sperm.

A Trisaccharide with a Terminal α3-Galactosyl Residue Has a Higher Inhibitory Activity than βG-βGN-βG-βGN—The conclusion by Bleil and Wassarman (4) that a sperm-binding oligosaccharide from ZP3 has a terminal α-galactosyl residue and our demonstration that oocytes transcriptionally express α1,3-galactosyltransferase (13) suggested to us that the product of this enzyme, αG-βG-βGN, would be an inhibitor of sperm-ZP binding. To determine how effective an inhibitor, sperm were incubated with 1, 5, 9, 18, and 36 µM αG-βG-βGN (Fig. 1C). The resulting dose-response curve was nearly linear from 0 to 9 µM where 53% inhibition was observed. Regression analysis indicated that αG-βG-βGN produced an estimated 78% maximal inhibition and an ED50 of 5.3 µM. Thus, the inhibitory activity of this trisaccharide was 8-fold greater than the activity of βG-βG-βGN-βG-βGN.

The Addition of an α3-Fucosyl Residue to αG-βG-βGN, Forming αG-βG-[F]-βG-βGN, Yields a Tetrascarhide with High Inhibitory Activity—There is circumstantial evidence that an α-fucosyl residue may also be a component of a sperm-binding oligosaccharide on murine ZP3. Digestion with α-fucosidase reduced the inhibitory activities of ZP3- or ZP-derived O-linked oligosaccharides in the competitive sperm-ZP binding assay (4). Additionally, millimolar amounts of two fucose-containing oligosaccharides inhibit sperm-ZP binding in vitro (12). Consequently, we examined the effect of adding an α3-fucosyl residue to the GlcNAc residue of αG-βG-βGN. αG-βG-[F]-βG-βGN was initially examined at 1, 9, 18, and 36 µM. Maximal inhibition (approximately 40%) was observed at 1 µM (Fig. 1D, inset), and no further inhibition was observed at concentrations as high as 140 µM (data not shown). To accurately define the dose response, this analysis was repeated with 0.25, 0.5, 0.75, 1, and 9 µM αG-βG-[F]-βG-βGN (Fig. 1D). Results show a linear dose response between 0 and 0.75 µM; maximal inhibition and ED50 values were calculated to be 46% and 430 nM, respectively. Thus, α3-fucosylation of the reducing terminal GlcNAc residue increased the inhibitory activity of αG-βG-βGN by 12-fold.

The Presence of an α3-Galactosyl Residue Is Not Obligatory for an Oligosaccharide with High Inhibitory Activity—Female mice that lack a functional α1,3-galactosyltransferase gene are still fertile (14), indicating that an α3-galactosyl residue is not obligatory in vivo for a functional sperm-binding oligosaccharide. To determine whether the presence of an α3-fucosyl residue is sufficient to generate a tetrascarhide with high inhibitory activity, βG-[F]-βG-βGN was titrated against sperm at 0.25, 0.5, 0.75, 1, and 9 µM concentrations. The resulting dose-response curve (Fig. 1E) was essentially identical to that for αG-βG-[F]-βG-βGN (Fig. 1D). At 1 µM, a maximal inhibition of 46% was obtained. The ED50 for this fucosylated, β-galactosylated oligosaccharide was 500 nM. Thus, α3-fucosylation of βG-βGN-βG-βGN increased its inhibitory activity approximately 85-fold. This result suggests that an α3-fucosyl and not an α3-galactosyl residue is required for forming an oligosaccharide with high inhibitory activity in the competitive sperm-ZP binding assay.

**Do Inhibitors Bind at the Same Oligosaccharide-binding Site? Additive Effects of Paired Oligosaccharides**

While both αG-βG-[F]-βG-βGN and βG-[F]-βG-βGN had low ED50 values (430 and 500 nM, respectively) in the competitive sperm-ZP binding assay, each only inhibited approximately 45% of sperm-ZP binding. In contrast, acid-solubilized ZP inhibited 90% of sperm-ZP binding. One potential explanation for this difference in maximal inhibition between solubilized ZP and the oligosaccharides examined is that the ZP binds two or more distinct sites with different oligosaccharide binding specificities on the sperm surface. To test this hypothesis, we added a saturating concentration of one oligosaccharide inhibitor to a second oligosaccharide and compared the inhibition achieved with the paired oligosaccharides with inhibition obtained with each oligosaccharide alone. Saturation of the first oligosaccharide was confirmed, since inhibition was not significantly increased when the concentration of the first oligosaccharide was doubled (Fig. 2, A–C, compare lanes 1 and 2).

Evidence That αG-βG-βGN, αG-βG-[F]-βG-βGN, and βG-[F]-βG-βGN Bind the Same Site on Sperm—Fig. 2A compares the effect on sperm-ZP binding of incubating sperm with αG-βG-βGN (lanes 1 and 2), αG-βG-[F]-βG-βGN (lane 3), or both oligosaccharides together (lane 4). No significant differences were observed among these four groups. Identical results were obtained when
sperm were incubated with αG-βG-[F]-GN, βG-[F]-βGN-GN, or both oligosaccharides together (n = 4; data not shown). These results suggest that αG-βG-GN, αG-βG-[F]-GN, and βG-[F]-βGN-GN bind to the same ZP-binding site on sperm.

Evidence for a Second Site on Sperm That Binds βG-βG-βGN-GN—Fig. 2B shows the effects on sperm-ZP binding of incubating sperm with αG-βG-GN, βG-βG-βGN-GN or both trisaccharides together. The 75% inhibition of sperm-ZP binding achieved with 9 μM αG-βG-GN plus 72 μM βG-βG-βGN-GN (lane 4) was significantly greater than the 45% inhibition achieved with either 18 μM αG-βG-GN (lane 2) or 72 μM βG-βG-βGN-GN (lane 3) alone.

Fig. 2C shows the effects on sperm-ZP binding of incubating sperm with αG-βG-[F]-GN, βG-βG-βGN-GN, or both oligosaccharides together. The 60% inhibition achieved with 36 μM αG-βG-[F]-GN plus 72 μM βG-βG-βGN-GN (lane 4) was significantly greater than the inhibition achieved with either 72 μM αG-βG-[F]-GN (40%) (lane 2) or 72 μM βG-βG-βGN-GN (36%) (lane 3) alone. In summary, these results suggest that βG-βG-βGN-GN binds to a second, independent oligosaccharide-binding site that is distinct from the binding site occupied by αG-βG-GN, αG-βG-[F]-GN, or βG-[F]-βGN-GN.

**DISCUSSION**

The impetus for this study is the current lack of a consensus on the molecular basis for mouse sperm-ZP binding. This lack of a consensus is reflected by the fact that different models have been proposed to account for this fundamental biological process. As outlined in the Introduction, each model under consideration in this study is based on the putative identification of a distinct sperm cell surface protein (β1,4-galactosyltransferase versus ssp6) that has the intrinsic ability to bind to a specific and dissimilar terminal carbohydrate sequence found on a subset of O-linked glycans on ZP3. Because the common denominator for both models is the binding of sperm to a glycan with specified sequence requirements, we have used the experimental strategy of examining a series of short oligosaccharides with differing nonreducing ends to competitively inhibit sperm-ZP binding in vitro. Dose-response curves (as opposed to single concentration assays) were carried out to establish a rank order for the effectiveness of the oligosaccharides as competitive inhibitors. Inherent in our approach is the assumption that the more effective the competitive inhibitor, the more closely the structure of the inhibitor mimics the essential intrinsic structure of the functional sperm-binding oligosaccharide ligand(s). By extrapolation, information on the essential monosaccharides in a functional sperm-binding ligand can aid in the identification of the cognate sperm surface protein.

This analysis required that the competitive sperm-ZP binding assay measures the relative affinities of specific oligosaccharides for ZP-binding sites on sperm. Based on the data presented in Fig. 1, this requirement was met. The relative effectiveness of a given inhibitor was specified by its structure, and the binding of each inhibitor was saturable. Both characteristics are hallmarks of the specific binding of a ligand to its receptor.

A Fucosyl Residue Is Required for an Oligosaccharide to Bind Acrosome-intact Sperm with High Affinity—The lack of inhibition of sperm-ZP binding with βGN-βGN-GN is in agreement with published reports that in vitro sperm-ZP binding is not inhibited by free N-acetylglucosamine or by a tetraantennary oligosaccharide with four terminal N-acetylgalactosaminyl residues (12, 24). Additionally, our results are consistent with two independent reports that male mice that lack a functional β4GT gene are fertile (7, 8). Collectively, in our view, these observations do not support the requirement for nonreducing terminal N-acetylgalactosaminyl residues on ZP3 to form a high affinity ligand for acrosome-intact sperm, nor do these observations support a role for sperm surface β1,4-galactosyltransferase in mediating high affinity murine sperm-ZP binding.
In contrast, our data indicate that αG-βG-GN binds, albeit with moderate affinity, a high affinity ZP-binding site on murine sperm. This observation is consistent with a role for terminal α-galactosyl residues on ZP3 and sperm surface protein, sp56 (4, 9–12). However, the major new insight from this study is that high affinity sperm binding oligosaccharides require a fucosyl residue and that it is the fucosyl and not α-galactosyl residue that is essential for the assembly of this high affinity sperm-binding ligand. Interestingly, the apparent affinities of the two fucosylated tetrasaccharides (ED₅₀ values in the 430 and 500 nM range) are comparable with the affinity reported for gp55 (ED₅₀ ≈ 200 nM), a functional sperm binding glycopeptide isolated from murine ZP3 (25).

The apparent high affinity of the two fucosylated oligosaccharides for ZP-binding sites on sperm raises the question of whether a fucosyl residue in the absence of either an α- or β-galactosyl residue is sufficient to create a sperm-binding ligand. To address this question, chemically synthesized Fuc(1,3)GlcNAc(β1,2Man was incubated with sperm and eggs. Preliminary results indicate that 9 μM of this oligosaccharide inhibited sperm-ZP binding by approximately 40%. This observation reinforces the conclusion that fucosyl residues may be important to forming high affinity sperm binding oligosaccharides on the ZP. Last, the potential importance of the fucosyl residue in sperm-ZP binding is relevant to considering why female mice, which lack a functional, α1,3-galactosyltransferase gene and consequently cannot synthesize α3-galactosyl-capped oligosaccharides, are nevertheless still fertile (14). The observation that a fucosyl-containing tetrasaccharide with or without a terminal α-galactosyl residue (αG-βG-β-[F]-GN and βG-β-[F]-βG-GN-GN) competes with comparable affinity for the same oligosaccharide-binding site on sperm provides a potential explanation for the observed fertility in these animals.

The α3-Fucosyl Residue in High Affinity Oligosaccharides Is Present in the Context of the LewisX Trisaccharide, Galβ1,4(Fuc(1,3)GlcNAc—It is noteworthy that both high affinity tetrasaccharides we have identified contain the LewisX trisaccharide. This trisaccharide is of interest because it has been implicated in other examples of cell-cell interactions, including embryonic compaction, nerve cell adhesion, and breast cancer invasiveness (26–29). Additionally, the sialylated LewisX oligosaccharide mediates binding of lymphocytes to the vascular endothelium (30). Thus, murine sperm-ZP binding may be another example of cell-cell interactions regulated by oligosaccharides containing the LewisX trisaccharide.

Evidence That the Second, Low Affinity Site on Sperm Participates in Murine Sperm-ZP Binding—Our data suggest that there are two independent oligosaccharide-binding sites on sperm with different binding specificities. One site binds αG-βG-GN and the two fucose-containing tetrasaccharides tested, with moderate and high affinity, respectively. The second site preferentially binds the linear β-galactosyl-capped oligosaccharide (βG-βG-GN-GN), with low affinity. The apparent low affinity of this second site raises the question of whether it, in fact, mediates sperm-ZP binding. The following considerations are relevant to this question. First, we have tested only a single β-galactosyl-capped oligosaccharide, and this compound may be a poor mimic for the naturally occurring ligand on ZP3. Second, for the sake of argument, let us assume that the relatively low binding affinity estimated for this test oligosaccharide reflects the affinity of the "unknown" intrinsic ligand. In model systems, it has been experimentally estimated that the adhesive strength of a noncovalent bond varies as a function of the logarithm of the affinity of the ligand for its binding site (31). Consequently, the predicted strength of the low affinity bond would be only 4-fold less than that achieved with the α3-fucosyl-containing, high affinity tetrasaccharides. However, independent of whether this second site mediates a low or high affinity interaction between a spermatozoan and an oligosaccharide on ZP3, our empirical data indicate that this site does, in fact, participate in sperm-ZP binding. When the high affinity site on sperm was saturated with either of the α3-fucosyl-containing tetrasaccharides, sperm-ZP binding was reduced by only 45%; sperm remaining on the ZP were bound with sufficient strength to resist the shear force produced by multiple pipettings. However, as shown in the mixing experiments in Fig. 2, in specific cases, two inhibitors are better than one. The addition of βG-βG-GN to αG-βG-β-[F]-GN further inhibited sperm-ZP binding by an additional 20%. Thus, the lower affinity site must create a sufficiently strong bond to participate in the tight binding of sperm to the ZP. The availability of both high and low affinity sites on mouse sperm, as demonstrated by binding studies with 125I-labeled solubilized ZP proteins, has been reported (32).

Are There Candidate Sperm Surface Molecules That Mediate the High and Low Affinity Binding Reactions between ZP3 and Sperm?—Our conclusion that there are two distinct oligosaccharide-ZP-binding sites on the surface of murine sperm raises the question of the identity of these sites. As already noted, our results are consistent with a role for sp56 as the high affinity ZP-binding site on sperm. If sp56 is responsible for the high affinity oligosaccharide-binding site, our results predict that this sperm surface protein would bind the two fucose-containing tetrasaccharides with high affinity.

There are at least two different candidates for the second binding site. A cell surface, calcium-dependent lectin with affinity for galactose has been described on sperm surface from mice, rats, and rabbits; in rabbits, this lectin has been shown to bind ZP3 (33–35). A sperm surface fucosyltransferase enzyme activity has been identified and suggested to mediate sperm-ZP binding (24, 36). However, we cannot exclude the possibility that these two oligosaccharide-binding sites on sperm represent unidentified receptors. While the identity of the authentic ZP-binding molecule(s) on the sperm surface has yet to be completely resolved, our findings demonstrate that in mice, sperm-ZP binding is a redundant process that involves at least two distinct oligosaccharide-binding sites on the plasma membrane of sperm.

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