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O-Linked Oligosaccharides of Mouse Egg ZP3 Account for Its Sperm Receptor Activity

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Summary

Previously, we reported that ZP3, one of three different glycoproteins present in the mouse egg’s zona pellucida, serves as a sperm receptor. Furthermore, small glycopeptides derived from egg ZP3 retain full sperm receptor activity, suggesting a role for carbohydrate, rather than polypeptide chain in receptor function. Here, we report that removal of O-Linked oligosaccharides from ZP3 destroys its sperm receptor activity, whereas removal of N-linked oligosaccharides has no effect. A specific size class of O-linked oligosaccharides present on ZP3 destroys its sperm receptor activity, whereas removal of N-linked oligosaccharides has no effect. A specific size class of O-linked oligosaccharides from ZP3 destroys its sperm receptor activity, whereas removal of N-linked oligosaccharides has no effect. A specific size class of O-linked oligosaccharides present on ZP3.

Introduction

Cellular adhesion is central to a range of morphogenetic, differentiative, and homeostatic processes. Consequently, considerable effort has been directed towards identification and characterization of species of macromolecules that mediate such associations. This is in general context that we have studied the interaction between mouse sperm and eggs just prior to fertilization.

For fertilization to occur, mammalian sperm must penetrate the egg’s extracellular coat, the zona pellucida. In the mouse, this coat is about 7 μm thick and consists of three different glycoproteins, ZP1, ZP2, and ZP3, that are coordinately synthesized and secreted by growing oocytes (Bleil and Wassarman, 1980b; 1980c; Greve et al., 1982; Salzmann et al., 1990; Roifer and Wassarman, 1990; Shimizu et al., 1983; Wassarman et al., 1984a; Greve and Wassarman, 1985). To penetrate the zona pellucida, sperm first bind to its outer margin. Subsequently, a secretory response, the acrosome reaction, is triggered, enabling passage of sperm through the extracellular coat. Finally, sperm contact and fuse with the egg’s plasma membrane (Gwatkin, 1977; Saling and Storey, 1979; Wassarman and Bleil, 1986; Florman and Storey, 1982; Bleil and Wassarman, 1983; Wassarman, 1983).

Several lines of evidence suggest that specific sperm receptors are present in zonae pellucidae and are necessary mediators of binding, the initial phase of gamete interaction (Gwatkin, 1977; Gulyas and Schmell, 1981; Yanagimachi, 1981; Wassarman and Bleil, 1982; Wassarman, 1983; Schmoll et al., 1983; Wassarman et al., 1984b).

Experimental Rationale

The mouse egg’s zona pellucida consists of three different glycoproteins, designated ZP1, (200 kd), ZP2 (120 kd), and ZP3 (83 kd) (Bleil and Wassarman, 1980c). Previously, we demonstrated that only ZP3 exhibits sperm receptor activity in an in vitro competition assay, and it accounts for virtually all sperm receptor activity present in egg zonae pellucidae (Bleil and Wassarman, 1980a). Furthermore, we found that even relatively small glycopeptides (1.5–6 kd) derived from ZP3, following extensive Pronase digestion, exhibit full sperm receptor activity in vitro (Florman et al., 1984). These and other observations (Wassarman et al., 1984b) suggest that the sperm receptor activity of egg ZP3 is dependent on its carbohydrate components rather than on polypeptide chain. To demonstrate this directly, and to identify the class of oligosaccharides involved, the experiments described here were carried out. In these experiments, a competition assay (Bleil and Wassarman, 1980a; Florman et al., 1984) was used to determine the ability of oligosaccharides, derived from either solubilized zonae pellucidae or purified zona pellucida glycoproteins, to inhibit the binding of sperm to eggs in vitro ("sperm receptor activity").

Effect of Trifluoromethane sulfonic Acid on Sperm Receptor Activity

The role of carbohydrate in sperm receptor activity was

1985). When constituents of mouse egg zonae pellucidae were assayed individually for sperm receptor activity in vitro, ZP3 alone was found to be functional (Bleil and Wassarman, 1980a; 1983; Wassarman and Bleil, 1982). This glycoprotein (83 kd) consists of a 44 kd molecular weight polypeptide chain, to which 3 or 4 N-linked oligosaccharides are added (Salzmann et al., 1983; Wassarman et al., 1984a). A variety of circumstantial evidence suggests that ZP3 also contains O-linked oligosaccharides (Wassarman et al., 1984a). Although embryo zonae pellucidae also contain ZP3, the glycoprotein does not have sperm receptor activity (Bleil and Wassarman, 1980a; 1983; Wassarman and Bleil, 1982). This behavior of ZP3 from mouse eggs and embryos is consistent with the fact that sperm bind to eggs, but not to embryos (Gwatkin, 1977; Yanagimachi, 1981; Wassarman, 1983).

Recently, we reported that small glycopeptides derived from egg ZP3 retain full sperm receptor activity (Florman et al., 1984). This, as well as other observations, suggests that the sperm receptor activity of ZP3 is attributable to its carbohydrate components, rather than to its polypeptide chain. Here, we describe results of experiments that examine directly the role of carbohydrate in ZP3 function. These results strongly suggest that O-linked oligosaccharides are present on ZP3 and are essential for its sperm receptor activity. A preliminary account of some of these results has appeared (Florman and Wassarman, 1983).
first evaluated by extensive deglycosylation of zona pellucida glycoproteins with trifluoromethanesulfonic acid (TFMS). This reagent breaks glucosidic bonds between adjacent monosaccharides, as well as O-glycosidic linkages between carbohydrate and amino acids (serine and threonine), but does not cleave asparaginyl N-acetyl-D-glucosaminy1 amide linkages (Edge et al., 1981). Consequently, glycoproteins treated with TFMS are virtually denuded of carbohydrate, with only asparagine-linked N-acetylgalactosamine remaining associated with the polypeptide chain.

The sperm receptor activity of egg zona pellucidae, as measured by an in vitro competition assay (see Experimental Procedures), is extremely sensitive to TFMS. Solubilized zona pellucidae (2(p)l) exposed only to TFMS buffers inhibited sperm binding by more than 60% (11.5 ± 4.6 sperm bound/egg) as compared with controls (i.e., no zona pellucidae; 29.7 ± 5.7 sperm bound/egg), a value similar to that observed with untreated zona pellucidae (8.7 ± 4.2 sperm bound/egg). Zona pellucidae treated with TFMS inhibited sperm binding by less than 10% (26.9 ± 5.1 sperm bound/egg). Electrophoretic analyses of TFMS-treated zona pellucidae confirmed that the mature form of ZP3 (83 kd) had been converted to a species with a molecular weight approximating that of the polypeptide chain (44 kd; Salzmann et al., 1983). These data indicate that removal of both N- and O-linked oligosaccharides from ZP3 results in elimination of its sperm receptor activity.

Effect of Endo-F-β-N-Acetyl-D-Glucosaminidase F on Sperm Receptor Activity

In view of the above results described above, endo-F-β N-acetyl-D-glucosaminidase F (Endo F) was used to determine whether or not removal of only N-linked oligosaccharides from ZP3 affected its sperm receptor activity. Endo F cleaves glycosidic bonds in the diacetyltchitobiosyl core region of both high-mannose and complex type N-linked oligosaccharides, but does not alter O-linked carbohydrates of glycoproteins (Elder and Alexander, 1982). Previously, we reported that there are two forms of mature ZP3; one form possessing 3, and the other possessing 4, N-linked oligosaccharides per polypeptide chain (Salzmann et al., 1983). In the experiments that follow, the behavior of Endo F-treated ZP3 was compared with another zona pellucida glycoprotein, ZP2 (6 N-linked oligosaccharides per polypeptide chain; Greve et al., 1982), and with total egg zona pellucidae (ZP1, ZP2, and ZP3).

Both purified ZP2 and ZP3 are substrates for Endo F. Following extensive digestion, both ZP2 and ZP3 migrated as broad bands on SDS-polyacrylamide gels, with their apparent molecular weights decreased by about 40 kd and 30 kd, respectively, as the result of Endo F treatment (Figure 1). As expected, the apparent molecular weights of Endo F-treated ZP2 and ZP3 were higher than those of their polypeptide chains (81 kd and 44 kd, respectively; Greve et al., 1982; Salzmann et al., 1983), since Endo F does not remove O-linked carbohydrate from glycoproteins.

Endo F-treated ZP3 was found to possess full sperm receptor activity, as compared with untreated ZP3 and egg zona pellucidae, when tested by the in vitro competition assay; similarly, Endo F-treated egg zona pellucidae retained full receptor activity (Table 1). It was noted that neither Endo F treated ZP3 nor Endo F treated zona pellucidae affected the fraction of motile sperm present in these experiments. Similarly, based simply on microscopic examination, the speed and patterns of movement of the sperm used in these experiments were apparently unaffected. Furthermore, BSA, hCG, ZP2, and embryo zona pellucidae, all of which lack any sperm receptor activity, continued to lack activity following treatment with Endo F (data not shown). Finally, sequential digestion of egg zona pellucidae with Endo F and Pronase had no effect on sperm receptor activity, minimizing the possibility that a peptide domain possessing sperm receptor activity is rendered resistant to proteolysis by the presence of N-linked oligosaccharides (Olden et al., 1982). These results strongly suggest that N-linked oligosaccharides are not involved in the receptor activity of ZP3.

Effect of Alkali on Zona Pellucida Glycoproteins

ZP2 and ZP3

The O-glycosidic bond between N-acetyl-D-galactosamine

![Figure 1. Electrophoretic Analysis of Endoβ-N-Acetyl-D-Glucosaminidase F-Treated Zona Pellucida Glycoproteins.](image-url)
Table 1. Effect of Endo-β-N-Acetyl-D-Glucosaminidase F on Sperm Receptor Activity

| Sample Assayed* | Sample Treatment† |
|-----------------|------------------|
|                 | Heat Inactivated Endo F* | Active Endo F† |
| [Sperm/Egg (% Control)] | [Sperm/Egg (% Control)] |
| Water           | 27 ± 5 (88 ± 13)     | 27 ± 5 (88 ± 13) |
| Egg Zonae       |                 |               |
| Pellucidae      | 10 + 1 (34 + 2)     | 6 + 4 (20 + 8) |
| Egg ZP3         | 10 ± 1 (31 ± 3)     | 10 ± 2 (31 ± 5) |

* Sperm receptor activity was assayed as described in Experimental Procedures. Egg zonae pellucidae and egg ZP3 were present at a concentration of 2 zonae pellucidae/μL. In these experiments, binding of sperm incubated with mSECM (control) and with mSECM containing 2 zonae pellucidae/μL was 30 ± 6 and 10 ± 2 sperm bound/egg, respectively.

† Samples (all in distilled water) were lyophilized, were resuspended in Endo F buffers, were either heat inactivated (100°C, 1 min) or heat active Endo F added, were incubated 4 hr at 37°C, and were boiled 1 min as described in Experimental Procedures. Samples were dialyzed, first against 7 M urea and then against distilled water, were lyophilized, were resuspended in mSECM, and were then assayed for sperm receptor activity.

And the β-hydroxyamino acids, serine and threonine, is relatively sensitive to alkaline cleavage by a β-elimination type reaction (Sharon, 1975). In view of the results obtained with TFMS and Endo F-treated ZP3 (described above), we examined whether or not removal of O-linked oligosaccharides by mild alkaline hydrolysis affected the sperm receptor activity of ZP3.

To find conditions under which alkali released carbohydrate from zona pellucida glycoproteins but did not break peptide bonds, egg zona pellucidae were lyophilized, were resuspended in various concentrations of NaOH, and were incubated at 37°C for 16 hr. Following alkaline hydrolysis, zona pellucida solutions were neutralized, were radiolabeled, and were subjected to SDS-PAGE, as described in Experimental Procedures. We found that concentrations of NaOH typically used in β-elimination reactions (50–100 mM) resulted in extensive degradation of the polypeptide chain of ZP2 and ZP3 under the conditions described here (data not shown). However, based on SDS-PAGE, 5 mM NaOH had the desired effect on ZP2 and ZP3, reducing their molecular weights by less than 10 kDa (Figure 2), whereas 0.5 mM and lower concentrations of NaOH did not have a detectable effect on the molecular weights of ZP2 and ZP3. When zona pellucidae were radiolabeled prior to treatment with 5 mM NaOH, 75–90% of the radiolabel probably reflects alkali catalyzed release of 14C, although the possibility of a low level of peptide hydrolysis has not been completely eliminated. Finally, it was noted that ZP1 largely disappeared following exposure to 5 mM NaOH (Figure 2). We attribute this apparent loss to alkali catalyzed reduction of the intermolecular disulfide bond of ZP1 (Putnam, 1954), resulting in formation of a species that comigrates with ZP2 and ZP3.

Figure 2. Electrophoretic and Competition Binding Analyses of Alkali-Treated Zona Pellucida Glycoproteins

Egg zona pellucidae were solubilized, were lyophilized, were resuspended in either 5 mM NaOH or distilled water, and were incubated for 16 hr at 37°C as described in Experimental Procedures. Radiolabeled samples were run on SDS-PAGE, under nonreducing conditions, and were autoradiographed. Shown are autoradiographs of untreated zona pellucidae (lane A), zona pellucidae incubated in the presence of distilled water (lane B), and zona pellucidae incubated in the presence of 5 mM NaOH (lane C). Positions of the origin of the gel (o), ZP1, ZP2, and ZP3 are indicated. Sperm receptor activity was measured by using the in vitro competition binding assay described in Experimental Procedures. Zona pellucidae incubated in the presence of either distilled water or 5 mM NaOH were lyophilized, were resuspended in culture medium, and were incubated with sperm, in the range of 1-6 zona pellucidae/μL, as described in Experimental Procedures. The ability of untreated (▲), 5 mM NaOH-treated (●), distilled-water-treated (□), and NaOH-treated (○) zona pellucidae to competitively inhibit binding of sperm to eggs is shown. These data represent the mean ± standard deviation of triplicate experiments in which the control level of sperm binding was 33.1 ± 5.4 sperm bound/egg (i.e., at 0 zona pellucidae/μL).
were exposed to 5 mM NaOH at 37°C for 16 hr and tested for sperm receptor activity in the in vitro competition as compared with untreated material recovered and, therefore, estimation of zona pellucida concentrations used in sperm receptor assays.

Effect of Alkali on Sperm Receptor Activity
Results of experiments presented above indicated that, by using mild alkali, O-linked oligosaccharides could be removed from ZP3 without causing extensive degradation of its polypeptide chain. Accordingly, egg zonae pellucidae were exposed to 5 mM NaOH at 37°C for 16 hr and tested for sperm receptor activity in the in vitro competition assay. These experiments, 1–2% of the zona pellucidae were radiolabeled with 3H-Bolton-Hunter reagent. This permitted determination of the percentage of treated material recovered and, therefore, estimation of zone pellucida concentrations used in sperm receptor assays.

We found that egg zonae pellucidae retained virtually full sperm receptor activity, as compared with untreated material, following incubation at 37°C for 16 hr in the presence of distilled water (Figure 2). Furthermore, addition of 5 mM NaOH, which had been neutralized and lyophilized, to culture medium had no effect on binding of sperm to eggs in the competition assay. On the other hand, eqq zonae pellucidae subjected to mild alkaline hydrolysis, under the conditions described above, lost approximately 90% of the sperm receptor activity present in untreated samples and in samples treated only with distilled water (Figure 2). Similarly, mild alkaline hydrolysis of Endo F-treated zonae pellucidae resulted in the loss of about 90% of sperm receptor activity in such samples (data not shown). In no case did the addition of untreated or treated zonae pellucidae affect sperm motility. The results of these experiments strongly suggest that removal of O-linked oligosaccharides from ZP3 causes the loss of sperm receptor activity.

Fractionation of O-Linked Oligosaccharides Possessing Sperm Receptor Activity
As a result of alkaline hydrolysis of serine/threonine: N-acetyl-b-galactosaminyl bonds in glycoproteins, liberated oligosaccharides undergo degradation stepwise from their reducing termini. This so-called "peeling reaction" can be minimized by including a strong reducing agent, such as NaBH₄, during the hydrolysis (Lloyd, 1978). Under these conditions, the reducing terminal of released oligosaccharides is rapidly converted to alkali-stable sugar alcohols. In the presence of 4H-NaBH₄, the released oligosaccharides are recovered as 4H-labeled alcohols. We used such a procedure to obtain radiolabeled oligosaccharides that possess sperm receptor activity from purified ZP3.

Initial experiments demonstrated that radiolabeled (4H-NaBH₄) oligosaccharides, possessing sperm receptor activity, could be recovered from alkaline hydrolysates of egg zona pellucidae. In these experiments, peptides, Na⁺, and borates were removed, samples were subjected to gel filtration, and fractions were assayed for sperm receptor activity in the in vitro competition assay as described in Experimental Procedures. Following gel filtration of zona pellucida hydrolysates on Bio-Gel P-2, sperm receptor activity was found associated with the pooled void volume, but not with the included volume material. Aliquots of void volume material decreased binding of sperm to eggs by

### Table 2. Radiolabeling of Amino Acids during Mild Alkaline Hydrolysis of Zona Pellucida Glycoproteins ZP2 and ZP3

| Glycoprotein | Tritium Incorporation Ratio† | Alanine | Other Amino Acids |
|--------------|------------------------------|---------|-----------------|
| ZP2          | 2.5 ± 0.4                    | 4.2 ± 0.3 | 1.1 ± 0.3       |
| ZP3          | 2.5 ± 0.7                    | 2.3 ± 1.3 | 1.1 ± 0.1       |

† Alkaline reduction and amino acid analysis was carried out as described in Experimental Procedures. Data expressing the ratio of cpm incorporated into the indicated amino acid following NaOH treatment as against that observed in distilled water controls was calculated as follows: [cpm(NaOH)/cpm(H₂O)]. Reported are the results of triplicate experiments.

These are the average values for 22 ninhydrin-positive spots representing either amino acids or their derivatives. The ranges of values for ZP2 and ZP3 were 0.0–1.0 and 0.3–1.3, respectively. The range of values for background (i.e., ninhydrin-negative regions of the chromatograms) was 0.7–1.4.
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Fraction Number

Figure 3. Gel Filtration and Competition Binding Analyses of O-Linked Oligosaccharides Released from ZP2

Egg ZP2 was purified and subjected to mild alkaline reduction in the presence of \(^{3}H\)-NaBH\(_4\), as described in Experimental Procedures. Oligosaccharides, separated from peptides, Na\(^{+}\), and borates (see Experimental Procedures), were first chromatographed on Bio-Gel P-2 (0.7 x 17 cm), void volume fractions were pooled, were lyophilized, were resuspended, and were then chromatographed on Bio-Gel P-6 (1.5 x 70 cm). Bio-Gel P-6 columns were developed with distilled water at 55°C and at a flow rate of 20 ml/hr. An elution profile for radiolabeled oligosaccharides was determined by counting 10 \(\mu\)l aliquots of each column fraction of 1.5 ml (+A). In addition, two 700 \(\mu\)l aliquots of each column fraction were lyophilized, were resuspended in culture medium, and each was assayed for sperm receptor activity by using the in vitro competition binding assay (W-O). The control level of sperm binding was 30.5 ± 8.9 sperm bound/egg in the experiment shown. Ferritin and \(^{3}H\)-borohydride eluted in regions I (void volume) and V, respectively.

75% relative to control samples (8.3 ± 4.7 versus 33.5 ± 5.8 sperm bound/degg, respectively). The latter samples included untreated sperm, as well as sperm exposed to aliquots of pooled void volume material obtained following gel filtration of alkaline-borohydride hydrolysates not containing zonae pellucidae (i.e., ‘sham hydrolysates’). It was noted in these experiments that, while void volume fractions had no effect on sperm motility, material comigrating with \(^{3}H\)-mannose on Bio-Gel P-2 completely inhibited sperm motility; this inhibitory effect was even seen with sham hydrolysates, suggesting that it can be attributed to contaminants present in \(^{3}H\)-NaBH\(_4\).

In view of the results just described, purified ZP2 and ZP3 were subjected to mild alkaline hydrolysis in the presence of \(^{3}H\)-NaBH\(_4\), followed by gel filtration. Void volume fractions recovered from Bio-Gel P-2 columns were then pooled (as above) and were applied to Bio-Gel P-6. The P-6 elution profiles for ZP2 and ZP3 are shown in Figure 3 and in Figure 4, respectively, together with the results of sperm receptor activity measurements for each fraction eluted. Receptor activity was determined on an aliquot of each fraction that had been lyophilized and had then been resuspended in culture medium, as described in Experimental Procedures. While the elution profile for ZP2 (Figure 3) was similar to that for ZP3 (Figure 4), as expected, only fractions from ZP3 possessed sperm receptor activity, and the activity was associated with a single region of the elution profile (region II) (Figure 4). In two independent experiments, oligosaccharides eluted in region II (3.4 kd-4.6 kd apparent molecular weight) accounted for about 35% of the sperm receptor activity associated initially with intact ZP3.

In a control experiment, hCG was subjected to alkaline-borohydride hydrolysis and to gel filtration on Bio-Gel P-2 and P-6 under the same conditions used for purified ZP3; fractions eluted from P-6 had no effect on binding of sperm to eggs in vitro. Furthermore, when egg zonae pellucidae were treated with borohydride in the absence of alkali, no sperm receptor activity was found in fractions eluted from P-6 columns (data not shown). Under these conditions, O-linked carbohydrate remained associated with peptide and was removed during cation exchange chromatography (see Experimental Procedures). These results strongly suggest that release of sperm receptor activity from ZP3 by mild alkaline reduction did not result from borohydride side reactions; these can include release of N-linked oligosaccharides (Rasio and Renkonen, 1981; Ogata and Lloyd, 1982) and cleavage of peptide bonds (Crestfield et al., 1963; Shimamura et al., 1984).

Binding of O-Linked Oligosaccharides to Sperm

The results described above strongly suggest that a specific size class of O-linked oligosaccharides, derived from
ZP3 and possessing sperm receptor activity, can be fractionated on Bio-Gel P-6. To determine whether or not these oligosaccharides bind to sperm, the experiments that followed were carried out using purified ZP2 and ZP3. These experiments involved incubation of sperm with radiolabeled oligosaccharides released from either ZP2 or ZP3, centrifugation of the sperm through dibutyl phthalate into sucrose-Tris-X-100, and gel filtration, first on Bio-Gel P-2 and then on P-6, as described in Experimental Procedures.

The Bio-Gel P-6 elution profiles for 3H-oligosaccharides released from ZP2 and ZP3 are shown in Figure 5 and in Figure 6, respectively. In each case, profiles are presented for both total oligosaccharides and for oligosaccharides associated with sperm following a brief incubation. The results obtained with ZP2 oligosaccharides were virtually identical with those presented in Figure 3; no particular size class of ZP2 oligosaccharides was selectively bound to sperm. On the other hand, while the profile of total ZP3 oligosaccharides closely resembled that presented in Figure 4, the profile of sperm-associated oligosaccharides differed. The latter material was significantly enriched in the region of the elution profile that had been shown to possess sperm receptor activity (designated as region II in Figure 4 and region IV in Figure 6). This enrichment of region IV was observed in three independent experiments. Although other size classes of ZP3 oligosaccharides were associated with sperm (Figure 6), the extent of their association simply reflected their relative abundance in the total population (i.e., no enrichment), as was the case with ZP2 oligosaccharides (Figure 5). Furthermore, selective binding of ZP3 oligosaccharides (region IV) appeared to be specific for sperm, since analogous experiments using mouse adipocytes did not demonstrate any selective binding of oligosaccharides to these cells (data not shown).

The implication that sperm-associated oligosaccharides found in region IV of Bio-Gel P-6 profiles should possess sperm receptor activity was tested directly. Sperm were incubated with radiolabeled ZP3 oligosaccharides, and bound oligosaccharides were then eluted from the sperm, were fractionated on Bio-Gel P-6, were pooled as indicated in Figure 6, and were tested for sperm receptor activity. When sperm were exposed to oligosaccharides released from ZP3 of apparent molecular weight, and were then incubated with unfertilized eggs, a 50% inhibition of sperm binding was observed. Other regions of the elution profile were without effect on sperm binding, even though they, too, had been adsorbed to sperm (see legend to Figure 6). Similarly, all regions of the elution profile of sperm-associated ZP2 oligosaccharides were tested for sperm receptor activity and were found to be completely inactive (see legend to Figure 5).

**Analysis of O-Linked Oligosaccharides Bound to Sperm**

Results presented above (Table 2) strongly suggest that ZP3 possesses O-linked oligosaccharides that are released on mild alkaline hydrolysis. Since a specific size class of ZP3 oligosaccharides was found associated with sperm (Figure 6), and this material possessed sperm receptor activity, we determined directly the linkage class of these oligosaccharides. Egg ZP3 was subjected to mild alkaline hydrolysis in the presence of 3H-NaBH₄, as before. Under these conditions, glycosidically linked sugars are converted to their respective sugar alcohols, 1H-N-acetyl-b-glucosaminitol and 1H-N-acetyl-b-galactosaminitol were converted from N- and O-linked oligosaccharides, respectively. Oligosaccharides were separated from peptides, from Na+, and from borates, were incubated in the presence or absence of sperm, were hydrolyzed, were re-N-acetylated, and were analyzed by descending paper chromatography as described in Experimental Procedures.

In the case of oligosaccharides not adsorbed to sperm, the majority of the tritium incorporated into hexosamines was found in N-acetyl-b-galactosaminitol (Figure 7). A small amount of radiolabel was observed comigrating with N-acetyl-b-glucosaminitol; however, this constituted 10%
or less of the radiolabel present in N-acetyl-D-galactosaminitol (Figure 7). When identical analyses were performed on oligosaccharides that had been bound to sperm, once again, the vast majority of radiolabel (>95%) was found in N-acetyl-D-galactosaminitol, rather than in N-acetyl-D-glucosaminitol (Figure 7). These results are consistent with those presented in Table 2 and provide additional support for our conclusion that O-linked oligosaccharides of ZP3 are involved in sperm receptor activity.

Discussion

We have found that mammalian sperm–egg interaction provides an attractive system within which to define the role of oligosaccharides in cellular adhesion. While a role for carbohydrates has been implicated in several other biological systems (Culp, 1978; Frazier and Glaser, 1979; Barondes, 1981; Ashwell and Hartford, 1982), it has often been difficult to distinguish between a direct effect of carbohydrates on cellular adhesion and an indirect, modulatory influence (Hoffmann and Edelman, 1963). In this connection, we recently reported that small glycopeptides derived from mouse egg ZP3 possess virtually all of the sperm receptor activity of the intact glycoprotein, suggesting that sperm receptor function is carbohydrate-mediated (Florman et al., 1984). Here, we have extended these observations by demonstrating directly that a specific size class of O-linked oligosaccharides derived from ZP3 binds to sperm and possesses receptor activity.

Carbohydrates conjugated to the β-hydroxyl groups of serine and threonine (O-linked) were first reported for bacterial enzymes (Hanauska et al., 1955) and were later shown to be constituents of mammalian mucins and proteoglycans (Anderson et al., 1964; Bhavanandan et al., 1964). To date, these glycoconjugates have been found on a large variety of both membrane-associated and secretory proteins (Sharon, 1975; Marshall, 1979; Kornfeld and Kornfeld, 1980). O-linked oligosaccharides are structurally diverse, consisting of linear to highly branched chains of 2 to 30 sugars that are added after translation to nascent polypeptide chains by stepwise transfer of monosaccharides (Kornfeld and Kornfeld, 1980). It is significant to note that the majority of O-linked oligosaccharides are involved in antifreeze glycoprotein function (Vanderheede et al., 1972), in the protection of mucins against proteolysis (Allen, 1983), in platelet adhesion (Judson et al., 1982; Tsuji et al., 1983), and in hemagglutination by Vaccinia virus (Shida and Dales, 1981). Several observations suggest that O-linked oligosaccharides are present on the mouse egg’s sperm receptor, ZP3. First, ZP3 exhibits both heterogeneity with respect to

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Figure 6. Gel Filtration, Competition Binding Analyses, and Determination of Sperm Association of O-Linked Oligosaccharides Released from ZP3

These experiments were carried out with purified ZP3 exactly as described in the legend to Figure 5. Shown are the Bio-Gel P-6 elution profile for radiolabeled oligosaccharides not incubated with sperm (M) and associated with sperm after a 1 hr incubation (W 0). The region of the elution profile from which was significantly enriched following incubation of oligosaccharides with sperm (Region IV) is stippled. In the case of radiolabeled oligosaccharides associated with sperm, fractions were pooled (Regions I–VIII), lyophilized, and were re-N-acetylated, and were resolved as sugar alcohols by descending paper chromatography, as described in Experimental Procedures. Shown are the profiles of radioactivity for purified ZP3 not incubated with sperm (M) and associated with sperm after a 1 hr incubation (W 0). The positions of N-acetyl-D-galactosaminitol (I) and N-acetyl-D-glucosaminitol (II) are indicated.

Figure 7. Chromatographic Analysis of the Reducing Terminus of Oligosaccharides Released from ZP3

Purified egg ZP3 was subjected to mild alkaline hydrolysis in the presence of 1 M NaOH, oligosaccharides were isolated and were analyzed either directly or following adsorption by sperm (see Experimental Procedures). Radiolabeled oligosaccharides were lyophilized, were resuspended in 200 μl of 4 N HCl, and were hydrolyzed for 4 hr at 100°C.Hexosamines were isolated by ion-exchange chromatography, were re-N-acetylated, and were resolved as sugar alcohols by descending paper chromatography. Shown are the profiles of radioactivity for purified ZP3 not incubated with sperm (M) and associated with sperm after a 1 hr incubation (W 0). The positions of N-acetyl-D-galactosaminitol (I) and N-acetyl-D-glucosaminitol (II) are indicated.
isoelectric point, and it exhibits a molecular weight higher than that of its polypeptide chain, following either removal of N-linked oligosaccharides with Endo F or inhibition of N-linked glycosylation with tunicamycin (Roller and Wassarman, 1983; Salzmann et al., 1983). Second, mild alkaline hydrolysis of ZP3, in the absence of NaBH₄, results in a slight decrease of its molecular weight (Figure 2). In the presence of NaBH₄, serine and threonine residues are converted to alanine and to α-aminobutyric acid, respectively (Table 2), and release of N-acetyl-α-galactosaminotol is observed (Figure 7). Third, treatment of ZP3 with either TFMS or with mild alkali (Figure 2), but not with Endo F (Table 1) or with Pronase (Florman et al., 1984), results in a loss of its sperm receptor activity. Finally, following mild alkaline hydrolysis of ZP3, in the presence of NaBH₄, sperm receptor activity is found associated with released oligosaccharides having N-acetyl-α-galactosaminotol at the reducing terminus (Figure 4, Figure 6, and Figure 7); strong evidence for the presence of O-linked oligosaccharides, since N-linked oligosaccharides do not contain N-acetyl-α-galactosamine (Sharon, 1975; Marshall, 1979).

Therefore, ZP3 apparently resembles secretory proteins such as hCG (Kessler et al., 1979a, 1979b) and fetuin (Spiro and Bhoyroo, 1974), membrane proteins such as glycoporin (Thomas and Winzler, 1969; Marchesi et al., 1978), LDL receptor (Cummings et al., 1983), and several viral coat proteins (Niemann et al., 1982; Johnson and Spear, 1983), in that it possesses both N- (Roller and Wassarman, 1983; Salzmann et al., 1983) and O-linked oligosaccharides.

A discrete size class of O-linked oligosaccharides derived from ZP3, but not from ZP2, inhibits binding of sperm to eggs in vitro (Figure 3 and Figure 4). This appears to be a direct effect on the adhesion process, since small glycopeptides derived from ZP3 do not trigger the acrosome reaction in vitro (Florman et al., 1984). Moreover, the same size class of ZP3 O-linked oligosaccharides (~3.9 kD apparent molecular weight) that inhibits binding of sperm to eggs, also binds preferentially to sperm (Figure 6); no particular size class of ZP2 oligosaccharides displays such a preference (Figure 5). These, as well as other observations presented here (Figure 2 and Table 1) and elsewhere (Biele and Wassarman, 1980a; Florman et al., 1984; Wassarman et al., 1984b, 1985), strongly suggest that mouse sperm recognize and bind to eggs via O-linked oligosaccharides present on ZP3. In particular, such a situation explains the unusual stability of the sperm receptor activity of ZP3 following exposure of the glycoprotein to extremes of temperature, denaturants, or detergents (Biele and Wassarman, 1980a; Florman and Wassarman, 1983); a property characteristic of a number of other putative receptors (Biele and Wassarman, 1980a).

It has been suggested previously that carbohydrate plays a role in binding sperm to zona pellucidae, since various lectins, monosaccharides, and glycoconjugates inhibit the binding of sperm to mammalian eggs (Ahuja, 1982; Okawa et al., 1973; Huang et al., 1982; Shur and Hall, 1982; Wassarman et al., 1984b). Similarly, it has been reported that various monosaccharides and poly- saccharides inhibit sperm–egg interaction in several invertebrate and plant species (Boiwell et al., 1979, 1980; Rosati and De Santis, 1980; Glabe et al., 1982; Barnum and Brown, 1983). In sea urchins, such observations are particularly relevant, since several lines of evidence suggest that gamete adhesion is mediated by “bindin”, a lectin-like sperm protein associated with acrosomes, and by a carbohydrate containing sperm receptor in the egg's vitelline envelope (Vacquier and Moy, 1977; Giabo and Vacquier, 1978; Glabe, 1979; Glabe and Lennarz, 1979, 1981; Glabe et al., 1982; Rossignol and Lennarz, 1983; Rossignol et al., 1984). In the case of one species of sea urchin, Strongylocentrotus purpuratus, galactosamine has been detected in glycoconjugates, which are derived from vitelline envelopes (Rossignol et al., 1984), having sperm receptor activity. This suggests possible structural analogies with the mouse egg's sperm receptor. In the mouse, it remains to be determined whether or not O-linked oligosaccharides on ZP3 are recognized by a lectin-like protein, analogous to bindin, on sperm. Should such a protein be present, it would have to be located on plasma membrane overlying the sperm head, rather than on the acrosomal membrane, since only sperm that have not undergone the acrosome reaction bind to mouse eggs (Saling and Storey, 1979; Florman and Storey, 1982; Biele and Wassarman, 1983).

The mouse egg's sperm receptor plays a multifaceted role in the regulation of fertilization. In addition to mediating binding of sperm to eggs, ZP3 induces bound sperm to undergo the acrosome reaction (Biele and Wassarman, 1983; Florman et al., 1984; Wassarman et al., 1985) and participates in the secondary block to polyspermy (Wolf, 1981; Schmoll et al., 1983; Wassarman, 1983; Wassarman et al., 1984b). The latter apparently involves modification of ZP3 following fertilization, such that it no longer possesses sperm receptor activity (Biele and Wassarman, 1980a, 1983). In sea urchins, it has been suggested that proteases, originating from the egg's cortical granules, release sperm receptors from the vitelline envelope following fertilization (Vacquier et al., 1972, 1973; Glabe and Vacquier, 1978). Since ZP3 purified from 2-cell embryo zonae pellucidae, as well as glycopeptides derived from embryo ZP3, do not possess receptor activity, it would appear that ZP3 throughout the zona pellucida is modified following fertilization. Although ZP2 does undergo limited proteolysis following either fertilization or parthenogenetic activation (Biele et al., 1981), there is no evidence as yet for proteolysis of ZP3 (Biele and Wassarman, 1980a; Biele et al., 1981). Whatever the nature of the modification, it is subtle, not rendering embryo ZP3 distinguishable from egg ZP3 by conventional electrophoretic analysis (Biele and Wassarman, 1980a; P Wassarman, unpublished results). We suggest that modification of the O-linked oligosaccharides described here, by a specific cortical granule glycosidase, could account for inactivation of ZP3 following either fertilization or parthenogenetic activation. Detailed characterization of O-linked oligosaccharides derived from both egg and embryo ZP3, as well as characterization of cortical granule glycosidases, will be necessary to resolve this issue.

Finally, it should be noted that mammalian sperm...
receptors exhibit a certain degree of species specificity (Bedford, 1981; Yanagimachi, 1984; Wassarman, 1983; Wassarman et al., 1984b). Fertilization of zona pellucida-free eggs by heterologous sperm in vitro is quite common, whereas hybrid fertilization of zona pellucida-intact eggs is rare (Adams, 1974; Yanagimachi, 1981, 1984; Barros and Leal, 1980; Gulyas and Schmell, 1981). The question of whether or not the O-linked oligosaccharides derived from ZP3 inhibit sperm binding in a species-specific manner has not been addressed in this investigation. In this connection, it has been demonstrated that, although sperm receptor activity is associated with glycoproteins from sea urchin vitelline envelopes, the glycoproteins do not exhibit the species specificity observed with high molecular weight, vitelline envelope glycoconjugates (Kinsey and Lennarz, 1981; Rossignol et al., 1983; Giabe and Lennarz, 1981). It will certainly be of interest to compare the structure of the ZP3 O-linked oligosaccharides described here with functionally analogous oligosaccharides from other mammalian species.

**Experimental Procedures**

**Collection and Culture of Mouse Gametes**

Gamete incubations were routinely carried out under oil. In a mouse gamete culture medium supplemented with 0.4% polyvinylpyrrolidone-40 (mSECM), as 37°C in an environment of 5% CO₂ in air. These conditions are capable of supporting mouse sperm capacitation and fertilization in vitro (Florman et al., 1984).

Mature (3-4 weeks old), female, Swiss albino mice (CD-1, Charles River Breeding Labs) were injected with 10 IU of pregnant mare's serum gonadotropin (PMSG, Sigma), followed in 48 hr by 10 IU of human chorionic gonadotropin (hCG, Sigma). Ovulated eggs, recovered from oviducts 13-16 hr after hCG injection, were removed of surrounding cumulus cells with hyaluronidase (0.1% in mSECM; type V ovine testicular hyaluronidase, Sigma). Epididymal tissue was removed, sperm collected, and sperm swimming into mSECM were used. After 10 min, epididymal sperm were resuspended in distilled water (pH 7.0) and centrifuged for 37°C. Incubations were carried out under conditions for 4 hr at 37°C was sufficient for complete digestion of zona pellucida glycoproteins (it was noted that under these conditions the electrophoretic mobility of BSA was unaffected, indicating the absence of protease contaminants). Control incubations received either 1 μl of 50% glyceral/25 mM EDTA or 1 μl of heat-inactivated (100°C for 1 min) Endo F. Samples were analyzed as described (Zill et al., 1953).

**Preparation of Zona Pellucidae**

Zona pellucidae were removed from eggs and embryos by micropipettes (100 μm internal diameter), were washed by transfer through PBS (pH 7.5) supplemented with 0.4% polyvinylpyrrolidone-40 (PBS-PVP), and were solubilized in 1-2 μl of 5 mM NaH₂PO₄ (pH 2.5). When required, zona pellucidae were radiolabeled with 125I-Bolton Hunter reagent (~4000 Cim/mole, New England Nuclear). Zona pellucidae were obtained by electroelution from gel slices, followed by electrodialysis, dialysis against 7 M urea and then against distilled water, and lyophilization (Bleil and Wassarman, 1980a).

**Modification of Zona Pellucida Glycoproteins**

Extensive deglycosylation of zona pellucida glycoproteins was carried out in the presence of trifluoromethanesulfonic acid (TFMS, Sigma), as described by Edge et al. (1981). In control experiments, distilled water was substituted for TFMS. Protein was recovered by extraction with diethyl ether and 50% (v/v) aqueous pyridine. The aqueous phase was dialyzed against distilled water, aliquots were taken for radiolabeling with 125I-Bolton Hunter reagent, followed by SDS-PAGE analysis, and determination of sperm receptor activity (see below). Under these conditions the electrophoretic mobility of BSA was unaffected.

Selective deglycosylation was achieved by two different procedures. To remove N-linked oligosaccharides, glycoproteins were treated with endo-N-acetyl-o-glucosaminidase F (Endo F; Elder and Alexander, 1982). Lymphoplated samples were resuspended in 25 μl of Endo F buffer (100 mM NaH₂PO₄, pH 6.1), 50 mM EDTA, 1% NP-40, 0.1% SDS, and 1% 2-mercaptoethanol, were boiled for 2 min, and were cooled to 37°C. Digestions were initiated by the addition of 1 μl Endo F (provided in a 50% glycerol 25 mM EDTA solution by Dr. J. H. Elder) and were terminated by boiling for 1 min. Incubation under these conditions for 4 hr at 37°C was sufficient for complete digestion of zona pellucida glycoproteins (it was noted that under these conditions the electrophoretic mobility of BSA was unaffected, indicating the absence of protease contaminants). Control incubations received either 1 μl of 50% glycerol/25 mM EDTA or 1 μl of heat-inactivated (100°C for 1 min) Endo F. Samples were analyzed as described (Bleil and Wassarman, 1980a).

O-linked oligosaccharides were removed by alkaline f-elimination. Zona pellucida glycoproteins were lyophilized and were resuspended in 25 μl of 5 mM NaH₂PO₄. To prevent alkaline degradation of released oligosaccharides when reducing conditions were required (Lloyd, 1976), reactions were carried out in the presence of 1 M H⁻NaBH₄ (≥100 mCi/mole, New England Nuclear). Samples were incubated for 16 hr at 37°C in a nitrogen atmosphere. In control samples, distilled water (pH 7.0) was substituted for NaOH. Reactions were terminated by cooling samples to 4°C, followed by acidification to pH 6.0 with 0.1 N acetic acid to eliminate excess H⁻NaBH₄. Samples containing oligosaccharides were neutralized with 1 N NaOH, were separated from peptide and from Na⁺ on 6 ml columns of Dowex 50X4-400 (H⁺ form; 200-400 mesh), were lyophilized, were resuspended in 1% acetic acid in methanol, and were dried under a stream of N₂. Methanol evaporations were repeated 4 times to remove excess borate as its volatile methyl ester derivative (Zill et al., 1953).

**Oligosaccharide Binding Studies**

Oligosaccharides derived from egg zona pellucidae and radiolabeled during mild alkaline reduction, were lyophilized and were resuspended in mSECM at a concentration of about 0.7 zona pellucida μl⁻¹. One μl was reserved for gel filtration (see below), and the remaining oligosaccharide solution was divided into 37.5 μl aliquots that were each added to 12.5 μl drops of sperm (4 x 10⁷/ml) in mSECM (2 μl zona pellucida μl⁻¹, final concentration). Sperm were incubated for 1 hr at 37°C, during which time motility was monitored as previously described (Florman et al., 1984). Experiments were discarded when oligosaccharide treatment resulted in decreased cell motility relative to untreated control incubations. Oligosaccharide binding was assessed by applying 40 μl of the sperm suspension to siliconized. 400 μl Eppendorf tubes containing step gradients of 200 μl dibutyl phthalate (Sigma) on top of 20 μl 1% Triton X-100 in 0.5 M sucrose (Cuatrecasas and Hollenberg, 1976). Since dibutyl phthalate has a density (1.043 g/ml) intermediate between that of mSECM and sperm, centrifugation (8000 g, 30 sec) of sperm yielded a pellet in the sucrose layer, whereas mSECM did not penetrate the oil phase. Aliquots were taken from the medium and from the sucrose layers for both determination of radioactivity and for gel filtration.

Zona pellucida oligosaccharides were also incubated with adipocytes isolated from mouse epididymal fat pads (Rodbell, 1964). After 1 hr incubation, 40 μl of adipocyte suspensions were layered on top of 200 μl of diocyl phthalate (Aodrich). Centrifugation (8000 g, 30 sec) displaced incubation medium to the bottom of the tube, while the less dense adipocytes remained as a layer of packed cells above the oil phase (Dubyak and Kleinzeiler, 1980).

**Gel Filtration of Oligosaccharides**

To analyze zona pellucida oligosaccharides following sperm binding, 5 μl samples of either the starting material (O-linked oligosaccharides in mSECM prior to the addition of sperm) or of the sucrose phase, following centrifugation (see above), were brought to 25 μl with distilled water.

Gel filtration of H-oligosaccharides was carried out at 55°C, on Bio-Gel P-4 (200-400 mesh; 1.5 x 70 cm) that had previously been
were tested for sperm receptor activity. Recovery from these columns varied from 80 to 97%.

**Glycoprotein Linkage Analysis**

Amino acid analysis was carried out after mild alkaline reduction of Glycoprotein Linkage Analysis varied from 80 to 97%.

200 μl of 6 N HCl. In some experiments samples were dissolved in 200 μl HCl and 2 μl of an amino acid mixture (5 mM of each of the biologically relevant amino acids, as well as of L-α-aminobutyric acid). Glycoproteins were hydrolyzed in vacuo at 100°C for 18 hr, were dried in a desiccator over NaOH, were washed 3 times by methanol evaporation to remove borates, and with distilled water to remove HCl. Undialyzed hydrolysates were resuspended in 10 μl of distilled water, insoluble material was removed by centrifugation (8,000 x g, 1 min), and 1.5 μl aliquots were applied to cellophane, thin-layer chromatography plates (20 x 20 cm; Chromogram, Eastman). Chromatograms were developed in an unsaturated atmosphere of n-butanol:acetonitrile:water (10:10:3; pH 12.0) in the first dimension and in an atmosphere of isopropanol:formic acid (99%)water (40:2:10; pH 2.5) in the second dimension (Brenner and Niederwieser, 1987). Amino acids were visualized with ninhydrin. The identity of carbohydrates, glycosidically linked to ZP3, was determined following mild alkaline reduction. Analysis was performed both on total H-oligosaccharides from ZP3 and on material bound by sperm (see above). Oligosaccharides were hydrolyzed in vacuo in 4 N HCl for 4 hr at 100°C, were deacetylated by repeated washes with distilled water under a stream of N2, and were resuspended in 1 ml distilled water. Amino sugars were eluted from 5 ml columns of Dowex-50 (H+ form; 200-400 mesh; 0.5 ml bed volume) and Dowex-1 (OH- form; 200-400 mesh; 1 ml bed volume) with 0.5 ml distilled water (30 min, 60°C), and associated 3H-radioactivity was determined by liquid scintillation spectroscopy with 10 ml Aquasol (New England Nuclear). In some experiments, the entire chromatogram was divided into a 1 x 1 cm grid, and the distribution of tritium was evaluated.

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SDS Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed according to the method of Laemmli (1970), by using a 10% polyacrylamide separating gel and a 4% polyacrylamide stacking gel. Nonreducing conditions were used where indicated.

**Determination of Sperm Receptor Activity**

Sperm receptor activity was determined in an in vitro competition binding assay (Blei and Wassarman, 1980a; Florman et al., 1984). Aliquots (10 μl) of preincubated sperm suspensions were added to 30 μl mSHEM containing substances to be tested for sperm receptor activity. After 60 min at 37°C, ten unfertilized eggs and three 2-cell embryos were added in 1-2 μl mSHEM. Thirty minutes later, eggs and embryos, with associated sperm, were removed with a wide-bore micropipette (internal diameter >100 μm) and were pipetted until no more than 1-2 sperm remained attached to embryo zona pellucida. Sperm associated reversibly and nonspecifically with embryo zona pellucidae, but established both nonspecific as well as tenacious, specific bonds to zona pellucidae of eggs; thus, these conditions serve to remove nonspecifically-associated sperm from egg zonae pellucidae (Blei and Wassarman, 1980a). Eggs and embryos were then transferred to microscope slides, were fixed with 3% glutaraldehyde in PBS-PVP, and the number of bound sperm was determined with an inverted phase microscope.

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