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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, recovered following mild alkaline hydrolysis and reduction of ZP3, is shown to possess sperm receptor activity and to bind to sperm. The results presented strongly suggest that mouse sperm bind to eggs via 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. It is in this 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., 1983; Roller and Wassarman, 1983, 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, 1982; 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; Schmell et al., 1983; Wassarman et al., 1984b, 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).

Results

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 Trifluoromethanesulfonic Acid on Sperm Receptor Activity

The role of carbohydrate in sperm receptor activity was

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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-glucosaminyl amide linkages (Edge et al., 1981). Consequently, glycoproteins treated with TFMS are virtually denuded of carbohydrate, with only asparagine-linked N-acetylglucosamine remaining associated with the polypeptide chain.

The sperm receptor activity of egg zonae pellucidae, as measured by an in vitro competition assay (see Experimental Procedures), is extremely sensitive to TFMS. Solubilized zonae pellucidae (2/μl) 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 zonae pellucidae; 29.7 ± 5.7 sperm bound/egg), a value similar to that observed with untreated zonae pellucidae (8.7 ± 4.2 sperm bound/egg). Zonae pellucidae treated with TFMS inhibited sperm binding by less than 10% (26.9 ± 5.1 sperm bound/egg). Electrophoretic analyses of TFMS-treated zonae 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-β-N-Acetyl-D-Glucosaminidase F on Sperm Receptor Activity

In view of the TFMS results described above, endo-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 diacetylchitabiosyl 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 zonae 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 zonae pellucidae, when tested by the in vitro competition assay; similarly, Endo F-treated egg zonae pellucidae retained full receptor activity (Table 1). It was noted that neither Endo F treated ZP3 nor Endo F treated zonae 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 zonae 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 zonae 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.

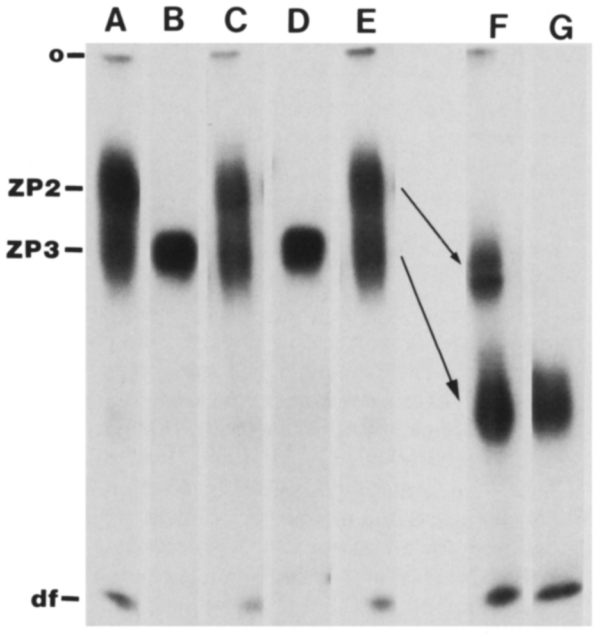

Figure 1. Electrophoretic Analysis of Endoglycosidase F-Treated Zona Pellucida Glycoproteins

Egg zonae pellucidae and electrophoretically purified ZP3 were prepared and were radioiodinated as described in Experimental Procedures. Following incubation in either the presence or absence of Endo F for 4 hr at 37°C, zona pellucida glycoproteins were run on SDS-PAGE, under reducing conditions, and were autoradiographed. Shown are autoradiographs of zonae pellucidae (lane A) and purified ZP3 (lane B) incubated in the presence of electrophoresis solubilization buffer alone; zonae pellucidae (lane C) and purified ZP3 (lane D) incubated in the presence of heat inactivated Endo F (1 min at 100°C); zonae pellucidae incubated in the presence of heat-inactivated Endo F (lane E); zonae pellucidae (lane F) and purified ZP3 (lane G) incubated in the presence of Endo F. Positions of the origin of the gel (o), ZP2, ZP3, and dye front (df) are indicated. The changes in electrophoretic mobilities of ZP2 and ZP3, as the result of Endo F treatment, are indicated by arrows between lanes F and G.

Effect of Alkali on Zona Pellucida Glycoproteins ZP2 and ZP3

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

Table 1. Effect of Endo-β-N-Acetyl-D-Glucosaminidase F on Sperm Receptor Activity

Sample Assayed*	Sample Treatment†	
	Heat Inactivated Endo F‡ [Sperm/Egg (% Control)]	Active Endo F‡ [Sperm/Egg (% Control)]
Distilled 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 had 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.

‡ These data represent the mean ± SD of 2 to 6 individual experiments.

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 zonae 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 radioiodinated, 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 chains 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 kd (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 zonae pellucidae were radiolabeled prior to treatment with 5 mM NaOH, 75–90% of the radiolabel initially associated with ZP2 and ZP3 was recovered in the lower molecular weight forms of the glycoproteins; the loss of radiolabel probably reflects alkali catalyzed release of ^{125}I, 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

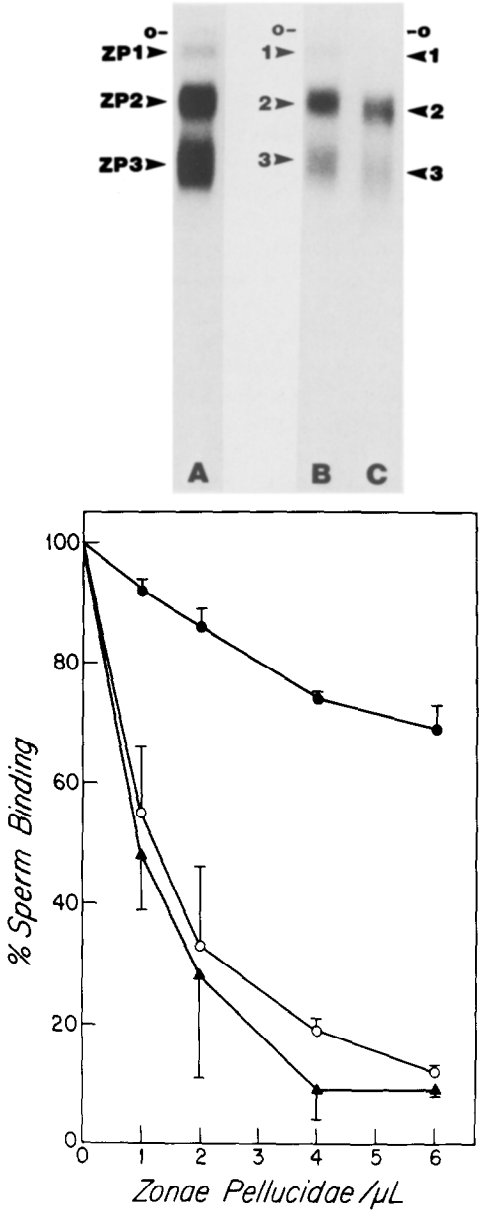

Figure 2. Electrophoretic and Competition Binding Analyses of Alkali-Treated Zona Pellucida Glycoproteins

Egg zonae 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 zonae pellucidae (lane A), zonae pellucidae incubated in the presence of distilled water (lane B), and zonae 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. Zonae 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 zonae pellucidae/μl, as described in Experimental Procedures. The ability of untreated (▲——▲), distilled-water-treated (○——○), and NaOH-treated (●——●) zonae 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 zonae pellucidae/ul).

ZP2 (Bleil and Wassarman, 1980c; Wassarman et al., 1984a).

Following β-elimination in the presence of a strong reducing agent such as $NaBH_4$, the elimination products of the glycosidically linked amino acids, serine and threonine, are alanine and α-aminobutyric acid, respectively (Sharon, 1975). In view of the molecular weight shifts of ZP2 and ZP3 following 5 mM NaOH treatment, we determined whether or not serine and threonine were converted into alanine and α-aminobutyric acid under these conditions. Purified egg ZP2 and ZP3 were incubated in the presence of 5 mM NaOH and 1 M ^{3}H-$NaBH_4$ at 37°C for 16 hr (mild alkaline reduction), were acid hydrolyzed, and were subjected to two-dimensional thin layer chromatography as described in Experimental Procedures. Incorporation of ^{3}H into alanine and α-aminobutyric acid was compared using zona pellucida glycoproteins exposed to ^{3}H-$NaBH_4$ in both the presence and absence of alkali. As a control glycoprotein, hCG was treated and was analyzed under identical experimental conditions.

Results of the analyses described above were expressed as incorporation ratios ($cpm_{(NaOH)}/cpm_{(H_2O)}$) for alanine, for α-aminobutyric acid, and for other amino acids. In the case of hCG, incorporation ratios of 2.19 and 0.93 were determined for alanine and α-aminobutyric acid, respectively, indicating the presence of serine-, but not threonine-linked oligosaccharides. This result is in agreement with reports that hCG is O-glycosylated only at serine residues (Kessler et al., 1979b). The average incorporation ratio for all other amino acids examined was 1.02 ± 0.23, indicating that no other amino acids in hCG were radiolabeled in an alkali-specific manner. Incorporation ratios for purified ZP2 and ZP3 are presented in Table 2. For both ZP2 and ZP3, ratios significantly greater than 1.0 were determined for alanine and for α-aminobutyric acid, but not for other amino acids. These results strongly suggest that both of these glycoproteins possess serine- and threonine-linked oligosaccharides that are released on mild alkaline hydrolysis. Experimental evidence concerning the nature of the carbohydrate released is described below.

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

Glycoprotein	Tritium Incorporation Ratio*		
	Alanine	α-Amino-butyric Acid	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: $[cpm_{(NaOH)}/cpm_{(H_2O)}]$. 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 0.6–1.6 and 0.9–1.3, respectively. The range of values for background (i.e., ninhydrin-negative regions of the chromatograms) was 0.7–1.4.

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. In these experiments, 1–2% of the zonae pellucidae were radiolabeled with ^{125}I-Bolton-Hunter reagent. This permitted determination of the percentage of treated material recovered and, therefore, estimation of zona 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, egg 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-D-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 $NaBH_4$, during the hydrolysis (Lloyd, 1976). Under these conditions, the reducing termini of released oligosaccharides are rapidly converted to alkali-stable sugar alcohols. In the presence of ^{3}H-$NaBH_4$, the released oligosaccharides are recovered as ^{3}H-labeled alcohols. We used such a procedure to obtain radiolabeled oligosaccharides that possess sperm receptor activity from purified ZP3.

Initial experiments demonstrated that radiolabeled (^{3}H-$NaBH_4$) oligosaccharides, possessing sperm receptor activity, could be recovered from alkaline hydrolysates of egg zonae 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

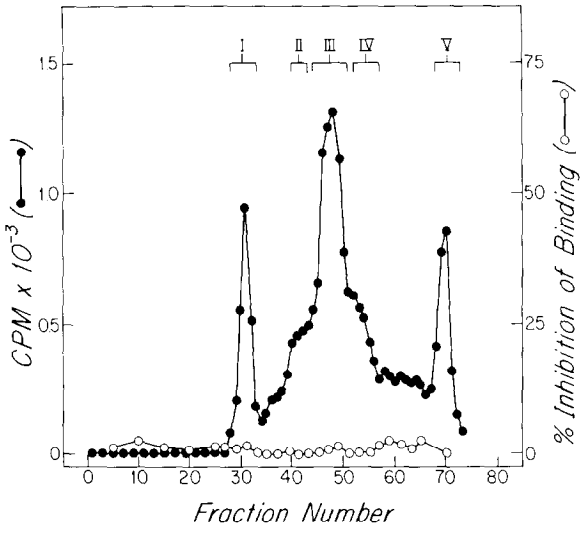

Figure 3. Gel Filtration and Competition Binding Analyses of O-Linked Oligosaccharides Released from ZP2

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 × 17 cm), void volume fractions were pooled, were lyophilized, were resuspended, and were then chromatographed on Bio-Gel P-6 (1.5 × 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 μl aliquots of each column fraction of 1.5 ml (●——●). In addition, two 700 μl 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 (○——○). 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.

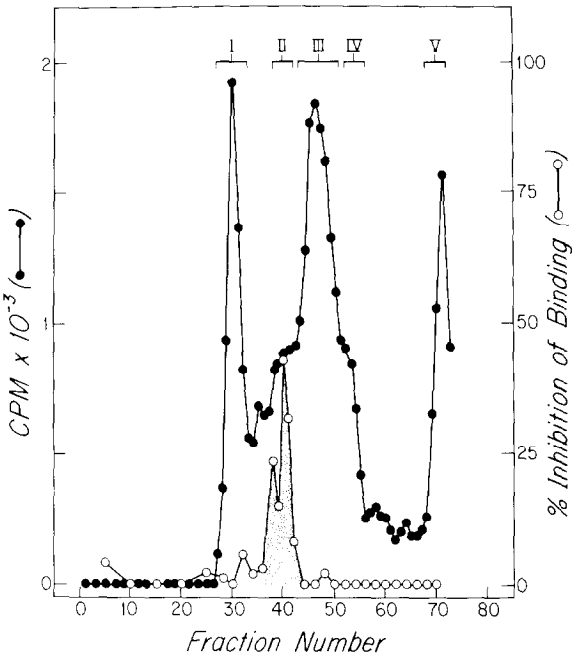

Figure 4. Gel Filtration and Competition Binding Analyses of O-Linked Oligosaccharides Released from ZP3

These experiments were carried out with purified ZP3, radiolabeled with ^{3}H-NaBH$_4$, exactly as described in the legend to Figure 3. Shown are the Bio-Gel P-6 elution profile for radiolabeled oligosaccharides (●——●) and the sperm receptor activity profile (○——○). The region of the elution profile displaying sperm receptor activity (Region II) is stippled. The control level of sperm binding was 53.9 ± 7.8 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 ± 5.6 sperm bound/egg, 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 (Rasilo 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 follow 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–Triton 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 ^{3}H-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 found in region IV (~3.4 kd–4.5 kd 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).

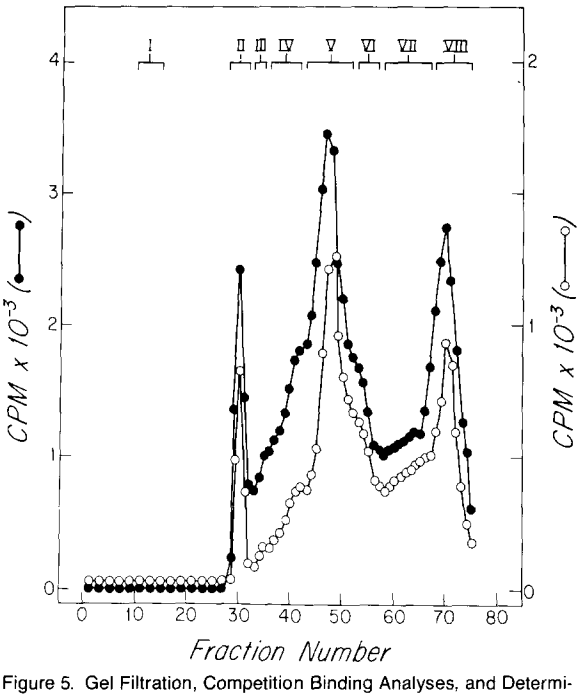

Figure 5. Gel Filtration, Competition Binding Analyses, and Determination of Sperm Association of O-Linked Oligosaccharides Released from ZP2

O-Linked oligosaccharides were released from purified ZP2, were radiolabeled with ^{3}H-$NaBH_4$, and were chromatographed on Bio-Gel P-2 and P-6 as described in Experimental Procedures and the legend to Figure 3. Prior to gel filtration, one portion of radiolabeled material was incubated with sperm for 1 hr at 37°C, the sperm were centrifuged through dibutyl phthalate into 0.5 M sucrose /1% Triton X-100, and the detergent phase was collected and was centrifuged to remove insoluble material (see Experimental Procedures). Shown are the Bio-Gel P-6 elution profiles for radiolabeled oligosaccharides not incubated with sperm (●——●) and associated with sperm after a 1 hr incubation (○——○). In the case of radiolabeled oligosaccharides associated with sperm, fractions were pooled (Regions I–VIII), were lyophilized, were resuspended in culture medium (30 μl), and were assayed for sperm receptor activity in the in vitro competition binding assay. None of the fractions examined exhibited sperm receptor activity. The control level of sperm binding was 32.2 ± 7.6 sperm bound/egg in the experiment shown. The values for Regions I–VIII, expressed as a percent of the control, were 100 ± 8, 101 ± 6, 94 ± 5, 98 ± 4, 102 ± 2, 105 ± 2, 99 ± 1, and 99 ± 1, respectively. Ferritin and ^{3}H-borohydride eluted in regions II (void volume) and VIII, respectively.

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 ^{3}H-$NaBH_4$ as before. Under these conditions, glycosidically linked sugars are converted to their respective sugar alcohols; ^{3}H-N-acetyl-D-glucosaminitol and ^{3}H-N-acetyl-D-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-D-galactosaminitol (Figure 7). A small amount of radiolabel was observed comigrating with N-acetyl-D-glucosaminitol; however, this constituted 10%

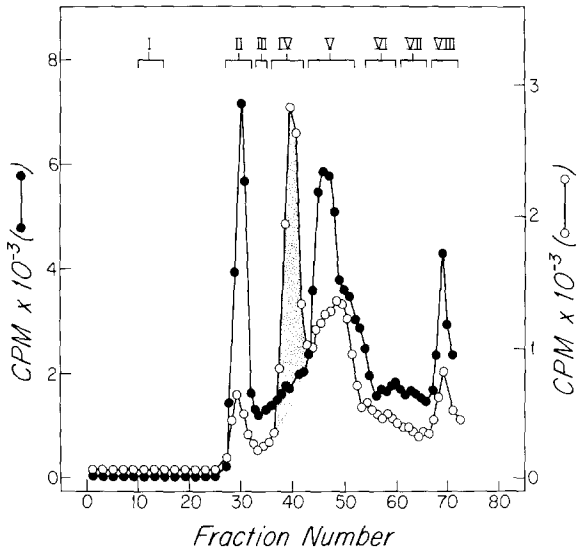

Figure 6. Gel Filtration, Competition Binding Analyses, and Determination of Sperm Association of O-Linked Oligosaccharides Released from ZP3

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 (●———●) and associated with sperm after a 1 hr incubation (○———○). The region of the elution profile that 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), were lyophilized, were resuspended in culture medium (30 μl), and were assayed for sperm receptor activity in the in vitro competition binding assay. Only Region IV exhibited sperm receptor activity, reducing sperm binding by more than 50%, as compared with controls. The control level of sperm binding was 29.1 ± 6.6 sperm bound/egg in the experiment shown. The values for Regions I–VIII, expressed as a percent of the control, were 93 ± 3, 93 ± 4, 94 ± 1, 49 ± 2, 95 ± 2, 97 ± 2, 100 ± 1, and 98 ± 5, respectively. Ferritin and ^{3}H-borohydride eluted in regions II (void volume) and VIII, respectively.

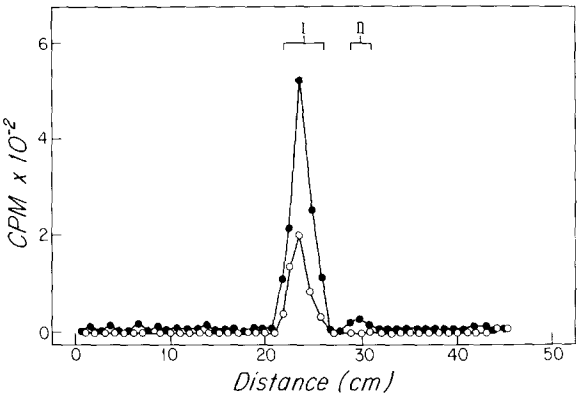

Figure 7. Chromatographic Analysis of the Reducing Terminus of Oligosaccharides Released from ZP3

Purified egg ZP3 was subjected to mild alkaline hydrolysis in the presence of 1 M ^{3}H-$NaBH_4$, 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, as described in Experimental Procedures. Shown are the profiles of radioactivity for purified ZP3 not incubated with sperm (●———●) and associated with sperm after a 1 hr incubation (○———○). The positions of N-acetyl-D-galactosaminitol (I) and N-acetyl-D-glucosaminitol (II) are indicated.

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 Harford, 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, 1983). 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 (Hanafusa et al., 1955) and were later shown to be constituents of mammalian mucins and proteoglycans (Anderson et al., 1964; Bhavanandan et al., 1964; Harbon et al., 1964; Tanaka 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 20 sugars that are added after translation to nascent polypeptide chains by stepwise transfer of monosaccharides (Kornfeld and Kornfeld, 1980; Hanover and Lennarz, 1981; Schachter and Williams, 1982). Studies of the functional significance of O-linked oligosaccharides have been severely hindered by the lack of any specific metabolic inhibitors of this type of glycosylation. However, evidence that O-linked carbohydrates may be involved in antifreeze glycoprotein function (Vanderheede et al., 1972), in 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) has been presented.

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

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 $NaBH_4$, results in a slight decrease of its molecular weight (Figure 2). In the presence of $NaBH_4$, serine and threonine residues are converted to alanine and to α-aminobutyric acid, respectively (Table 2), and release of N-acetyl-D-galactosaminitol 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 $NaBH_4$, sperm receptor activity is found associated with released oligosaccharides having N-acetyl-D-galactosaminitol 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-D-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 glycophorin (Thomas and Winzler, 1969; Marchesi et al., 1976), 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 ($\sim$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 (Bleil 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 (Bleil and Wassarman, 1980a; Florman and Wassarman, 1983); a property characteristic of a number of other putative receptors (Bleil and Wassarman, 1980a).

It has been suggested previously that carbohydrate plays a role in binding sperm to zonae pellucidae, since various lectins, monosaccharides, and glycoconjugates inhibit the binding of sperm to mammalian eggs (Ahuja, 1982; Oikawa et al., 1973; Huang et al., 1982; Shur and Hall, 1982; Wassarman et al., 1984b). Similarly, it has been reported that various monosaccharides and polysaccharides inhibit sperm–egg interaction in several invertebrate and plant species (Bolwell 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; Glabe 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; Bleil 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 (Bleil and Wassarman, 1983; Florman et al., 1984; Wassarman et al., 1985) and participates in the secondary block to polyspermy (Wolf, 1981; Schmell 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 (Bleil 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 (Bleil et al., 1981), there is no evidence as yet for proteolysis of ZP3 (Bleil and Wassarman, 1980a; Bleil et al., 1981). Whatever the nature of the modification, it is subtle, not rendering embryo ZP3 distinguishable from egg ZP3 by conventional electrophoretic analysis (Bleil 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 glycopeptides derived from sea urchin vitelline envelopes, the glycopeptides do not exhibit the species specificity observed with high molecular weight, vitelline envelope glycoconjugates (Kinsey and Lennarz, 1981; Rossignol et al., 1983; Glabe 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), at 37°C in an environment of 5% CO_2 in air. These conditions are capable of supporting mouse sperm capacitation and fertilization in vitro (Florman et al., 1984).

Mature (>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, were freed of surrounding cumulus cells with hyaluronidase (0.1% in mSECM; type V ovine testicular hyaluronidase; Sigma). Embryos at the 2-cell stage were flushed from oviducts 37–40 hr after hCG injection.

The caudae epididymes of mature (retired breeders), male, Swiss albino mice were punctured with sterile, steel needles, releasing sperm into mSECM. After 10 min, epididymal tissue was removed, sperm concentrations were adjusted to 4×10^6/ml, and sperm were preincubated for 30 min. Sperm motility was assessed with an inverted phase microscope; preparations with less than 70% of the sperm motile at the end of the preincubation period were discarded.

Preparation of Zonae Pellucidae

Zonae pellucidae were removed from eggs and embryos with micropipettes (≅60 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 NaH_2PO_4 (pH 2.5). When required, zonae pellucidae were radiolabeled with ^{125}I-Bolton-Hunter reagent (~4000 Ci/mmole, New England Nuclear), as previously described (Greve et al., 1982). After SDS-PAGE, zona pellucida glycoproteins 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 ^{125}I-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-β-N-acetyl-D-glucosaminidase F (Endo F; Elder and Alexander, 1982). Lyophilized samples were resuspended in 25 μl of Endo F buffer (100 mM NaH_2PO_4 (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 electrodialyzed, were dialyzed exhaustively against 7 M urea and then against distilled water, and aliquots were taken for electrophoretic analysis and for evaluation of sperm receptor activity.

O-linked oligosaccharides were removed by alkaline β-elimination. Zona pellucida glycoproteins were lyophilized and were resuspended in 50 μl of 5 mM NaOH. To prevent alkaline degradation of released oligosaccharides when reducing conditions were required (Lloyd, 1976), reactions were carried out in the presence of 1 M ^{3}H-$NaBH_4$ (≅100 mCi/mmole, New England Nuclear). Samples were incubated for 16 hr at 37°C in a toluene 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 ^{3}H-$NaBH_4$. Samples containing oligosaccharides were neutralized with 1 N NaOH, were separated from peptides and from Na^+ on 5 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 N_2. Methanol evaporations were repeated 4 times to remove excess borate as its volatile methyl ester derivative (Zill et al., 1953).

Conditions for the extensive proteolysis of zona pellucida glycoproteins with CMC-conjugated Pronase (Sigma) have previously been described (Florman et al., 1984).

Oligosaccharide Binding Studies

Oligosaccharides derived from egg zonae pellucidae and radiolabeled during mild alkaline reduction, were lyophilized and were resuspended in mSECM at a concentration of about 2.7 zonae pellucidae/μl. One μl was removed 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 (4×10^6/ml) under oil (2 zonae pellucidae/μl, 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 (8500 × 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 dioctyl phthalate (Aldrich). Centrifugation (8500 × 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 Kleinzeller, 1980).

Gel Filtration of Oligosaccharides

To analyze zona pellucida oligosaccharides following sperm binding, 5 μl samples of either the starting material (^{3}H-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 ^{3}H-oligosaccharides was carried out at 55°C, on Bio-Gel P-6 (200–400 mesh; 1.5 × 70 cm) that had been previously

equilibrated in distilled water (Heyraud and Rinaudo, 1978; Yamashita et al., 1982). Columns were developed in distilled water, 1.5 ml fractions were collected, were lyophilized, were resuspended in mSECM, and 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 zona pellucida glycoproteins. Lyophilized samples were resuspended in 2 μl of a 5 mM L-alanine, 5 mM L-α-aminobutyric acid solution, and 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 110°C for 18 hr, were dried in a dessicator over NaOH, were washed 3 times by methanol evaporation to remove borates, and with distilled water to remove HCl. Dried hydrolysates were resuspended in 10 μl of distilled water, insoluble material was removed by centrifugation (8500 × g, 1 min), and 1–5 μl aliquots were applied to cellulose, thin-layer chromatography plates (20 × 20 cm; Chromogram, Eastman). Chromatograms were developed in an unsaturated atmosphere of n-butanol:acetone:diethylamine:water (10:10:2:5, 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, 1967). Amino acids were visualized with ninhydrin, were scraped into scintillation vials, were extracted with 0.5 ml distilled water (30 min, 60°C), and associated ^{3}H-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 × 1 cm grid, and the distribution of tritium was evaluated.

The identity of carbohydrates, glycosidically linked to ZP3, was determined following mild alkaline reduction. Analysis was performed both on total ^{3}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 deacidified by repeated washes with distilled water under a stream of N_2, and were resuspended in 1 ml distilled water. Amino sugars were eluted from 5 ml columns of Dowex-50 (H^+ form; 200–400 mesh) with 2 N HCl, were deacidified as previously described, were resuspended in 0.2 ml saturated $NaHCO_3$, and re-N-acetylated with acetic anhydride (Takasaki and Kobata, 1975). The fraction eluted from coupled 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 distilled water was then dried under N_2, was resuspended in 10 μl 35% ethanol, and was applied to borate impregnated, Whatmann #1 paper. Chromatograms were developed, in the descending direction, with ethyl acetate:pyridine:water (2:1:2; Cabib et al., 1953), were cut into 1 cm long strips, and the associated radioactivity was determined by liquid scintillation spectroscopy. Standards were chromatographed in adjacent lanes and were visualized by staining with silver nitrate.

SDS Polyacrylamide Gel Electrophoresis

SDS-PAGE was performed according to the method of Laemmli (1970), by using a 10% acrylamide separating gel and a 4% acrylamide 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 (Bleil and Wassarman, 1980a; Florman et al., 1984). Aliquots (10 μl) of preincubated sperm suspensions were added to 30 μl mSECM 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 mSECM. 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 zonae pellucidae. Sperm associate reversibly and nonspecifically with embryo zonae pellucidae, but establish both nonspecific, as well as tenacious, specific bonds to zonae pellucidae of eggs; thus, these conditions serve to remove nonspecifically-associated sperm from egg zonae pellucidae (Bleil 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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