Structural Analysis of Murine Zona Pellucida Glycans

EVIDENCE FOR THE EXPRESSION OF CORE 2-TYPE O-GLYCANS AND THE Sd<sup>a</sup> ANTIGEN*<sup>†</sup>

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Murine sperm initiate fertilization by binding to specific oligosaccharides linked to the zona pellucida, the specialized matrix coating the egg. Biophysical analyses have revealed the presence of both high mannose and complex-type N-glycans in murine zona pellucida. The predominant high mannose-type glycan had the composition Man<sub>9</sub>GlcNAc<sub>2</sub>, but larger oligosaccharides of this type were also detected. Biantennary, triantennary, and tetraantennary complex-type N-glycans were found to be terminated with the following antennae: Galβ1-4GlcNAc, NeuAcα2–3Galβ1–4GlcNAc, NeuGcα2–3Galβ1–4GlcNAc, the Sd<sup>a</sup> antigen (NeuAcα2–3[GalNAcβ1–4Galβ1–4GlcNAc], NeuGcα2–3GalNAcβ1–4Galβ1–4GlcNAc), and terminal GlcNAc. Polyolactosamine-type sequence was also detected on a subset of the antennae. Analysis of the O-glycans indicated that the majority were core 2-type (Galβ1–4GlcNAcβ1–6Galβ1–3GalNAc). The β1–6-linked branches attached to these O-glycans were terminated with the same sequences as the N-glycans, except for terminal GlcNAc. Glycans bearing Galβ1–4GlcNAcβ1–6 branches have previously been suggested to mediate initial murine gamete binding. Oligosaccharides terminated with GalNAcβ1–4Gal have been implicated in the secondary binding interaction that occurs following the acrosome reaction. The significant implications of these observations are discussed.

The initial event in the life of all sexually reproducing metazoans is the fertilization of an individual egg by a single sperm. Murine sperm begin this process by binding to the specialized extracellular matrix of the egg known as the mZP. This matrix has been shown to be composed of three major glycoproteins (designated mZP1, mZP2, and mZP3) (2). There is strong evidence to suggest that binding occurs via the interaction of mZP3-associated glycans with lectin-like proteins on the sperm surface (3, 4) that in turn induce a signal transduction event known as the acrosome reaction (5). During this reaction, the plasma membrane of the sperm fuses with the outer membrane of a lysosome-like organelle known as the acrosome lying just beneath the surface of the sperm. The resulting membrane complex then blebs off to expose the inner acrosomal membrane. In the mouse model, this inner acrosomal membrane then undergoes secondary binding to mZP2. Sperm move through the mZP and fuse with the egg, thus completing the process of fertilization.

Initial studies performed by Wassarman and co-workers (3) indicate that either Pronase glycopeptides (3) or O-linked oligosaccharides (4) obtained from mZP3 block murine sperm-egg binding. Several major models for the initial murine gamete binding interaction have subsequently been proposed that are based upon specific carbohydrate recognition (6–9). A recent study involving recombinant mZP3 synthesized in murine F9 embryonal carcinoma cells suggests that vicinal presentation of O-linked oligosaccharides within a specific region of mZP3 is necessary for initial sperm-egg binding (10). mZP2 has been proposed to be the glycoprotein that mediates the secondary binding interaction involving binding to the inner acrosomal membrane (11). This binding event has also been postulated to rely upon carbohydrate-mediated interactions (12).

Structural analysis of mZP2 and mZP3-associated glycans has previously been performed using radioactive (13) and fluorescence (14) detection methods. However, precise compositional and linkage data related to these oligosaccharides could not be obtained by employing such techniques. The present study was undertaken to obtain this information using high sensitivity mass spectrometric techniques. We report evidence for the presence of the Sd<sup>a</sup> antigen in both the N- and O-linked oligosaccharides derived from murine ZP. The O-glycans detected in this analysis are primarily of the core 2 glycan type. Potential structure-function relationships emerging from this new structural information will be discussed in depth.

EXPERIMENTAL PROCEDURES

Purification of Murine ZP—The method that was employed for zona purification was a modification of an existing procedure kindly provided by Dr. Jeffrey Bleil.‡ Flash-frozen mouse ovaries were obtained from Harlan Bioproducts for Science (Indianapolis, IN) and stored at –80 °C until processed. The total number of ovaries used in this study was 4,000, processed in batches of 40–50 ovaries each. Each batch was thawed and suspended in 6 mL of ice-cold triethylenemethylenediamine-NaCl buffer (25 mM triethylenemethylenediamine, pH 8.5, containing 150 mM NaCl, 1 mM CaCl<sub>2</sub>, and 0.02% NaN<sub>3</sub>). Turkey egg white trypsin inhibitor, DNase I, and hyaluronidase (Sigma) were added to this solution to give final concentrations of 1, 0.1, and 0.1 mg/mL, respectively. The solution was homogenized with a Polytron homogenizer until the ovaries were particulate. The mixture was adjusted to 1% (v/v) Nonidet P-40 by the addition of a 20%
solution of this detergent in triethylamine-NaCl buffer. The solution was treated briefly with the Polytron homogenizer and then transferred to a Dounce homogenizer. The solution was further homogenized until liquefied and adjusted to a 1% concentration of deoxycholate by the addition of a 20% solution of this detergent in triethylamine-NaCl buffer. The solution was further homogenized until the mixture became opalescent. 3.8 ml of 90% Percoll in triethylamine-NaCl buffer was added to a 10-ml sealable Ultracentrifuge tube. The homogenate was carefully layered on the Percoll solution without mixing. A solution of 35% Percoll in triethylamine-NaCl buffer was carefully layered on top of the homogenate. The tube was centrifuged in Sorvall SS-34 rotor at 19,000 rpm for 60 min at 18 °C. An identical sham tube containing the same solutions was prepared and supplemented with Percoll gradient indicator beads. The length and/or the speed of the run was adjusted so that the top blue bands of beads (specific gravity = 1.018) migrated at least 2/3 of the way to the top of the gradient. The position of the blue beads matched the migration of the zona in the Percoll gradient. The zona (4,000–5,000) were carefully removed with a syringe in a volume of roughly 0.5 ml.

A small aliquot (5–10 μl) of each preparation was subjected to visual inspection under an Olympus phase contrast microscope to confirm the presence of zonae. The purity of the preparation was also confirmed by inspection under an Olympus phase contrast microscope to confirm the presence of zonae. The purity of the preparation was also confirmed by

Tryptic Digestion of Murine ZP—Suspensions of murine ZP were centrifuged at 2600 × g for 30 min to precipitate this extracellular matrix. The ZP pellet was resuspended in 800 μl of 50 mM ammonium hydrogen carbonate, pH 8.5. This ZP suspension was digested with 20 μg of trypsin (EC 3.4.21.4, Sigma) for 5 h at 37 °C. 20 μg of trypsin was added, and the digestion was allowed to proceed at 37 °C for 16 h. The sample was placed in boiling water for 2 min to terminate the reaction and lyophilized.

PNGase F Digestion—The trypsic digest was dissolved in 200 μl of 50 mM ammonium hydrogen carbonate, pH 8.5, and incubated with PNGase F (EC 3.5.1.52, Roche) at 37 °C overnight to release N-linked oligosaccharides. The products were lyophilized and subjected to reverse phase chromatography on a C18 Sep-Pak cartridge exactly as described previously (15) to separate released oligosaccharides from peptides and O-glycopeptides.

Reductive Elimination—The 20% and 40% 1-propanol Sep-Pak fractions obtained from the purification of the PNGase F digestion were dissolved in 200 μl of a solution of sodium borohydride (10 mg/ml) in 0.05 M NaOH and incubated at 45 °C for 16 h. The reactions were terminated by the addition of glacial acetic acid. Released O-glycans were purified by Dowex as described previously (15).

Permethylation of Released Glycans and Preparation of Partially Methylated Alditol Acetates—Permethylation was performed using the sodium hydroxide/methyl iodide procedure exactly as established previously (15). The permethylated N- and O-glycans were purified by Sep-Pak chromatography using an established method (15). Preparation of partially methylated alditol acetates was performed as described (16).

Glycosidase Digestions—The purified N-glycans were subjected to digestion with specific glycosidases to discern relevant structural features. Digestion with endo-β-galactosidase from Bacteroides fragilis (EC 3.2.1.103, Roche Molecular Biochemicals) was performed with 10 milliunits of enzyme in 200 μl of 50 mM ammonium acetate, pH 5.5, for 24 h at 37 °C. Terminal Galα1–3Gal sequences were hydrolyzed by treatment with 10 milliunits of α-galactosidase from green coffee beans (EC 3.2.1.22, Roche Molecular Biochemicals) in 200 μl of 50 mM ammonium acetate, pH 6.0, for 24 h at 37 °C. Jack bean α-mannosidase (EC 3.2.1.24, Roche Molecular Biochemicals) (0.5 units) digestion was performed under conditions identical to those used for endo-β-galactosidase digestion. The α-sialidase from Arthrobacter ureafaciens (EC 3.2.1.18, Glyko, Inc.) (0.2 units) was incubated with the glycans in 200
was performed using VG Analytical Opus® software. Solvent spectrometer fitted with a cesium ion gun operating at 30 kV. Data rides were acquired using a ZAB-2S.E.-2FPD double-focusing mass and then increased to 290 °C at a rate of 8 °C/min. 

Methylated alditol acetates were dissolved in hexanes and loaded di-

and matrices were as described previously (15).

Streptomyces plicatus

isolation. SDS-gel electrophoresis of the isolated ZP using the

CA). Visual analysis of the preparation indicated the presence 

of 100 mM sodium acetate, pH 5.0, at 37 °C for 48 h. Digestion with Streptomyces plicatus β-N-acetylhexosaminidase (recombinant fusion protein, New England Biolabs) was performed with 50 units of enzyme in 200 μl of 50 mM ammonium acetate, pH 4.5, at 37 °C for 24 h.

FAB-MS Analysis—FAB mass spectra of permethylated oligosaccharides were acquired using a ZAB-28 E2-FPD double-focusing mass spectrometer fitted with a cesium ion gun operating at 30 kV. Data analysis was performed using VG Analytical Opus® software. Solvent and matrices were as described previously (15).

GC-MS Analysis—GC-MS analysis was performed on a Fisons Instruments MD800 machine. Separation was achieved using an RTX-5 fused silica capillary column (30 m × 0.25 mm, Restek Corp). Partially methylated alditol acetates were dissolved in hexanes and loaded directly onto the column at 65 °C. The column was held at 65 °C for 1 min and then increased to 290 °C at a rate of 8 °C/min.

RESULTS

Isolation and Characterization of ZP—Murine ZP were iso-

lated by a modification of an original procedure (2) provided to us by Dr. Jeffrey Bleil ( Scripts Research Institute, La Jolla, CA). Visual analysis of the preparation indicated the presence of individual zonae exactly as was observed in the original isolation. SDS-gel electrophoresis of the isolated ZP using the same conditions employed in this earlier study indicated the presence of three major diffuse bands consistent with molecular weights of mZP1, mZP2, and mZP3 proposed earlier (2) (data not shown). Based on these criteria, the isolated zonae were subjected to carbohydrate structural analysis.

Mapping of the N-Glycan Population—Purified ZP were di-

gested with trypsin and incubated with PNGase F to release N-glycans. The oligosaccharides were separated from peptides and glycopeptides via reverse phase chromatography on a C18 Sep-Pak cartridge. The N-glycans were permethylated and separated from other reactants and products by a second step of Sep-Pak reverse phase purification (15).

The permethylated glycans were subjected to FAB-MS analysis using an established method (15). The data indicated the presence of a range of N-glycans of both the high mannos and complex-types (Fig. 1, Table I). The following structural features were evident. (i) The most abundant component was the high mannos structure Hex5HexNAc2 indicated by the molecular ion at m/z 1580. Lower levels of larger high mannos-type glycans (Hex6–9HexNAc2) were also indicated by the presence of molecular ions at m/z 1784, 1988, 2192, and 2396 (ii) Complex-type biantennary N-glycans decorated with fucose and N-acetyllneuraminic acid were also found (Hex5HexNAc2Fuc at m/z 2244; NeuAcHex5HexNAc2 at m/z 2432, NeuAcHex5HexNAc2Fuc at m/z 2968). (iii) Some of these biantennary glycans contain N-glycolyneuraminic acid, as indicated by signals consistent with NeuGcHex5HexNAc2 at m/z 2044; NeuAcHex5HexNAc2 at m/z 2244; NeuAcHex5HexNAc2Fuc at m/z 2968, and NeuGcHex5HexNAc2Fuc at m/z 3027. The presence of N-glycolyneuraminic acid was also confirmed by the detection of A-type fragment ions at m/z 855 (NeuGcHex5HexNAc2) and m/z 406 (NeuGc-). (iv) Both N-acetyllneuraminic acid and N-glycolyneuraminic acid can be located on the same glycan, as indicated by the molecular ion at m/z 2996, consistent with NeuAcNeuGcHex5HexNAc2. (v) Minor amounts of lactosamine repeats are present, as indicated by the ion at m/z 913 (Hex5HexNAc2). (vi) A fragment ion at m/z 668 (Hex5HexNAc2) is consistent with the presence of terminal Galα1–3Gal. (vii) The presence of A-type ions at m/z 1070 and m/z 1100 suggest unusual terminal epitopes with the compositions NeuAcHex5HexNAc2 and NeuGcHex5HexNAc2, respectively.

Linkage Analysis of Released Murine ZP N-Glycans—Perm-

ethylated N-glycans were acid-hydrolyzed, deuteroreduced, and peracetylated. The resulting partially methylated alditol acetates were analyzed by GC-MS (Table II). Notable features of the methylation analysis were the presence of 3,4-disubstituted galactose and terminal N-acetyllgalactosamine. The detection of 2 (major)-, 2,4 (minor)-, and 2,6 (minor)-linked man-
nose indicated that tri- and tetraantennary structures were present in addition to the major biantennary structures. Bisection-type glycan structures also exist, as shown by the presence of 3,4,6-linked mannose, albeit as very minor components.

Endo-β-galactosidase Digestion of Murine ZP N-Glycans—To analyze the terminal structures of the minor polylactosamine-containing N-glycans, PNGase F-released N-linked oligosaccharides were digested with endo-β-galactosidase. After digestion, a fraction of the reaction products was permethylated, separated from contaminants by reverse phase separation on a Sep-Pak cartridge, and analyzed by FAB-MS. The data (Fig. 2, Table III) indicate the presence of several components not observed before digestion. These components represent the antennae of complex N-glycans bearing a range of different terminal structures. The high mannose-type structures and shorter complex structures were unaffected by this enzyme treatment. Because of the specificity of the enzyme, the oligosaccharides released from the nonreducing termini of polylactosamine-containing antennae all have a reducing Gal residue. Thus, significant signals were observed at m/z 926 (Hex,HexNAc,−Gal), which likely represents structures terminating in α-linked galactose, m/z 1328 (NeuAcHex,HexNAc,−Gal), and its N-glycolyneuraminic acid counterpart at m/z 1358 (NeuGcHex,HexNAc,−Gal). The large signal at m/z 518 represents the disaccharide GlcNAc-Gal, the major product expected after endo-β-galactosidase of polylactosamine-type chains.

Confirmation That α-Linked Galactose Is Associated with Murine ZP Glycans—The products of endo-β-galactosidase digestion were further digested with α-galactosidase, permethylated, and analyzed by FAB-MS. The signal at m/z 926 (Fig. 2)

![Fig. 2](image-url)

**Fig. 2.** FAB mass spectra of the products of digestion of N-glycans from murine zona pellucida with endo-β-galactosidase. a, 35% acetonitrile Sep-Pak fraction; b, 50% acetonitrile Sep-Pak fraction. After digestion, the products were permethylated and purified by Sep-Pak before mass spectrometric analysis.

| Signal Assignment | m/z |
|-------------------|-----|
| 35% acetonitrile fraction |     |
| 518 HexNAcHex + Na⁺ |     |
| 700 HexHexNAc + H⁺ |     |
| 722 HexHexNAc + Na⁺ |     |
| 904 HexHexNAc + H⁺ |     |
| 926 HexHexNAc + Na⁺ |     |
| 1083 NeuAcHexHexNAc + Na⁺ | 1306 NeuAcHexHexNAc + H⁺ |
| 1113 NeuGcHexHexNAc + Na⁺ | 1328 NeuGcHexHexNAc + Na⁺ |
| 1306 NeuGcHexHexNAc + H⁺ | 1336 NeuGcHexHexNAc + H⁺ |
| 1336 NeuGcHexHexNAc + Na⁺ | 1358 NeuGcHexHexNAc + Na⁺ |
| 50% acetonitrile fraction |     |
| 1580 HexHexNAc + Na⁺ |     |
| 1784 HexHexNAc + Na⁺ |     |
| 1988 HexHexNAc + Na⁺ |     |
| 2081 HexHexNAcFuc + Na⁺ |     |
| 2192 HexHexNAc + Na⁺ |     |
| 2244 HexHexNAcFuc + Na⁺ |     |
| 2285 HexHexNAcFuc + Na⁺ |     |
| 2396 HexHexNAc + Na⁺ |     |
| 2490 HexHexNAcFuc + Na⁺ |     |
| 2531 HexHexNAcFuc + Na⁺ |     |
| 2560 NeuAcHexHexNAcFuc + Na⁺ | 2606 NeuAcHexHexNAcFuc + Na⁺ |
| 2636 NeuGcHexHexNAcFuc + Na⁺ | 2735 NeuGcHexHexNAcFuc + Na⁺ |
| 2832 NeuGcHexHexNAcFuc + Na⁺ | 2922 NeuGcHexHexNAcFuc + Na⁺ |
| 3126 NeuGcHexHexNAcFuc + Na⁺ | 3517 NeuGcHexHexNAcFuc + Na⁺ |
| 3517 NeuGcHexHexNAcFuc + Na⁺ |     |
was absent after α-galactosidase digestion (data not shown), demonstrating that it contained α-linked galactose.

**Confirmation of the Presence of High Mannose-type N-Glycans on Murine ZP**—Another aliquot of the endo-β-galactosidase-treated N-glycans was digested with α-mannosidase. The products of the digestion were analyzed by FAB-MS after permethylation. All signals corresponding to the high mannose-type sequences were absent, but those of the complex structures were still detected (Fig. 3). This result is consistent with the presence of high mannose-type sequences in this mixture.

**Identification of the Sdα Determinant on Murine ZP N-Linked Oligosaccharides**—The A-type fragment ions at m/z 1070 and m/z 1100 observed in the N-glycan mapping experiment have compositions consistent with the Sdα determinant (GalNAcβ1–4(NeuAca2–3)Galβ14GlcNAc) and its N-glycolylneuraminic acid counterpart. In addition, the results of the linkage analysis and endo-β-galactosidase experiments support the presence of this epitope (see above). Further information on the structures of these epitopes was obtained from the following experiments. FAB-MS of the permethylated reaction products of α-sialidase digestion of the PNGase F-released murine ZP N-glycans showed the release of both N-acetylneuraminic acid and N-glycolyneuraminic acid (Fig. 4, Table IV). Thus the fragment ions attributable to NeuAc-containing structures originally detected in the undigested N-glycans at m/z 375, m/z 825, and m/z 1070 were absent after digestion. The NeuGc-containing structures showed differing susceptibilities to the sialidase. The linear structure NeuGeHexHexNAc\(^{−}\) was fully digested as shown by the absence of m/z 855, but the potentially branched sequence, NeuGc[HexNAc]HexHexNAc, which is detected as an A-type ion at m/z 1100, was still present after digestion. A new fragment ion was present at m/z 709, corresponding to HexHexNAc\(^{−}\), the expected ion produced after the removal of N-acetylneuraminic or N-glycolyneuraminic acid from the Sdα determinant. The small signal present at m/z 1362 demonstrated the presence of low levels of polygalactosamine repeats. This signal was not observed before sialidase digestion, demonstrating that the polygalactosamine repeats are capped with N-acetylneuraminic acid or N-glycolyneuraminic acid. Linkage analysis of the products of digestion confirmed the reduction in abundance of 3,4-linked galactose and an increase in 4-linked galactose.

To provide further evidence for the putative Sdα structure, an aliquot of the endo-β-galactosidase-treated murine ZP N-glycans was digested with α-sialidase. A portion of this reaction mixture was permethylated and analyzed by FAB-MS (Fig. 5a). The data indicated that N-acetylneuraminic acid had been removed (loss of m/z 1328), the signal at m/z 1358 (NeuGcHex\(_3\)HexNAc\(_2\)-Gal) had been reduced in intensity, and a new signal had appeared at m/z 967, corresponding to Hex\(_5\)HexNAc\(_2\)-Gal+Na\(^+\).

The remainder of the α-sialidase-treated fraction was further digested with β-N-acetylhexosaminidase. An aliquot was removed for permethylation and FAB-MS analysis (Fig. 5b). The data showed significant reduction of the signal at m/z 967 (Hex\(_5\)HexNAc\(_2\)-Gal+Na\(^+\)) and the loss of the signal at m/z 518 (GlcNAc-Gal+Na\(^+\)). The new minor signal at m/z 1171 (Hex\(_5\)HexNAc\(_2\)-Na\(^−\)) is due to the loss of N-acetylglucosamine from truncated complex structures. To confirm that N-acetylgalactosamine was removed, linkage analysis was performed on the α-sialidase-treated N-glycans and on the α-sialidase plus β-N-acetylhexosaminidase-treated glycans. Comparison of the data indicated that terminal N-acetylgalactosamine was present before β-N-acetylhexosaminidase digestion but was absent afterward. N-Acetylgalactosamine continued to be detected after β-N-acetylhexosaminidase digestion, data that are consistent with the assignments of structures present in the FAB-MS spectrum after this full range of enzyme digests.
Taken together, the above data provide convincing evidence for the presence of the Sd\(^a\) epitope and its N-glycolylneuraminic acid counterpart on polylactosamine antennae of complex-type N-glycans in the murine ZP.

**Assignment of N-Glycan Structures**—Taking into account all the above data, the major N-glycans in the murine ZP preparation are assigned the structures in Fig. 6.

**Mapping of the O-Glycan Population**—Murine ZP were digested with trypsin and subjected to N-glycanase digestion. After separation of released N-glycans from peptides and glycopeptides on a Sep Pak C18 cartridge, the glycopeptides eluting in 20% and 40% n-propanol were subjected to reductive elimination, Dowex purification, borate removal, permethylation, reverse phase separation, and FAB-MS analysis. The data obtained (Fig. 7, Table V) were consistent with the following structural assignments: (i) the presence of a range of reduced O-glycans, the majority of which are composed of from 3 to 6 monosaccharide residues; (ii) pairs of molecular ions separated by 30 mass units whose compositions are consistent with the presence of both N-acetylneuraminic acid and N-glycolylneuraminic acid (m/z 873/903, 1256/1286, 1344/1374, 1590/1620); fragment ion data gives further evidence for the presence of these monosaccharides; (iii) ions at m/z 1070 and 1100 whose compositions suggest the possible presence of the Sd\(^a\) epitope, which is of particular interest; (iv) no observation of fucosylated O-glycans nor any evidence for the presence of lactosamine repeats; and (v) a weak signal at m/z 668 (Hex\(_2\)HexNAc\(^c\)), most likely derived from the molecular ion at m/z 1165, providing evidence for the presence of the Gal\(^\alpha1–3\)Gal epitope.

**Linkage Analysis of Released Murine ZP O-Glycans**—Permethylated O-glycans were acid-hydrolyzed, deuteroreduced, and permethylated with Dowex resin. After borate removal, the permethylated glycopeptides were separated by reverse phase chromatography and their molecular ions were measured by FAB-MS. The data obtained (Fig. 4, Table IV) were consistent with the following structural assignments: (i) the presence of a range of reduced O-glycans, the majority of which are composed of from 3 to 6 monosaccharide residues; (ii) pairs of molecular ions separated by 30 mass units whose compositions are consistent with the presence of both N-acetylneuraminic acid and N-glycolylneuraminic acid (m/z 873/903, 1256/1286, 1344/1374, 1590/1620); fragment ion data gives further evidence for the presence of these monosaccharides (m/z 825/855); (iii) ions at m/z 1070 and 1100 whose compositions suggest the possible presence of the Sd\(^a\) epitope, which is of particular interest; (iv) no observation of fucosylated O-glycans nor any evidence for the presence of lactosamine repeats; and (v) a weak signal at m/z 668 (Hex\(_2\)HexNAc\(^c\)), most likely derived from the molecular ion at m/z 1165, providing evidence for the presence of the Gal\(^\alpha1–3\)Gal epitope.

**Table IV**

| Signal Assignment | Assignment |
|-------------------|------------|
| 1558              | HexHexNAc\(_c\) + H\(^{+}\) |
| 1580              | HexHexNAc\(_c\) + Na\(^{+}\) |
| 1672              | HexHexNAc\(_c\) + H\(^{+}\) |
| 1784              | HexHexNAc\(_c\) + Na\(^{+}\) |
| 1966              | HexHexNAc\(_c\) + H\(^{+}\) |
| 1988              | HexHexNAc\(_c\) + Na\(^{+}\) |
| 2040              | HexHexNAc\(_c\)Puc + Na\(^{+}\) |
| 2070              | HexHexNAc\(_c\) + Na\(^{+}\) |
| 2192              | HexHexNAc\(_c\) + Na\(^{+}\) |
| 2222              | HexHexNAc\(_c\)Puc + H\(^{+}\) |
| 2244              | HexHexNAc\(_c\)Puc + Na\(^{+}\) |
| 2285              | HexHexNAc\(_c\)Puc + Na\(^{+}\) |
| 2396              | HexHexNAc\(_c\) + Na\(^{+}\) |
| 2448              | HexHexNAc\(_c\)Puc + Na\(^{+}\) |
| 2490              | HexHexNAc\(_c\)Puc + Na\(^{+}\) |
| 2694              | HexHexNAc\(_c\)Puc + Na\(^{+}\) |
| 3143              | HexHexNAc\(_c\)Puc + Na\(^{+}\) |
| 3592              | HexHexNAc\(_c\)Puc + Na\(^{+}\) |
| Loss of methanol from m/z 464 | HexHexNAc\(^c\) |
| Loss of methanol from m/z 668 | HexHexNAc\(^c\) |
| Loss of methanol from m/z 709 | HexHexNAc\(^c\) |
| Loss of methanol from m/z 913 | HexHexNAc\(^c\) |
| NeuGcHexHexNAc\(^c\) | HexHexNAc\(^c\) |
| HexHexNAc\(^c\) | HexHexNAc\(^c\) |

**Fig. 4.** FAB mass spectrum of the products of digestion of N-glycans from murine zona pellucida with α-sialidase. **a**, molecular ion region; **b**, fragment ion region. After digestion, the products were permethylated and purified by Sep-Pak before mass spectrometric analysis.
acetylated, and analyzed by GC-MS (Table VI). The presence of 3-linked galactose and the absence of 6-linked galactose indicates that both N-acetylneuraminic acid and N-glycolyneuraminic acid are 3-linked to this monosaccharide. A weak spectrum was obtained for 3,4-linked galactose, providing further evidence that the Sda structure is expressed in the O-glycans. Although the O-glycan preparation is contaminated with low levels of N-glycans as indicated by the signals for high mannose structures in Fig. 6 and the variously linked mannoses in the linkage data (Table VI), it is unlikely that the 3,4-linked galactose is derived from N-glycan contaminants. This conclusion is arrived at by taking into consideration the low levels of Sdα containing N-glycans in the total N-glycan population and the fact that complex-type N-glycans were not detectable in the FAB spectra of the O-glycan preparation.

Assignment of O-Glycan Structures—Rigorous characterization of each of the O-glycans is particularly challenging because of the low levels of material available and the complexity of the O-glycan population. Nevertheless some firm conclusions can be drawn from the data. In particular the observation of 3,6-linked GalNAcitol and the absence of detectable levels of 3-linked GalNAcitol in the linkage analysis indicate that the majority of O-glycans are likely to have core-type 2 structures. Fig. 8 shows the sequences that have been assigned from the FAB and linkage data.

**DISCUSSION**

This report outlines the first characterization of murine ZP glycans using very sensitive biophysical methods of analysis to determine their precise structural features. Structural analysis of glycans derived from mZP2 and mZP3 have been performed in previous studies (13, 14). However, because of the analytical methods used, these investigations did not provide the explicit kind of data that was generated in the present study. The findings reported in this study are crucial because the mouse is the most flexible mammalian model for experimental manipulation. Therefore this study provides complementary data that may now finally enable the murine gamete binding interaction to be understood at the molecular level. As will be discussed, this information may also provide more insight into potential immunological relationships present in the eutherian reproductive system.

The proposed structures for the major N-linked oligosaccharides in mZP are shown in Fig. 6. Based on the current study, the majority of the glycans associated with this extracellular matrix are primarily high mannose and biantennary complex glycans, with lesser amounts of tri- and tetraantennary complex-type oligosaccharides. The complex N-glycans exhibit a range of terminal structures, the great majority of which have been previously identified during an investigation of mZP2- and mZP3-derived N-glycans (14). Our data confirm the conclusion of Noguchi and Nakano (14) that polylactosamine sequences and terminal α-linked galactose are associated with mZP glycoproteins. An additional structural feature revealed in our work is the presence of the Sdα antigen on a subset of the glycans, a sequence not reported in the earlier structural analysis (14). However, Noguchi and Nakano were required to remove sialic acid from the acidic glycans associated with mZP2 and mZP3 before obtaining structural analysis of the core sequences (14). These investigators reported the presence of terminal GalNAcβ1-Galβ1-4GlcNAc (terminal GalNAc linkage not defined) on 4% of the terminal sequences associated with mZP3 (14). This nonreducing trisaccharide sequence...
would be the predicted product resulting from desialylation of the Sda antigen. In addition, the current analysis has provided strong biophysical evidence for the Sda antigen containing the less common N-glycolyneuraminic acid derivative. This findings highlight the importance of performing structural analysis on intact carbohydrate structures rather than after specific glycosidase treatments.

The proposed structures for the O-linked oligosaccharides are shown in Fig. 8. Like the N-linked oligosaccharides, O-glycans of the murine ZP are very heterogeneous and carry the same range of terminal structures as the complex-type N-glycans. The majority of the glycans are core 2 structures (Gal[1–4]GlcNAc[1–6]Gal[1–3]GlcNAc) terminated with one or two N-acetyl or N-glycolyneuraminic acid residues. A notable feature of the O-glycan population is the presence of components containing the Sda antigen and its N-glycolyneuraminic acid-containing counterpart.

The results of the present structural analysis are also consistent with lectin binding studies previously performed on mZP. A potential carbohydrate ligand was identified for virtually all the lectins that have been shown to bind to this extracellular matrix (Table VII) (17–19). For example, oligosaccharides terminated with the Sda antigen have previously been shown to bind to Dolichos biflorus agglutinin (20). In addition, lectins that did not bind to ZP (e.g. Ulex europaeus) do not have a corresponding ligand expressed in the mZP glycans (17).

Another very significant observation is that the lectins from D. biflorus and Griffonia simplicifolia do not bind to the outer surface of the mZP, inferring that oligosaccharides terminated with either the Sda antigen or α-1,3-linked Gal are not expressed in this region of initial sperm contact (17–19). Skutelsky et al. (17) also report that Ricinus communis agglutinin-I, a lectin specific for terminal β-linked Gal, was specifically bound to the outer surface of the mZP but not the inner ZP (17). Therefore the outer surface of the mZP could be differentially glycosylated from the inner ZP. Another possibility is that murine ZP glycans are modified before fertilization. In the human, there is evidence suggesting that a sperm surface-associated neuraminidase exists that is activated by a specific uterine glycoprotein (21). Removal of sialic acid from the surface of the human ZP results in a 3-fold increase in sperm binding to this matrix in the hemizona assay system (22). By comparison, neuraminidase treatment of murine eggs results in only a 30–40% increase in binding (23). Therefore it would not be unreasonable for murine ZP glycans to undergo modification in the interrum between ovulation and fertilization that would promote initial sperm-egg binding.

Structural analysis of the oligosaccharides associated with porcine ZP has been carried out in several laboratories. Groups led by Yurewicz (24), Kobata (25, 26), Vliegenthart (27, 28), Nakano (14, 29–37), and Takasaki (38) have analyzed the carbohydrate sequences associated with this matrix. The majority of the N- and O-linked oligosaccharides express extended polylactosamine chains of heterogeneous size that are highly substituted with sulfate groups and sialic acid at their terminal ends. Both N-linked (30) and O-linked oligosaccharides (24) have been proposed to act as ligands for sperm binding. The exact oligosaccharide sequences that mediate binding have not been determined unequivocally. However, it is certainly apparent from this study and from previous analyses (13, 14) that the carbohydrate chains linked to porcine and murine ZP glycoproteins are very different. Therefore some specificity of binding may be mediated by differential glycosylation.

Evidence obtained in many studies indicates that the carbohydrate sequences associated with mZP play significant functional roles in murine gamete binding and fertilization. Wassarman and co-workers (3, 4) provide the first evidence that initial murine gamete binding involves carbohydrate recognition. This finding led to the development of several different hypothetical models to explain the molecular basis for this interaction.

Bleil and Wassarman (7) originally proposed that initial binding was mediated by ZP3-associated O-glycans terminated with Galα1–3Gal sequences. Our study provides further evidence for the presence of these sequences in mZP. However, transgenic mice lacking the α-1–3-galactosyltransferase retain their fertility (39) and display normal gamete binding (40). Therefore it is doubtful that this terminal sequence is obligatory for binding.

Wassarman and co-workers have also isolated biologically active recombinant mZP3 from murine F9 embryonal carcinoma cells (41) and prepared specific mutant ZP3 proteins that lack biological activity (42). This group has recently suggested that the presentation of vicinal O-linked oligosaccharides at Ser-332 and Ser-334 is responsible for the binding interaction (10). The data we have obtained on the structures of the O-linked oligosaccharides will facilitate future experiments addressing this important issue of vicinal presentation. However, it is also necessary to recognize that the genetic manipulations in F9 embryonal carcinoma cells may engender unexpected structural changes that do not reflect the native state of glyco-
sylation of mZP glycoproteins.

Shur and co-workers (6, 43) propose that a sperm-specific \(\beta_1\text{--}4\text{-galactosyltransferase}\) mediates binding by recognizing mZP3-associated \(\text{O}\)-glycans terminated with GlcNAc. Our current results indicate the presence of minor amounts of GlcNAc-terminated N-glycans, whereas terminal GlcNAc was not detectable in the O-glycans. Terminal GalNAc was also found, but it is presented in the context of Sd\(^a\) antigen. In addition, the lack of \(D.\ biflorus\) agglutinin binding to the mZP surface (Table VII) suggests that this \(\beta\)-galactosyltransferase does not have immediate access to Sd\(^a\)-terminated oligosaccharides. Finally, studies performed by Lu and Shur (41) indicate that transgenic mice lacking this specific \(\beta_1\text{--}4\text{-galactosyltransferase}\) display 3–4-fold higher binding to eggs compared with control mice (44). Therefore, based on all available data, it is very unlikely that this galactosyltransferase plays any significant role in the initial binding process.

Johnston et al. (9) recently proposed that glycans terminated with either Gal\(\beta_1\text{--}3\text{Gal}\beta_1\text{--}4\text{[Fuc}\alpha_1\text{--}3]\text{GlcNAc}\) or Lewis\(^x\)-active sequences (Gal\(\beta_1\text{--}4\text{[Fuc}\alpha_1\text{--}3]\text{GlcNAc}\beta_1\text{--}4\text{GlcNAc}\)) could act as high affinity ligands that mediate initial sperm-egg binding. In addition, these investigators also report that the trisaccharide Gal\(\beta_1\text{--}4\text{GlcNAc}\beta_1\text{--}4\text{GlcNAc}\) maximally inhibited sperm-egg binding by 47\% at the highest tested concentration (72 \(\mu\text{M}\)). A synergistic effect was observed when Gal\(\alpha_1\text{--}3\text{Gal}\beta_1\text{--}4\text{[Fuc}\alpha_1\text{--}3]\text{GlcNAc}\) and Gal\(\beta_1\text{--}4\text{GlcNAc}\beta_1\text{--}4\text{GlcNAc}\) were included together. Based upon this evidence, these investigators proposed that Gal\(\alpha_1\text{--}3\text{Gal}\beta_1\text{--}4\text{[Fuc}\alpha_1\text{--}3]\text{GlcNAc}\) could be binding to sp56, a sperm protein previously implicated in binding to ZP3 (45). They also suggested that another sperm-associated calcium-dependent lectin that binds terminal Gal\(\beta_1\text{--}4\text{GlcNAc}\) sequences (46, 47) interacted with low affinity ligands like Gal\(\beta_1\text{--}4\text{GlcNAc}\beta_1\text{--}4\text{GlcNAc}\) (9).

Our work indicates that glycans carrying terminal Gal\(\alpha_1\text{--}3\text{Gal}\beta_1\text{--}4\text{[Fuc}\alpha_1\text{--}3]\text{GlcNAc}\) or Gal\(\beta_1\text{--}4\text{[Fuc}\alpha_1\text{--}3]\text{GlcNAc}\) are not expressed in murine ZP, consistent with the results of a previous study (14). We have found that fucose is attached to N-glycans but apparently only via \(\alpha_1\text{--}6\) linkage to the chitobiose core. No evidence for the fucosylation of O-glycans was found. In addition, antibodies directed against the Lewis\(^x\) sequence did not bind to eggs obtained from transgenic mice lacking \(\alpha\)-galactosyltransferase.\(^3\) Therefore, although the inhibition mediated by the small fucosylated oligosaccharides is genuinely interesting, it is very unlikely that such ligands are physiologically relevant in the murine gamete binding system.

Tulsiani and co-workers (8) suggest that initial sperm-egg binding is mediated via recognition of terminal \(\alpha\)-mannosyl residues by a specific sperm surface \(\alpha\)-mannosidase. High mannos-type glycans are located in mZP, based on the results of the present study. The physiological relationship between this \(\alpha\)-mannosidase and sperm adhesion needs to be more fully investigated.

It is also significant that murine sperm will bind to surfaces other than their homologous eggs. For example, murine sperm undergo rapid and very tight binding to rabbit erythrocytes (48, 49). Electron microscopy of sperm-erythrocyte interaction indicates that this binding is between the plasma membranes of

\(^3\) A. Thall, personal communication.
**TABLE V**
Assignment of FAB-MS peaks observed for the molecular and fragment ions of the permethylated O-glycans released from murine zona pellucida

| Signal | Assignment |
|--------|------------|
| m/z    |            |
| 793    | Loss of methanol from m/z 825 |
| 825    | NeuAcHexHexNAc+ |
| 855    | NeuGcHexHexNAc- |
| 873    | NeuAcHexHexNAcitol + H+ |
| 895    | NeuAcHexHexNAcitol + Na+ |
| 903    | NeuAcHexHexNAcitol + H+ |
| 925    | NeuAcHexHexNAcitol + Na+ |
| 983    | HexHexNAcHexNAcitol + Na+ |
| 1070   | NeuAcHexHexNAc+ |
| 1100   | NeuGcHexHexNAc- |
| 1140   | NeuAcHexHexNAcHexNAcitol + Na+ |
| 1165   | HexHexNAcHexNAcitol + H+ |
| 1187   | HexHexNAcHexNAcitol + Na+ |
| 1234   | NeuAcHexHexHexNAcitol + H+ |
| 1256   | NeuAcHexHexHexNAcitol + Na+ |
| 1264   | NeuAcNeuGcHexHexNAcitol + H+ |
| 1286   | NeuAcNeuGcHexHexNAcitol + Na+ |
| 1294   | NeuGcHexHexNAcitol + H+ |
| 1316   | NeuGcHexHexNAcitol + Na+ |
| 1344   | NeuAcHexHexNAcHexNAcitol + Na+ |
| 1374   | NeuGcHexHexHexNAcHexNAcitol + Na+ |
| 1432   | HexHexNAcHexNAcHexNAcitol + Na+ |
| 1531   | NeuAcNeuGcHexHexNAcHexNAcitol + Na+ |
| 1549   | NeuAcHexHexHexNAcHexNAcitol + Na+ |
| 1590   | NeuAcHexHexHexNAcHexNAcitol + Na+ |
| 1595   | HexHexNAcHexNAcHexNAcitol + Na+ |
| 1620   | NeuGcHexHexHexNAcHexNAcitol + Na+ |
| 1800   | HexHexNAcHexNAcHexNAcitol + Na+ |
| 1824   | NeuGcHexHexHexNAcHexNAcitol + Na+ |
| 1865   | NeuGcHexHexHexNAcHexNAcitol + Na+ |

**TABLE VI**
GC-MS analysis of the partially methylated alditol acetates obtained from the O-glycan preparation of murine zona pellucida

| Elution time (min) | Characteristic fragment ions | Assignment |
|-------------------|-----------------------------|------------|
| 18.83             | 102, 118, 129, 145, 161, 162, 205 | Terminal mannose |
| 19.13             | 102, 118, 129, 145, 161, 162, 205 | Terminal galactose |
| 20.05             | 129, 139, 161, 190             | 2-Linked mannose |
| 20.38             | 118, 129, 161, 234             | 3-Linked galactose |
| 21.07             | 118, 129, 143, 185, 203, 232, 305, 35 | 3,4-Linked galactose |
| 21.93             | 118, 129, 189, 234             | 3,6-Linked mannose |
| 23.40             | 130, 246, 318                 | 3,6-Linked GalNAcitol |
| 23.89             | 117, 159, 233                 | 4-Linked GlcNAc |

the two cell types (50). Periodate oxidation of rabbit erythrocytes (10 mM NaIO4, 0.15 M NaCl, 1 h, 23 °C) results in a >98% reduction in binding. Thus sperm binding to rabbit erythrocytes (49) is both carbohydrate-dependent and requires acroseome-intact sperm, precisely the same requirements associated with murine sperm-ZP binding. This result suggests that both sperm-erythrocyte binding and initial sperm-egg binding involve specific lectins associated with the plasma membrane of murine sperm.

Previous studies indicate that the O-glycans associated with mZP3 are responsible for mediating adhesion (4, 43). Based on the current data, the carbohydrate sequence common to mZP O-glycans and rabbit erythrocytes is the β1–6-linked N-acetyllactosamine (Galβ1–4GlcNAcβ1–6) sequence. Unlike red blood cells from other species, rabbit erythrocytes profusely express branches of this type on polyvalosamine sequences associated with their glycolipids (51) and possibly their N-glycans but not on their O-linked oligosaccharides (52). Virtually all of the rabbit erythrocyte glycolipids are also terminated with Galα1–3Gal sequences, but murine sperm bind to rabbit erythrocytes even after exhaustive digestion with α-galactosidase (49). As stated beforehand, murine sperm-egg binding is also not dependent upon the presence of terminal α1–3-linked galactose (39, 40). If the mutation studies involving recombinant mZP3 are indeed correct (10), then vicinal presentation of core 2 O-glycans (each presenting terminal β1–6-linked N-acetyllactosamine units) at Ser-332 and Ser-334 could be responsible for mediating the initial binding interaction. This hypothesis must be thoroughly investigated.

Cahova and Draber (12) report that an IgM monoclonal antibody (Tec-02) directed against terminal GalNAcβ1–4Gal binds to murine ZP and inhibits fertilization in a concentration-dependent manner. However, Tec-02 did not interfere with the initial sperm-ZP binding but did inhibit secondary binding that occurs after the induction of the acrosome reaction. Cahova and Draber (12) therefore proposed that glycans terminated with GalNAcβ1–4Gal sequences mediate secondary gamete binding in the mouse. In our study, we have clearly shown that the glycans bearing terminal GalNAcβ1–4Gal sequences are present but are specifically associated with the Sd<sup>a</sup> antigen. Thus the structural data in combination with the antibody inhibition data indicates that the secondary binding interaction may be dependent upon the presence of the sequences terminated with the Sd<sup>a</sup> antigen. Again, this hypothesis needs to be thoroughly tested (12).

4. M. Patankar and G. Clark, unpublished observation.
**TABLE VII**

Lectin binding to murine zona pellucida reported in previous studies

| Species of origin | Location of binding | Potential ligands detected in the current study |
|-------------------|---------------------|-----------------------------------------------|
| *Triticum vulgaris* | E                   | Sialylated glycans, polylactosamine sequences (63–65) |
| *Triticum vulgaris* (succinylated) | E                   | Polylactosamine sequences (66) |
| *Arachis hypogaea* | E                   | Galβ1–3GalNAc sequences (67) |
| *D. biflorus* | I                   | Sdα determinant (20) |
| *Maackia amurensis* | I                   | Terminal Neu5Acα2–3Gal (68) |
| *G. simplicifolia* | I                   | Terminal Galα1–3Gal sequences (69) |
| *Concanavalin enisformis* | E                   | N-Linked glycans with fucose linked α1–6 to the core GlcNAc covalently bound to Asn (72) |
| *Helix pomatia* | I                   | Sdα determinant (71) |
| *Aleuria aurantia* | E                   | N-Linked glycans with fucose linked α1–6 to the core GlcNAc covalently bound to Asn (72) |
| *Fucus serratus* | E                   | Tri- and tetraantennary complex N-linked glycans with α1–6- or α1–4-linked N-acetyllactosamine units; polylactosamine sequences (73) |
| *R. communis* | O                   | Terminal Galβ1–4GlcNAc (74, 75) |
| *Limmus flavus* | E                   | Neu5Acα2–3Galβ1–3GalNAc (76) |

*a* E, binding to entire ZP; I, binding to inner ZP but not to outer surface; O, binding to outer surface only.  
*b* Entire ZP (18); outer surface (17).

Another very significant recent observation involves the induction of the acrosome reaction. Bovine serum albumin (BSA)-based neoglycoproteins terminated at multiple positions with a single monosaccharide (GalNAc-BSA, GlcNAc-BSA, or Man-BSA) induce the acrosome reaction in mice (53). The current study indicates that oligosaccharides with GalNAc, GlcNAc, or Man at the nonreducing ends are also present in the mZP. More study will be required to determine if mZP glycans participate in mediating signal transduction events during murine fertilization.

Initial human sperm binding to homologous zona pellucida is inhibited at low concentrations by glycodelin-A (54), a uterine glycoprotein with potent immunosuppressive activities (55). Oligosaccharides of this type have previously been shown to be potent inhibitors of selectin-mediated adhesions (57). Based upon these both functional and structural studies, we suggested the possibility that similar carbohydrate sequences are utilized during immune and gamete recognition events in the human (56). It is therefore significant to note in this context that the expression of core 2 O-glycan sequences and the Sdα antigen are significantly up-regulated on interleukin-2-stimulated T lymphocytes (58, 59) and cytotoxic T lymphocytes (60–62) in this context that the expression of core 2 O-glycan sequences and the Sdα antigen are significantly up-regulated on interleukin-2-stimulated T lymphocytes (58, 59) and cytotoxic T lymphocytes (60–62) in the mouse, respectively. Another major goal in the future is to determine if the shared expression of these carbohydrate sequences on murine ZP and activated lymphocytes has any relevant physiological implications in the mouse.

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