Gender-specific Glycosylation of Human Glycodelin Affects Its Contraceptive Activity*

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We have recently demonstrated that a human amniotic fluid-derived glycoprotein, glycodelin-A (GdA previously known as PP14 or PAEP), potently inhibits gamete binding in an established sperm-egg binding system and expresses immunosuppressive activities directed against a variety of different immune cell types. GdA has high mannose-, hybrid-, and complex-type bi-antennary oligosaccharides including structures with fucosylated or sialylated N,N'-diacetyllactosediamine (GalNAcβ1-4GlcNAc) sequences, which are rare in other human glycoproteins. We now report the characterization of glycodelin-S (GdS). This is a human seminal plasma glycoprotein that is immunologically indistinguishable from GdA, but unlike the latter, does not inhibit human sperm-zona pellucida binding under hemizona assay conditions. Analysis of the N-glycans of GdS by mass spectrometry revealed that all glycoforms of GdS are different from those of GdA. GdS glycans are unusually fucose-rich, and the complex-type structures are biantennary glycans with Lewisα (Galβ1-4(Fucα1-3)GlcNAc) and Lewisβ (Fucα1-2Galβ1-4(Fucα1-3)GlcNAc) antennae. It is probable that these highly fucosylated epitopes contribute to the immunosuppressive activity of human seminal plasma and to the low immunogenicity of sperm. This study provides the first evidence for gender-specific glycosylation that may serve to regulate key processes involved in human reproduction.

Evidence obtained from diverse species in both the plant and animal kingdoms indicates that appropriate recognition of surface carbohydrates is required for initial gamete binding (1–3). We have utilized the hemizona assay system to determine the potential inhibitory effects of different glycoconjugates on human gamete binding (4–6). We recently showed that PP14 (human placental protein 14) (7); also referred to as PAEP (progesterone-associated endometrial protein) (8) inhibits human sperm-zona pellucida binding under hemizona assay conditions in an immediate and dose-dependent fashion (9). This glycoprotein also suppresses responses by a variety of different immune effector cell types (10–13). We have characterized the glycoforms of the immunotic fluid form of PP14 and have introduced new nomenclature for this family of proteins to reflect their varied tissue origin and the fact that synthesis does not occur in the placenta. Thus, the amniotic fluid form of the glycoprotein that was shown to inhibit sperm-zona pellucida binding has been designated glycodelin-A (GdA).1 During the menstrual cycle, GdA is not expressed in the proliferative endometrium, but increases significantly from the 4th post-ovulatory day, peaking around the 12th day (14). Thus, GdA is expressed at a minimum during the peri-ovulatory period of the cycle. Following implantation of the embryo, GdA synthesis is in the decidua is induced to very high levels (4–10% of total protein) (15). GdA is also secreted into the amniotic fluid in concentrations sufficient to manifest immunosuppressive effects in vitro, increasing up to 232 μg/ml by mid-trimester (15). Therefore, the human embryo/fetus is surrounded by and bathed in a glycoprotein with potent immunosuppressive activities. The temporal and spatial expression of GdA in the female reproductive tract suggests that this glycoprotein could mediate contraceptive effects during specific stages of the menstrual cycle and suppress the maternal immune response during pregnancy.

Immunological and molecular biological analyses have suggested that glycodelin is expressed in tissues other than the endometrium (16, 17), and significantly, a glycoprotein that cross-reacted with antibodies to GdA was detected in human seminal plasma over a decade ago (16). The role of this seminal plasma form of glycodelin (GdS) remains unknown, but the expression of a potential contraceptive glycoprotein in the seminal plasma of the human male does not make physiological sense. To address this anomaly, we have initiated comparative structure/function studies of GdA and GdS. GdA, whose protein sequence has been determined by cDNA sequencing (18), is a 162-amino acid glycoprotein that gives a 28-kDa band upon SDS gel electrophoresis (16). GdS shows a major band at 27 kDa and a minor band at 30 kDa when analyzed in the same manner (19). Despite this, there is complete identity in both

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§The abbreviations used are: GdA, glycodelin-A; GdS, glycodelin-S; FAB-MS, fast atom bombardment mass spectrometry; LC-ES-MS, liquid chromatography-electrospray mass spectrometry; GC-MS, gas chromatography-mass spectrometry; PNGase F, peptide N-glycosidase F.
immunodiffusion and tandem-crossed electrophoresis studies, which suggests that GdS and GdA are very closely related proteins (16, 19). Furthermore, recent analysis of GdS indicates that the protein component of GdS is very similar to that of GdA based upon peptide mapping, N-terminal sequencing, and immune binding studies using specific polyclonal and monoclonal antibodies (19). Lectin binding and endo/exoglycosidase digestion studies (19) suggested that the observed differences in size and charge between GdS and GdA could be due to differential glycosylation, implying that human male and female reproductive tissues are capable of glycosylating the protein differently.

In previous work, we have utilized powerful FAB-MS and LC-ES-MS techniques to examine the glycosylation of GdA (20). GdA has three consensus sites for N-linked glycosylation, and we have shown that the first two of these sites are glycosylated with defined and substantially different heterogeneous populations of glycans. Among the glycans expressed on this glycoprotein are complex-type biantennary structures whose antennae are composed of fucosylated or sialylated N,N,N-di-acetyllactosediaamine (GalNAcβ1–4GlcNAc) sequences, which are rare in other human glycoproteins. In this work, we have employed similar strategies to define the N-glycan structures in GdS and their sites of attachment. We now report that the N-linked glycans on GdS are remarkably different from those on GdA. More important, none of the N,N,N-di-acetyllactosedia- mine structures in GdA is present in GdS, and the latter carries abundant Lewis epitopes, which are absent in GdA. This study provides the first evidence for gender-specific glycosylation that may serve to regulate key processes involved in human reproduction.

EXPERIMENTAL PROCEDURES

Purification of Glycodelin-S—GdS was purified from seminal plasma using antibody affinity chromatography as described for the purification of GdA (21), followed by HiTrap Q anion-exchange chromatography. Full details of the purification of GdS and assessment of its purity are given in Ref. 19.

Analysis of the Effects of GdS on Human Sperm Binding to Zona Pellucida—The hemizona assay and the assignment of the hemizona index were carried out exactly as described previously (9). There was no significant effect of GdA or GdS on sperm motility parameters or viability under the assay conditions.

Tryptic Digestion—Glycodelin-S (230 μg) was dialyzed against 4 x 2.0 liters of 50 mM ammonium bicarbonate, pH 8.5, at 4 °C, with each change occurring after stirring gently for 12 h. The sample was then lyophilized prior to tryptic digestion. Digestion was performed in 50 mM ammonium bicarbonate, pH 8.5, with bovine trypsin (EC 3.4.21.4, Sigma) for 3 h at 37 °C using an enzyme/substrate ratio of 1:50 (w/w). The reaction was terminated by placing in boiling water for 2 min, followed by lyophilization.

Staphylococcus aureus V8 Digestion—Glycodelin-S peptides (125 μg), produced by sequential digestion of glycodelin-S with trypsin and PNGase F (EC 3.2.2.18; Boehringer Mannheim), were digested in 100 mM ammonium bicarbonate, pH 7.9, and digested with S. aureus V8 protease (staphylococcal serine proteinase, EC 3.4.21.19; Boehringer Mannheim) for 2.25 h at 40 °C using an enzyme/substrate ratio of 1:30 (w/w). The reaction was terminated by placing in boiling water for 2 min, followed by lyophilization.

Preparation of CNBr Fragments—Glycodelin-S (140 μg) was digested again with 4 x 2.0 liters of 50 mM ammonium bicarbonate, pH 8.5, at 4 °C for 48 h and then lyophilized as described above. The lyophilized sample was dissolved in 100 μl of a solution of CNBr in 70% formic acid and left in the dark for 12 h. The reaction was terminated by drying in vacuo. An additional 25 μl of water was added, and the sample was again dried in vacuo. The sample was then dissolved in 25 μl of water and 2.5 μl of triethylamine and reduced using a 4-fold molar excess of dithiothreitol over the number of S-S bridges. The sample was briefly flushed under nitrogen, and the reaction was allowed to proceed for 30 min at 37 °C, after which it was dried in vacuo. An additional 25 μl of water was added, and the sample was again dried in vacuo.

PNGase F Digestion—PNGase F digestion was carried out on tryptic digests of glycodelin-S in 50 mM ammonium bicarbonate buffer, pH 8.5, for 16 h at 37 °C using 0.6 unit of the enzyme. The reaction was terminated by lyophilization, and the released peptides were separated from the carbohydrates on Sep-Pak C8 cartridges (Waters Associates) as described (22), with the carbohydrates eluting in the void volume.

Sequential Exoglycosidase Digestions—These were performed on N-glycans released from glycodelin-S (130 μg) by sequential digestion with bovine trypsin and PNGase F with the carbohydrates isolated on Sep-Pak cartridges: α-mannosidase (from jack bean, EC 3.2.1.24; Boehringer Mannheim), 0.5 unit in 200 μl of 50 mM ammonium acetate buffer, pH 4.5, for a total of 48 h, with a fresh aliquot of enzyme being added after each 12 h; β-galactosidase (from bovine testes, EC 3.2.1.23; Boehringer Mannheim), 10 milliunits in 100 μl of 50 mM sodium/citrate/phosphate buffer, pH 4.6, for 12 h and then for another 12 h with another aliquot of fresh enzyme; and N-acetyl-β-D-glucosaminidase (from bovine kidney, EC 3.2.1.30; Boehringer Mannheim), 0.2 unit in 100 μl of sodium/citrate/phosphate buffer, pH 4.6, initially for 18 h and then for another 18 h with another aliquot of fresh enzyme. All enzyme digestions were incubated at 37 °C and terminated by boiling for 3 min before lyophilization. An appropriate aliquot was taken after each digestion and permethylated for FAB-MS analysis.

Chemical Removal of Fucose—Fucose residues were removed from the released N-glycans of glycodelin-S using the method of Reason et al. (23), with the exception of an increased incubation time. Briefly, the isolated N-glycans were dissolved in 200 μl of 0.05 M methanolic HCl (prepared by adding 37% hydrochloric acid to methanol). The sample was incubated at 65 °C for 14 h, after which the reaction was terminated by drying under nitrogen. The sample was then washed with 2 x 200 μl of water, being dried under nitrogen after each addition.

Methanolysis—The reagent was prepared by bubbling dry HCl gas into methanol until hot to the touch. After cooling, a 20-μl aliquot of this reagent was added to the permethylated sample (derived from 100 μg of glycodelin-S), which was then heated for 5 min at 40 °C. A 1-μl aliquot was removed for FAB-MS analysis, and the remainder of the sample was dried under nitrogen.

Chemical Derivatization for FAB-MS and GC-MS Analysis—Permethylated using the sodium hydroxide procedure was performed as described previously (22). Briefly, sodium hydroxide pellets were crushed with dimethyl sulfoxide to form a slurry. An aliquot of this slurry was added to the dried carbohydrate preparation along with methyl iodide, and the reaction mixture was shaken for 10 min, after which the reaction was terminated by addition of water. Chloroform was then added to extract the permethylated carbohydrates. The chloroform layer was subsequently washed four times with water to remove any impurities. Partially methylated alditol acetates were prepared from permethylated samples for GC-MS linkage analysis as described (24). Briefly, the permethylated samples were hydrolyzed with 2 M trifluoroacetic acid for 2 h at 121 °C, reduced with 10 mg/ml sodium borodeuteride in 2 M aqueous ammonia at room temperature for 2 h, and then acetylated with acetic anhydride at 100 °C for 1 h.

Methyl Esterification of Peptides—Methanolic HCl was prepared by bubbling HCl gas through methanol until hot to the touch. The cooled solution was then added to the dried peptides, and the reaction was allowed to proceed for 30 min, after which it was terminated by drying under nitrogen (25).

GC-MS Analysis—GC-MS analysis was carried out on a Fisons Instruments MD800 apparatus fitted with a DB-5 fused silica capillary column (30 m x 0.32 mm internal diameter; J & W Scientific). The partially methylated alditol acetates were dissolved in hexanes prior to
on-column injection at 65 °C. The GC oven was held at 65 °C for 1 min before being increased to 290 °C at a rate of 8 °C/min.

**FAB-MS Analysis**—FAB mass spectra were acquired using a Fisons Instruments VG ZAB-2SE-2FPD mass spectrometer fitted with a cesium gun operated at 30 kV. Data acquisition and processing were performed using VG Analytical Opus software. Dried peptide samples were dissolved in 5% acetic acid, and dried permethylated carbohydrates were dissolved in methanol prior to loading. The matrix used was thioglycerol.

**LC-ES-MS Analysis**—LC-ES-MS analysis of CNBr-digested glycode- lin-S was performed using an on-line microbore reverse-phase high performance liquid chromatography system (Brownlee C18 Aquapore column) coupled to a VG BioQ triple quadrupole electrospray mass spectrometer. The sample was dissolved in 0.1% trifluoroacetic acid (solvent system A) for injection on the column. The column was held for 5 min at 0% solvent system B (90% acetonitrile in 0.1% trifluoroacetic acid), followed by an increase to 100% solvent system B over 90 min. The flow rate was 50 μl/min. After passage through an ultraviolet spectrophotometer with a microflow cell, with monitoring at 214 nm, the eluant was mixed with a 1:1 mixture of 1-propanol and 2-methoxyethanol prior to stream splitting 1:9 for ES-MS analysis and collection, respectively.

**RESULTS**

**GdS as an Inhibitor of Sperm-Zona Pellucida Binding**—GdS was tested in the hemizona assay system to determine if it blocked human sperm-zona pellucida binding (9). Comparison of sperm binding to the human zona pellucida at three different concentrations of GdS and GdA indicated that only the endometrially derived GdA was capable of blocking this interaction (Fig. 1). At lower concentrations, GdS slightly stimulated binding. These findings indicate that, unlike GdA, GdS likely does not function as an inhibitor of human gamete binding in seminal plasma.

**Characterization of GdS N-Glycans**—Oligosaccharides were isolated from GdS and permethylated for FAB-MS analysis under the identical conditions utilized for the structural definition of GdA glycans (20). Analysis of GdS permethylated N-glycans suggested that the oligosaccharides either were of the high mannose type (Man$_5$−7GlcNAc$_2$; signals at m/z 1557.1, 1761.3, and 1655.5, respectively) or were highly fucosylated complex-type biantennary glycans (Hex$_5$−HexNAc$_4$).
Glycodelin-S-derived N-Glycans

Fuc1–5; signals at m/z 2222.2 (minor), 2396.1, 2570.3, 2745.2, and 2920.2, respectively) (Fig. 2a). Major A-type fragment ions were present at m/z 812.3 (Fuc2–Hex–HexNAc) and 638.2 (Fuc2–Hex–HexNAc+) (Fig. 2b). The data are summarized in Table I. The secondary cleavage ions at m/z 606.2 and 432.1 were shown by linked scanning experiments to be predominantly derived from m/z 812.3 and 638.2, respectively (data not shown). These data indicate that the majority of monofucosylated antennae giving the A-type ion at m/z 638.2 have the fucose linked at the 3-position of the HexNAc residue. Likewise, the difucosylated antennae have a 3-linked fucose. A minor portion of the m/z 606.2 signal was observed when the signal at m/z 638.2 was subjected to linked scanning, possibly suggesting that, in some monofucosylated antennae, HexNAc is not substituted by fucose.

The positions of substitution for all monosaccharides in the intact and defucosylated glycans were determined by GC-MS analysis of partially methylated alditol acetates (20). Defucosylation was carried out by hydrolysis in methanolic HCl for 14 h under established conditions (23). Linkage analysis indicated the presence of 2-linked galactose and 3,4-linked GlcNAc before defucosylation and their subsequent loss coupled with an increase of terminal galactose and 4-linked GlcNAc after removal of fucose. The data from linkage analysis are summarized in Table II. Sequential digestion of GdS glycans with β-galactosidase, β-hexosaminidase, and α-mannosidase under established conditions (20) coupled with FAB-MS monitoring of the digests confirmed that Gal and GlcNAc were β-linked and that mannos was α-linked, except for the β-linked mannos of the trimannosyl core. The linkage and exoglycosidase results are consistent with the FAB-MS fragmentation data of intact glycans and provide unambiguous evidence for the presence of both Lewisα-type (Galβ1–4Fuc1–3GlcNAc) and Lewisα-type (Fuc1–2Galβ1–4(Fuc1–3)GlcNAc) antennae.

To address site occupancy, GdS was digested with cyanogen bromide exactly as described previously for GdA (20). The resultant mixture of peptides was analyzed by on-line microbore LC-ES-MS using established elution conditions (20) (Fig. 3). The data allowed 96% of the molecule to be mapped, including the glycosylation sites. No substantial differences were observed when comparing the peptides maps generated from GdS and GdA in either the respective on-line LC-ES-MS or direct FAB-MS mapping data (Tables III and IV). Scans 127–147, upon transformation, gave signals at m/z 2050.5, 2212.1, and 2374.4 (Fig. 4). These signals are consistent, within the experimental mass accuracy, with masses computed for the peptide Ala25–Hse32–Hse33 (Table II and Fig. 2). Confirmation of this glycosylation was obtained by PNGase F digestion of the collected fractions representing this peak, followed by separation of peptides and N-glycans using a Sep-Pak cartridge and permethylation of the N-glycans present (20). FAB-MS analysis of the glycan fraction gave signals at m/z 1579.9, 1784.1, and 1988.6, consistent with the sodiated adducts of the predicted high mannose structures (Fig. 5 and Table V). FAB-MS analysis of the peptide fraction from the Sep-Pak purification showed a signal at m/z 856.2, corresponding to the mass of the sodiated adduct ion of peptide Ala25–Hse32, converted to an aspartic acid residue during the PNGase F digestion. Taken together, these data show that Asn28 is occupied exclusively by high mannose structures in GdS and that these glycans have between 5 and 7 mannose residues. In contrast, GdA has high mannose, hybrid, and complex structures at Asn28, and high mannose structures are restricted to 4 (very minor) and 5 mannose residues (20).

Combining scans 263–277 produced a complex series of peaks indicative of the presence of glycopeptide heterogeneity. Transformation of these spectra gave rise to signals of 11–12 kDa, consistent with glycosylation of the peptide Ala33–Hse117. Digestion of the relevant fractions with PNGase F and subsequent FAB-MS analysis of the isolated permethylated N-glycans revealed the presence of signals at m/z 2221.9 (minor), 2396.5 (minor), 2571.0, 2745.1, and 2919.6 (Fig. 6 and Table V), corresponding to the complex-type components with 1–5 fucose residues (Hex5–7–HexNAc5–Fuc1–5), respectively.

### Table I

Assignments of FAB-MS peaks observed for the molecular and fragment ions of the permethylated N-glycans released from glycodelin-S

| Signal (m/z) | Assignment |
|-------------|------------|
| High mass region |
| 1452.2 | Loss of methanol from Hex5–HexNAc+ |
| 1484.1 | Hex5–HexNAc+ |
| 1557.1 | Hex5–HexNAc+ + H+ |
| 1656.1 | Loss of methanol from Hex5–HexNAc+ |
| 1688.2 | Hex5–HexNAc+ |
| 1792.9 | Hex5–HexNAc5+ |
| 1761.3 | Hex5–HexNAc+ + H+ |
| 1933.4 | Hex5–HexNAc5+ |
| 1965.5 | Hex5–HexNAc+ + H+ |
| 2222.2 | Hex5–HexNAcFuc+ + H+ |
| 2396.1 | Hex5–HexNAcFuc+ + H+ |
| 2570.3 | Hex5–HexNAcFuc+ + H+ |
| 2745.2 | Hex5–HexNAcFuc+ + H+ |
| 2920.6 | Hex5–HexNAcFuc+ + H+ |
| Low mass region |
| 432.1 | Loss of methanol from 464.1 and fucose from 638.2 |
| 450.1 | β-Cleavage of fucose from 638.2 |
| 464.1 | Hex5–HexNAc+ |
| 606.2 | Loss of methanol from 638.2 |
| 624.2 | β-Cleavage of fucose from 812.3 |
| 638.2 | Fuc–Hex–HexNAc+ |
| 812.3 | Fuc2–Hex–HexNAc+ |
| 1280.5 | Hex5–HexNAc+ |

### Table II

GC-MS analysis of partially methylated alditol acetates obtained from the PNGase F-released N-glycans of glycodelin-S

| Elution time (min) | Characteristic fragment ions | Assignment |
|--------------------|-----------------------------|------------|
| 16.38              | 115, 118, 131, 162, 175     | Terminal Fuc |
| 17.98              | 102, 118, 129, 145, 161, 162, 205 | Terminal Man |
| 18.25*             | 102, 118, 129, 145, 161, 162, 205 | Terminal Gal |
| 19.21              | 129, 130, 161, 190          | 2-Linked Man |
| 19.50              | 129, 130, 161, 190          | 2-Linked Gal |
| 21.07              | 118, 129, 189, 234          | 3,6-Linked Man |
| 22.98*             | 117, 159, 233               | 4-Linked GlcNAc |
| 23.88*             | 117, 159, 346               | 3,4-Linked GlcNAc |
| 24.37              | 117, 159, 261               | 4,6-Linked GlcNAc |

* Signals increase after methanolysis.

* Signals disappear after methanolysis.
In a single ion-monitoring data-processing experiment screening the LC-ES-MS data of a tryptic digest of GdS in which the Asn63 and Asn85 sites exist on separate tryptic peptides, only the tryptic peptide bearing free Asn85 was observed via signals at m/z 1585.1 and m/z 1189.0, corresponding to the triple and quadruple charge states, respectively. These data suggest that Asn63 is fully glycosylated, whereas a principal proportion of Asn85 is not.

In a separate experiment, GdS was digested with trypsin and PNGase F sequentially (20). The N-glycans and peptides were separated using a Sep-Pak cartridge as before (20), and the peptides were analyzed by FAB-MS, both in the native state and after methyl esterification (25). The data showed, among other signals, the presence of a weak signal at m/z 923.2, consistent with the expected mass for the peptide Ile84–Glu91, produced during the series of digestions. The remainder of this digest mixture was subject to methyl esterification. FAB analysis of the methyl-esterified peptides revealed the presence of a signal at m/z 951.2, consistent with the expected addition of two methyl groups to the two carboxylic acid groups in the peptide Ile84–Glu91 (data not shown). A smaller signal was present at m/z 966.2, corresponding to the addition of three methyl groups to peptide Ile84–Glu91, the third esterifiable group presumably being produced by the conversion of asparagine to aspartic acid by the action of PNGase F. These data again may be interpreted as evidence of substoichiometric N-glycosylation at Asn85 of GdS or simply partial deamidation.

**TABLE III**

FAB-MS of tryptic peptides after PNGase F digestion of glycodelin-S

| Signal (m/z) | Sequence assignment | Comments |
|-------------|---------------------|---------|
| 4750.2      | Ile84–Arg24         | Contains consensus site for Asn85 glycosylation. |
| 2363.7      | Leu15–Lys36         | Only seen after PNGase F digestion; contains consensus site for Asn28 glycosylation. |
| 2296.8      | Val18–Arg50         |        |
| 1663.7      | Val135–Arg136       |        |
| 1200.5      | His146–Lys154       |        |

In a single ion-monitoring data-processing experiment screening the LC-ES-MS data of a tryptic digest of GdS in which the Asn63 and Asn85 sites exist on separate tryptic peptides, only the tryptic peptide bearing free Asn85 was observed via signals at m/z 1585.1 and m/z 1189.0, corresponding to the triple and quadruple charge states, respectively. These data suggest that Asn63 is fully glycosylated, whereas a principal proportion of Asn85 is not.

In a separate experiment, GdS was digested with trypsin and PNGase F sequentially (20). The N-glycans and peptides were separated using a Sep-Pak cartridge as before (20), and the peptides were analyzed by FAB-MS, both in the native state and after methyl esterification (25). The data showed, among other signals, the presence of a signal at m/z 4750.2, which, upon methyl esterification and FAB-MS analysis, was converted principally to a species at m/z 4833.9, corresponding to the mass computed for the tryptic peptide Ile84–Arg24. However, a smaller signal was detected at m/z 4848.0, containing one additional esterified carboxyl group. This would be consistent with either conversion of Asn to Asp in the PNGase F procedure and/or a minor amount of chemically induced deamidation expected during the esterification reaction. Therefore, the majority of Asn85 is not N-glycosylated, but the possibility exists that a small proportion of GdS may carry N-glycans at this position.

In another experiment, GdS was digested with trypsin and PNGase F as before (20). The released N-glycans were separated from peptides using a Sep-Pak cartridge (20). The 40% 1-propanol Sep-Pak fraction was subdigested with protease V8 from S. aureus. FAB mapping of a portion of the resulting peptide mixture revealed the presence of a weak signal at m/z 923.2, consistent with the expected mass for the peptide Ile84–Glu91, produced during the series of digestions. The remainder of this digest mixture was subject to methyl esterification. FAB analysis of the methyl-esterified peptides revealed the presence of a signal at m/z 951.2, consistent with the expected addition of two methyl groups to the two carboxylic acid groups in the peptide Ile84–Glu91 (data not shown). A smaller signal was present at m/z 966.2, corresponding to the addition of three methyl groups to peptide Ile84–Glu91, the third esterifiable group presumably being produced by the conversion of asparagine to aspartic acid by the action of PNGase F. These data again may be interpreted as evidence of substoichiometric N-glycosylation at Asn85 of GdS or simply partial deamidation.
during esterification. It is nonetheless clear from these results that the great majority of GdS molecules are glycosylated at the same positions as GdA.

**DISCUSSION**

The proposed structures for the oligosaccharides present on GdS are shown in Fig. 7. Two distinct populations were shown to exist. At Asn<sup>28</sup>, only high mannose structures were found, and at Asn<sup>63</sup>, only complex N-linked glycans were observed, demonstrating the highly site-specific nature of N-glycan biosynthesis in this molecule. More than 80% of the complex glycans at Asn<sup>63</sup> have between 3 and 5 fucose residues/glycan, and none of the glycans is sialylated, which is unusual for a human secreted glycoprotein. Each biantennary glycan is core-fucosylated, and the remaining fucoses are attached to N-acetyllactosamine antennae, resulting in Lewis<sup>x</sup> and Lewis<sup>y</sup> blood group epitopes. Linked scanning experiments suggest that a minor proportion of the monofucosylated antennae have the Fuc attached to Gal (i.e. Fucα1–2Gal) rather than GlcNAc, and this is corroborated by GdS showing some cross-reactivity with a monoclonal antibody specific for the H2 antigen (data not shown).

Our structural studies provide clear evidence that GdA and GdS are different sets of glycoforms of the same protein. All the complex-type glycans linked to GdS are of the common N-acetyllactosamine type (Galβ1–4GlcNAc). In contrast, rare (GalNAcβ1–4GlcNAc) and fucosylated (GalNAcβ1–4(Fucα1–3)GlcNAc) N,N<sup>-</sup>diacetyllactosediame sequences are abundant in GdA (20). Oligosaccharides with at least one fucosylated N,N<sup>-</sup>diacetyllactosediame sequence have been shown to be potent inhibitors of selectin-mediated adhesions (26). Both Lewis<sup>x</sup>- and Lewis<sup>y</sup>-active antennae associated with GdS react very weakly if at all with the known selectins (27). We have previously shown that human sperm-zona pellucida binding can be inhibited by selectin ligands, suggesting that human egg-binding protein(s) on sperm can interact with such oligosaccharides (28, 29). The lack of such glycans on GdS correlates with its inability to significantly inhibit initial sperm-zona pellucida binding in the hemizona assay.

**TABLE IV**

| Scan No. | Mass assigned | Peptide                  |
|----------|---------------|--------------------------|
| 127–147  | Heterogeneous<sup>a</sup> | Glycosylated Ala<sup>25</sup>–Hse<sup>32</sup> |
| 146–156  | 780.3         | Glu<sup>157</sup>–Phe<sup>162</sup>        |
| 192–199  | 2377.9        | Asp<sup>7</sup>–Hse<sup>22</sup>         |
| 198–205  | 1766.8        | Cys<sup>119</sup>–Hse<sup>133</sup>     |
| 232–239  | 2866.7        | Gln<sup>134</sup>–Hse<sup>156</sup>     |
| 240–243  | 2897.7        | Gln<sup>134</sup>–Hse<sup>156</sup> (formylated) |
| 263–277  | Heterogeneous<sup>a</sup> | Glycosylated Ala<sup>53</sup>–Hse<sup>177</sup> |

<sup>a</sup> See “Results.”

<sup>b</sup> C terminus.
60% of all GdA oligosaccharides, but no GdS-derived glycans, contain at least one antenna terminated with a -2,6-linked sialic acid linked to either Gal or GalNAc (NeuAc a 2–6Gal(NAc)) (20). Oligosaccharides with these terminal ends bind to CD22, the human B cell receptor glycoprotein (30). CD22 also binds to CD45, the leukocyte-specific receptor-linked phosphotyrosine phosphatase involved in T cell activation (31). Therefore, it is unlikely that GdS mediates its immunosuppressive or anti-inflammatory effects via the same carbohydrate sequences as GdA does.

The oligosaccharides associated with GdS are unusually rich in fucose. The Lewis x/y epitope, which is a difucosylated tetrasaccharide (Fig. 7), is a relatively rare sequence in humans. It was first identified in ovarian cyst mucin (32), was subsequently found on a variety of different tumors (33), and is generally considered to be a cancer-associated antigen. However, it is significant that Lewis x/y is known to occur on normal glycoproteins in seminal plasma. Thus, O-linked oligosaccharides expressing the Lewis x/y sequence have been derived from mucin-like proteins in this secretion (34, 35). Furthermore, γ-seminoprotein isolated from seminal plasma carries the Lewis x/y epitope on its N-linked glycans (36). However, the Lewis x/y-containing glycans in γ-seminoprotein are not the same as those in GdS because they are tri- and tetraantennary and also carry sialic acid on at least one antenna.

Although the precise function of GdS remains to be determined, it is interesting that analysis of the immunosuppressive activity of human seminal plasma in mixed lymphocyte culture indicates that a portion of this effect could be selectively removed by immunoprecipitation using an anti-PP14 antibody (37) (PP14 has now been renamed glycodelin to avoid confusion over misleading abbreviations (20)). We consider it probable that GdS manifests its immunosuppressive effects through its abundant Lewis x/y sequences. It is possible that the immunosuppressive properties of the Lewis x/y epitopes in GdS contribute to the low immunogenicity of sperm in women in spite of
their frequent exposure to antigens on the spermatozoa and in seminal plasma. It is notable that the Lewisx epitope has been shown to stimulate murine B lymphocytes to proliferate and produce factors implicated in the shift from a Th-1 to Th-2 response (38). It has also been demonstrated that the percentage of circulating human CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes bearing the Lewisy sequence increases from 3–5 to 20–25% in human immunodeficiency virus-infected individuals (39). Interestingly, the Lewisy sequence has also been implicated in programmed cell death (33, 40, 41).

In conclusion, the results presented in this study indicate that the tissue-specific glycosylation machinery found in the human reproductive tract, which results in novel gender-specific glycosylation, may play an essential role in regulating the biological activities of a key functional glycoprotein involved in human reproduction.

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