Structural Analysis of the Oligosaccharides Derived from Glycodelin, a Human Glycoprotein with Potent Immunosuppressive and Contraceptive Activities*

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Glycodelin, also known as placental protein 14 (PP14) or progesterone-associated endometrial protein (PAEP), is a human glycoprotein with potent immunosuppressive and contraceptive activities. In this paper we report the first characterization of glycodelin-derived oligosaccharides. Using strategies based upon fast atom bombardment and electrospray mass spectrometry we have established that glycodelin is glycosylated at Asn-28 and Asn-63. The Asn-28 site carries high mannose, hybrid and complex-type structures, whereas the second site is exclusively occupied by complex-type glycans. The major non-reducing epitopes in the complex-type glycans are: Galβ1-4GlcNAc (IacNAc), GalNAcβ1-4GlcNAc (IacdiNAc), NeuAcα2-6Galβ1-4GlcNAc (sialylated IacNAc), NeuAcα2-6GalNAcβ1-4GlcNAc (sialylated IacdiNAc), Galβ1-4(Fucα1-3)GlcNAc (Lewisβ), and GalNAcβ1-4(Fucα1-3)GlcNAc (IacdiNAc analogue of Lewisβ). It is possible that the oligosaccharides bearing sialylated IacNAc or IacdiNAc antennae may manifest immunosuppressive effects by specifically blocking adhesive and activation-related events mediated by CD2, the human B cell associated receptor. Oligosaccharides with fucosylated IacdiNAc antennae have previously been shown to potently block selectin-mediated adhesions and may perform the same function in glycodelin. The potent inhibitory effect of glycodelin on initial human sperm-zona pellucida binding is consistent with our previous suggestion that this cell adhesion event requires a selectin-like adhesion process. This result also raises the possibility that a convergence between immune and gamete recognition processes may have occurred in the types of carbohydrate ligands recognized in the human.

PP14* (Bohn et al., 1982). PP14 was subsequently found to be synthesized not by the placenta but by the secretory and decidual tissues. PP14 was therefore also referred to as progesterone-associated endometrial protein or PAEP in accordance with its endometrial origin. More recent evidence indicates that PAEP is also synthesized by the hematopoietic tissues of the bone marrow (Kamarainen et al., 1994) and perhaps other tissues. Since the glycoprotein referred to as PP14 or PAEP is not of placental origin nor is it exclusively synthesized in the endometrium, previous designations may not truly reflect its diverse sites of synthesis or its function. Therefore in this paper, we have designated PP14/PAEP as "glycodelin" to eliminate confusion over these issues and to emphasize the unique nature of oligosaccharides in this glycoprotein.

The temporal and spatial expression of glycodelin in the reproductive organs of the human female is highly regulated. During the menstrual cycle, glycodelin is not expressed in the reproductive organs of the human female and may perform the same function in glycodelin. The potent inhibitory effect of glycodelin on initial human sperm-zona pellucida binding is consistent with our previous suggestion that this cell adhesion event requires a selectin-like adhesion process. This result also raises the possibility that a convergence between immune and gamete recognition processes may have occurred in the types of carbohydrate ligands recognized in the human.
Glycodelin-derived Oligosaccharides

EXPERIMENTAL PROCEDURES

Isolation of Glycodelin—Isolation and purification procedures were the same as those described elsewhere (Riittinen et al., 1991) using 140 ml of midtrimester amniotic fluid as starting material.

Tryptic Digestion—Glycodelin (250 μg) was dialyzed against 4 × 2.0 liters of 50 mM potassium bicarbonate, at 4°C for 48 h. After lyophilization, trypsin digestion was carried out as described (Dell et al., 1994).

Preparation of CNBr Fragments—Glycodelin (160 μg) was dialyzed against 2 liters of 50 mM potassium bicarbonate buffer, pH 8.5 at 4°C for 12 h, after which time it was dialyzed against 2 liters of water for another 12 h at 4°C and then lyophilized. 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 5 μl of water was added and the sample dried in vacuo. The sample was then dissolved in 25 μl of triethylamine, 2.5 μl of water and reduced using a 4-fold molar excess of diithiothreitol over the number of S-S bridges. The reaction was allowed to proceed for 30 min at 37°C, after which time it was dried in vacuo. An additional 5 μl of water was added and the sample dried in vacuo.

PNGase F Digestion—PNGase F (EC 3.2.2.18, Boehringer Mannheim) digestion was carried out on tryptic digests of glycodelin (250 μg) in ammonium bicarbonate buffer (50 mM, pH 8.4) for 16 h at 37°C using 0.6 unit of the enzyme. The reaction was terminated by lyophilization and the products were purified on C18-Sep-Pak (Waters Ltd.) as described (Dell et al., 1994).

Sequential Exoglycosidase Digestions—These were carried out on glycans released from 250 μg of glycodelin except for the α-mannosidase digest, where 80 μg was used. N-Acetyl-β-D-galactosaminidase (from bovine kidney, EC 3.2.1.30, Boehringer Mannheim): 0.2 unit in 100 μl of 50 mM sodium-citrate-phosphate buffer, pH 4.6, initially for 18 h and then for another 18 h with another aliquot of fresh enzyme. 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. α-1-fucosidase (from bovine kidney, EC 3.2.1.51, Boehringer Mannheim): 0.2 unit in 200 μl of 100 mM ammonium acetate buffer, pH 4.5–5.0, for 24 h; neuraminidase (from Vibrio cholerae, EC 3.2.1.18, Boehringer Mannheim): 25 milliunits in 100 μl of 50 mM ammonium acetate buffer, pH 5.5, for 24 h; 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, a fresh aliquot of enzyme being added after each 12 h. All enzyme digestions were incubated at 37°C and terminated by boiling for 3 min before lyophilization. For sequential enzyme digestions, an appropriate aliquot was taken after each digest and permethylated for FAB-MS analysis.

Methodology—The reagent was prepared by bubbling dry HCl gas into methanol as described (Dell et al., 1994). After cooling, a 20-μl aliquot of this reagent was added to the permethylated sample, which was then heated for 2 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—Permethylation using the sodium hydroxide procedure was performed as described (Dell et al., 1994). Partially methylated alditol acetates were
Assignments of FAB-MS peaks observed for the molecular and fragment ions of the permethylated N-glycans released from glycodelin. The signal at m/z 2090.7 is produced by A-type cleavage from the structure at m/z 2368.4.

| Signal | Assignment |
|--------|------------|
| 1557.3 | Hex$_6$HexNAc$_2$H$^+$ (major) |
| 1574.2 | Hex$_6$HexNAc$_2$NH$_2^+$ |
| 1579.2 | Hex$_6$HexNAc$_2$Na$^+$ |
| 1813.3 | NeuAHex$_2$HexNAc$_2$H$^+$ |
| 1959.4 | NeuAHex$_2$HexNAc$_2$H$^+$ |
| 2000.6 | NeuAHex$_2$HexNAc$_2$H$^+$ |
| 2017.5 | Hex$_2$HexNAc$_2$Fu$_3$H$^+$ |
| 2090.7 | NeuAcHex$_2$HexNAc$_2$ |
| 2164.3 | NeuAcHex$_2$HexNAc$_2$H$^+$ |
| 2205.6 | NeuAcHex$_2$HexNAc$_2$H$^+$ |
| 2220.2 | Hex$_2$HexNAc$_2$Fu$_3$H$^+$ |
| 2268.3 | NeuAcHex$_2$HexNAc$_2$H$^+$ (major) |
| 2368.4 | NeuAcHex$_2$HexNAc$_2$H$^+$ (major) |
| 2409.1 | NeuAcHex$_2$HexNAc$_2$H$^+$ |
| 2420.9 | NeuAcHex$_2$HexNAc$_2$Fu$_3$H$^+$ |
| 2437.1 | Hex$_2$HexNAc$_2$Fu$_3$H$^+$ (major) |
| 2450.2 | NeuAcHex$_2$HexNAc$_2$H$^+$ |
| 2478.2 | Hex$_2$HexNAc$_2$Fu$_3$H$^+$ |
| 2582.9 | NeuAcHex$_2$HexNAc$_2$Fu$_3$H$^+$ |
| 2624.2 | NeuAcHex$_2$HexNAc$_2$Fu$_3$H$^+$ (major) |
| 2685.2 | NeuAcHex$_2$HexNAc$_2$Fu$_3$H$^+$ |
| 2798.4 | NeuAcHex$_2$HexNAc$_2$Fu$_3$H$^+$ (major) |
| 2839.9 | NeuAcHex$_2$HexNAc$_2$Fu$_3$H$^+$ |
| 2944.2 | NeuAc$_2$Hex$_2$HexNAc$_2$Fu$_3$H$^+$ |
| 3189.8 | NeuAc$_2$Hex$_2$HexNAc$_2$Fu$_3$H$^+$ (major) |
| 3230.6 | NeuAc$_2$Hex$_2$HexNAc$_2$Fu$_3$H$^+$ |
| 3345.3 | NeuAc$_2$Hex$_2$HexNAc$_2$Fu$_3$H$^+$ (minor) |
| 3625.6 | NeuAc$_2$Hex$_2$HexNAc$_2$Fu$_3$H$^+$ (minor) |
| 3813.4 | NeuAc$_2$Hex$_2$HexNAc$_2$Fu$_3$H$^+$ (minor) |
| 4000.7 | NeuAc$_2$Hex$_2$HexNAc$_2$Fu$_3$H$^+$ (minor) |

### RESULTS

A unique feature of mass spectrometry, namely the ability to derive definitive structural information from mixtures (in contrast to other spectroscopies normally requiring pure samples for study), was recognized and exploited by us in specifically designed strategies for “mixture analysis” some years ago (Geddes et al., 1969; Morris et al., 1971; Morris et al., 1978). The masses of component peaks alone (even in the absence of...
FAB-MS of tryptic peptides after PNGase F digestion of glycodelin

| Signal | Sequence assignment | Comments |
|--------|---------------------|----------|
| m/z    |                     |          |
| 4750.9 | Ile-84–Arg-124      | Contains consensus site for Asn-85 glycosylation. |
| 2363.9 | Leu-15–Lys-36       | Only seen after PNGase F digestion. Contains consensus site for Asn-28 glycosylation. |
| 2296.5 | Val-41–Arg-60       |          |
| 1663.4 | Val-125–Arg-138     |          |
| 1200.3 | His-146–Lys-154     |          |
| 856.2  | Ala-139–Arg-145     |          |
| 842.2  | Glc-8–Lys-1        |          |
| 832.1  | Met-1–Lys-7         |          |
| 545.1  | Val-71–Lys-76       |          |

These strategies have been applied here to glycodelin to map the A-type fragment ions in Fig. 2 have compositions consistent with biantennary structures; (iv) minor fragment ions in Table III are indicative of low levels of poly- and hybrid types of complex structures present; (v) the Lac and Lac di antenna may be substituted with either sialic acid or fucose but not both; (vi) minor fragment ions of composition HexXHexNAc2 and HexXHexNAc3 are indicative of low levels of poly-N-acetylglactosamine.

FAB Mapping of Tryptic Peptides—FAB-MS was carried out on the tryptic digest before and after PNGase F digestion (Table II). The following conclusions may be drawn from these data. (i) The molecular ion for the non-glycosylated peptide spanning the consensus site at Asn-28 was observed only after PNGase F digestion and there was no evidence for the formation of its Asp-85 analogue after PNGase F digestion, indicating that Asn-85 is unlikely to be glycosylated; (ii) the tryptic peptide corresponding to the third consensus site at Asn-85 was observed prior to digestion and its mass was consistent with conversion of Asn to Asp during the digestion, consistent with Asn-28 being glycosylated; (iii) the tryptic peptide corresponding to the third consensus site was not observed in these experiments.

| Elution time | Characteristic fragment ions | Assignment |
|--------------|------------------------------|------------|
| min          |                              |            |
| 16.27        | 115, 118, 131, 162, 175      | Terminal fucose (major) |
| 17.87        | 102, 118, 129, 145, 161, 162, 205 | Terminal mannose (major) |
| 18.15        | 102, 118, 129, 145, 161, 162, 205 | Terminal galactose (major) |
| 19.10        | 129, 130, 161, 190           | 2-Linked mannose (major) |
| 19.20        | 118, 233                     | 4-Linked galactose (minor) |
| 19.32        | 118, 129, 161, 234           | 3-Linked mannose (minor) |
| 19.40        | 118, 129, 161, 234           | 3-Linked galactose (minor) |
| 19.54        | 99, 102, 118, 129, 162, 189, 233 | 6-Linked mannose (minor) |
| 19.97        | 99, 102, 118, 129, 162, 189, 233 | 6-Linked galactose (major) |
| 20.35        | 130, 190, 233                | 2,4-Linked mannose (minor) |
| 20.77        | 129, 130, 189, 190           | 2,6-Linked mannose (minor) |
| 20.95        | 118, 129, 189, 234           | 3,6-Linked mannose (minor) |
| 21.42        | 118, 333                     | 3,4,6-Linked mannose (minor) |
| 21.94        | 117, 159, 203, 205           | Terminal GlcNAc (minor) |
| 22.42        | 117, 159, 203, 205           | Terminal GlcNAc (major) |
| 22.89        | 117, 159, 233                | 3,4-Linked GlcNAc |
| 23.77        | 117, 159, 346                | 3,4-Linked GlcNAc |
| 23.92        | 117, 159, 203, 233           | 6-Linked GlcNAc |
| 24.27        | 117, 159, 261                | 4,6-Linked GlcNAc |
these data are as follows. (i) The majority of the complex glycans are biantennary but low levels of 2,4-Man and 2,6-Man suggest that minor tri- and/or tetraantennary structures are present; (ii) GalNAc, Gal, and Man are the major non-reducing sugars; (iii) after desialylation, 6-linked GalNAc and 6-linked Gal disappear and there is a concomitant increase in terminal GalNAc and terminal Gal, indicating that sialic acid residues were attached to the 6-positions of Gal and GalNAc prior to desialylation; (iv) some terminal GlcNAc is present but most of the GlcNAc is 4-linked, 3,4-linked, or 4,6-linked; (v) a very minor amount of 3-linked Gal is present, the majority of which is retained after desialylation and is therefore likely to be derived from the minor poly-N-acetyllactosamine moieties suggested by the FAB data (m/z 913 and 1362 in Fig. 2, see above); (vi) the minor 3,4,6-linked Man is indicative of some bisected structures; (vii) 3-linked Man and 6-linked Man are present but only as very minor components; they are indicative of hybrid and/or high mannose structures.

Determination of Fucosyl Linkages—The attachment sites of the fucosyl residues were established by linkage analysis after removal of the fucoses by mild methanolyis and remethylation of the newly formed hydroxyl groups (see footnotes to Table III). Comparison of linkage data before and after mild methanolyis indicates that loss of fucosyl residues from the antennae is accompanied by loss of the 3,4-linked GlcNAc and a concomitant increase in 4-linked GlcNAc. Importantly no 3-linked GlcNAc was observed after methanolyis. These data establish that fucose is attached to the 3-position of 3,4-linked GlcNAc.

**FIG. 3.** UV chromatogram (upper trace) and TIC (lower trace) of glycodelin peptides and glycopeptides analyzed by on-line microbore LC-ES-MS.

**FIG. 4.** Transformed electrospray mass spectrum of the glycopeptides spanning Asn-28 of glycodelin. Glycodelin was digested overnight with CNBr. The dried sample was then reduced with dithiothreitol in triethylamine and dried again. The sample was analyzed by LC-ES-MS.
Exoglycosidase Digestions—The glycan mixture was treated sequentially with \(\alpha\)-sialidase, \(\alpha\)-fucosidase, \(\beta\)-hexosaminidase, and \(\beta\)-galactosidase, and the reactions were monitored by FAB-MS after permethylation. The FAB spectrum of the fully digested sample was dominated by an A-type ion at m/z 872 (Hex\textsubscript{6}HexNAc\textsuperscript{+}) and an \([M + H]^+\) ion at m/z 1149 corresponding to Hex\textsubscript{6}HexNAc\textsuperscript{+} (data not shown), confirming that the majority of the complex structures can be degraded to the trimannosyl core by this series of exoglycosidases. Thus the NeuAc and Fuc residues are in normal \(\alpha\) linkages and the Gal, GaINAc, and GlcNAc residues are all \(\beta\) linked. The signal at m/z 1557 (see Fig. 2) was unaffected by the above exoglycosidase digestions, a result that is consistent with the assignment of a high mannose structure to this ion. This was corroborated in a separate experiment in which the intact glycans were subjected to \(\alpha\)-mannosidase digestion. The resulting FAB spectrum was very similar to Fig. 2 except that m/z 1557 had disappeared and a new signal was present at m/z 1149 corresponding to Man\textsubscript{2}GlcNAc\textsubscript{2} (data not shown). In addition the signal at m/z 2368 was no longer present, consistent with the proposed composition of NeuAcHex\textsubscript{5}HexNAc\textsubscript{3} (Table I), which corresponds to a hybrid structure.

LC-ES Mapping of Glycodelin—In the analysis of tryptic digests of glycodelin, a well resolved intense late-eluting peak in the UV chromatogram gave electrospray data transformed to a mass of 4749, which maps onto the non-glycosylated disulfide bridged peptide Ile\textsubscript{84} to Arg\textsubscript{124}. Subtraction of the mass of this peptide from each of the observed signals in the ES spectrum yields the tentative glycan sequence (Fig. 1), it was considered likely that this early eluting glycopeptide peak comprised glycoforms of the peptide spanning residues 25–32, which includes the consensus site at Asn-28. Subtraction of the mass of this peptide from each observed signal in the ES spectrum yields the tentative glycan

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**Table V**

Masses and assignments of oligosaccharide compositions for the glycopeptide containing Asn-28

| Observed mass | Assignment |
|---------------|------------|
| 2050.3        | Hex\textsubscript{6}HexNAc\textsuperscript{+} |
| 2278.3        | Hex\textsubscript{5}HexNAc\textsuperscript{+}Fuc |
| 2381.0        | NeuAcHex\textsubscript{5}HexNAc\textsuperscript{+} |
| 2423.4        | NeuAcHex\textsubscript{5}HexNAc\textsuperscript{+} |
| 2543.5        | NeuAcHex\textsubscript{5}HexNAc\textsuperscript{+} |
| 2585.9        | NeuAcHex\textsubscript{5}HexNAc\textsuperscript{+} |
| 2624.1        | NeuAcHex\textsubscript{5}HexNAc\textsuperscript{+} |
| 2705.9        | NeuAcHex\textsubscript{5}HexNAc\textsuperscript{+} |
| 2747.5        | NeuAcHex\textsubscript{5}HexNAc\textsuperscript{+} |
| 2788.1        | NeuAcHex\textsubscript{5}HexNAc\textsuperscript{+} |
| 2789.3        | Hex\textsubscript{6}HexNAc\textsuperscript{+}Fuc |
| 2830.1        | NeuAcHex\textsubscript{5}HexNAc\textsuperscript{+} |
| 2893.5        | NeuAcHex\textsubscript{6}HexNAc\textsuperscript{+}Fuc |

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**Table VI**

Assignments of FAB-MS peaks observed for the molecular ions of the permethylated N-glycans released from Asn-28 of glycodelin by PNGase F

| Mass       | Assignment                                |
|------------|-------------------------------------------|
| 1557.1     | Hex\textsubscript{6}HexNAc\textsuperscript{+}Na\textsuperscript{+} (major) |
| 1579.1     | Hex\textsubscript{6}HexNAc\textsuperscript{+}Na\textsuperscript{+} |
| 1835.2     | Hex\textsubscript{6}HexNAc\textsuperscript{+}Fuc\textsuperscript{+}Na\textsuperscript{+} |
| 1959.7     | NeuAcHex\textsubscript{6}HexNAc\textsuperscript{+}Na\textsuperscript{+} |
| 1982.2     | NeuAcHex\textsubscript{6}HexNAc\textsuperscript{+}Na\textsuperscript{+} |
| 2023.2     | NeuAcHex\textsubscript{6}HexNAc\textsuperscript{+}Na\textsuperscript{+} |
| 2185.8     | NeuAcHex\textsubscript{6}HexNAc\textsuperscript{+}Na\textsuperscript{+} |
| 2227.3     | NeuAcHex\textsubscript{6}HexNAc\textsuperscript{+}Na\textsuperscript{+} |
| 2367.5     | NeuAcHex\textsubscript{6}HexNAc\textsuperscript{+}Na\textsuperscript{+} |
| 2390.3     | NeuAcHex\textsubscript{6}HexNAc\textsuperscript{+}Na\textsuperscript{+} |
| 2408.6     | NeuAcHex\textsubscript{6}HexNAc\textsuperscript{+}H\textsuperscript{+} (minor) |
| 2431.4     | NeuAcHex\textsubscript{6}HexNAc\textsuperscript{+}Na\textsuperscript{+} |
| 2437.4     | Hex\textsubscript{6}HexNAc\textsuperscript{+}Fuc\textsuperscript{+}H\textsuperscript{+} (minor) |
| 2450.1     | NeuAcHex\textsubscript{6}HexNAc\textsuperscript{+}H\textsuperscript{+} |
| 2472.0     | NeuAcHex\textsubscript{6}HexNAc\textsuperscript{+}Na\textsuperscript{+} |
| 2490.3     | NeuAcHex\textsubscript{6}HexNAc\textsuperscript{+}H\textsuperscript{+} (minor) |
| 2513.3     | NeuAcHex\textsubscript{6}HexNAc\textsuperscript{+}Na\textsuperscript{+} |
| 2583.2     | NeuAcHex\textsubscript{6}HexNAc\textsuperscript{+}Fuc\textsuperscript{+}H\textsuperscript{+} (minor) |
| 2624.1     | NeuAcHex\textsubscript{6}HexNAc\textsuperscript{+}Fuc\textsuperscript{+}H\textsuperscript{+} |
| 2646.9     | NeuAcHex\textsubscript{6}HexNAc\textsuperscript{+}Fuc\textsuperscript{+}Na\textsuperscript{+} |
compositions shown in Table V. In order to confirm these assignments, including resolving the NeuAc/Fuc2 ambiguity (see legend to Table V), and to check for additional minor components, the collected fractions 41–43 were pooled and digested with PNGase F. Thereleased glycans were permethylated and analyzed by FAB-MS (Fig. 5, Table VI). The FAB data show that, with the exception of Hex5HexNAc6Fuc2, all the fucosylated glycans contain only a single fucose residue ruling out the other tentative Fuc2 assignments in Table V. The FAB spectrum contains one minor molecular ion not observed in the ES spectrum (corresponding to NeuAcHex5HexNAcFuc), but otherwise all molecular ion signals observed in the FAB spectrum (Fig. 5, Table VI) have their counterparts in the ES spectrum (Fig. 4, Table V).

The peptide released in the glycanase experiment was found to produce a quasimolecular ion at m/z 834, corresponding to peptide residues 25–32. Glycosylation site 28 is thus proven to carry the glycans in Table VI, and the LC-ES-MS data show it to be well separated from other molecular species.

Combining scans 139–145 produces the raw data (multiply charged) shown in Fig. 6. The complex appearance of this spectrum, which contrasts with the clean single signals of different charge states expected for a peptide, is immediately indicative of the expected heterogeneity seen in a glycopeptide. Computing the charge states shown, and transformation of the data produces component masses of 11,835.5, 11,879.3, and 12,126.8 for the most abundant peaks. These masses are approximately 2000 Da higher than the anticipated mass of peptide Ala-33 to Met-117, indicating glycosylation of the peptide. The peptide contains potential glycosylation sites Asn-63 and Asn-85, but since we have already proven that Asn-85 is not glycosylated (see earlier FAB and ES mapping experiments), it follows that the glycans on peptide Ala-33 to Met-117 are attached to Asn-63. Their identities were studied in detail by FAB-MS analysis after their release by PNGase F from the glycopeptides in collected fractions 56–57 (see Fig. 7 and Table VII). It is noteworthy that the majority of glycans in this sample are different from those attached at Asn-28 (see below).

**Occupancy of Consensus Sites**—Unequivocal evidence for full glycan occupancy of Asn-28 was provided by a combination of the FAB Mapping experiments on tryptic and PNGase F digests of glycodein (Table IV) and the on-line LC-ES-mapping experiments on tryptic and CNBr digests (Fig. 3). The latter experiments also established that only one (Asn-63) of the remaining two consensus sites is glycosylated at observable levels (Fig. 6). The separation of the occupied sites in the LC-ES mapping experiments on reduced CNBr digests of glycodein allowed identification of the differing oligosaccharide structures at Asn-28 and Asn-63 (see below).

**Assignment of Oligosaccharide Structures**—The proposed structures for the major oligosaccharides are shown in Fig. 8. The glycans fall into three classes, namely high mannose (i), hybrid (ii and iii) and complex (iv–xx), of which the first two classes are only found at Asn-28, whereas complex structures occur at both glycosylation sites. Among the complex structures only (ix and xiii) are common to both sites. The most notable differences between the complex structures at Asn-28 and Asn-63 are the increased levels of sialylation and fucosylation of the glycans at the latter site. The high sensitivity achieved in the FAB-MS analyses of the total glycan population allowed the detection of very minor components giving molecular ions at masses above m/z 3000 (see Fig. 2 and Table I). These correspond to tri- and tetraantennary structures and/or bi- and tri-antennary structures with N-acetyllactosamine repeats in their antennae. The FAB fragmentation data and the linkage analysis results suggest that both types of structure are present (see above). The very low abundance of these components has to date precluded precise structural analysis or determination of attachment sites.

The major non-reducing epitopes in the glycodein complex-type glycans are: (i) Galβ1–4GlcNAc (IacNAC), (ii) GalNAcβ1–4GlcNAc (lactdNAC), (iii) NeuAcα2–6Galβ1–4GlcNAc (sialylated IacNAC), (iv) NeuAcα2–6GalNAcβ1–4GlcNAc (sialylated lactdNAC), (v) Galβ1–4(Fucα1–3)GlcNAc (Lewis\(^{\text{a}}\)), and (vi) GalNAcβ1–4(Fucα1–3)GlcNAc (the lactdNAC analogue of Lewis\(^{\text{a}}\)). The relative abundances of molecular ions in the ES and FAB spectra indicated that IacNAC- and lactdNAC-containing epitopes are of comparable abundance and that approxi-
approximately 60% of the glycans are sialylated and about 20% of the glycans have fucosylated antennae. It is notable that about 30% of the biantennary glycans bear lacNAc and lacdiNAc antennae within a single structure. Additional quantitative information was obtained from sugar analysis of trimethylsilyl ether methylglycosides of the total glycan population (data not shown). These experiments gave a Gal:GalNAc ratio (translating into a lacNAc: lacdiNAc ratio) of 1.2:1, which supports the conclusions from the MS data.

**DISCUSSION**

The majority of the glycodegin N-linked oligosaccharides characterized in this study are not typically found in mammalian glycoproteins. In particular, the presence of lacdiNAc-containing antennae is unusual because, with the exception of the pituitary glycohormones, this sequence has been rarely observed in the glycoproteins of higher animals (see Dell and Khoo (1993), vandenEijnden et al. (1995), and references cited therein). The best characterized family of mammalian lacdiNAc glycoproteins are the pituitary glycohormones, which contain sulfated lacdiNAc structures (Baenziger and Green, 1988). The GalNAc transferase, which adds GalNAc to these glycoproteins, recognizes the tripeptide motif Pro-Xaa-Arg/Lys (PXR/K) located 6–9 residues NH₂-terminal to an Asn glycosylation site (Smith and Baenziger, 1992). This GalNAc transferase, together with the sulfotransferase responsible for synthesizing the unique sulfated epitope on the pituitary glycohormones, is present in a number of tissues other than the pituitary, and the two enzymes appear to be co-ordinately expressed (Dharmesh et al., 1993). However, we consider it unlikely that glycodegin is a substrate for the PXR/K-specific GalNAc transferase because it does not contain a recognition motif 6–9 residues upstream of either glycosylation site. Furthermore, we were not able to detect sulfated structures in glycodegin using acetylation/FAB-MS strategies, which are optimized for the detection of sulfated oligosaccharides (data not shown) (Khoo et al., 1993).

Non-sulfated lacdiNAc structures of the type present in glycodegin have previously been found in a three categories of mammalian glycoproteins. The first comprises glycoproteins produced by bovine mammary glands, including lactotransferrin (Coddeville et al., 1992), CD36 (Nakata et al., 1993), and butyrophilin (Sato et al., 1995). The second contains three human glycoproteins, all of which are serine proteases with important physiological functions, namely Bowes melanoma tissue plasminogen activator (Chan et al., 1991) and urinary type plasminogen activator (urokinase) (Bergwerff et al., 1992), both of which convert plasminogen to plasmin, and urinary kallidinogenase (Tomiya et al., 1993), which cleaves kallikreinogenes to liberate lysyl-bradykinin, a vasoactive peptide. The third category contains only a single glycoprotein at present, namely human recombinant Protein C (rHPC) expressed in human kidney 293 cells (Yan et al., 1993), but we anticipate the discovery of many more examples with the increasing use of this human cell line for the expression of recombinant glycoproteins. Glycodegin, rHPC carries a heterogeneous population of complex-type oligosaccharides composed of lacNAc and lacdiNAc building blocks, which are substituted with either sialic acid or fucose (Yan et al., 1993). Interestingly, the sialylated lacdiNAc antennae in rHPC have both α2-3 and α2-6 linked sialic acid, but the the former linkage was not observed in the lacdiNAc antennae. It is notable that NeuAcα2-
3GalNAcβ1–4GlcNAc has not, to our knowledge, been found in mammalian glycoproteins, although this structure has been identified in serine proteases derived from snake venoms (Pfeiffer et al., 1992; Lochnit and Geyer, 1995). It is possible that, by analogy with PXR/K-specific GalNAc transferase and sulfotransferase (Dharmesh et al., 1993), there could be co-ordinate expression of GalNAc transferase and α2–6-sialyltransferase in mammalian cell lines that synthesize sialylated lacdINAc structures.

Several lines of evidence indicate that oligosaccharides are essential recognition sequences in cell-mediated adhesions in both inflammatory and immune responses (Phillips et al., 1990; Springer, 1990; Lasky, 1992; Bevilacqua, 1993). Oligosaccharides terminated with sialylated or sulfated Lewisx/a type sequences have been shown to act as specific ligands for selectin-mediated adhesions (Berg et al., 1991; Yuen et al., 1992). Other oligosaccharide sequences can, however, act as selectin ligands (Varki, 1994). Importantly, in the rHPC study (see above) it was shown that a bi-antennary N-linked oligosaccharide bearing GalNAcβ1–4(Fucα1–3)GlcNAc antennae is a potent inhibitor of E-selectin-mediated adhesion (Grinnell et al., 1994). Since the same fucosylated epitope is also expressed on glycodelin, it is possible that a component of the immunosuppressive effect exhibited by glycodelin is mediated via blocking of the selectin-like binding sites by this carbohydrate sequence.

Other specific antennae associated with glycodelin may also interact with alternative bioactive receptor proteins of the human immune system. CD22 is a B cell-associated receptor of the immunoglobulin superfamily that acts as both an adhesion molecule and an activation molecule (Clark and Lane, 1991; Clark, 1993; Ledbetter et al., 1993; Peaker, 1994). Transfected cells that stably express CD22 on their surfaces show greatly

![Fig. 8. Structures of the major N-glycans present at Asn-28 (a) and Asn-63 (b) of glycodelin. Panel a, superscript a indicates that minor forms may exist with different arm structures as indicated by presence of 3- and 6-linked mannose; superscript b indicates that fucose residue may be 3-linked to the GlcNAc on either arm. Panel b, superscript c indicates that the fucose residue may be 3-linked to the GlcNAc on either arm, but is not on the arm bearing the sialic acid.](image-url)
enhanced binding to T and B lymphocytes (Wilson et al., 1991). CD22 is closely associated with the subset of responsive B lymphocytes as defined by stimulation with anti-μ (Pezzutto et al., 1988). CD22 also binds to CD45, the leukocyte-specific receptor-linked phosphotyrosine phosphatase involved in T-cell activation (Stamenkovic et al., 1991). Previous studies have revealed that CD22 binds to NeuAcα2–6Galβ1–4GlcNAc sequences (Powell and Varki, 1994). More recent studies indicate that CD22 also binds the NeuAcα2–6Galβ1–4GlcNAc disaccharide with approximately equal affinity as it does the NeuAcα2–6Galβ1–4GlcNAc sequences (Powell et al., 1995). Therefore we believe that glycolipid may bind to CD22 via its NeuAcα2–6Galβ1–4GlcNAc and/or NeuAcα2–6GalNAcβ1–4GlcNAc antennae and may inhibit specific immune cell adhesion and activation events mediated via this receptor protein.

We also find it significant that glycolipid has glycoforms carrying NeuAcα2–6GalNAcβ1–4GlcNAc and GalNAcβ1–4(Fucα1-3)Galβ1–3GlcNAc antennae on a single biantennary oligosaccharide (structure xviii; Fig. 8). Although this structure has been previously observed in rHPC (Yan et al., 1993), we are now the first to demonstrate its expression in a naturally occurring glycoprotein. The biological activities expressed by this glycan remain to be determined. It is possible that this oligosaccharide could interact with the selectins or other adhesion molecules with selectin-like specificity via the fucosylated antenna whereas its sialylated antenna could bind to CD22. Such an oligosaccharide could manifest multiple biological effects, including blocking inflammatory responses, attenuating CD22-dependent immune responses or perhaps inhibiting other selectin-like adhesion processes. It is also possible that certain carbohydrate-binding proteins associated with either the immune or reproductive systems may require the precise spatial arrangement of fucose and sialic acid provided by the antennae for optimal binding.

Evidence collected from diverse species in both the plant and animal kingdoms indicates that the appropriate recognition of surface carbohydrates is a crucial event in the binding of sperm to the eggs during fertilization (Macek and Shur, 1988; Miller and Ax, 1990; Wassarman, 1990). In the mouse, oligosaccharides associated with the zona pellucida glycoprotein ZP3 have been shown to be recognized by specific egg-binding proteins located on the sperm plasma membrane (Wassarman, 1990). It is probable that a similar paradigm is utilized in the human system.

We have previously suggested that initial human sperm-zona pellucida binding involves a selectin-like adhesion (Patanak et al., 1993a, 1993b). This proposed specificity was initially based upon our observation that fucoidan blocked initial human sperm-zona pellucida binding (Oehninger et al., 1990) and a selectin-mediated adhesion process (lymphocyte homing) in the same concentration range (Yednock and Rosen, 1989). Fucoidan also blocked induction of the sperm's acrosome reaction by solubilized human zona pellucida, consistent with its ability to block sperm-zona pellucida binding (Mahony et al., 1991). We recently reported in a preliminary study that sialyl-Lewis* oligosaccharide and human orosomucoid also inhibit initial human sperm-zona pellucida binding in the same concentration-dependent manner as is observed for E-selectin-mediated adhesion (Clark et al., 1995a). Although these studies suggest that the egg-binding protein is a selectin, our preliminary studies using specific anti-selectin monoclonal antibodies indicate that these adhesion proteins are not expressed on human sperm (Clark et al., 1995b). Therefore, we have hypothesized that the human egg-binding protein, though not itself a selectin, may have converged with the selectins in its carbohydrate binding specificity. Glycolipid also inhibits initial human sperm-zona pellucida binding in a potent concentration-dependent manner (Oehninger et al., 1995). The expression of putative selectin ligands on this glycoprotein provides further evidence supporting our hypothesis that initial human sperm-zona pellucida binding is dependent upon a selectin-like adhesion process.

The structural studies reported in this paper provide the necessary foundation for effectively addressing the above issues. We are now investigating the potential contribution of the variety of glycans attached to glycolipid to its immunosuppressive and contraceptive activities. Finally, since glycolipid is expressed in bone marrow (Kamarainen et al., 1994; Morrow et al., 1994) and perhaps other tissues, it will be of great interest to see whether their glycosylation and function are the same. Until that information is available we propose to designate this glycoprotein isolated from amniotic fluid “glycolipid-A.”
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