Structural Analysis of Sequences O-Linked to Mannose Reveals a Novel Lewis X Structure in Cranin (Dystroglycan) Purified from Sheep Brain*

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The Lewis X epitope, Galβ1–4(Fucα1–3)GlcNAc-R, has been implicated in cell-cell recognition events in a number of systems including the central nervous system and is expressed on diverse glycoconjugates including cell adhesion molecules, glycolipids, and the proteoglycan phosphacan. Although Lewis X sequences 3-linked to mannose have been described within proteoglycan fractions of mammalian brain, these have not been reported in other contexts and have been widely believed to be peculiar constituents of brain proteoglycans. In the present paper, we confirm the existence of Lewis X structures O-linked to mannose within the mammalian brain, demonstrate that these structures are present on a well defined mucin-like glycoprotein, cranin (dystroglycan), and report studies suggesting that the linkages involved may be predominantly 2-linked to mannose. Mannose-linked Lewis X is the latest in an increasing list of glycosaccharide recognition “tags” that have been shown to be expressed on cranin (dystroglycan) purified from brain.

The Lewis X epitope, Galβ1–4(Fucα1–3)GlcNAc-R, has been implicated in cell-cell recognition events in a number of systems. For example, Lewis X structures appear to participate in calcium-dependent compaction of 8-cell embryos (1) and neural tube closure (2). In brain, Lewis X epitopes are expressed on the proteoglycan phosphacan, several glycolipids, and a number of cell adhesion molecules (3, 4). Lewis X antibodies reveal distinctive spatial and temporal patterns of expression that strongly suggest a role for Lewis X epitopes in forebrain development (5) and that correlate with patterns of selective calcium-dependent adhesion of cells taken from different telencephalic regions (6).

Margolis and co-workers (7, 8) were the first to report the existence of Lewis X sequences linked to mannose in mammalian glycoconjugates. In studies of rat brain chondroitin sulfate proteoglycans they found mannitol-containing oligosaccharides among the products of mild alkaline borohydride treatment. These were identified as small neutral and sialylated oligosaccharides attached to the proteoglycan by a 3-substituted mannose residue: GlcNAcβ1–3Man-Ser/Thr, NeuAcα2–3Galβ1–4GlcNAcβ1–3Man-Ser/Thr, and the Lewis X sequence Galβ1–4(Fucα1–3)GlcNAcβ1–3Man-Ser/Thr. No similar mannose-linked oligosaccharides were identified in mammalian tissues in the ensuing 10 years. Indeed, recently, mannose-linked oligosaccharides were widely believed to be very rare constituents of brain proteoglycans.

Within the past two years, however, it has become clear that O-linked mannose is not as rare as previously thought and is present in glycoproteins as well as proteoglycans. Thus Endo and co-workers (9) have shown that bovine peripheral nerve α-dystroglycan is rich in sialylated oligosaccharides of sequence NeuAcα2–3Galβ1–4GlcNAcβ1–2Man-Ser/Thr, whereas Feizi and co-workers (10) have found that the major HNK-1-immunoreactive O-linked oligosaccharides from total rabbit brain extracts terminate with 2-substituted mannose (HSO₃⁺Galβ1–3Galβ1–4GlcNAcβ1–2Man-Ser/Thr). This latter group also reported preliminary evidence for the presence of fucosylated oligosaccharides linked through mannose in the rabbit brain extracts.

In this paper we identify the mucin-like extracellular matrix receptor cranin (dystroglycan) as an important glycoprotein carrier of O-linkedmannosyl oligosaccharides in the brain, and we provide evidence for sheep brain cranin having the same sialylated O-linked mannose structure, NeuAcα2–3Galβ1–4GlcNAcβ1–2Man-Ser/Thr, as bovine peripheral nerve α-dystroglycan (11). Importantly we also demonstrate a novel Lewis X-containing oligosaccharide (Gal1–4[Fuc1–3]GlcNAc1–2Man-Ser/Thr) as a minor neutral component of cranin glycans; this structure has not yet been localized upon any protein.

EXPERIMENTAL PROCEDURES

Purification and Characterization of Cranin—Cranin was purified exactly as described previously (11), and several of the same preparative runs characterized in the previous paper were also analyzed here. Briefly, sheep brains were homogenized in ice-cold 50 mM Tris, 5 mM EDTA, 150 mM NaCl, 2 mM pheymethylsulfonyl fluoride, 10 mM N-ethylmaleimide, pH 7.6. Pellets were rinsed in high salt-urea solutions and solubilized in 2% Triton X-100 in TEA buffer1 (0.01 M triethanolamine, 137 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, pH 7.4). Extracts were incubated with DEAE-Sepharose CL-6B, rinsed, and eluted in 0.5 mM NaCl, 0.1% Triton X-100, TEA buffer. DEAE eluates were adjusted to 1 mM in MnCl₂, passed over a concanavalin A-lectin affinity column,

¹ The abbreviations used are: TEA, triethanolamine; CAD, collision-activated dissociation; GC, gas chromatography; MS, mass spectrometry; FAB, fast atom bombardment.
rinsed, and eluted with 0.4 α-methyl-mannoside, 0.1% Triton X-100, TEA buffer. Concanavalin A eluates were passed over a Jacalin lectin affinity column and eluted with 1 M NaCl, 10 mM EDTA, 10 mM EGTA, 0.1% Triton X-100, TEA buffer. The laminin column eluates were treated to remove excess salt, passed over a laminin affinity column again, and eluted with 10 mM EDTA, 10 mM EGTA, 0.1% Triton X-100, TEA buffer. Purified cranin gave two bands (corresponding to its alpha and beta subunits) on one- and two-dimensional polyacrylamide gel electrophoresis gels, as assessed by silver staining and lectin-blotting. The bands were confirmed as cranin (dystroglycan) by amino acid sequencing and immunoblotting with anti-peptide antibodies (11).

Removal of traces of contaminating detergent was achieved by acetone precipitation. Acetone (high performance liquid chromatography grade) was cooled to 4 °C. The cranin sample was dissolved in 1 ml of 5% acetic acid and placed on ice before adding 2 ml of cold acetone. After standing at 4 °C for 1 h, the mixture was centrifuged at 3000 rpm for 10 min. The supernatant was removed, and the precipitated cranin was used for the reductive elimination experiments described below.

Release of Oligosaccharides—O-Glycans were released from 100 mg samples of cranin by reductive elimination (1 M NaBH4 in 0.05 M NaOH at 45 °C for 16 h) and desalted through a Dowex 50W-X8(H) column. Excess borates were then removed by co-evaporation with 10% acetic acid in methanol under a stream of nitrogen.

Structural Analysis Strategy—O-Linked oligosaccharides were liberated from cranin by reductive elimination, and their methylated derivatives were characterized by FAB-MS before and after mild methanolysis and by linkage analysis. Because of the availability of only limited amounts of material, the oligosaccharides were analyzed as mixtures. Structural assignments were based on molecular weight and fragment ion information (the latter derived from CAD experiments on a tandem mass spectrometer as well as unassisted fragmentation in the normal FAB-MS experiment), susceptibility to mild methanolysis, and linkage data.

FAB-MS of Methylated Products of Reductive Elimination—The FAB spectrum of the methylated products of reductive elimination is shown in Fig. 1, and assignments are given in Table I. All assignments were corroborated by observing mass shifts after deuteromethylation (data not shown). The compositions in Table I suggest the presence of two families of O-glycans. Members of the first family have compositions Hex1HexNAc1-ol, NeuAc 1Hex1HexNAc1-ol and NeuAc 2Hex1HexNAc-ol, which are consistent with non-, mono- and disialylated type 1 cores ((±NeuAc-Galβ1–3(±NeuAc)GalNAc); note that anomeric configurations were not defined in our study because of limited material. CAD-MS/MS experiments on the mono- and disialylated molecular ions gave fragment ions consistent with this interpretation (data not shown). Data from linkage analysis (not shown) were also consistent with core type 1 structures being abundant. This is not unexpected because sialylated core type 1 structures are commonly found in mam-
malian glycoproteins. Members of the second family are of greater interest because their compositions are indicative of unusual hexose-rich core structures. This family has \([M + H]^+/[M + Na]^+\) quasimolecular ion pairs at \(m/z\) 716/738, 890/912, and 1077/1099 corresponding to compositions \((\text{Hex}_2\text{HexNAc}_1)\)-ol, \((\text{Fuc}_1\text{Hex}_2\text{HexNAc}_1)\)-ol, and \((\text{NeuAc}_1\text{Hex}_2\text{HexNAc}_1)\)-ol, respectively. Evidence for these glycans being attached to cranin via 2-substituted mannose was provided by the following experiments.

Collisional activation of the \([M + H]^+\) ions for \((\text{Hex}_2\text{HexNAc}_1)\)-ol and \((\text{NeuAc}_1\text{Hex}_2\text{HexNAc}_1)\)-ol gave major A-type fragment ions at \(m/z\) 464 \((\text{HexHexNAc}_1)^+\) and 825 \((\text{NeuAcHexHexNAc}_1)^+\), respectively, indicating that both oligosaccharides have hexitol at the proximal position (data not shown). Both \(m/z\) 464 and 825 were accompanied by secondary fragment ions 32 mass units lower (minus methanol). Formation of these ions requires the 3-position of the HexNAc to be unsubstituted. Collisional activation of the \([M + Na]^+\) ion of \((\text{NeuAc}_1\text{Hex}_2\text{HexNAc}_1)\)-ol, \(m/z\) 1099, gave the data shown in Fig. 2. It is known that ring fragmentation of permethylated oligosaccharides is enhanced and A-type fragmentation is reduced when \([M + Na]^+\) quasimolecular ions are substituted for \([M + H]^+\) in the CAD experiment (14). In this instance, ring fragmentation of HexNAc (Fig. 2, inset), together with the A-type fragmentation data described earlier, provided unequivocal evidence for the sequence NeuAc-Hex-HexNAc-hexitol in which the HexNAc is 4-substituted.

The structure of the oligosaccharide giving quasimolecular ions consistent with a fucosylated analogue of Hex-HexNAc-hexitol \([M + H]^+/[M + Na]^+\) at \(m/z\) 890/912, Fig. 1, Table I) was deduced from chemical and mass spectrometric degradation. The presence of fucose was confirmed by mild methanolysis of the methylated products of reductive elimination using conditions that are known to liberate fucose but not other potentially acid labile sugars such as sialic acids. In this experiment the methanolysis reagent is added to the sample at room temperature, and an aliquot was taken immediately for FAB analysis. Further aliquots are analyzed at intervals of 1 to 3 min, with the reaction being terminated by removal of the reagents under nitrogen as soon as the FAB data indicate a successful outcome of the experiment. Typically, 3-linked fucose residues are liberated within the first few minutes of methanolysis, whereas fucoses in other linkages are released.

**TABLE I**

| Signal | Assignments |
|--------|-------------|
| 260    | HexNAc$^+$  |
| 344    | NeuAc$^+$ minus methanol |
| 376    | NeuAc$^+$   |
| 464    | HexHexNAc$^+$ |
| 512    | (Hex$_2$HexNAc$_1$)-ol + H$^+$ |
| 534    | (Hex$_2$HexNAc$_1$)-ol + Na$^+$ |
| 716    | (Hex$_2$HexNAc$_1$)-ol + H$^+$ |
| 738    | (Hex$_2$HexNAc$_1$)-ol + Na$^+$ |
| 793    | NeuAcHexHexNAc$^+$ minus methanol |
| 825    | NeuAcHexHexNAc$^+$ |
| 873    | (NeuAc$_1$Hex$_2$HexNAc$_1$)-ol + H$^+$ |
| 890    | (Fuc$_1$Hex$_2$HexNAc$_1$)-ol + H$^+$ |
| 895    | (NeuAc$_1$Hex$_2$HexNAc$_1$)-ol + Na$^+$ |
| 912    | (Fuc$_1$Hex$_2$HexNAc$_1$)-ol + Na$^+$ |
| 1077   | (NeuAc$_1$Hex$_2$HexNAc$_1$)-ol + H$^+$ |
| 1099   | (NeuAc$_1$Hex$_2$HexNAc$_1$)-ol + Na$^+$ |
| 1234   | (NeuAc$_1$Hex$_2$HexNAc$_1$)-ol + H$^+$ |
| 1256   | (NeuAc$_1$Hex$_2$HexNAc$_1$)-ol + Na$^+$ |

**Fig. 2.** Positive ion CAD mass spectra of the sodiated molecular ion of \((\text{NeuAc}_1\text{Hex}_2\text{HexNAc}_1)\)-ol. Sequence and/or linkage-informative fragment ions are assigned in the inset. Fragment ions were magnified ×20.
more slowly. All [M + H]^+ molecular ions observed before mild methanolysis (Table I) were preserved in the FAB spectrum taken after 3 min of methanolysis, with the notable exception of m/z 890 (Fig. 3). The disappearance of this ion correlated with the appearance of a new [M + H]^+ signal at m/z 702, consistent with the loss of methylated fucose. It should be noted that analysis of the zero time point is important despite little chemical activity having occurred at this point. This is because the acidity of the reagent enhances A-type fragmentation, and the first aliquot may give fragment information additional to that found in the spectrum before addition of the reagent. Significantly the A-type ion at m/z 638 ([Fuc, Hex]-HexNAc) was enhanced immediately after addition of the reagent (data not shown) and disappeared, concomitant with an increase of m/z 450 ([OH], Hex)-HexNAc) after 3-min methanolysis (Fig. 3). Notably m/z 638 did not show a loss of methanol (m/z 606), indicating that the HexNAc has a substituent at position 3.

Taking into account the molecular and fragment ion information as well as the rate of fucose release, these data suggest the sequence Hex-(Fuc-1–3)HexNAc-hexitol for m/z 890 (Fig. 3). This sequence was corroborated by CAD-MS/MS of the sodiated molecular ion (m/z 912) in the unmethanolyzed sample (Fig. 4, inset).

Linkage Analysis—The hexitol residue observed in the FAB-MS experiments was shown to be 2-linked mannitol from GC-MS linkage analysis data obtained from the methylated products of reductive elimination. A peak was observed in the GC chromatogram at the elution time of authentic 2-substituted mannitol. This gave the mass spectrum in Fig. 5, which is identical to that derived from linkage analysis of Manα1–2Man-ol (data not shown).

Mannitol-containing Oligosaccharides in the Products of Reductive Elimination—The FAB and linkage data provide convincing evidence for the presence of both GalNAc- and Man-linked oligosaccharides in the cranin sample. Those linked through GalNAc have compositions consistent with type 1 structures with and without sialylation. The Man-linked oligosaccharides are assigned the following sequences: Gal1–4GlcNAc–2Man-ol, NeuAc2–3Gal1–4GlcNAc–2Man-ol, and Gal1–4(Fuc-1–3)GlcNAc–2Man-ol. In addition it is probable that GlcNAc–1–2Man-ol is present because terminal GlcNAc is observed in the linkage analysis (data not shown) and an A-type ion for HexNAc is present in the FAB spectra (Figs. 1 and 3). However, its abundance cannot be assessed because it is isobaric to Gal1–3GalNAc-ol, which is abundant in cranin.

DISCUSSION

In the present paper, we (a) confirm the existence of Lewis X structures O-linked to mannose within the mammalian brain, (b) demonstrate that these structures are present not only on proteoglycans but on a well defined mucin-like glycoprotein as well, and (c) report studies suggesting that the linkages involved may be predominantly 2-linked to mannose.

Mannotyl-linked Lewis X Oligosaccharides—In the original study of Finne et al. (7), mass spectroscopic data indicated that the linkage to mannose was either 1–2 or 1–3; based on fragment ion shifts upon deuteration, they concluded that a 1–2 linkage was unlikely (7). In contrast, in our studies and in the recent studies of glycoproteins performed by others (9, 10), the mannose residue has been clearly 2-substituted. Moreover, it is
interesting to note that each 3-substituted mannosyloligosaccharide identified by Margolis and co-workers (7, 8) in proteoglycans has now been shown to have its 2-substituted mannosyl counterpart in brain glycoproteins. A reassessment of proteoglycans may be warranted to confirm whether they contain 3-substituted mannose, in contrast to the predominance of 2-substituted mannose in glycoprotein-linked species.

In previous studies, neither we (11) nor Yamada et al. (15) detected Lewis X epitopes on cranin (dystroglycan) by immunoblotting with Lewis X antibodies. Antibody binding might not have been sensitive enough to detect minor constituents; alternatively, antibody binding might have been inhibited by steric hindrance within regions of highly clustered mucin-like saccharides, particularly when the epitope is located relatively close to the peptide linkage site.

Significance of Carbohydrate Modifications of Cranin (Dystroglycan)—Cranin (dystroglycan) is a widely expressed extracellular matrix receptor synthesized as a single precursor that is cleaved into an extracellular subunit (\(\alpha\)) and a transmembrane subunit (\(\beta\)) (Ref. 16; reviewed in Ref. 17). The \(\alpha\)-subunit is highly glycosylated and exhibits high affinity calcium-dependent binding to a set of matrix proteins including laminins, agrin, and perlecan (16–18). The primary sequence of cranin (dystroglycan) is known from cDNA cloning (16), and the protein has been strongly implicated in morphogenesis, cell adhesion, and human disease (19–22).

The carbohydrate moieties of cranin have become the focus of heightened attention as being critical for regulating the func-
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isolated from sheep brain is highly susceptible to O-ans previously found to bear Lewis (dystroglycan) is demonstrably similar to typical mucin-like dance, mobility, and ability to bind laminin (11). Thus, cranin

which has been implicated in cell-cell adhesion in both neural and nonneural tissues (1, 2), in view of the observation that cranin (dystroglycan) can be facultatively recruited to sites of cell-cell contact (22). If such a recruitment should occur in cells whose cranin expresses Lewis X structures, the latter would thereby be recruited to the contact site as well.

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