Structures of Sialylated O-Linked Oligosaccharides of Bovine Peripheral Nerve a-Dystroglycan

THE ROLE OF A NOVEL O-MANNOSYL-TYPE OLIGOSACCHARIDE IN THE BINDING OF a-DYSTROGLYCAN WITH LAMININ*

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α-Dystroglycan is a heavily glycosylated protein, which is localized on the Schwann cell membrane as well as the sarcolemma, and links the transmembrane protein β-dystroglycan to laminin in the extracellular matrix. We have shown previously that sialidase treatment, but not N-glycanase treatment, of bovine peripheral nerve α-dystroglycan greatly reduces its binding activity to laminin, suggesting that the sialic acid of O-glycosidically-linked oligosaccharides may be essential for this binding. In this report, we analyzed the structures of the sialylated O-linked oligosaccharides of bovine peripheral nerve α-dystroglycan by two methods. O-Glycosidically-linked oligosaccharides were liberated by alkaline-borotritide treatment or by mild hydrazinolysis followed by 2-aminobenzamide-derivatization. Acidic fractions obtained by anion exchange column chromatography that eluted at a position corresponding to monosialylated oligosaccharides were converted to neutral oligosaccharides by exhaustive sialidase digestion. The sialidases from Arthrobacter ureafaciens and from Newcastle disease virus resulted in the same degree of hydrolysis. The neutral oligosaccharide fraction, thus obtained, gave a major peak with a mobility of 3.8–3.9 kDa and a trace peak with a mobility of 9.2 kDa, suggesting that the sialic acid moiety of this sugar chain was involved in the interaction of the α-dystroglycan with laminin.

α-Dystroglycan is an extracellular peripheral membrane glycoprotein anchored to the cell membrane by binding to a transmembrane glycoprotein, β-dystroglycan (1, 2). These two dystroglycan subunits were originally identified as members of a sarcolemmal dystrophin-associated (glyco)protein complex. α- and β-dystroglycans are encoded by a single gene and cleaved into two proteins by posttranslational processing (3). Based on recent amino-terminal sequencing of β-dystroglycan, it was proposed that the serine residue at position 654 of the precursor protein of dystroglycan is the cleavage site (4, 5). The α-dystroglycan/β-dystroglycan complex is widely expressed in many tissues (6) and thought to act as a transmembrane linker between the extracellular matrix and intracellular cytoskeleton (7), because α-dystroglycan binds extracellular matrix components, laminin-1 and -2 with high affinity (7–10), and the intracellular domain of β-dystroglycan binds cytoskeletal proteins, dystrophin or its autosomal homologue, utrophin (2, 11, 12).
The amino acid sequence of α-dystroglycan shows one feature of a mucin type O-glycosylation site (21) in the central region of the molecule. Threonine, serine, and proline are densely distributed between the 317th and the 488th amino acid residues, often clustering, and over one-half of the proline residues in this region are at positions −1 or +3 relative to the threonine or serine residues. In addition, the susceptibility of α-dystroglycan to O-sialoglycopeptidase supports the hypothesis that α-dystroglycan is a sialylated mucin-type glycoprotein (4). Recently, we demonstrated that exhaustive sialidase treatment of α-dystroglycan or the addition of sialic acid to the incubation medium diminished the laminin binding activity of α-dystroglycan, suggesting that the sialic acid residues of α-dystroglycan, which are probably attached to O-linked oligosaccharides, were essential for this binding (10).

In this study, we have analyzed the structures of sialylated O-linked oligosaccharides of bovine peripheral nerve α-dystroglycan and demonstrate that a novel O-linked mannose-type oligosaccharide, Siaα2–3Galβ1–4GlcNAcβ1–2Man-Ser-Thr, is the major component. The results of a binding-inhibition study suggest that this unique oligosaccharide contributes to the laminin binding activity of α-dystroglycan.

EXPERIMENTAL PROCEDURES

**Chemicals, Enzymes, Lectins, and Oligosaccharides**—NaB3H4 (1000 mCi/mmol) was purchased from DuPont NEN; 1,2-diamino-4,5-methylenedioxybenzene (DMB) from Dojindo Laboratories (Kumamoto, Japan); 2-AB labeling kit from Oxford Glycosystems (Oxon, United Kingdom); bis(dodecamethylene)triamine, and 6-aminoisobutyric acid from Sigma; A. ureafaciens (22).

**Glycosidase Digestion**—Glycosidase digestion (250 mU for 18 h at 37°C with 0.8% Triton X-100) or by acid hydrolysis (0.1 N HCl for 1 h at 80°C) (28). To this was added 240 µl of DMB solution (4 µM DMB, 2 µM acetic acid, 0.45 µM β-mercaptoethanol, and 11 mN Na2SO4), and the mixture was incubated for 2.5 h at 50°C in the dark. A 60-µl aliquot of the sample was applied to the Cosmostail 5C18-AR reversed-phase HPLC column (4.6 × 250 mm), and the column was eluted with acetonitrile:water (9:7.84, v/v) at a flow rate of 0.75 ml/min at room temperature. The fluorescence was monitored at 448 nm (excitation, 373 nm). Authentic N-acetylgalactosamine acid treated in the same way was used to make a standard curve.

**Libration of O-Glycosidically-linked Sugar Chains of α-Dystroglycan as Oligosaccharides**—The sugar chains of α-dystroglycan were converted to oligosaccharide derivatives by two chemical methods. In the first procedure, an α-dystroglycan sample (31.0 µg of protein) was thoroughly dried and subjected to hydrazinolysis for 5 h at 60°C according to the method of Patel et al. (23). The sample was subjected to N-acetylation followed by paper chromatography using 1-butanol:ethanol:water (4:1:1, v/v) for 18 h. The area of the paper from the origin to the position of authentic lactose was extracted with water, and the extracted oligosaccharides were labeled with 2-aminobenzamide (2AB) using the 2AB labeling kit (29). As for the 2AB-labeled oligosaccharides, fluorescence was monitored at 430 nm (excitation, 330 nm).

In the second procedure, a lyophilized α-dystroglycan sample (201.5 µg of protein) was dissolved in 400 µl of 0.05 N NaOH and 1 x NaBH4 containing 25 mCi of NaB3H4 and incubated for 16 h at 45°C (30, 31). After adjusting the pH to 6 by adding acetic acid, the solution was passed through a column containing 1 ml of Pellicon (31) and the column was washed with 10 ml of water. The effluent and the wash volume were combined and evaporated to dryness. After the remaining borate was removed by repeated evaporation with methanol, the residue was subjected to paper chromatography using 1-butanol:ethanol:water (4:1:1, v/v) for 18 h. The area of the paper from the origin to the position of authentic GlcNAcβ1–2Man was extracted with water.

**Analytical Methods**—The oligosaccharides samples dissolved in distilled water were applied to the Mono Q HR5/5 column. After elution of neutral oligosaccharides with 10 ml of water, acidic oligosaccharides were eluted with a 0–1 M gradient of ammonium acetate, pH 4.0, at a flow rate of 1 ml/min at room temperature. Neutral oligosaccharides were applied to the Superdex Peptide HR10/30 gel filtration column (1 × 80 cm) and eluted with distilled water at a flow rate of 0.4 ml/min at 60°C. PVL-Affi-Gel 10 column chromatography was performed as described previously (32). Reducing termini of tritium-labeled oligosaccharides were determined by using the Shodex SUGAR SP-1010 column (8 × 300-mm) (33). Reversed-phase HPLC was carried out on the Cosmosil 5C18-AR column by eluting with a 50 mM acetate-acetonic acid gradient solvent system at a flow rate of 1.0 ml/min at 40°C. The 60-cm HPLC column was eluted with a 0.05 M sodium acetate-acetonic acid gradient solvent system at a flow rate of 0.55 (v/v) over 50 min from 5 min after injection for 2AB-labeled disaccharide analysis and from 100 to 98.5:1.5 (v/v) over 50 min from 10 min after injection and then maintained at that ratio for 2AB-labeled monosaccharide analysis.

**Glycosidase Digestion**—Oligosaccharides were incubated with one of the following mixtures for 18 h at 37°C (40). A. ureafaciens sialidase (200

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1 The abbreviations used are: Sia, sialic acid; DMB, 1,2-diamino-4,5-methylenedioxybenzene; PVL, Psathyrella velutina lectin; HPLC, high-performance liquid chromatography; 2AB, 2-aminobenzamide; GU, glucose unit(s). Subscripts OT and OH are used to indicate NaB3H4- and NaBH4-reduced saccharides, respectively. All sugars mentioned in this study are of the β configuration except for fucose, which has an α configuration.
mU) in 80 μl of 0.5 mM sodium acetate or ammonium acetate buffer, pH 5.0; (ii) Newcastle disease virus sialidase (10 mU) in 40 μl of 50 mM sodium acetate buffer, pH 5.5; (iii) diplococcal β-galactosidase (5 mU) in 40 μl of 0.5 mM citrate phosphate buffer, pH 6.0; and (iv) jack bean β-N-acetylhexosaminidase (0.5 U) in 55 μl of 0.3 mM citrate phosphate buffer, pH 5.0. One drop of toluene was added to all reaction mixtures to inhibit bacterial growth during incubation. Digestions were terminated by heating the reaction mixture in a boiling water bath for 3 min. DIGESTED samples were desalted using ion exchange resin (300 μl of AG3-X4(OH−) and 300 μl of AG50-X12(H+) for neutralized tritium-labeled oligosaccharides or using the Sep-Pack C18 (washing with 8 ml of distilled water and elution with 6 ml of acetonitrile:water (2:8, v/v)) for neutralized 2AB-labeled oligosaccharides. As for samples after sialidase digests, ammonium acetate was removed by extensive evaporation, and sodium acetate was removed by passing the sample through a AG50-X12(H+) column (300 μl) and evaporation.

Laminin Binding Inhibition Assay—Sixteen ng of α-dystroglycan were dispensed to each well of a polystyrene microtiter plate and dried under blowing cold air. After rinsing with LBB (10 mM triethanolamine, pH 7.6, 140 mM NaCl, 1 mM CaCl2, and 1 mM MgCl2), the wells were blocked with LBB containing 3% bovine serum albumin (3% BSA-LBB). Fifty μl of the biotinylated laminin-1 (2 μM in 3% BSA-LBB) containing various saccharides were added to the wells and incubated overnight at room temperature. After rinsing with LBB, the binding of laminin-1 was measured using the avidin-biotin complex (ABC) kit. α-Phenylendiamine dihydrochloride was used as a coloring substrate, and absorbance was measured at 492 nm. The experiments were done in triplicate for each added saccharide.

RESULTS

Quantification and Characterization of Sialic Acids—The enzymatic cleavage and acid hydrolysis released about 14.9 and 13.8 mol of free sialic acids from 1 mol of α-dystroglycan, respectively. When the sialic acid residues thus obtained were analyzed by HPLC, N-acetyllactosaminic acid and N-glycolylneuraminic acid were detected in a ratio of 4:1 by both methods. Although the DMB labeling method can distinguish O-acetyl derivatives of sialic acid from N-acetyl or N-glycolyl ones (27), no O-acetyl derivative was detected in α-dystroglycan.

Structural Analysis of Oligosaccharides Released by Mild Hydrazinolysis and Labeled with 2AB—α-Linked oligosaccharides of bovine peripheral nerve α-dystroglycan, released by mild hydrazinolysis and labeled with 2AB, were subjected to Mono Q column chromatography at pH 4.0 (Fig. 1A). The acidic fraction was exhaustively digested by A. ureafaciens sialidase, and the neutral oligosaccharides, thus obtained, were subjected to Superdex Peptide gel filtration column chromatography. Three major peaks eluting at 3.9 glucose units (GU) (F-I, 2.9 GU (F-II), and 0.9–1.0 GU (F-III) were detected (Fig. 1B). Their percentage molar ratios calculated on the basis of their peak areas were 54, 30, and 16%, respectively.

The peak F-I released one galactose residue upon incubation with diplococcal β-galactosidase, which cleaves only the Galβ1–4GlcNAc linkage (34) (Fig. 2A), and subsequently one N-acetylhexosamine residue was released upon jack bean β-N-acetylhexosaminidase digestion (Fig. 2B). The product eluted with 0.8 GU in Fig. 2B was identified to be Man-2AB by 2AB-labeled monosaccharide analysis using reversed-phase HPLC (Fig. 3A). The component in Fig. 2A was eluted at the same retention time as that of authentic GlcNAcβ1–2Man-2AB standard in the reversed-phase HPLC (Fig. 4A), and more than 90% of it was retarded in the PVL-Affi-Gel 10 column, which specifically binds α-2,6-sialyl type oligosaccharide. The component in Fig. 2A was eluted at the same retention time as that of authentic GlcNAcβ1–2Man-2AB on reversed phase HPLC (Fig. 4B). This fraction released one galactose residue upon diplococcal β-galactosidase digestion (Fig. 2C). The digestion product corresponded to authentic GlcNAc-2AB in the monosaccharide-2AB analysis (Fig. 3B). The fraction F-III was separated into two peaks corresponding to Gal-2AB and Glc-2AB, respectively, in the monosaccharide-2AB analysis (Fig. 3C). Based on these data, the following structures were proposed for the components of F-I, F-II and F-III: F-I, Galβ1–4GlcNAcβ1–2Man-2AB; F-II, Galβ1–4GlcNAc-2AB; F-III, Galβ1–4GlcNAc-2AB and Glc-2AB.

Structural Analysis of Sialylated Oligosaccharides Released by β-Elimination Using Sodium Borotritide—The above results showed that the major sialylated O-linked oligosaccharide in bovine peripheral nerve α-dystroglycan was an O-mannosyl-type oligosaccharide. However, the oligosaccharides in the fractions F-II and F-III, except for Glc-2AB, may be the peeling reaction products of the major component found in the fraction F-I. This raised a question whether the major component of the apparent O-mannosyl-type oligosaccharide also might be a peeled product of larger oligosaccharides. To solve this problem, we investigated whether oligosaccharides with the same composition could be obtained from the same material by the conventional β-elimination method, which releases O-linked oligosaccharides from the polypeptide backbone. Triterium-labeled O-linked oligosaccharides released by alkaline β-elimination from the bovine peripheral nerve α-dystroglycan were separated by Mono Q column chromatography at pH 4.0 into neutral and acidic fractions (21 and 79%, respectively, based on the radioactivities). Three main peaks of the
acidic fractions (T-A1, T-A2, and T-A3) were obtained (Fig. 6A). By exhaustive A. ureafaciens sialidase digestion, the peak T-A1 was completely converted to neutral oligosaccharides, whereas the peaks T-A2 and T-A3 were not digested (Fig. 6B), indicating that most of the sialylated O-linked oligosaccharides of the bovine peripheral nerve α-dystroglycan are monosialylated and do not contain any other anionic residues such as sulfated sugars and uronic acids. Sialidases from both A. ureafaciens and Newcastle disease virus gave the same results (data not shown), indicating that the sialic acid residues are linked at the C-3 position of the galactose (36). The predominance of α2–3-linked sialic acid is compatible with the previous results of a lectin blot study in which bovine peripheral nerve α-dystroglycan was stained with Siaα2–3Gal-specific Maackia amurensis agglutinin but not with Siaα2–6Gal-specific Sambucus nigra.
**FIG. 6. Mono Q column chromatography of tritium-labeled oligosaccharides obtained by β-elimination of bovine peripheral nerve α-dystroglycan.** The tritium-labeled oligosaccharide mixture was applied to the anion exchange column and eluted with a 0–1 M gradient of ammonium acetate, pH 4.0, at a flow rate of 1.0 ml/min at room temperature (A). The broken line indicates the concentration of ammonium acetate in the elution buffer. The acidic fraction indicated by the solid bar in A was exhaustively digested by A. ureafaciens sialidase and then subjected to the same column chromatography (B).

The neutral fraction obtained by sialidase digestion was then subjected to Superdex Peptide gel filtration column chromatography. A peak eluting at 3.8 GU with a slight shoulder was detected (Fig. 7). It must be stressed here that no radioactive peak around 1 GU was detected. The shoulder portion was separated into two peaks of 3.8 and 2.7 GU by a second gel filtration. The combined fraction of the peaks of 3.8 GU was designated as fraction T-II. The percentage molar ratios of T-I and T-II were 78 and 22%, respectively, on the basis of their radioactivities.

The peak around 1 GU was detected. The shoulder portion was separated into two peaks of 3.8 and 2.7 GU by a second gel filtration. The combined fraction of the peaks of 3.8 GU was designated as fraction T-II. The percentage molar ratios of T-I and T-II were 78 and 22%, respectively, on the basis of their radioactivities.

Further structural studies of the oligosaccharides in fraction T-II could not be performed because of the limited amount of the sample.

Based on these results, the following structures were proposed for the major components of the fractions T-I and T-II: T-I, Galβ1–4GlcNAc-ManOT, and T-II, hexose-GlcNAcOT. Based on the radioactivities of the fractions obtained by the Superdex Peptide and PVL-Affi-Gel 10 column chromatographies, the amount of the Galβ1–4GlcNAc-ManOT was estimated to be at least 66% of the neutral oligosaccharides obtained by sialidase digestion.

**Inhibition of Laminin Binding of α-Dystroglycan by Sialic Acid Compounds**—We investigated the effect of sialic acid compounds, especially those having configurations similar to that of the major O-linked sialylated oligosaccharide of α-dystroglycan, on its laminin binding activity (Fig. 8). 3'-Sialyl N-acetyllactosamine, Neu5Acα2–3Galβ1–4GlcNAc, which is the trisaccharide terminal portion of Neu5Acα2–3Galβ1–4GlcNAcβ1–2Man, inhibited the binding of laminin, whereas both 3'-sialyllactose and 6'-sialyllactose did not. 6'-Sialyl N-acetyllactosamine, Neu5Acα2–6Galβ1–4GlcNAc, which is an isomer of 3'-sialyl N-acetyllactosamine in the sialic acid linkage, also reduced the binding of laminin, but its effect was weaker than that of 3'-sialyl N-acetyllactosamine. These results indicate that the interaction between laminin and sialylated oligosaccharides of α-dystroglycan are not simply dependent on the anionic charge of sialic acid residues but also on the structure comprising the neutral sugar portion and the sialic acid linkage.

**DISCUSSION**

By two different analytical methods, we identified a sialic acid-sensitive oligosaccharide with the same configuration as the major component. Based on these results, we conclude that the major sialylated O-linked oligosaccharide of the bovine peripheral nerve α-dystroglycan has the following structure: Neu5Acα (and Neu5Gcα)2–3Galβ1–4GlcNAcβ1–2Man-Ser/Thr (where Neu5Gc is N-glycolyneuraminic acid). Based on the radioactivity incorporated by the β-elimination method, these oligosaccharides constitute at least 66 molar percent of the total sialylated O-linked sugar chains. The components corresponding to fractions F-II and F-III (Fig. 1B), obtained by mild hydrazinolysis, were not detected by the β-elimination method. Therefore, Galβ1–4GlcNAc-2AB and Gal-2AB in these fractions were considered to be the degradation products of the major oligosaccharide by a peeling reaction during hydrazinolysis. No reasonable explanation could be presented for the detection of Glc-2AB. Analysis of the fraction T-II was incomplete because of the limited amount of the radioactive sample. In view of the previous reports that lectin blots revealed the presence of a Galβ1–3GlcNAc group in α-dystroglycan (4, 7, 10), the hexose-GlcNAcOT in the fraction T-II may represent this disaccharide. Mild hydrazinolysis has not been applied to...
Sialylated O-Linked Oligosaccharides of α-Dystroglycan

In the presence of various concentrations of α2–3 sialyl N-acetyllactosamine (3'-SLNac), α6-sialyl N-acetyllactosamine (6'-SLNac), 3'-sialylactose (3'-SL), 6'-sialylactosyl (6'-SL), and N-acetyllactosaminyl (LNac), biotinylated laminin-1 (2 nm) was incubated with α-dystroglycan (16 ng) coated onto microtiter wells. The bound laminin was detected using the avidin-biotin complex (ABC) kit and α-phenylenediamine dihydrochloride as a coloring substrate. Points are the mean percentage values obtained by triplicate experiments compared with the values obtained in the absence of the sugars.

Various samples as frequently as conventional β-elimination to release their O-linked oligosaccharides. Although our results suggest the occurrence of a peeling reaction to some extent in the mild hydrazinolysis, this method has great advantages in that it requires a smaller amount of sample and allows a greater choice of labeling method. In the mild hydrazinolysis followed by 2AB derivatization, we could determine the major oligosaccharide structure by using less than one-sixth the amount of sample compared with the conventional alkaline borotritide treatment method. The fluorescent derivatives generally allow us to apply reversed-phase HPLC analysis, by which we could separate the disaccharide derivatives of the four possible GlcNAcβ1-Man isomers (Fig. 4A), and determine the exact linkage between the N-acetyllactosamine and mannosic residues by using only about 2 pmol of the oligosaccharide derivative.

O-Mannosyl-type linkages, Man-Ser/Thr, have been found in the cell walls of microorganisms (37–39) and the skin collagen derivative. This would have a strong influence on the three-dimensional structure of the glycosylation of proteins is both tissue- and species-specific (50).

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