α2,3-Sialylation of Terminal GalNAcβ1-3Gal Determinants by ST3Gal II Reveals the Multifunctionality of the Enzyme

THE RESULTING Neu5Acα2-3GalNAc LINKAGE IS RESISTANT TO SIALIDASES FROM NEWCASTLE DISEASE VIRUS AND STREPTOCOCCUS PNEUMONIAE

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Enzymatic α2,3-sialylation of GalNAc has not been described previously, although some glycoconjugates containing α2,3-sialylated GalNAc residues have been reported. In the present experiments, recombinant soluble α2,3-sialyltransferase ST3Gal II efficiently sialylated the X₂ pentasaccharide GalNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc, globo-N-tetraose GalNAcβ1-3Galα1-4Galβ1-4Glc, and the disaccharide GalNAcβ1-3GlcNAcβ1-4Glc in vitro. The purified products were identified as Neu5Acα2-3GalNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc, Neu5Acα2-3GlcNAcβ1-3Galα1-4Galβ1-4Glc, and Neu5Acα2-3GalNAcβ1-3Gal, respectively, by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, enzymatic degradations, and one- and two-dimensional NMR-spectroscopy. In particular, the presence of the Neu5Acα2-3GalNAc linkage was firmly established in all three products by a long range correlation between Neu5Ac C2 and GalNAc H3 in heteronuclear multiple bond correlation spectra. Collectively, the data describe the first successful sialyltransfer reactions to the 3-position of GalNAc in any acceptor. Previously, ST3Gal II has been shown to transfer to the Galβ1-3GalNAc determinant. Consequently, the present data show that the enzyme is multifunctional, and could be renamed ST3Gal(Nac) II. In contrast to ST3Gal II, ST3Gal III did not transfer to the X₂ pentasaccharide. The Neu5Acα2-3GalNAc linkage of sialyl X₂ was cleaved by sialidases from Arthrobacter ureafaciens and Clostridium perfringens, but resisted the action of sialidases from Newcastle disease virus and Streptococcus pneumoniae. Therefore, the latter two enzymes cannot be used to differentiate between Neu5Acα2-3GalNAc and Neu5Acα2-6GalNAc linkages, as has been assumed previously.

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1 The abbreviations used are: GalNAc, N-acetyl-D-glucosamine; DQF-COSY, double-quantum-filtered correlated spectroscopy; Gal, D-galactose; Glc, D-glucose; GlcNAc, N-acetyl-D-glucosamine; HexNAc, N-acetylgalactosamine; HMBD, heteronuclear multiple bond correlation; HMBC, heteronuclear multiple quantum coherence; HSQC, heteronuclear single quantum coherence; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; 1/z, mass to charge ratio; Neu5Ac, N-acetyllactosaminic acid; TOCSY, total correlation spectroscopy; X₂, GalNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc; X₃, GalNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc; HPLC, high performance liquid chromatography; MOPS, 4-morpholinoephopropanesulfonic acid.

2 The abbreviated nomenclature of sialyltransferases is based on Ref. 33.
Escherichia freundii
endo-
products of X2 (essentially as described in Ref. 18, using a distinct ammonium sulfate
ST3Gal II (H9251 CMP-Neu5Ac, bovine milk
enzymatically synthesized as described in Ref. 17. UDP-GalNAc, Corp. (Tokyo, Japan).
N-capable of transferring to different monosaccharide residues.

it one of the few glycosyltransferases reported to date that are
Capable of sialylating X2 core structures by using a distal Gal. The product, representing the putative X2 pentasaccharide
was isolated from the reaction mixture by gel filtration chromatography. Takeya et al. (18) have shown by methylation analysis that the product formed from lactose under these reaction conditions is GalNAcβ1→3Galβ1→4Glc. We established the structure of the putative X2 pentasaccharide by the following set of experiments. First, its MALDI-TOF mass spectrum revealed two peaks at m/z 933.33 and 949.33, which were assigned to [M + Na]⁺ and [M + K]⁺ of HexNAcβ1→4GlcNAcβ1→3Galβ1→4Glc, respectively (calculated m/z 933.32 and 949.29, respectively).

Second, the terminal HexNAc was sensitive to jack bean β-N-acetylhexosaminidase (data not shown), but it was not GlcNAc as it could not be galactosylated by β-1,4-galactosyltransferase from bovine milk (data not shown). Finally, the HMBC spectrum of the sialyl X2 revealed that the distal β-GalNAc and the peridistal Gal of the X2 core were joined by a 1,3-bond (Fig. 7a).

Enzymatic Methods

ST3Gal II Reactions—600 nmol of acceptor oligosaccharide and 2.4 μmol of CMP-Neu5Ac were incubated with 40 milliunits of rat recombinant ST3Gal II (α2,3-(O)-sialyltransferase, EC 2.4.99.4) in 50 mM sodium cacodylate, pH 6.0, 0.02% NaN₃, 0.05% bovine serum albumin, and 8 mM MnCl₂ in a reaction volume of 600 μl for 6 days at room temperature. 20 milliunits of fresh enzyme was added on day 3. The reaction was terminated by boiling for 3 min.

ST3Gal III Reactions—49 nmol of acceptor oligosaccharide and 100 nmol of CMP-Neu5Ac were incubated with 3.2 milliunits of rat recom-

Materials

UDP-Gal was a gift from Prof. B. Ernst (University of Basel, Basel, Switzerland). Lacto-N-neotetraose was a gift from Prof. R. D. Cummings (University of Oklahoma, Oklahoma City, OK). Globo-N-tetraose was from Accurate Chemical and Scientific Corporation (Westbury, NY), GalNAcβ1→3Gal was from Dextra (Reading, United Kingdom). Neu5Acα2→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAc was enzymatically synthesized as described in Ref. 17. UDP-GalNAc, CMP-Neu5Ac, bovine milk β1,4-galactosyltransferase, and jack bean β-N-acetyhexosaminidase were from Sigma. Rat recombinant ST3Gal II (α2,3-(O)-sialyltransferase), rat recombinant ST3Gal III (α2,3-N-sialyltransferase), and Streptococcus pneumoniae sialidase were from Calbiochem (La Jolla, CA). Jack bean β-galactosidase and Escherichia freundii endo-β-galactosidase were from Seikagaku Corp. (Tokyo, Japan). Bacteroides fragilis endo-β-galactosidase and Newcastle disease virus sialidase were from Roche Molecular Biochemicals (Basel, Switzerland). Arthrobacter ureafaciens sialidase was from Glyko (Novato, CA). Clostridium perfringens sialidase was from New England Biolabs (Beverly, MA). Dowex AG-50, Dowex AG-1, and Bio Gel P-4 were from Bio-Rad. Superdex Peptide HR 10/30 and Mono-Q columns were from Amersham Pharmacia Biotech (Uppsala, Sweden). D₂O was from Cambridge Isotope Laboratories (Woburn, MA).

Synthesis of the X2 Pentasaccharide

GalNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc (X2) was synthesized essentially as described in Ref. 18, using a distinct ammonium sulfate
binant ST3Gal III (α2,3-(N)-sialyltransferase, EC 2.4.99.5) in 100 mM MOPS-NaOH, pH 7.5, 0.02% NaN₃, and 8 mM MnCl₂ in a reaction volume of 12.5 μl for 6 days at room temperature. The reaction was terminated by boiling for 3 min.

Other Enzymatic Methods—β1,4-Galactosyltransferase reactions were performed with bovine milk β1,4-galactosyltransferase (EC 2.4.1.90) essentially as described in Ref. 21. A. perfringens sialidase reactions in 50 mM sodium phosphate buffer, pH 5.5, with 8 milliunits per ml were performed essentially as described in Ref. 23. Sialidase reactions were terminated by boiling for 3 min.

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Chromatographic Methods

The sialyltransferase reaction products were purified by gel filtration HPLC in a column of Superdex Peptide HR 10/30 with 50 mM NH₄HCO₃—H₂O and 8 mM MnCl₂ in a reaction volume of 12.5 μl for 6 days at room temperature. The reaction was terminated by boiling for 3 min.

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Chromatographic Methods

The sialyltransferase reaction products were purified by gel filtration HPLC in a column of Superdex Peptide HR 10/30 with 50 mM NH₄HCO₃ as eluant, followed by anion exchange HPLC in a column of MonoQ (5/5) essentially as described in Ref. 24. The oligosaccharides were quantitated by comparing their UV 214 absorbance to external GlcNAc and Neu5Ac.

Mass Spectrometry

Matrix-assisted laser desorption/ionization mass spectrometry of reaction products was performed with a BIFLEX™ mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany). The neutral oligosaccharides were analyzed essentially as described in Ref. 25 and the sialylated oligosaccharides as in Refs. 26 and 27.

NMR Spectroscopy

Prior to NMR experiments the saccharides (400–600 nmol) were lyophilized twice from D₂O and then dissolved in 40 μl of D₂O (99.996 atom %). The NMR experiments were carried out on a Varian Unity 500 spectrometer at 23 °C using a gHX nano-NMR probe (Varian). A spinning rate of 2000 Hz was used. In recording one-dimensional proton spectra, a modification of the water-eliminated Fourier transformation sequence (28) was used. The DQF-COSY and TOCSY experiments were carried out essentially as in Ref. 29.

For the gradient HMQC (30) and gradient HMBC experiments (31, 32) (32 and 128 scans/#, value, respectively), matrices of 2K×256 and 2K×128 points were recorded and zero-filled to 2K×512 and 2K×256 points, respectively and a shifted sine-bell function was used. The average ¹H-¹³C coupling constant was estimated to be 140 Hz, and ¹J₁₂ = 8.5 Hz.

RESULTS

The present report describes sialylation reactions catalyzed by the α2,3-sialyltransferase known as ST3Gal II (33) with oligosaccharide acceptors containing the terminal GalNAcβ1-3Gal determinant. The acceptors and products are depicted in Fig. 1, which also shows the one-letter symbols of the constituent monosaccharides, used for describing the NMR data.

Sialylation of the X₂ Pentasaccharide, GalNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc—The X₂ pentasaccharide GalNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc (600 nmol) was incubated with CMP-Neu5Ac (2.4 μmol) and rat recombinant sialyltransferase ST3Gal II (α2,3-(O)-sialyltransferase) as described under “Experimental Procedures.” The sialylated product (590 nmol) was isolated by gel filtration and anion exchange HPLC in pure form. In negative ion mode MALDI-TOF mass spectrometry, the purified product gave a peak at m/z 1200.44, which was assigned to [M – H]⁻ of Neu5Ac[GalNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc] (calculated monoisotopic m/z 1200.42) (Fig. 2a).

In contrast to ST3Gal II, rat recombinant ST3Gal III (α2,3-(N)-sialyltransferase) sialylated less than 0.5% of the X₂ pentasaccharide when incubated under conditions that completely sialylated Galβ1-4GlcNAc (data not shown).

N-Acetyhexosaminidase Did Not Cleave Sialyl X₂—Sialyl X₂ was incubated with jack bean β-N-acetyhexosaminidase under conditions that completely released the terminal N-acetylgalactosamine from non-sialylated X₂. No cleavage product was seen.
in a MALDI-TOF mass spectrum of the desalted reaction mixture of sialyl X₂ (Fig. 3a). The data suggest that the sialic acid is linked to the terminal N-acetylgalactosamine.

Endo-β-galactosidases from B. fragilis and E. freundii Cleaved Sialyl X₂ at Two Sites—The X₂ lipid-linked pentasaccharide is known to be cleaved at both internal galactosidic linkages by endo-β-galactosidase from E. freundii, yielding GalNAcβ1–3Gal, GlcNAcβ1–3Gal, and Glc (2, 34). In the present experiments, both B. fragilis and E. freundii endo-β-galactosidases also cleaved unconjugated sialyl X₂ hexasaccharide completely at two sites. MALDI-TOF mass spectrometry of the desalted digest revealed a sialic acid-containing trisaccharide of the composition Neu5AcHexNAcHex₃, and a neutral disaccharide of the composition HexNAcHex₂ (Fig. 3b). Additionally, Glc was probably formed in the reaction, but it could not be identified in the spectrum among the matrix peaks. In view of the NMR data described below, the oligosaccharide products were identified as Neu5Acα2–3GalNAcβ1–3Gal and GlcNAcβ1–3Gal (Fig. 4).

**NMR Spectroscopy of Sialyl X₂ Hexasaccharide**—The ¹H and ¹³C signals of sialyl X₂ were assigned from the one-dimensional and from DQFCOSY, TOCSY, HSQC, HMQC, and HMBC spectra (Tables I–III).

Some features of the structural reporter group area in the...
one-dimensional proton spectrum are significant for the structure of the glycosidic bonds of sialic acids, including the Neu5Ac2–6GalNAc bond (37–39). By contrast, less than 10% of sialyl X₂ was desialylated by the sialidases from Newcastle disease virus (Fig. 8) and S. pneumoniae, which are able to cleave the Neu5Acα2–3Gal linkage, hydrolyzed poorly the Neu5Acα2–3GalNAc linkage.

**Sialylation of Globo-N-tetraose, GalNAcβ1–3Gala1–4Glc**—Unconjugated globo-N-tetraose GalNAcβ1–3Gala1–4Glcβ1–4Galβ1–4Glc (600 nmol) was incubated with CMP-Neu5Ac (2.4 μmol) and rat recombinant ST3Gal II as described under “Experimental Procedures.” The sialylated product (450 nmol) was isolated by gel filtration and anion exchange HPLC in pure form. In negative ion mode MALDI-TOF mass spectrometry, the purified product gave a peak at m/z 997.28, which was assigned to [M – H]⁻ of Neu5Ac;[GalNAcβ1–3Gala1–4Glcβ1–4Galβ1–4Glc] (calculated monoisotopic m/z 997.34) (Fig. 2b).

**NMR Spectroscopy of Sialylated Globo-N-tetraose**—The one-dimensional proton NMR spectrum (Fig. 5b), the TOCSY spectrum (Fig. 6b), and ¹H and ¹³C resonances (Tables I–III) show that the Neu5Ac and the GalNAc residues of sialyl globo-N-tetraose are virtually identical to their counterparts in sialyl X₂. This suggests that unconjugated globo-N-tetraose was sialylated by ST3Gal II in the same way as the X₂ pentasaccharide, at position 3 of the terminal GalNAc residue. This notion was confirmed by the downfield shift of GalNAc H3 of globo-N-tetraose (40, 22) that was caused by sialylation (Table I). The best proof of the presence of Neu5Acα2–3GalNAc linkage in the sialyl globo-N-tetraose was obtained from the HMBC spectrum (Fig. 7b). This spectrum shows a correlation between Neu5Ac FC2 and GalNAc EH3. The correlations EH1–DC3, DH1–BC4, and BH1–AC4 in the HMBC spectrum of sialyl globo-N-tetraose identified correctly the glycosidic linkages of the globo-
N-tetraose core of the sialylated product.

Sialylation of the Free Disaccharide GalNAcβ1–3Gal—The disaccharide GalNAcβ1–3Gal (600 nmol) was incubated with CMP-Neu5Ac (2.4 μmol) and rat recombinant ST3Gal II as described under “Experimental Procedures.” The sialylated product (400 nmol) was isolated by gel filtration and anion exchange HPLC in pure form. In negative ion mode MALDI-TOF mass spectrometry, the purified product gave a peak at m/z 673.06, which was assigned to [M – H] of Neu5Ac1GalNAcβ1–3Gal] (calculated monoisotopic m/z 673.23) (Fig. 2c).

NMR Spectroscopy of Sialylated GalNAcβ1–3Gal—The 1H and 13C signals of the sialylated GalNAcβ1–3Gal were fully assigned (Tables I–III). The structural reporter group resonances of the 1D proton spectrum of the sialylated GalNAcβ1–3Gal (Fig. 5c and Table I) resemble closely their counterparts in sialyl X2 and sialyl globo-N-tetraose. The TOCSY spectrum (Fig. 6c) reveals that the GalNAc spin system, too, is virtually identical with those of sialyl X2 and sialyl globo-N-tetraose. Finally, the HMBC spectrum (Fig. 7c) confirms the presence of Neu5Ac from the reducing end, several GalNAc protons resonate at different fields (Table I). The two signals belong to the α

Desialylation of sialyl X2 and a control oligosaccharide by different sialidases

| Oligosaccharide                                      | A. urogficusine sialidase | C. perfringens sialidase | Newcastle disease virus sialidase | S. pneumoniae sialidase |
|------------------------------------------------------|---------------------------|--------------------------|----------------------------------|-------------------------|
| Neu5Acα2–3GalNAcβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glc (sialyl X2) | 100%                      | 100%                     | <10%                             | <10%                    |
| Neu5Acα2–3Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc            | 100%                      | 100%                     | 100%                             | 100%                    |

Fig. 7. HMBC spectra of X2 sialylated by ST3Gal II (a), globo-N-tetraose sialylated by ST3Gal II (b), and the disaccharide GalNAcβ1–3Gal sialylated by ST3Gal II (c). The one-letter symbols of the monosaccharide residues are shown in Fig. 1.
(Gal Doα) and β (Gal Dβ) anomic forms of the oligosaccharide. In the HMBC spectrum (Fig. 7c), interglycosidic correlations between the GalNac E H1α and Gal Do C3, as well as between GalNac E H1β and Gal Dβ C3 are visible.

**DISCUSSION**

The present report describes α,2,3-sialylation of the distal GalNAc residue of the free X2 pentasaccharide GalNAcβ–3Galβ–1–4GlcNAcβ–3Galβ–1–4Glc, globo-N-tetraose GalNAcβ–3Gala1–4Galβ–1–4Glc, and the disaccharide GalNAcβ–3Gal by the recombinant sialyltransferase ST3Gal II, as shown in Fig. 1. These reactions represent the first successful enzymatic in vitro syntheses of the Neu5Ac2–3GalNAc linkage. The structure of the purified sialyl X2 hexasaccharide was characterized by its molecular mass as obtained from MALDI-TOF mass spectrometry experiments, by enzymatic degradations, and by one- and two-dimensional NMR spectroscopy. The presence of a Neu5Ac2–3GalNAc bond was firmly established by the HMBC spectrum, which revealed a long range correlation between Neu5Ac C2 and GalNAc H3. The sialylated products obtained from globo-N-tetraose and the disaccharide GalNAcβ–3Gal were characterized in the same way, but enzymatic degradations were not performed. Compared with previous reports on structural analysis of Neu5Ac2–3GalNAc determinants of naturally expressed glycans, the experiments of the present report are more NMR-oriented, and are not based on methylation analysis.

Our present data, showing that, in addition to the Galβ1–3GalNAc determinant (14), ST3Gal II also transfers to the isomeric GalNAcβ–3Gal determinant, establish that the enzyme represents one of the few glycosyltransferases that are capable of transferring to different monosaccharide residues in the acceptor, challenging the dogma "one glycosyltransferase, one glycosidic linkage" (41). The best known of these is β1,4-galactosyltransferase, which is induced by α-lactalbumin to transfer to glucose instead of N-acetylgalactosamine (20). Glycosyltransferases that transfer to different monosaccharide residues without requiring an additional modifier molecule include the β1,3-galactosyltransferase βGalT-V, which transfers to both the terminal GalNAc of GalNAcβ1–3Gala1–4Galβ1–4Glc and the terminal GlcNAc of GlcNAcβ1–3Gala1–4Glc (42), and the Core2GlcNAcTs, which transfer to the GalNAc of Galβ1–3Gala1–4GlcNAc-R and GlcNAcβ1–3Gala1–4GlcNAc1-R, as well as to the Gal of GlcNAcβ1–3Gala1–4GlcNAc1-R (43–45). A fourth example is the human fucosyltransferases III, V, and VI, which transfer to the Glc of lactose as well as to the GlcNAc of N-acetyllactosamine, generating Galβ1–4(Fucα1–3)Glc and Galβ1–4(Fucα1–3)GlcNAc, respectively (46–48). Finally, the bovine colostrum α,2,6-sialyltransferase has also been shown to tolerate N-acetylation of C2 of the acceptor monosaccharide; it sialylates both the Gal of Galβ1–4GlcNAc-R and the GalNAc of GalNAcβ1–4GlcNAc-R (49), suggesting an acceptor recognition mechanism similar to that of ST3Gal II discussed here. We suggest that ST3Gal II, and the other multifunctional glycosyltransferases, may bind their multiple acceptors by recognizing identical sets of saccharide atoms that belong to several monosaccharide residues, and form identical patterns.

The Neu5Ac2–3GalNAc linkage in the sialylated X2 hexasaccharide resisted cleavage by sialidases from Newcastle disease virus and S. pneumoniae. The Newcastle disease virus sialidase is known to exhibit strict specificity for hydrolysis of the Neu5Acα2–3Gal linkage under conditions that leave Neu5Acα2–6Gal and Neu5Acα2–6GalNAc bonds intact (50). Similar data have been reported for the sialidase from S. pneumoniae (manufacturer’s specifications). Neu5Ac-GalNAc linkages that resist the action of Newcastle disease virus sialidase have been regarded as Neu5Acα2–6GalNAc bonds (51–53). Our cleavage data show that this conclusion is not necessarily valid.

Globoside is expressed abundantly in human tissues. Therefore, it is remarkable that, although globo-N-tetraose is readily sialylated by ST3Gal II, as shown by the present experiments, sialyl globoside appears to be rare; its presence has been reported only in human embryonal carcinoma cells (5) and in muscles affected by amyotrophic lateral sclerosis (6). The reasons for the low expression levels of sialyl globoside are unknown, but association of globoside with other biomolecules than ST3Gal II, or low expression levels of ST3Gal II in cells expressing globoside may be involved.

α,2,3-Sialylation of the X2 structure may play a role in bacteria-host interactions. The X2 structure occurs in the lipooligosaccharide of N. gonorrhoeae strain F62 (10). Sialylation of lipo-oligosaccharide converts gonococci into serum resistance (reviewed in Ref. 54), possibly by camouflaging bacterial surface structures that resist the action of molecular mimicry of human cell surface glycoconjugates (55). The N. gonorrhoeae α2,3-sialyltransferase, Lst, has relaxed acceptor specificity; it is able to use N-acetyllactosamine, lactose, and globotriose (Galα1–4Galβ1–4Glc) as acceptors (56), and Lst from the strain 126E/L1 can even make both Neu5Acα2–3Gal and Neu5Acα2–6Gal linkages (57). To our knowledge it has not been tested whether the N. gonorrhoeae sialyltransferase uses X2-like structures as acceptors, but considering its relaxed acceptor specificity, it seems possible.

It has been suggested that the X2 epitope on intestinal epithelium is the human receptor for C. difficile toxin A. X2 glycosphingolipid has been shown to bind toxin A, but α2,3-sialylation of X2 abolishes the binding (9). Therefore, sialylation of X2-like structures might be a protective measure against adhesion, and thus internalization and cytotoxic effects of C. difficile toxin A.

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