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Negative-ion Electrospray Tandem Mass Spectrometry and Microarray Analyses of Developmentally-regulated Antigens Based on Type 1 and Type 2 Backbone Sequences

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Abstract

Type 1 (Gal β 1-3GlcNAc) and type 2 (Gal β 1-4GlcNAc) sequences are constituents of the backbones of a large family of glycans of glycoproteins and glycolipids whose branching and peripheral substitutions are developmentally-regulated. It is highly desirable to have micro-sequencing methods that can be used to precisely identify and monitor these oligosaccharide sequences with high sensitivity. Negative-ion electrospray tandem mass spectrometry with collision-induced dissociation has been used for characterization of branching points, peripheral substitutions and partial assignment of linkages in reducing oligosaccharides. We now extend this method to characterizing entire sequences of linear type 1 and type 2 chain-based glycans, focusing on the type 1 and -2 units in the internal regions including the linkages connecting type 1 and type 2 disaccharide units. We apply the principles to sequence analysis of closely related isomeric oligosaccharides and demonstrate by microarray analyses distinct binding activities of antibodies and a lectin toward various combinations of type 1 and 2 units joined by 1,3- and 1,6-linkages. These sequence-specific carbohydrate-binding proteins are in turn valuable tools for detecting and distinguishing the type 1 and type 2-based developmentally-regulated glycan sequences.

Abstract

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ASSOCIATED CONTENT

Supporting Information

The supporting information is available as noted in the text.

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INTRODUCTION

Two types of glycan backbones, the so-called type 1 (Gal β 1-3GlcNAc) and type 2 (Gal β 1-4GlcNAc) sequences, are common disaccharide units that occur on *N*- and *O*-glycans, glycolipids, and secreted free oligosaccharides. Type 2 units connected to each other through β 1,3-linkage constitute linear poly-*N*-acetylglucosamine chains (poly-LacNAc). Glycans of this type can be branched at Gal residues by addition of β 1,6-linked *N*-acetylglucosamine to form branched poly-LacNAcs. In contrast, tandem repeats and branching of type 1 chains are less common, although type 1 units can occur at the non-reducing ends of chains of poly-LacNAc type. Repeated type 1 chains have been detected mainly on glycosylceramides of human meconium, small intestine and colonic adenocarcinoma cell line Colo205. Here we refer to glycans of this type as poly-LacNAcs irrespective of their content of type 1 or type 2 units or the presence of reducing terminal lactose (Gal β 1-4Glc) instead of Gal β 1-4GlcNAc.

Type 1 and type 2-terminating backbones may be capped with sialic acid, fucose, galactose, *N*-acetylglucosamine, and sulfate group, and these constitute many antigenically or biologically active carbohydrate determinants. The poly-LacNAc backbones *per se* are implicated in biological and pathological processes such as development, differentiation, immune responses and cancer metastasis through interactions with endogenous carbohydrate-binding proteins such as galectins.

Type 1 and type 2-based sequences have long been known to be differentiation antigens of murine and human cells, as detected by natural and hybridoma derived monoclonal antibodies (mAbs). The linear and branched poly-LacNAc backbones, recognized by anti-i and anti-I antibodies, are prominently expressed on human fetal and adult erythrocytes, respectively. Changes also occur in the branching patterns of poly-LacNAc chains during the stages of embryogenesis. The type 1-terminating tetrasaccharide sequence Gal β 1-3GlcNAc β 1-3Gal β 1-4GlcNAc/Glc recognized by a mAb Fc10.2 was found to be a marker of human fetal endoderm. More recently, the same sequence has been suggested to be the glycan epitope recognized by two mAbs Tra-1-60 and Tra-1-81 that are widely used to assess pluripotency of human embryonic stem cells and induced pluripotent stem cells.

Evaluation of the occurrence and distribution of variant forms of type 1 and type 2-based sequences on cells and tissues is challenging. This is due to the lack of reliable and highly sensitive microscale methods to determine precisely the sequences of oligosaccharide chains isolated from complex mixtures. Current methods rely on a combination of analytical approaches such as mass spectrometry (MS), methylation analysis, glycosidase digestion, and immunochemical detection using mAbs and lectins with defined specificities. Although NMR can be used to determine complete sequences, the amounts of glycans typically required for analysis (hundreds of micrograms) preclude its use in most cases. Recent development in mass spectrometry has opened up new possibilities to elucidate these complex sequences. Electrospray ionization tandem MS with collision-induced dissociation (ESI-CID-MS/MS) has been exploited successfully in oligosaccharide sequence analysis. In negative-ion mode, acidic oligosaccharides containing sialic acid, sulfate and carboxyl group give abundant fragment ions that can be used for sequence assignment. Neutral

oligosaccharides can also be analyzed in negative-ion mode ESI-CID-MS/MS with sufficient sensitivity without the requirement of prior derivatization. The fragmentation pattern can be used for differentiation of the peripheral type 1 and type 2 units, different fucosylation patterns, and partial assignment of linkages⁻. In addition, combinations of MS/MS of singly and doubly charged molecular ions readily afford information on branching pattern⁻. Recently, this method has been extended to blood-group typing and mapping of glucan oligosaccharides isolated from various sources of plant, fungal and bacterial origins.

The characteristic fragmentation patterns of the internal domains of backbones have not yet been described. We now evaluate the negative-ion ESI-CID-MS/MS method in sequence determination on the internal type 1 and type 2 units and also the β 1,3- or β 1,6-linkages joining together the type 1 and type 2 disaccharide units (referred to as 'linkers' in this paper). We demonstrate by microarray analysis the distinct antigenic activities conferred by these isomeric sequences using two sequence-specific human mAbs anti-I Ma, anti-i P1A ELL, a hybridoma derived mAb Fc10.2, and a plant lectin *Ricinus communis* agglutinin I (RCA-120).

EXPERIMENTAL SECTION

Oligosaccharides and Neoglycolipid Probes

Oligosaccharides sequences investigated are in Table 1. The sources of the oligosaccharides and the preparation of the neoglycolipid (NGL) probes derived from them are described in Support Information.

Antibodies and Plant Lectin

The human sera containing anti-I and anti-i IgM autoantibodies anti-I Ma and anti-i P1A ELL, not described previously, and the mouse monoclonal antibody Fc10.2 were from the collection in Glycosciences Laboratory. The specificities of anti-I Ma and Fc10.2 had been assigned on the basis of inhibition of binding assays. Anti-I Ma binding was inhibited by the trisaccharide sequence Gal β 1-4GlcNAc β 1-6Gal- on a branched backbone, whereas binding by mAb Fc10.2 was inhibited by the tetrasaccharide LNT: Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc. Biotinylated anti-human IgM (μ -chain specific) and anti-mouse IgM were from Vector Laboratories (Cambridge, UK) and Sigma (Dorset, UK), respectively. Biotinylated RCA-120 was from Vector Laboratories.

Electrospray Mass Spectrometry

Negative-ion ESI-MS and CID-MS/MS were carried out on a Synapt G2-S instrument (Waters, Manchester, UK). Cone voltage was generally kept at 80 eV for MS and CID-MS/MS. In some cases for quasi-MS to encourage in-source fragmentation the cone voltage was increased to 200 eV. The source temperature was at 120 °C and the desolvation temperature at 150 °C. Product-ion spectra were obtained using argon as the collision gas at a pressure of 7.3×10^{-3} mbar. The collision energy was adjusted between 7 and 33 eV for optimal fragmentation; detailed specific voltages used for individual samples are listed in Supplemental Table S1. A scan rate of 1.0 s/scan was used for both ESI-MS and MS/MS experiments and the acquired spectra were summed for presentation. For analysis,

oligosaccharides were dissolved in H₂O at a concentration of ~10 pmol/μl, of which 1 μl was injected via a loop-injector by a syringe. Solvent (ACN/H₂O 1:1) was delivered by a peristaltic pump at a flow rate of 10 μl/min.

Carbohydrate Microarray Analysis

The DH- and AO-NGLs derived from the 11 Glc-terminating oligosaccharides were used for microarray construction. The NGLs were robotically arrayed in duplicate at concentrations of 2 and 5 fmol/spot on nitrocellulose-coated glass slides (Sartorius Stedim, Epsom, U.K.) using a non-contact arrayer (Gesim, Germany). Fluorescent dye Cy3 (GE Healthcare) was included for quality control of the arraying process and for localization of the arrayed spots.

Microarray analyses were performed essentially as described. In brief, the slides were overlaid with 3% bovine serum albumin (Sigma) in HEPES-buffered saline (HBS, 5 mM HEPES pH 7.4, 150 mM NaCl, 5 mM CaCl₂). After brief washing with HBS, the arrayed slides were probed with anti-I Ma or anti-i P1A ELL sera at 1:100 dilution, or with mAb Fc10.2 culture supernatant undiluted. MAb binding was detected using biotinylated anti-human IgM or biotinylated anti-mouse IgM (1:200) followed by an Alexa Fluor-647-labelled streptavidin (Molecular Probes, 1 μg/ml). Binding by the biotinylated RCA-120 was examined at 5 μg/ml followed by Alexa Fluor-647-labelled streptavidin directly. The analyses with anti-I Ma and anti-i P1A ELL were performed at 4°C. The other analyses were at ambient temperature. Imaging and data analysis was as described¹. Binding signals were probe dose-dependent.

RESULTS

Assignment of Type 1 and Type 2 Units in Linear Poly-LacNAc Sequences

The diagnostic fragmentations of type 1 and type 2 units at non-reducing termini have been established. Using a panel of tetra- to octasaccharides composed of 'hybrid' type 1 and type 2 disaccharide units (Table 1), we investigated whether the characteristic fragmentation patterns for these disaccharide units can be employed for their location at internal and reducing terminal positions in order to assign the entire oligosaccharide backbone sequences.

The non-reducing terminal type 2 unit of LNnT is characterized by the ion doublet: ^{0,2}A and the associated dehydrated ion at *m/z* 263 and 281, respectively (thereafter defined as ^{0,2}A-doublet) whereas the type 1 counterpart LNT is indicated by the unique D-ion (D₁₋₂ at *m/z* 202) (Supplemental Fig S1). Here using two isomeric GlcNAc-terminating tetrasaccharides, LN-Tetra and LN-Tetra-b (Table 1), as examples, the different chain types at the reducing termini could also be unambiguously assigned. In the spectrum of LN-Tetra (Figure 1a), apart from the full set of glycosidic C ions (C₁ to C₃) defining the linear sequence, the characteristic ^{0,2}A doublet (^{0,2}A₄ at *m/z* 628/646) dominated the product-ion spectrum indicative of the reducing terminal 1,4-linked GlcNAc, hence a type 2 chain. LN-Tetra-b contains type 1 chains at both termini and this is clearly shown by the lack of any A-type ions (Figure 1b). In addition, the unique D-ion at *m/z* 202 is apparent. This ion was derived from double cleavage of the non-reducing (D₁₋₂) and reducing terminal (D₃₋₄) 3-linked

GlcNAc. Importantly, a further D-ion at m/z 142 was also identified accompanying the ion m/z 202, produced by loss of further two carbons at the saccharide ring in the form of $-2\text{CH}_2\text{O}$ through multiple cleavages (indicated by the wavy line in the structure of Figure 1b). This satellite ion with 60 mass units lower than the typical D-ion in linear sequence has not been identified previously and this ion pair at m/z 202/142 is useful for reliable assignment of a type 1 chain. Thus, in the internal and reducing regions, the presence of the $^{0,2}\text{A}$ -doublet indicates a type 2 unit, whereas the lack of this characteristic ion pair and the presence of the D-ion pair at m/z 202/142 are suggestive of a type 1 unit.

For the extended tandem type 2 sequences of hexa- and octasaccharides LN-Hexa and LN-Octa (Table 1), the three and four repeating type 2 chains can be unambiguously identified at each positions in the product-ion spectra. As shown in Figure 2a, the positions of the type 2 units at non-reducing, internal and reducing positions can be determined by the A-type ions $^{0,2}\text{A}_2$ (m/z 263/281), $^{0,2}\text{A}_4$ (m/z 628/646), $^{0,2}\text{A}_6$ (m/z 993/1011). A single ion $^{2,4}\text{A}_6$ at m/z 951 is also observed at the reducing end GlcNAc residue. LN-Octa (Table 1, Figure 2b) contains a further type 2 unit and thus produced an additional $^{0,2}\text{A}$ -doublet ($^{0,2}\text{A}_8$ at m/z 1358/1376). The $^{2,4}\text{A}_8$ at m/z 1316 is consistent with the reducing terminal fragmentation observed in the spectrum of LN-Hexa (Figure 2a). The octasaccharide LNO (Table 1) with a reducing terminal Glc instead of GlcNAc showed a similar fragmentation feature (Figure 2c) as LN-Octa: the three consecutive type 2 chains can be identified at the non-reducing side and a 4-linked Glc at the reducing end. The fragment ions of the longer chain oligosaccharides, e.g. LN-Octa and LNN-O, are weak or absent in the lower mass regions and thus insufficient to make unambiguous assignment for the non-reducing terminal sequences. Further product-ion scanning using C-ions as the precursors (e.g. quasi MS m/z 909) gave fragmentation information on the chain type on the non-reducing side of these oligosaccharides (lower panels of Figure 2b and 2c, respectively).

Assignment of the 'Linker' between the Type 1 and Type 2 Units

Repeating Type 2 units joined together by β 1,3-linkage (-GlcNAc β 1-3Gal-) form linear poly-LacNAc backbone sequences of the i antigen type. Hybrid chains with type 1 and type 2 units are also typically linked by the β 1,3-glycosidic bond. Other linkages, e.g. β 1,6-, generally occur at branching points of poly-LacNAc chains but instances of β 1,6-glycosidic bond have been described as part of an unbranched sequence. We next investigated the fragmentation patterns of the β 1-3 and β 1-6 linkers connecting the type 1 or type 2 disaccharide units. Four synthetic trisaccharides Orsay-1, -2, -3 and -4 with various combinations of type 1 and type 2 units and β 1-3 and β 1-6 linkers (Table 1) were analyzed (Figure 3). Here also, for the type 1 and type 2 units, spectra of Orsay-4 (Figure 3a) and Orsay-2 (Figure 3b) afforded the characteristic D-type ion pair and $^{0,2}\text{A}$ -doublet at m/z 202/142 and 263/281, respectively. No fragment ions were observed from the common reducing terminal 3-linked Gal. In contrast, the other two isomers, Orsay-3 and Orsay-1, both with reducing terminal β 1,6-linked Gal share the same set of ions at m/z 424, 454 and 484 (Figure 4c and 4d), derived from A-type cross-ring cleavage $^{0,4}\text{A}_3$, $^{0,3}\text{A}_3$ and $^{0,2}\text{A}_3$, respectively. This set is absent in the spectra of the two isomers with β 1,3-linked Gal residue. The data above indicated that $^{0,4}\text{A}$, $^{0,3}\text{A}$ and $^{0,2}\text{A}$ ion set can be used for identifying a 1-6 linker while the absence of these ions indicates a 1-3 linker.

Characterization of Isomeric Poly-LacNAc Sequences

Having established principles for assignment of the type 1 and type 2 units in an entire oligosaccharide sequence and of the linkages between these disaccharide units, we evaluated their use for sequence determination of four isomeric hexasaccharides pLNH, pLNnH, pLNH-b and GSC-915-4 (Table 1). The product-ion spectra of pLNH (Figure 4a) and pLNnH (Figure 4b) are almost identical except for the D-ion pair at m/z 202/142 in pLNH and the $^{0,2}A_2$ -doublet at m/z 263/281 in pLNnH, consistent with the non-reducing end type 1 unit in pLNH and type 2 unit in pLNnH. In both spectra $^{0,2}A_4$ and $^{0,2}A_6$ are indicative of the internal and reducing terminal type 2 units. The MS/MS spectrum of pLNH-b (Figure 4c) is distinct from that of pLNH in the lack of $^{0,2}A_4$ -doublet at m/z 628/646 and more intense D-ion pair m/z 202/142, indicating the presence of an internal type 1 unit. None of the three spectra (Figure 4a, b, and c for pLNH, pLNnH and pLNH-b, respectively) gave additional diagnostic ions between C_2 and C_3 , and between C_4 and C_5 , consistent with the presence of the 1-3 linker in both places in all three oligosaccharides. In contrast, the spectrum of GSC-915-4 contained a set of A type ions $^{0,4}A_3$, $^{0,3}A_3$ and $^{0,2}A_3$ at m/z 424, 454 and 484, respectively (Figure 4d). This ion set is suggestive of a 1-6 linker in GSC-915-4. Thus, the four isomeric glycans can be differentiated and sequenced unambiguously using the distinct fragment patterns established.

Distinct Binding Signals Elicited by Isomeric Poly-LacNAc Sequences in Microarray Analyses of Monoclonal Antibodies and a Plant Lectin

We then used three antibodies directed at developmentally regulated antigens and the plant lectin RCA-120 to demonstrate the different binding patterns with the isomeric poly-LacNAc glycans characterized, and arrayed as NGL probes (Supplemental Table S2).

When the microarray was probed with anti-I Ma, only Orsay-1 and GSC-915-4 were strongly bound (Figure 5a and Supplemental Table S2). Both glycans contain a peripheral type 2 unit with a β 1-6 linker. The trisaccharide Orsay-3 with a peripheral type 1 unit and a β 1-6 linker was not bound by anti-I Ma. The hexasaccharide pLNnH which differs from GSC-915-4 in having a β 1-3 linker instead of a β 1-6 linker between the two type 2 units was also not bound. Removal of the terminal Gal from GSC-915-4 abolished the binding of anti-I Ma (data will be described elsewhere). Thus the non-reducing terminal type 2 unit and the adjoining β 1-6 linkage to Gal constitutes the recognition motif for anti-I Ma and the minimum sequence required for binding is a trisaccharide.

Linear and longer poly-LacNAc sequences consisting of type 2 units have been shown previously to inhibit the binding of several anti-i antibodies. The anti-i antibody P1A ELL showed selective binding to the hexasaccharide pLNnH and the octasaccharide LNnO (Figure 5b; Supplemental Table S2). These two glycans are composed of repeating type 2 units joined by β 1-3 linker. LNnO, with four repeating type 2 units, was more strongly bound by anti-i P1A ELL than pLNnH with three type 2 units. Other analogs shorter than hexasaccharide were not bound. Interestingly, the hexasaccharide GSC-915-4 which contains three repeating type 2 units (including the reducing-terminal Gal β 1-4Glc) as in pLNnH but with a β 1,6 rather than β 1-3 linker elicited no binding signals with P1A ELL. The hexasaccharides pLNH and pLNH-b with β 1-3 linkers but terminating in type 1 unit

were not bound. Collectively these results show that the minimum sequence that anti-i P1A ELL binds is a linear poly-LacNAc composed of three type 2 units joined by two β 1-3 linkers, where the reducing terminal disaccharide unit may be lactose.

In the microarray analysis, mAb Fc10.2 showed binding restricted to pLNH (Figure 5c) which is a hybrid poly-LacNAc with a type 1 unit at the non-reducing terminus (Supplemental Table S2). No binding was detected to the type 2-terminating analogues, pLNnH and GSC-915-4. Importantly, there was also no binding to pLNH-b in which two repeated type 1 units are present at the non-reducing terminus. This shows the essential requirement by mAb Fc10.2 of a type 2 unit adjoining the non-reducing end type 1 unit. It should be noted that although the DH-NGL derivative of LNT is not bound by mAb Fc10.2, binding was detected previously (by chromatogram-binding assay) to the glycolipid Gal β -3GlcNAc β -3Gal β -4Glc-Cer which contains the same carbohydrate sequence as LNT but linked to ceramide. The lack of binding of mAb Fc10.2 to the DH-NGL can be attributed to the ring-opening of the terminal Glc in the NGL generated by reductive amination. In sum, these results show that the minimum sequence bound by mAb Fc10.2 is tetrasaccharide having type 1 unit at the non-reducing end linked to a type 2 or lactose disaccharide by a β 1-3 linker, as in Gal β -3GlcNAc β -3Gal β -4GlcNAc or Gal β -3GlcNAc β -3Gal β -4Glc, respectively.

RCA-120 is known to preferentially bind to peripheral type 2 unit but can also weakly bind to terminal type 1 unit. As predicted, the strongest binding was to NGL probes terminating with type 2 units, Orsay-1, -2, LNnT, GSC-915-4, pLNnH and LNnO were all strongly bound (Figure 5d; Supplemental Table S2). Those with a type 1 unit at the non-reducing end: Orsay-4, -3, LNT and pLNH-b were only weakly bound. It is deduced that a type 2 unit located at an internal position along a glycan chain, as in the case of pLNH, is not recognized by RCA-120 as only negligible binding was observed for pLNH. The lectin could bind to the probes with a type 2 unit at their non-reducing ends irrespective of whether they were joined by a β 1-3 or a β 1-6 linker to an adjoining disaccharide unit as in pLNnH and GSC-915-4, respectively.

DISCUSSION

In the present study we elaborate on the power and complementarity of negative-ion ESI-CID-MS/MS and oligosaccharide microarrays in carbohydrate sequencing and assignments of specificities of carbohydrate-protein interactions. Both methods require only minute amounts (low picomole level) of carbohydrates materials.

We have identified fragmentation patterns of the internal and reducing terminal type 1 and type 2 disaccharide units and of the β 1-3 or β 1-6 linkers connecting these units. As observed in earlier studies, these unique fragmentations appear only in negative-ion mode of ESI-CID-MS/MS and are restricted to reducing oligosaccharides which contain the terminal hemiacetal functionality. Under the established MS conditions, only a single glycosidic cleavage occurs and the product-ion spectra present a full set of C-type ions for sequence determination and various A- and D- type fragments which are very useful for linkage assignment. This rapid, highly sensitive method offers an additional dimension to metadata-

assisted sequencing of bioactive glycans, which combines lectin-binding, glycosidase treatment and MALDI-TOF-MSⁿ using permethylated glycans.

To facilitate the sequence assignment of oligosaccharides by ESI-CID-MS/MS, the characteristic fragmentation patterns observed so far for the commonly encountered monosaccharide residues in linear poly-LacNAc sequences can be summarized (Supplemental Scheme S1). The neutral loss between two adjacent C-type ions indicates the identity of the residue in terms of Hex and HexNAc. A set of diagnostic A- and D-type fragments can be used to differentiate some specific linkages. The diagnostic fragment pattern and formulas for calculation of the m/z value of the particular fragment ions are given and discussed in detail in Supplemental Discussion. Particularly relevant to the study shown here are: the D-ion pair, the ^{0,2}A-doublet and the characteristic ion cluster ^{0,4}A, ^{0,3}A and ^{0,2}A indicating the commonly occurring 1-3,1-4 and 1-6 linkages, respectively.

We have also demonstrated here that various combinations of type 1 and type 2 units and the β 1-3 and β 1-6 linkers can be robustly distinguished with the three mAbs and the lectin. The distinctive features can be summarised as follows. Glycans terminating in type 2 unit, whether joined by a β 1-3 or β 1-6 linker, can be bound by RCA-120. Type 2 units with a β 1-6 linker (Gal β 1-4GlcNAc β 1-6Gal-) constitute the antigenic determinant for anti-I Ma. Glycan sequences longer than three type 2 units linked by β 1-3 linker (Gal β 1-4GlcNAc β 1-3Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc/GlcNAc-) are recognized by anti-i P1A ELL. These specificities are distinct from that of mAb Fc10.2 which prefers a non-reducing end type 1 unit with an adjoining type 2 unit joined by a β 1-3 linker (Gal β 1-3GlcNAc β 1-3Gal β 1-4Glc/GlcNAc).

Previously, antibodies of the I/i type used in this study have proven to be invaluable in monitoring changes that occur in the backbones and branching patterns of carbohydrates in the course of embryonic development, cell differentiation and malignancy. Now that the recognition sequences of these antibodies and lectin have been elucidated in greater detail, they can be more confidently used in analysis of mixtures of poly-LacNAc chains as well as detecting their presence on whole cells at different stages of differentiation. Results of the negative-ion ESI-CID-MS/MS and NGL-based microarrays jointly applied as in the present study show that these methods can be confidently used for the microscale sequencing of, diverse isomeric forms of poly-LacNAc chains.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Negative-ion ESI-CID-MS/MS product-ion spectra of LN-Tetra (a) and LN-Tetra-b (b)
The proposed fragmentation patterns are illustrated based on the simplified structures in which the OH group at the end of each chemical bond is not shown.

Figure 2. Negative-ion ESI-CID-MS/MS product-ion spectra of LN-Hexa (a), LN-Octa (b), and LNnO (c)

For LN-Octa and LNnO, quasi MS were performed using the C type ion at m/z 909 as precursor to reveal the fragmentation patterns between m/z 100 and 900. The spectra are shown as lower panels. The proposed fragmentation patterns are illustrated based on the simplified structures in which the OH group at the end of each chemical bond is not shown.

Figure 3. Negative-ion ESI-CID-MS/MS product-ion spectra of four isomeric trisaccharides Orsay-4 (a), -2 (b), -3 (c) and -1 (d)

The proposed fragmentation patterns are illustrated based on the simplified structures in which the OH group at the end of each chemical bond is not shown.

Figure 4. Negative-ion ESI-CID-MS/MS product-ion spectra of four isomeric hexasaccharides pLNH (a), pLNnH (b), pLNH-b (c) and GSC-915-4 (d)

The proposed fragmentation patterns are illustrated based on the simplified structures in which the OH group at the end of each chemical bond is not shown.

Figure 5. Microarray analysis of three monoclonal antibodies that recognize developmentally regulate antigens anti-I Ma (a), anti-i P1A ELL (b) and Fc10.2 (c) and a plant lectin RCA-120 (d) The results are the means of fluorescence intensities of duplicate spots, printed at 2 fmol (blue) or 5 fmol (red) with error bars representing half of the difference between the two values. The carbohydrate sequences of the probes and the binding intensities are given in Supplemental Table S1.

Table 1
Oligosaccharides used in the negative-ion ESI-CID-MS/MS sequence analysis

Designation	Oligosaccharide sequences
LNT	Gal β -3GlcNAc β -3Gal β -4Glc
LNnT	Gal β -4GlcNAc β -3Gal β -4Glc
LN-Tetra-b	Gal β -3GlcNAc β -3Gal β -3GlcNAc
LN-Tetra	Gal β -4GlcNAc β -3Gal β -4GlcNAc
LN-Hexa	Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc
LNnO	Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4Glc
LN-Octa	Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4GlcNAc
Orsay-4	Gal β -3GlcNAc β -3Gal
Orsay-2	Gal β -4GlcNAc β -3Gal
Orsay-3	Gal β -3GlcNAc β -6Gal
Orsay-1	Gal β -4GlcNAc β -6Gal
pLNH	Gal β -3GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4Glc
pLNnH	Gal β -4GlcNAc β -3Gal β -4GlcNAc β -3Gal β -4Glc
pLNH-b	Gal β -3GlcNAc β -3Gal β -3GlcNAc β -3Gal β -4Glc
GSC-915-4	Gal β -4GlcNAc β -6Gal β -4GlcNAc β -3Gal β -4Glc