Unusual N-Glycan Structures in \(\alpha\)-Mannosidase II/IIX Double Null Embryos Identified by a Systematic Glycomics Approach Based on Two-dimensional LC Mapping and Matrix-dependent Selective Fragmentation Method in MALDI-TOF/TOF Mass Spectrometry*

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\(\alpha\)-Mannosidase IIX (MX) is an enzyme closely related to \(\alpha\)-mannosidase II (MII), a key enzyme in N-glycan biosynthesis that catalyzes the first step in conversion of hybrid- to complex-type N-glycans in Golgi apparatus. Recently, we generated MII/MX double knock-out mice and found that double nulls completely lack the complex-type N-glycans (Akama, T. O., Nakagawa, H., Wong, N. K., Sutton-Smith, M., Dell, A., Morris, H. R., Nakayama, J., Nishimura, S.-I., Pai, A., Moremen, K. W., Marth, J. D., and Fukuda, M. N. (2008) Essential and mutually compensatory roles of \(\alpha\)-mannosidase II and \(\alpha\)-mannosidase IIX in N-glycan processing in vivo in mice. Proc. Natl. Acad. Sci. U. S. A. 103, 8983–8988). In the present study, we determined minor but unusual N-glycan structures found in MII/MX double knock-out mice. We identified such N-glycans by a systematic glycomics approach applying a two-dimensional LC mapping database and matrix-dependent selective fragmentation technique in MALDI-TOF/TOF MS, a highly sensitive and reliable technique that provides specific fragmentations enabling the determination of precise oligosaccharide structures including regioisomers (Kurogochi, M., and Nishimura, S.-I. (2004) Structural characterization of N-glycopeptides by matrix-dependent selective fragmentation of MALDI-TOF/TOF tandem mass spectrometry. Anal. Chem. 76, 6097–6101). Quantitative profiling of all N-glycan structures including minor components from MII/MX nulls, MII nulls, MX nulls, and wild-type mice at embryonic day 15.5 yielded a total of 37 species when structural heterogeneity was reduced by the removal of the sialic acids. Among six unusual N-glycan structures, two glycoforms were novel and were found only in MII/MX double nulls. We characterize such structure as pseudocomplex-type N-glycans. The present study demonstrated that use of the versatile matrix-dependent selective fragmentation method in MALDI-TOF/TOF MS greatly accelerates detailed structural analysis of a trace amount of N-glycans. Molecular & Cellular Proteomics 5:2146–2157, 2006.

N-Glycosylation of proteins is one of the most ubiquitous post-translational modifications in eukaryotes and is thought to play an important role in development, differentiation, tumor metastasis, immunity, intracellular transport, cell adhesion, and aging (1). Insufficiency of human N-glycan biosynthesis leads to diseases such as congenital disorders of glycosylation (2) and hereditary erythroblastic multinuclearity with positive acidifies serum lysis test (3). N-Glycan biosynthesis involves five steps: (i) formation of a dolichol-linked oligosaccharide intermediate in the endoplasmic reticulum (ER), (ii) en bloc transfer of oligosaccharide Glc3Man9GlcNAc2 from dolichol to Asn residue in the consensus sequence of Asn-X-Ser/Thr on the nascent polypeptide chain by oligosaccharyltransferase that catalyzes the first step in conversion of hybrid- to complex-type N-glycans in Golgi apparatus. Recently, we generated MII/MX double knock-out mice and found that double nulls completely lack the complex-type N-glycans (Akama, T. O., Nakagawa, H., Wong, N. K., Sutton-Smith, M., Dell, A., Morris, H. R., Nakayama, J., Nishimura, S.-I., Pai, A., Moremen, K. W., Marth, J. D., and Fukuda, M. N. (2008) Essential and mutually compensatory roles of \(\alpha\)-mannosidase II and \(\alpha\)-mannosidase IIX in N-glycan processing in vivo in mice. Proc. Natl. Acad. Sci. U. S. A. 103, 8983–8988). In the present study, we determined minor but unusual N-glycan structures found in MII/MX double knock-out mice. We identified such N-glycans by a systematic glycomics approach applying a two-dimensional LC mapping database and matrix-dependent selective fragmentation technique in MALDI-TOF/TOF MS, a highly sensitive and reliable technique that provides specific fragmentations enabling the determination of precise oligosaccharide structures including regioisomers (Kurogochi, M., and Nishimura, S.-I. (2004) Structural characterization of N-glycopeptides by matrix-dependent selective fragmentation of MALDI-TOF/TOF tandem mass spectrometry. Anal. Chem. 76, 6097–6101). Quantitative profiling of all N-glycan structures including minor components from MII/MX nulls, MII nulls, MX nulls, and wild-type mice at embryonic day 15.5 yielded a total of 37 species when structural heterogeneity was reduced by the removal of the sialic acids. Among six unusual N-glycan structures, two glycoforms were novel and were found only in MII/MX double nulls. We characterize such structure as pseudocomplex-type N-glycans. The present study demonstrated that use of the versatile matrix-dependent selective fragmentation method in MALDI-TOF/TOF MS greatly accelerates detailed structural analysis of a trace amount of N-glycans. Molecular & Cellular Proteomics 5:2146–2157, 2006.

\(N\)-Glycosylation of proteins is one of the most ubiquitous post-translational modifications in eukaryotes and is thought to play an important role in development, differentiation, tumor metastasis, immunity, intracellular transport, cell adhesion, and aging (1). Insufficiency of human N-glycan biosynthesis leads to diseases such as congenital disorders of glycosylation (2) and hereditary erythroblastic multinuclearity with positive acidifies serum lysis test (3). N-Glycan biosynthesis involves five steps: (i) formation of a dolichol-linked oligosaccharide intermediate in the endoplasmic reticulum (ER). (ii) en bloc transfer of oligosaccharide Glc3Man9GlcNAc2 from dolichol to Asn residue in the consensus sequence of Asn-X-Ser/Thr on the nascent polypeptide chain by oligosaccharyltransferase that catalyzes the first step in conversion of hybrid- to complex-type N-glycans in Golgi apparatus.

1 The abbreviations used are: ER, endoplasmic reticulum; MII, \(\alpha\)-mannosidase II; MX, \(\alpha\)-mannosidase IIX; GnT, N-acetylgalactosaminyltransferase; LA, lactoalbumin; Man, mannose; Glc, glucose; Gal, galactose; Fuc, fucose; GlcNAc, N-acetylgalactosamine; GalNAc, N-acetyl-l-galactosamine; LacNAc, N-acetyllactosamine; Hex, hexose; HexNAc, N-acetylenoxosamine; GFC, gel filtration chromatography; PA, pyridylaminino; MDSF, matrix-dependent selective fragmentation; CHCA, \(\alpha\)-cyano-4-hydroxycinnamic acid; DHB, 2,5-dihydroxybenzoic acid; E, embryonic day; 2D, two-dimensional; ACTH, adrenocorticotropic hormone.
ferase, (iii) removal of Glc moieties from the oligosaccharide intermediate by \(\alpha\)-glucosidases I and II in the ER, (iv) further trimming of four Man residues by \(\alpha\)-mannosidase I in the Golgi apparatus, and (v) modification of the Man5GlcNAc2 structure by a series of glycosidases and glycosyltransferases. Consequently many diverse N-glycan structures arise from this key heptasaccharide intermediate. The earliest biosynthetic steps in all eukaryotic cells occur in the ER; however, some interspecies differences exist in the Golgi processing pathway depending on the kinds and amounts of expressed enzymes. N-Glycans are classified broadly into three categories based on the structures, which also reflect their biosynthetic pathway. High mannose types have only Man, complex types have an \(N\)-acetyllactosamine (LacNAc, \(\text{Gal}(1\rightarrow4)\text{GlcNAc}\)) unit included in some modification, and hybrid types have both Man and LacNAc components. In the biosynthetic pathway, N-glycans change in type from high mannose to hybrid and then to complex types sequentially.

The in vivo role of N-glycans has been evaluated using mouse mutants: the GnT-I null mutation leads to embryonic lethality (4), demonstrating that the high mannose-type N-glycans cannot support embryogenesis. GnT-II null embryos survive, but GnT-II nulls die within several weeks after birth, phenotypes attributable to congenital disorders of glycosylation type IIa (5). On the other hand, \(\alpha\)-mannosidase II (MII) null mice show mild phenotypes or dyserythropoiesis (6, 7). Structural analysis of N-glycans from MII null mouse tissues indicates the existence of an alternate pathway for detoxifying defective MII. \(\alpha\)-Mannosidase IIx (MX) is an enzyme closely related to MII as indicated in Fig. 1 (8, 9). Although MX null mice show no gross abnormalities, MX null males are infertile (10). MII/MX double knock-out mice show neonatal lethality. We have profiled major N-glycan structures from MII/MX double knock-out mice at embryonic day (E) 15.5 by means of a two-dimensional (2D) mapping based on a general approach using HPLC with pyridylamination (11, 12). The results of 2D LC mapping analysis and MALDI-TOF MS of the per-O-methylated N-glycan derivatives indicated that MII/MX double nulls do not synthesize the complex-type N-glycans (13).

In this study, we carried out extensive structural analysis of N-glycans from MII/MX double null embryos. Although their expression levels of unusual N-glycans were too low to be identified by 2D LC approach (11) or chemical/enzymatic modifications and MALDI-TOF MS, the MDSF technique of MALDI-TOF/TOF MS (14) enabled us to determine the trace amount of unknown oligosaccharides synthesized by MII/MX double nulls. The MDSF method gives highly sensitive and reliable product ion peaks suited for the efficient sequencing of highly complicated oligosaccharide structures as well as \(N\)/\(O\)-glycopeptides (14–17). Especially this technique allows for selective ionization/fragmentation of the target compound by means of two distinct matrices such as 2,5-dihydroxybenzoic acid (DHB) and \(\alpha\)-cyano-4-hydroxycinnamic acid (CHCA). It was demonstrated that TOF/TOF experiments from each precursor ion generated by DHB or CHCA provide highly informative fragmentation patterns dependent upon individual “excited electronic state” of the original precursor ion (14). In this method, it should be emphasized that two different excited electronic states derived from a target molecule by using DHB or CHCA afford completely different and characteristic fragmentation patterns. In general, fragmentation of a protonated precursor ion obtained in the presence of DHB causes exo-type degradation to give sequential information, whereas TOF/TOF of a sodiated ion generated in the presence of CHCA predominantly produces relatively large characteristic fragment ions by endo-type degradation suited for the identification of stable core structures. As a result, combined use of the two informative and different types of fragmentation patterns allows for the systematic structural characterization of common N-glycans on the basis of a versatile database for
mass spectral matching analysis. Here we report the feasibility of the MDSF method in the precise structural identification of these unknown and trace amounts of N-glycans synthesized in MII and/or MX knock-out mouse embryos.

**EXPERIMENTAL PROCEDURES**

**Materials**—Trypsin, sodium cyanoborohydride, and α-lactoalbumin were purchased from Sigma. Chymotrypsin and Pronase were purchased from Calbiochem. α-Mannosidase and β-galactosidase from jack bean were purchased from Seikagaku Co. (Tokyo, Japan). 2,5-Dihydroxybenzoic acid, α-cyano-4-hydroxycinnamic acid, human angiotensin II, bombesin, ACTH, and somatostatin 28 were purchased from Bruker Daltonics Gmbh (Bremen, Germany). Other materials were purchased from the sources indicated: Sephadex G-15, Amersham Biosciences; Bio-Gel P-4, Bio-Rad; peptide-N-glycosidase F, Roche Applied Science; β1,4-galactosyltransferase, Toyobo Co. Ltd. (Osaka, Japan); TSKgel Amide-80 (4.6 × 250 mm), Tosoh (Tokyo, Japan); ShimPack HRC-ODS (6.0 × 150 mm), Shimadzu (Kyoto, Japan); and Develosil UG-C30 (4.0 × 10 mm), Nomura Chemical Co. Ltd. (Seto, Japan). 2-Aminopyridine and other chemical reagents were obtained from Wako Pure Chemical Co. Ltd. (Osaka, Japan). UDP-N-acetylgalactosamine was a gift from Yamasa Corp. (Choshi, Japan).

**Generation of α-Mannosidase II/Ix Double Knock-out Animals**—Mice lacking a functional MII allele and MX allele (10) were mated, and resulting heterozygotes were then crossed to obtain double-null animals as described previously (13). Genotypic proportions of pups born from MII−/−MX+/+ parents were: MII−/−MX−/−:MII−/+MX−/+: MII−/−MX+/−:MII+/−MX−/+: MII+/−MX−/+; MII+/−MX+/+ = 20:47:20:35:33:15:33:1. Genotypic proportions of embryos (E15.5) produced from MII−/−MX+/+ parents were: MII−/−MX−/−:MII−/+MX−/+: MII−/−MX+/−:MII+/−MX−/+: MII+/−MX−/+ = 12:26:8, and those of E18.5 were 11:18:10, respectively.

**Preparation and HPLC Analysis of PA-derivatized N-glycans from Mouse Embryos**—PA-derivatized N-glycans from mouse embryos were prepared by the procedure reported previously (10). MII/MX double knock-out mice (n = 2), MII knock-out mice (n = 2), MX knock-out mice (n = 2), and wild-type (n = 1) mouse embryos (~10 mg/sample) were homogenized in 0.1 M ammonium acetate and digested with 0.1 μg each of trypsin (Sigma) and chymotrypsin (Calbiochem) at 37 °C for 16 h. After heating at 90 °C for 10 min to inactivate the enzymes, the residues were treated with 10 units of sodium cyanoborohydride (Sigma) at 90 °C for 16 h. After heating at 90 °C for 10 min to inactivate the enzymes, the residues were treated with 10 units of sodium cyanoborohydride (Sigma) at 90 °C for 10 min to inactivate the enzymes. The two-dimensional mapping analysis of PA-derivatized N-glycans was performed by using a fluorescence detector under an excitation at 320 nm and an emission at 400 nm, respectively.

**Two-dimensional Mapping Analysis Using HPLC of the PA-derivatized N-Glycans Isolated from Mouse Embryos**—The two-dimensional mapping technique established by Tomiya et al. (11) was used to identify the structures of the PA-derivatized N-glycans isolated from mouse embryos. Major N-glycan structures were determined by plotting the elution positions under two independent chromatography modes of HPLC analysis on the basis of a database consisting of about 480 kinds of N-glycan standards as determined by Takahashi et al. (11). First the mixture of the neutral PA-derivatized N-glycans was subjected to and analyzed on a reverse-phase column (Shim-Pack HRC-ODS, 6 × 150 mm, Shimadzu). Elution was performed at a flow rate of 1.0 ml/min at 55 °C using a linear gradient elution system. Solvent C was 10 mM sodium phosphate buffer (pH 3.8), and

![Fig. 2. Chromatograms of PA-derivatized N-glycans on ODS column. Analyses of PA-glycans released from mouse embryos were first performed on ODS columns. Each peak of glycans was fractionated and subsequently analyzed on an amide column. Peak labels correspond with Table I. Elution conditions were as follows: column, ShimPack HRC-ODS (6 × 150 mm); eluent, solvent C, 10 mM sodium phosphate buffer (pH 3.8); solvent D, 0.5% (v/v) 1-butanol in solvent C; gradient condition, a linear gradient from 20 to 50% solvent D in 60 min. KO, knock-out; DKO, double knock-out.](image-url)
Unusual \( \alpha \)-Mannosidase II/Lx Knock-out Mice

TABLE I

| Compound | GU | Mass measured | Composition | Structure code no. | Relative ratio [%] | Mass digestion sensitivity |
|----------|----|--------------|-------------|-------------------|---------------------|--------------------------|
| Peak A   | 4.9 | 9.0          | 1798        | 8 2 0             | M8.1                | 8.2                      | S                        |
| Peak B-1 | 5.1 | 8.1          | 1636        | 7 2 0             | M7.2                | 3.5                      | S                        |
| Peak B-2 | 5.2 | 9.7          | 1961        | 9 2 0             | M9.1                | 7.4                      | S                        |
| Peak C-2 | 5.8 | 8.0          | 1637        | 7 2 0             | M7.1                | 1.4                      | S                        |
| Peak C-3 | 6.4 | 8.5          | 1799        | 8 2 0             | M8.2                | 1.3                      | S                        |
| Peak D   | 6.8 | 7.7          | 1636        | 7 2 0             | M7.7                | 2.1                      | S                        |
| Peak E   | 6.1 | 7.1          | 1474        | 6 2 0             | M6.1                | 7.4                      | S                        |
| Peak F-2 | 6.3 | 10.3         | 2122        | 10 2 0            | M9.2                | 1.6                      | S                        |
| Peak G   | 7.2 | 6.2          | 1312        | 5 2 0             | M5.1                | 4.8                      | S                        |
| Peak H   | 8.0 | 7.4          | 1678        | 6 3 0             | H5.12               | 1.3                      | S                        |
| Peak I-1 | 8.0 | 6.4          | 1515        | 5 3 0             | M4.12               | 0.0                      | S                        |
| Peak I-2 | 7.8 | 6.5          | 1459        | 5 3 0             | M5.12               | 0.0                      | S                        |
| Peak J-1 | 9.8 | 6.8          | 1662        | 5 3 1             | M5.12               | 0.0                      | S                        |
| Peak J-2 | 9.8 | 7.4          | 1864        | 5 4 1             | Unknown             | 4.2                      | S                        |
| Peak K-1 | 10.2| 4.7          | 1134        | 3 2 1             | 010.1               | 1.3                      | S                        |
| Peak K-2 | 9.9 | 5.9          | 1864        | 5 4 1             | Unknown             | 0.0                      | S                        |
| Peak L-1 | 10.2| 7.0          | 1719        | 5 4 0             | 200.4               | 6.9                      | S                        |
| Peak L-2 | 10.6| 7.4          | 1865        | 5 4 1             | Unknown             | 0.0                      | S                        |
| Peak L-3 | 10.0| 7.8          | 1823        | 6 3 0             | 200.4               | 6.9                      | S                        |
| Peak M-1 | 10.2| 6.8          | 1661        | 5 3 1             | M5.12               | 0.0                      | S                        |
| Peak M-2 | 10.2| 8.7          | 1986        | 5 3 1             | M5.12               | 0.0                      | S                        |
| Peak M-3 | 11.0| 8.0          | 1659        | 7 3 1             | 200.4               | 6.9                      | S                        |
| Peak N   | 11.1| 9.0          | 1865        | 5 4 1             | Unknown             | 0.0                      | S                        |
| Peak O   | 11.8| 7.2          | 1865        | 5 4 1             | Unknown             | 0.0                      | S                        |
| Peak P   | 12.7| 8.2          | 2026        | 6 4 1             | Unknown             | 0.0                      | S                        |
| Peak Q-1 | 13.1| 6.3          | 1703        | 4 4 1             | 210.2               | 2.0                      | S                        |
| Peak Q-2 | 13.4| 7.7          | 1795        | 4 5 0             | Unknown             | 1.8                      | S                        |
| Peak R   | 13.4| 9.2          | 2188        | 7 4 1             | Unknown             | 0.0                      | S                        |
| Peak S   | 13.8| 8.9          | 2188        | 7 4 1             | Unknown             | 0.0                      | S                        |
| Peak T   | 14.1| 7.4          | 1864        | 5 4 1             | 210.4               | 14.3                     | S                        |
| Peak U   | 14.1| 8.1          | 2027        | 6 4 1             | 210.22              | 6.4                      | S                        |
| Peak V   | 14.5| 7.2          | 1922        | 5 5 0             | 201.4               | 1.3                      | S                        |
| Peak W   | 15.1| 8.0          | 2027        | 6 4 1             | Unknown             | 0.0                      | S                        |
| Peak X   | 17.8| 5.8          | 1743        | 3 5 1             | 211.1               | 1.8                      | S                        |
| Peak Y   | 17.7| 8.7          | 2230        | 6 5 1             | 310.8               | 0.0                      | S                        |
| Peak Z   | 18.7| 6.6          | 1906        | 4 5 1             | 211.2               | 3.0                      | S                        |
| Peak AA  | 20.2| 7.5          | 2068        | 5 5 1             | 211.4               | 14.3                     | S                        |

Digestion of PA-derivatized N-Glycans Using \( \alpha \)-Mannosidase and \( \beta \)-Galactosidase—PA-derivatized N-glycans isolated by two successive HPLC modes were subjected to digestion with 50 milliunits of \( \alpha \)-mannosidase (jack bean) (Seikagaku Kogyo Co., Tokyo, Japan) in 0.1 M sodium acetate (pH 5.0), 40 mM ZnCl\(_2\) (final volume, 20 \( \mu \)l) at 37°C for 15 h. In some cases, PA-derivatized N-glycans were digested with 5 milliunits of \( \beta \)-galactosidase from jack bean (Seikagaku Kogyo Co.) in 0.1 M citrate-phosphate buffer (pH 4.1) (final volume, 15 \( \mu \)l) at 37°C for 15 h.

MALDI-TOF/TOF MS of PA-derivatized N-Glycans Using the MDSF Method—Isolated PA-derivatized N-glycans were analyzed by MALDI-LIFT-TOF/TOF MS using MDSF, a method reported by Kuroguchi and Nishimura (14). Matrix solutions were prepared as follows: DHB (Bruker Daltonics) was dissolved in water at 10 mg/ml, and CHCA (Bruker Daltonics) was prepared as a saturated solution in 3:1 (v/v) of acetonitrile:water. Samples were dissolved in water, applied on the target spot of an AnchorchipTM plate (Bruker Daltonics), mixed with 1 \( \mu \)l of matrix solution, and dried at room temperature. Approximately a 1–2 pmol scale of the sample was used for the preparation of the test solution. PA-derivatized N-glycans were desalted by HPLC by using a Develosil UG-C30 column (4.0 \( \times \) 10 mm, Nomura Chem-
ical Co. Ltd.) at a flow rate of 0.2 ml/min at 25 °C. The elution was performed stepwise with water for 0–5 min and 20% methanol:water (v/v) for 5–20 min.

All measurements were performed using an Ultraflex TOF/TOF mass spectrometer equipped with a reflector and controlled by the FlexControl 2.2 software package (Bruker Daltonics). In MALDI-TOF MS reflector mode, ions generated by a pulsed UV laser beam (nitrogen laser, λ = 337 nm, 5 Hz) were accelerated to a kinetic energy of 23.5 kV. Metastable ions generated by laser-induced decomposition of the selected precursor ions were analyzed without any additional collision gas. In MALDI-LIFT-TOF/TOF mode, precursor ions were accelerated to 8 kV and selected in a timed ion gate. The fragments were further accelerated by 19 kV in the LIFT cell (LIFT means “lifting” the potential energy for the second acceleration of ion source), and their masses were analyzed after the ion reflector passage. Masses were automatically annotated by using the FlexAnalysis 2.2 software package. External calibration of MALDI mass spectra was carried out using singly charged monoisotopic peaks of a peptide mixture of human angiotensin II (m/z 1046.542), bombesin (m/z 1619.823), ACTH-(18–39) (m/z 2465.199), and somatostatin 28 (m/z 3147.472).

**Enzymatic Introduction of N-Acetyl-α-galactosamine into PA-deri-vatized N-Glycan Using β1,4-Galactosyltransferase in the Presence**

**Fig. 3. N-Glycan structures determined by two-dimensional mapping technique.** Peak numbers are indicated at left, while code numbers of N-glycans are at right. The code number of N-glycans follows the previous report (11).
of α-Lactoalbumin (α-LA)—To prepare a model oligosaccharide structure as a matching standard compound, a N-acetyl-α-galactosamine (GalNAc)-modified PA-derivatized N-glycan, termed as HF5.12 (this structure is shown in Fig. 6), was obtained from MII knock-out mouse serum according to the method described above. PA-derivatized N-glycan (HF5.12) was then digested with 5 milliunits of β-galactosidase from jack bean (Seikagaku Kogyo Co.) to afford HF5.11 (this structure is represented as J-2:HF5.11 in Fig. 3). Removal of the terminal D-galactose residue by β-galactosidase was carried out in 0.1 M citrate-phosphate buffer (pH 4.0) at 37 °C for 15 h in a total volume of 10 μL. Finally GalNAc residue was transferred in a regio- and stereoselective manner to the intermediate HF5.11 by treating with β1,4-galactosyltransferase (human, recombinant, Toyobo Co. Ltd.) and UDP-GalNAc (Yamasa Corp.) in the presence of bovine α-LA (Sigma) (18, 19). Approximately 70 pmol of HF5.11 was incubated with 8 milliunits of β1,4-galactosyltransferase, 500 pmol of UDP-GalNAc (a gift from the Yamasa Corp.), and 4 μg of α-LA in 10 μL of 25 mM Tris-HCl buffer (pH 7.4) containing 10 mM MnCl₂ at 37 °C for 20 h. To thoroughly transfer GalNAc residue into HF5.11, an additional 500 pmol of UDP-GalNAc was added to the reaction mixture and incubated for 48 h. Modified PA-derivatized N-glycan was isolated from the reaction mixture by HPLC using a Develosil UG-C30 column (4.0 × 10 mm, Nomura Chemical Co. Ltd.) at a flow rate of 0.2 mL/min at 25 °C. The column was first equilibrated with water, and the chromatography was performed stepwise using 100% water for 0–5 min and 20% methanol for 5–20 min. The target compound, a PA-derivatized N-glycan bearing a terminal GalNAc residue (termed L-2; represented in Fig. 6), was finally isolated by using HPLC under the condition described above. The structure of this standard material (L-2) was elucidated by MALDI-TOF/TOF MS analysis, and the detailed result is summarized in the supplemental information.

RESULTS

N-Glycan Profiling by 2D LC Mapping Technique—All isolated PA-derivatized N-glycans prepared from 10 mg of the lyophilized material were subjected to the analytical procedure using a two-dimensional mapping technique based on HPLC (11). The typical chromatograms on the ODS column are shown in Fig. 2. Because duplicate trials of each knock-out mouse embryo showed completely the same chromatographic profile, we decided to use the average values in terms of the relative ratio (%) of each oligosaccharide structure estimated from the fluorescence intensity. Herein the oligosaccharides with a relative molar ratio of over 1% were collected, and 37 kinds of N-glycans were obtained from the
mouse embryos as listed in Table I together with N-glycans identified in wild-type, MX knock-out, and MII knock-out mouse embryos as controls. The structures of 27 of them were successfully determined by comparing with an elution profile database of the authentic samples both on the ODS and the amide columns (Fig. 3) and further confirmed by their precursor ions in MALDI-TOF MS. Next all N-glycans from MII/MX double knock-out mice were then subjected to digestion by α-mannosidase (jack bean) to elucidate the existence of α-mannose residues at the non-reducing end. It was demonstrated by α-mannosidase digestion that MII/MX double knock-out mice seemed to synthesize both high mannose- and hybrid-type oligosaccharides, and they do not produce any complex-type N-glycans. As a result, it was suggested that 10 kinds of N-glycans could not be identified on the basis of the two-dimensional mapping and α-mannosidase digestion strategy. Although unknown N-glycans have been generally characterized by a combination of the conventional 2D HPLC analysis and the stepwise exoglycosidase digestion, the quantity of the obtainable unknown N-glycans made further structural characterization impossible.

### Table II
Remarkable fragment ion and its composition (the m/z numbers indicated in bold type are important for determining the structure of peak L-2)

Intensities are normalized by base ion intensity. M₁, molecular mass of HF5.12; M₂, molecular mass of peak L-2; N.D., not detected. * and ** refer to the peaks indicated in Fig. 5.

| PrecursorIon | FragmentIon | HF5.12 | PeakL-2 |
|-------------|-------------|--------|---------|
| [M+Na]+     | [HexNAc+H]+ | 204    | 63      |
|             | [GlcNAcPA+H]+ | 360   | 48      |
| 366**       | [HexNAc+Hex+H]+ | 100   | 17      |
| 407**       | [2HexNAc+H]+ | N.D.  | 48      |
| 448         | [Fuc+GlcNAc(PA)+H]+ | 97    | 100     |
| 388         | [HexNAc+Hex+Na]+ | 23    | N.D.    |
| 429         | [2HexNAc+Na]+  | N.D.  | 27      |
| 525**       | [GlcNAc=GlcNAc(PA)+Na]+ | 18    | 18      |
| 671**       | [4Hex+Na]+ or [GlcNAc=Fuc=GlcNAc(PA)+Na]+ | 51    | 28      |
| 833**       | [5Hex+Na]+ | 24    | 25      |
| 874**       | [4Hex=HexNAc+Na]+ | 22    | 31      |
| 1036*       | [5Hex=HexNAc+Na]+ | 63    | 71      |
| 1198        | [6Hex=HexNAc+Na]+ | 31    | N.D.    |
| 1239        | [5Hex+2HexNAc+Na]+ | N.D.  | 82      |
| 1401**      | [5Hex+3HexNAc+Na]+ | 100   | N.D.    |
| 1442**      | [5Hex+3HexNAc+Na]+ | N.D.  | 100     |
| 1481*       | [5,6-Gal-GlcNAc+Na]+ or [M₁-2HexNAc+Na]+ | 31    | 36      |
| 1684        | [5,6-HexNAc+Na]+ or [M₁-HexNAc+Na]+ | 14    | 49      |
| 1701**      | [M₁-Fuc+Na]+ | 31    | N.D.    |
| 1742**      | [M₁-Fuc+Na]+ | N.D.  | 43      |

Fig. 5. LIFT-TOF/TOF spectra of HF5.12 and peak L-2 using MDSF. Remarkable fragment ions are assigned in Table II. A, precursor ions are m/z 1846 (HF5.12) and m/z 1888 (peak L-2) as [M + Na]+. B, precursor ions are m/z 1824 (HF5.12) and m/z 1866 (peak L-2) as [M + H]+.
these unknown $N$-glycans obtained from mouse embryos. It has been demonstrated that MDSF using two typical matrices, DHB and CHCA, gives characteristic and completely different fragmentation patterns from the same compound due to the difference in the individual activated electronic state generated through the laser desorption ionization process (14). It seems likely that common CID fragmentation requires large amounts of oligosaccharides due to the labile glycosidic linkages, and high energy induced by CID gives extremely complicated fragmentation with low sensitivity. In the present study, we used the MDSF-based “spectral matching analysis” with characteristic fragment ion peaks due to the partial and typical oligosaccharide structures found in naturally occurring glycoproteins. We thought that these characteristic ions indicating oligosaccharide units would become key structural reporting groups for the identification of the total structures. In fact, DHB matrix can produce a protonated fragment ion at $m/z$ 366 corresponding to the non-reducing LacNAc disaccharide unit with great intensity. In cases of some fractions containing the bisecting-type GlcNAc residue (e.g., peaks represented as O, P, V, X, Z, and AA in Table I), TOF/TOF MS includes a protonated fragment ion at $m/z$ 569 corresponding to the typical trisaccharide component, GlcNAcβ-4Manβ-4GlcNAc (Fig. 4). Fragment ions at $m/z$ 731 in peaks S and W indicate the existence of a poly-$N$-acetyllactosamine unit (Galβ-4GlcNAcβ-3Galβ-4GlcNAc), and $m/z$ 528 found in the spectra of peak M-2 revealed the $\alpha$-galactosyl epitope structure (Galα-3Galβ-4GlcNAc). It should also be noted that we must use both the enzymatic digestion analyses and spectral matching analyses to conclude a precise structure among some plausible candidates estimated by TOF/TOF fragmentation (Table I and supplemental information).

On the other hand, CHCA makes some important fragment ions at $m/z$ 366 and 569 correspond to the characteristic fragment ions indicating oligosaccharide units.
FIG. 8. Relative ratios of each $N$-glycan from mouse embryos. Peak labels are the same as those used in Figs. 3 and 7. KO, knock-out; DKO, double knock-out.
ions reflecting general subtype structures of N-glycans. In the typical hybrid-type structures containing five mannose residues, sodium adduct fragment ions at m/z 833 and 1036 corresponding to Manα-6(Manα-3)Manα-6(Manα-3)Man and Manα-6(Manα-3)Manα-6(GlcNAcβ-2Manα-3)Man, respectively, were observed simultaneously. Conversely hybrid-type structures containing four mannose residues produced a sodium adduct fragment ion at m/z 712 corresponding to Manα-6(GlcNAcβ-2Manα-3)Man. In the case of the complex-type structures, sodium adduct fragment ions observed at m/z 712 and 915 due to Manα-6(GlcNAcβ-2Manα-3)Man and GlcNAcβ-2Manα-6(GlcNAcβ-2Manα-3)Man were observed simultaneously.

Precise Structural Characterization of Unknown Glycoforms—These important and informative fragment ions generated by means of MDSF method allowed for a more precise structural characterization of unknown oligosaccharide structures found in this study. For example, an unknown structure in peak O was identified by comparing TOF/TOF MS of this sample with that of standard materials, H5.1 and H5.3, containing a bisecting-type trisaccharide unit (data shown in Fig. 4 measured in the presence of DHB matrix) in addition to their TOF/TOF MS data measured in the presence of CHCA. In this case, the precise structure of peak O was successfully concluded by the existence of some key fragment ions detected at m/z 569 due to the bisecting trisaccharide component, GlcNAcβ-4Manβ-4GlcNAc (Fig. 4), and at m/z 833 and 1036 due to the pentamannooligosaccharide structures. Similarly, it was suggested that MALDI-TOF MS of peak L-2 in the presence of two distinct precursor ions at m/z 1866 as a protonated ion by DHB and m/z 1888 as a sodium adduct ion by CHCA corresponding to Hex3HexNAc6FucPA. It seemed that the LIFT-TOF/TOF fragmentation patterns derived from \([M + H]^+\) and \([M + Na]^+\) are quite similar to those of the known HF5.12 (Fig. 5). In the LIFT-TOF/TOF spectra of m/z 1846 (HF5.12) and m/z 1888 (peak L-2) as \([M + Na]^+\), fragment ions at m/z 525, 671, 833, 874, 1036, 1481, and 1684 were clearly observed in both spectra (Fig. 5A and Table II). Here it was demonstrated that ion peaks observed at m/z 833 and 1036 are characteristic fragment ions corresponding to the general hybrid-type N-glycans including five mannose residues. In addition, the LIFT-TOF/TOF spectra of HF5.12 and L-2 revealed that a terminal \(\alpha\)-galactose residue found in HF5.12 must be \(\alpha\)-acetyl-\(\alpha\)-galactosamine residue as detected by the meaningful mass difference in the two materials. These significant ion pairs could be observed at m/z 388 and 429, 1198 and 1239, 1401 and 1442, and 1701 and 1742 with a respective +41 mass shift. In contrast, precursor ions at m/z 1824 (HF5.12) and m/z 1866 (peak L-2) detected as \([M + H]^+\) in the presence of DHB produced fragment ions m/z 204, 300, 366, and 446 (Fig. 5B). Although the fragment ion at m/z 446 means that both structures have an \(\alpha\)-fucose residue attached to the GlcNAc residue at the reducing end, a fragment ion at m/z 407 was observed only in the case of peak L-2 instead of a significant degree of decrease in the intensity of the ion peak at m/z 366 due to \([\text{HexNAc} + \text{Hex} + \text{H}]^+\). This result also suggests that an oligosaccharide in peak L-2 includes a di-\(\alpha\)-acetylgalactosamine unit, GalNAcβ-4GlcNAc, which has been reported in some glycoprotein hormones (20, 21).

Finally it was concluded that the oligosaccharide structure of peak L-2 fraction can be identified as Manα-6(Manα-3)Manα-6(GalNAcβ-4GlcNAcβ-2Manα-3)Manα-6(GlcNAcβ-4(Fucα-6)GlcNAc-PA) and was termed HF5.12a (Fig. 6). This structure was unquestionably confirmed by comparing with an authentic standard compound derived by enzymatic synthesis from the known HF5.11 as a starting material (Manα-6(Manα-3)Manα-6(GalNAcβ-4GlcNAcβ-2Manα-3)Manβ-4GlcNAcβ-4(Fucα-6)GlcNAc-PA) according to the previous reports (18, 19). It was reported that this N-glycan structure can be detected as a partially degraded product of bovine lutropin N-glycans (20) but has not been isolated in an intact form.

In a similar manner, other unknown N-glycans were also systematically characterized by means of the MDSF method. Including peaks O and L-2, seven of 10 unknown N-glycan structures indicated in Table I were fully characterized, and partial structures of the other three (peaks J-3, K-2, and Q-2)
were estimated as shown in Fig. 7 (TOF/TOF MS data for all unknown glycoforms, see materials in supplemental information). Fig. 8 summarizes structures and relative molar ratios of N-glycans investigated by combined use of the two-dimensional mapping technique and the MDSF method of MALDI-LIFT-TOF/TOF mass spectrometry. The versatility of this novel analytical approach is evident because MALDI-LIFT-TOF/TOF analysis using MDSF method does not require any authentic samples and can be used for precise structural characterization of oligosaccharide materials obtained only in extremely small quantities.

**DISCUSSION**

In the present study, we established a novel strategy for high throughput structural characterization of N-glycans by combining a conventional 2D LC mapping technique and MALDI-TOF/TOF MS analysis. MDSF allowed us to elucidate novel oligosaccharide structures even though they were extremely small quantities. In this study, we succeeded in the identification of new structures using the limited amount (~10 pmol of unknown N-glycans) of the samples obtainable only from MII/MX double knock-out mice. It should be noted that differences in the activated electronic states of the precursor ions produced by two distinct matrices, CHCA and DHB, seem to be quite useful for further structural identification by means of the MS/MS spectral matching analyses. The versatility of this strategy was clearly demonstrated by profiling complicated N-glycan structures derived from four different types of mouse embryos from wild type, MX null, MII null, and MII/MX double nulls.

As summarized in Figs. 8 and 9, the ratio of hybrid and complex types varies significantly depending on the genotypes of these mice (Fig. 9A), whereas the relative quantity of high mannose type is constant. This suggests that disruption of MII or MX does not affect the upstream products but does affect the downstream products. The ratio of complex-type oligosaccharides is ~60% of total N-glycans both in wild type and MX nulls, and that of MII nulls is reduced to 20%. This observation suggests that at least 20% of complex-type N-glycans in MII nulls were produced by MX.

Interestingly, seven of 10 unknown oligosaccharides derived in this study were identified as hybrid type, and three (peaks M-2, S, and W) of them were completely new structures, namely pseudocomplex-type N-glycans. MDSF-assisted glycoform analysis revealed that MII/MX double knock-out mice synthesized four unusual hybrid-type (peaks O, R, P, and L-2) and two novel pseudocomplex-type N-glycans (peaks M-2 and S) as summarized in Fig. 10.

In conclusion, we demonstrated the feasibility of a novel strategy for the high throughput structural characterization of N-glycans by combining a conventional two-dimensional HPLC mapping technique and MALDI-TOF MS analysis. Re-

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**Fig. 10.** N-Glycans identified by combining 2D LC mapping and MDSF-MALDI-TOF/TOF MS strategy.
cently tissue-specific knock-outs of genes relevant to glyco-
sylation have been generated using Cre/loxP systems (22, 23). For example, Ye and Marth (24) have constructed neuron-
specific Gnt-I and Gnt-II knock-out mice and revealed an
essential role for hybrid-type N-glycan branching in the vi-
ability of neurons and the survival of mice during early postna-
tal development. Although we have succeeded in profiling the
gross glycoform of the N-glycans obtained from whole organs
tissues of mouse embryos, a glycoform-focused reverse
proteomics/genomics approach (25) will be required for fur-
ther detail discussion on the functional roles of each glyco-
protein in normal development or diseases caused by glyco-
sylation defects in human. The MDSF method should become
a powerful tool for reliable structural analysis of glycoproteins
because this method can be used concomitantly for gross
glycoform profiling (glycomics) and proteomics of trace
amounts of glycopeptides (13, 25).

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