Novel Poly-GalNAcβ1–4GlcNAc (LacdiNAc) and Fucosylated Poly-LacdiNAc N-Glycans from Mammalian Cells Expressing β1,4-N-Acetylgalactosaminyltransferase and α1,3-Fucosyltransferase*

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Glycans containing the GalNAcβ1–4GlcNAc (LacdiNAc or LDN) motif are expressed by many invertebrates, but this motif also occurs in vertebrates and is found on several mammalian glycoprotein hormones. This motif contrasts with the more commonly occurring Galβ1–3GalNAc (LacNAc or LN) motif. To better understand LDN biosynthesis and regulation, we stably expressed the cDNA encoding the Caenorhabditis elegans β1,4-N-acetylgalactosaminyltransferase (GalNAcT), which generates LDN in vitro, in Chinese hamster ovary (CHO) Lec8 cells, to establish L8-GalNAcT CHO cells. The glycan structures from these cells were determined by mass spectrometry and linkage analysis. The L8-GalNAcT cell line produces complex-type N-glycans quantitatively bearing LDN structures on their antennae. Unexpectedly, most of these complex-type N-glycans contain novel “poly-LDN” structures consisting of repeating LDN motifs (3GalNAcβ1–4GlcNAcβ1–4). These novel structures are in contrast to the well-known poly-LN structures consisting of repeating LN motifs (3Galβ1–4GalNAcβ1–4). We also stably expressed human α1,3-fucosyltransferase IX in the L8-GalNAcT cells to establish a new cell line, L8-GalNAcT-FucT. These cells produce complex-type N-glycans with α1,3-fucosylated LDN (LDNF) GalNAcβ1–4(Fucα1–3)GalNAc, as well as novel “poly-LDNF” structures (3GalNAcβ1–4(Fucα1–3)GalNAcβ1–4). The ability of these cell lines to generate glycoprotein hormones with LDN-containing N-glycans was studied by expressing a recombinant form of the common α-subunit in L8-GalNAcT cells. The α-subunit N-glycans carried LDN structures, which were further modified by co-expression of the human GalNAc 4-sulfotransferase I, which generates SO4–4GalNAcβ1–4GlcNAC-N-R. Thus, the generation of these stable mammalian cells will facilitate future studies on the biological activities and properties of LDN-related structures in glycoproteins.

There is a growing appreciation of the important roles of glycopeptides in modulating a wide variety of biological processes. A critical limiting factor in exploring glycan functions is the difficulty of obtaining many glycans either in a purified chemical form or within a relatively homogenous biological system. A common nonreducing terminal modification of glycans in mammalian glycoproteins is the N-acetyllactosamine (LN)1 disaccharide Galβ1–4GlcNAc-R, which may be sialylated, fucosylated, sulfated, or modified by other sugars to generate a wide range of terminal structures. However, another type of nonreducing terminal glycan structure that is less well understood but now appears to also be expressed by many organisms, including mammals, is based on the LacdiNAc (LDN) sequence GalNAcβ1–4GlcNAc-R, which can also occur within 4-O-sulfated, α1,3-fucosylated, or α2,6-sialylated derivatives. LDN-type glycans play vital roles in regulating the circulatory half-life of pituitary glycoprotein hormones (1–3) and other glycoproteins (4, 5), including tenascin-R produced by oligodendrocytes and small internurons in the hippocampus and cerebellum (6). Other glycoproteins containing LDN-type glycans include human glycoconjugates, a human glycoprotein with potent immunosuppressive and contraceptive properties (7, 8), and zona pellicula glycoproteins from murine eggs (9). Many human pathogens synthesize LDN and fucosylated LDN glycans, such as GalNAcβ1–4(Fucα1–3)GlcNAc-R, termed LDNF. Both LDN and LDNF as well as other modified LDN-type glycans are important determinants recognizable by our adaptive immune system and various carbohydrate-binding proteins within the innate immune system, such as DC-SIGN (10–17).

Although some LDN-based structures have been synthesized in vitro, there is no convenient biological system available to produce these structures in vivo to facilitate studies of their biological properties, and little is understood about the relationship of LDN expression to tissue glycosylation in general. We recently identified a cDNA encoding the Caenorhabditis elegans β1,4-N-acetylgalactosaminyltransferase (Ceβ4GalNAcT) (18) and found that the recombinant Ceβ4GalNAcT was active and capable of promoting LDN synthesis in vitro. Here we have explored whether mamma-
Celiac cells stably expressing the Ceβ4GalNAcT can be used to generate LDN-type N-glycans on cellular and recombinant glycoproteins. To this end, we generated a cell line derived from CHO Lec8 cells, termed L8-GalNAcT, that expresses Ceβ4GalNAcT and produces complex N-glycans containing LDN structures on their antennae. CHO Lec8 cells were chosen because they lack a functional UDP-Gal transporter and synthesize complex N-glycans with terminal β-linked GlcNAc residues (19, 20). Unexpectedly, L8-GalNAcT cells also produced novel poly-LDN-type structures with the repeating LDN structure (-3GalNAcβ1→4GlcNAcβ1), indicating that at least one member of the mammalian β1,3,4-acetylgalactosaminyltransferase family, which is responsible for poly-N-acetyllactosamine synthesis through the addition of β3-linked GlcNAc to terminal β4-linked Gal residues, also recognizes terminal β4-linked GalNAc to allow formation of the poly-LDN structures. Furthermore, we found that the poly-LDN backbone was efficiently fucosylated by recombinant human α1,3-fucosyltransferase IX (FucT 9) when it was stably co-expressed with the Ceβ4GalNAcT in a new cell line, termed L8-GalNAcT-FucT. The ability to generate such novel LDN-related glycans should aid in future studies to define the biological roles of LDN-containing glycans in a variety of biological systems, including bioactive glycoproteins in humans, such as pituitary hormones and fertility-related glycoproteins like glycodelin as well as in human immune responses to parasitic infections.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals and reagents used in this study, unless otherwise indicated, were from Sigma. FuGENE 6 and Complete Protease Inhibitor Mixture were from Roche Applied Science. Genetin and zeocin were from Invitrogen. HighSignal West Pico Chemiluminescent Substrate was from Pierce. Radiolabeled nucleotide sugars were from PerkinElmer Life Sciences. GalNAc51–GlcNAc55-S-pNP was synthesized from GlcNAc55-S-pNP using Ceβ4GalNAcT as previously described (18). The plasmid encoding full-length human GalNAc 4-sulfotransferase I was a kind gift from Dr. Jacques Baenziger.

**Establishment of Cell Lines**—CHO Lec8 cells (19, 20) were transfected with a plasmid encoding the complete open reading frame of GalT and GalNAcT from PerkinElmer Life Sciences. GalT and GalNAcT activities toward GlcNAc-terminating glycan were dissolved in 10 μl of matrix (2,5-dihydrobenzoic acid), incubated with loading buffer, resolved by SDS-PAGE (4–20% gradient), and transferred to a nitrocellulose membrane. For Western blot analysis, the membrane was blocked with 5% bovine serum albumin in a buffer of 20 mM Tris-HCl, pH 7.2, 150 mM NaCl, 2 mM CaCl2, 0.05% Tween 20 for 5 h at 4 °C. It was then incubated with primary antibody (mouse monoclonal anti-LDN IgM SMLDN1.1 or anti-LDNF IgM SMLDFN (11)) in the same buffer (without bovine serum albumin) for 1 h at room temperature. The membrane was then washed in the same buffer, and incubated with the secondary antibody (horseradish peroxidase-conjugated, goat anti-mouse IgM) as before. The membrane was then washed again, incubated in HighSignal West Pico Chemiluminescent Substrate for 2 min at room temperature, and exposed to a BioMax film (Eastman Kodak Co.) for 1 min. The film was then developed using a processing machine (Konica SRX-101). For Ponceau staining, the membrane was rinsed in water and stained for 3 min with 0.2% Ponceau S in 3% trichloroacetic acid. The membrane was then rinsed again in water and air-dried.

**Preparation of N-Glycans and MALDI-TOF MS Analysis**—N-Glycans were prepared from 3 × 107 cells of each cell line as previously described (22). Briefly, proteins extracted from washed cell pellets were reduced and carboxymethylated and then digested with trypsin or lysylglycine and analyzed by MALDI-TOF MS. 

**RESULTS**

**Establishment of L8-GalNAcT and L8-GalNAcT-FucT Cell Lines**—CHO Lec8 cells were chosen as founder cells for a new cell line to explore the expression of glycans with the LDN motif GalNAc51–4GlcNAcβ1. The CHO Lec8 cells lack functional UDP-Gal transporter, thus, they are expected to synthesize complex N-glycans with terminal β-linked GlcNAc residues at the nonreducing termini of their antennae (19, 20). The recombinant Ceβ4GalNAcT has β1,4-N-acetylgalactosaminyltransferase (GalNAcT) activity toward GlcNAc-terminating glycans (18). CHO Lec8 cells were transfected with a plasmid encoding Ceβ4GalNAcT under the control of the CMV promoter as well as a Genetin resistance gene. A stable, clonal cell line (L8-GalNAcT) was established from these cells by antibiotic selection and limiting-dilution culturing, as previously described (18).

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**Expression and Analysis of the Glycoprotein Hormone α-Subunit**—A peptide encoding an HPC4 linear version of the human glycoprotein hormone α-subunit under the control of the CMV promoter was constructed. A PCR-amplified DNA fragment starting at bp 72 of the open reading frame of the human α-subunit and extending beyond the stop codon was subcloned into the BamHI site of the pcDNA3.1(+) vector. The resulting vector (pHPC4-α-subunit) encodes a fusion protein consisting of a signal peptide at the N terminus, followed by an HPC4 linear peptide epitope (23, 24), which is followed by the mature peptide of the α-subunit (beginning at Ala72, the first amino acid after the endogeneous, cleavable, signal peptide). The mature HPC4-tagged α-subunit protein (after the removal of the signal peptide) has a calculated molecular mass of 11,785 ± 0.1 Da, but the mature protein contains two N-glycosylation sites and migrates anomalously on reducing SDS-PAGE as a glycoprotein with an apparent molecular mass of 25 kDa (25). CHO Lec8 and L8-GalNAcT cells were transfected with pHPC4-α-subunit, with or without α-transfection with a plasmid encoding full-length human GalNAc 4-sulfotransferase I (26). The cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum for 36 h post-transfection. The α-subunit was then affinity-purified from the extracellular medium and analyzed by SDS-PAGE and Western blotting using the HPC4 monoclonal antibody, as previously described (18).

**RESULTS**

**Establishment of L8-GalNAcT and L8-GalNAcT-FucT Cell Lines**—CHO Lec8 cells were chosen as founder cells for a new cell line to explore the expression of glycans with the LDN motif GalNAc51–4GlcNAcβ1. The CHO Lec8 cells lack functional UDP-Gal transporter, thus, they are expected to synthesize complex N-glycans with terminal β-linked GlcNAc residues at the nonreducing termini of their antennae (19, 20). The recombinant Ceβ4GalNAcT has β1,4-N-acetylgalactosaminyltransferase (GalNAcT) activity toward GlcNAc-terminating glycans (18). CHO Lec8 cells were transfected with a plasmid encoding Ceβ4GalNAcT under the control of the CMV promoter as well as a Genetin resistance gene. A stable, clonal cell line (L8-GalNAcT) was established from these cells by antibiotic selection and limiting-dilution culturing, as described under “Experimental Procedures.”
Glycosyltransferase assays. Extracts of CHO Lec8, L8-GalNAcT, and L8-GalNAcT-FucT cells were assayed for GalT (clear bars), GalNAcT (gray bars), and FucT (black bars) activities as described under “Experimental Procedures.” The activity is indicated in pmol of donor sugar transferred per hour per 1.5 × 10^6 cells, and each bar represents the average of two duplicate reactions.

We also wanted to explore the possibility that the Lec8-GalN-AcT cells could be used for stably producing N-glycans containing the LDN or LDNF motifs.

We expected that the LDN and LDNF structures in the new cell lines should be expressed on N- rather than O-glycans, as we have previously demonstrated in transiently transfected CHO-Lec8 cells (18). This is to be predicted, since CHO-Lec8 cells produce O-glycans consisting primarily of GalNAcα1Ser/Thr (30), which may or may not be α2-6-sialylated (31).

Mass Spectrometric Analysis of N-Glycans Produced by L8-GalNAcT and L8-GalNAcT-FucT Cells—To obtain a more detailed view of the carbohydrate structures produced by the cell lines in this study, MALDI-TOF-MS analyses were carried out on N-glycans released from the endogenous glycoproteins of these cells (Fig. 3 and Table 1). Signals consistent with compositions of HexNAc,Fuc,Hex3-HexNAc,Fuc,Hex10 (m/z 1591.5–2571.7) detected from CHO-Lec8 cells indicate the presence of truncated complex N-glycans. The spectra of the N-glycans from the Lec8-GalNAcT cells clearly indicate the presence of larger complex structures with compositions of HexNAc,Fuc,Hex3-HexNAc,Fuc,Hex3 (m/z 1590.9–4775.8). The spectra of the N-glycans from the Lec8-GalNAcT-FucT cells is dominated by highly fucosylated complex structures with compositions of HexNAc,Fuc,Hex10-HexNAc,Fuc,Hex (m/z 1836.5–5983.5). Additional MALDI-TOF MS experiments at higher mass ranges detected signals consistent with com-
sitions up to HexNAc$_{24}$Fuc$_1$Hex$_3$ and HexNAc$_{18}$Fuc$_9$Hex$_3$ in the Lec8-GalNAcT and Lec8-GalNAcT-FucT, respectively (data not shown).

**Linkage Analysis**—The N-glycans from the three cell lines were subjected to gas chromatography-MS linkage analysis (Table II). The presence of terminal GlcNAc, 2-linked mannose, and 2,4- and 2,6-linked mannose in the CHO-Lec8 cells is consistent with the presence of truncated bi-, tri-, and tetraantennary complex glycans. Additionally, the small amounts of 3,4,6-linked mannose and 4,6-linked GlcNAc residues indicate that the N-glycan core can also be modified by fucosylation and bisecting GlcNAc. The L8-GalNAcT cells gave additional signals for termi-
nal GalNAc and 3-linked GalNAc, which are consistent with complex N-glycans with LDN and poly-LDN units. Finally, linkage analysis of L8-GalNAcT-FucT cells showed an increase in the relative abundance of terminal fucose and a new signal for 3,4-linked GlcNAc, which is fully consistent with complex N-glycans with LDNF and poly-LDNF units.

Taken together, these data demonstrate that expression of GalNAcT in CHO-Lec8 cells allows for the production of complex N-glycans with LDN and poly-LDN antennae and that co-expression of GalNAcT and FucT allows for the production of N-glycans with LDNF and poly-LDNF antennae (Fig. 4).

### TABLE I

| Composition a | m/z b | Composition | m/z | Composition | m/z |
|---------------|-------|-------------|-----|-------------|-----|
| HexNAc,Hex   | 1580.5| HexNAc,Hex  | 1579.9| HexNAc,Hex  | 1580.4|
| HexNAc,Fuc,Hex | 1591.5| HexNAc,Fuc,Hex | 1590.9| HexNAc,Fuc,Hex | 1784.5|
| HexNAc,Hex   | 1662.5| HexNAc,Hex  | 1661.9| HexNAc,Hex  | 1986.8|
| HexNAc,Hex   | 1784.5| HexNAc,Hex  | 1783.8| HexNAc,Hex  | 2010.6|
| HexNAc,Fuc,Hex | 1836.5| HexNAc,Fuc,Hex | 1835.9| HexNAc,Fuc,Hex | 2040.6|
| HexNAc,Hex   | 1400.5| HexNAc,Hex  | 1386.8| HexNAc,Hex  | 2081.6|
| HexNAc,Fuc,Hex | 2040.5| HexNAc,Fuc,Hex | 2039.8| HexNAc,Fuc,Hex | 2192.7|
| HexNAc,Fuc,Hex | 2186.1| HexNAc,Fuc,Hex | 2069.8| HexNAc,Fuc,Hex | 2244.7|
| HexNAc,Hex   | 2152.6| HexNAc,Hex  | 2080.8| HexNAc,Hex  | 2255.7|
| HexNAc,Hex   | 2182.6| HexNAc,Hex  | 2151.8| HexNAc,Hex  | 2257.8|
| HexNAc,Hex   | 2285.6| HexNAc,Hex  | 2191.7| HexNAc,Hex  | 2396.8|
| HexNAc,Fuc,Hex | 2326.6| HexNAc,Fuc,Hex | 2325.7| HexNAc,Fuc,Hex | 2500.8|
| HexNAc,Fuc,Hex | 2396.6| HexNAc,Fuc,Hex | 2396.6| HexNAc,Fuc,Hex | 2571.9|
| HexNAc,Fuc,Hex | 2530.6| HexNAc,Fuc,Hex | 2370.7| HexNAc,Fuc,Hex | 2674.9|
| HexNAc,Fuc,Hex | 2571.7| HexNAc,Fuc,Hex | 2815.6| HexNAc,Fuc,Hex | 2920.0|
| HexNAc,Hex   | 2886.6| HexNAc,Hex  | 3080.5| HexNAc,Hex  | 2991.0|
| HexNAc,Fuc,Hex | 3060.5| HexNAc,Fuc,Hex | 3131.5| HexNAc,Fuc,Hex | 3319.1|
| HexNAc,Fuc,Hex | 3305.4| HexNAc,Fuc,Hex | 3350.3| HexNAc,Fuc,Hex | 3410.1|
| HexNAc,Fuc,Hex | 3795.2| HexNAc,Fuc,Hex | 4040.2| HexNAc,Fuc,Hex | 3952.9|
| HexNAc,Fuc,Hex | 4530.8| HexNAc,Fuc,Hex | 4575.8| HexNAc,Fuc,Hex | 4391.3|

**Largest N-glycan detected**

HexNAc 12Fuc 5Hex 3

### TABLE II

| Elution time a | Characteristic fragment ions | Assignment | Relative abundance/cell line | L8 b | L8GT | L8GTFT |
|----------------|-----------------------------|------------|-------------------------------|------|------|--------|
| 17.09–17.61   | 115, 118, 131, 162, 175    | Terminal fucose | 0.128 0.159 0.757 |
| 18.62–19.10   | 102, 118, 129, 145, 161, 205 | Terminal mannose | 1.000 0.785 0.664 |
| 19.33         | 102, 118, 129, 145, 161, 205 | Terminal galactose | ND ND Trace |
| 19.77–20.26   | 129, 130, 161, 190         | 2-Linked mannose | 0.666 1.000 1.000 |
| 19.95–20.41   | 118, 129, 161, 234         | 3-Linked mannose | Trace Trace Trace |
| 20.04–20.50   | 118, 129, 161, 234         | 3-Linked galactose | Trace Trace Trace |
| 20.17–20.64   | 102, 118, 129, 162, 189, 233 | 6-Linked mannose | Trace ND Trace |
| 20.96–21.42   | 130, 190, 233              | 2,4-Linked mannose | 0.074 0.092 0.472 |
| 21.35–21.82   | 129, 130, 189, 190         | 2,6-Linked mannose | 0.102 0.163 0.626 |
| 21.52–22.01   | 118, 129, 189, 234         | 3,6-Linked mannose | 0.615 0.620 0.958 |
| 21.99–22.42   | 118, 139                  | 3,4,6-Linked mannose | Trace Trace Trace |
| 22.44–22.93   | 117, 159, 203, 205        | Terminal GlcNAc | 0.028 0.023 0.238 |
| 22.59–23.40   | 117, 159, 203, 205        | Terminal GalNAc | ND ND 0.561 |
| 23.33–23.85   | 117, 159, 233             | 4-Linked GlcNAc | 0.223 0.374 0.748 |
| 23.89–24.33   | 117, 159, 318             | 3-Linked GalNAc | ND ND 0.155 |
| 24.69         | 117, 159, 301, 346        | 4,6-Linked GlcNAc | ND ND 0.776 |
| 24.64–25.10   | 117, 159, 261             | 4,6-Linked GlcNAc | 0.001 0.024 0.239 |

**a** Elution times are given as a range to cover the individual readings observed in each of the three analyses.

**b** L8, CHO Lec8; L8GT, L8-GalNAcT; L8GTFT, L8-GalNAcT-FucT.

**c** ND, none detected.
Expression of a Recombinant α-Subunit of the Glycoprotein Hormones and GalNAc 4-Sulfotransferase in L8-GalNAcT Cells—We then asked whether the recombinant GalNAcT in L8-GalNAcT cells could act on the common α-subunit of human glycoprotein hormones, which normally contains two complex-type N-glycans. These N-glycans from pituitary hormones are typically characterized by the presence of the terminal sulfated sequence $\text{SO}_4\text{-4GalNAcβ1-4GlcNAcβ1-2Manα-R}$ (32, 33). The addition of the $\beta1-4$-linked GalNAc to the GlcNAcβ1-2Manα-R acceptor to generate the LDN determinant may be catalyzed by a GalNAcT that utilizes UDP-GalNAc (34) and may specifically recognize N-glycans on glycoprotein hormones. The LDN sequence is then sulfated by a glycoprotein hormone GalNAc-4-sulfotransferase.

**Fig. 4. N-Glycan structures.** Shown are proposed complex N-glycan structures from CHO Lec8, L8-GalNAcT, and L8-GalNAcT-FucT cells.
that utilizes the donor phosphoadenosine phosphosulfate (26, 35).

We transiently transfected both parental CHO Lec8 cells and L8-GalNAcT cells with a cDNA encoding an N-terminal HPC4 epitope-tagged form of the α-subunit, as described under "Experimental Procedures." It has been shown that the free α-subunit expressed in CHO cells acquires an O-glycan, whereas this is not normally observed when the α-subunit is co-expressed with specific β-subunits of glycoprotein hormones (36). Medium from the transfected cells was recovered and the secreted HPC4-tagged α-subunit was purified and analyzed by Western blot. The recombinant α-subunit synthesized by parental CHO Lec8 cells migrated as a broad band with an apparent approximate molecular mass of ~25 kDa (Fig. 5), which is similar in mobility to the free α-subunit observed for recombinant α-subunit expressed in insect cells (25), which have some similarity to Lec8 CHO cells in being unable to terminally glycosylate either N- or O-glycans. By contrast, the recombinant α-subunit expressed in L8-GalNAcT cells migrated as a broad band with higher apparent molecular mass extending to ~32 kDa (Fig. 5). Western blotting with anti-LDN showed that the α-subunit from parental CHO Lec8 cells lacks LDN determinants as expected, whereas the α-subunit expressed in L8-GalNAcT cells is well recognized by anti-LDN (data not shown). Thus, the α-subunit can be modified by the GalNAcT in L8-GalNAcT cells to generate the LDN determinant. The increase in apparent Mr of the recombinant α-subunit from L8-GalNAcT cells suggests that multiple sugar residues were added, consistent with the presence of poly-LDN structures, such as those observed in the analysis of the total N-glycans. We further explored the N-glycosylation and potential sulfation of the α-subunit, by co-expressing the α-subunit in L8-GalNAcT cells with a recombinant form of the human GalNAc-4-sulfotransferase (GalNAc4-ST) that recognizes terminal LDN sequences on the N-glycans of pituitary hormones (26, 35). Co-expression of the GalNAc4-ST with the α-subunit in L8-GalNAcT cells caused a dramatic decrease in its apparent Mr, and sharpening of its mobility on SDS-PAGE (Fig. 5), which is probably due to a truncation of poly-LDN structures by sulfation of terminal GalNac residues within LDN. Only a small amount of recombinant α-subunit was generated, and further extensive studies will be required to purify the protein from multiple cell lines and sequence the N-glycans. The studies demonstrate that the N-glycans of the common α-subunit of pituitary hormones can be modified with LDN and possibly poly-LDN sequences and that co-expression with the GalNAc4-ST alters the biosynthesis of the LDN-containing N-glycans.

The analyses of the N-glycans from L8-GalNAcT and L8-GalNAcT-FucT cells indicate that the terminal GlcNac residues found in CHO Lec8 cells are converted into LDN or LDNF structures in L8-GalNAcT and L8-GalNAcT-FucT cells, respectively. Unexpectedly, we found that L8-GalNAcT and L8-GalNAcT-FucT cells contain N-glycans bearing repeating poly-LDN or poly-LDNF units, respectively. N-Glycans from CHO Lec8 cells are approximately equally distributed between bi-, tri-, and tetraantennary species (Figs. 3A and 4A), represented by the glycans with m/z of 1836.5, 2081.6, and 2326.6, respectively. The largest N-glycan mass detected in L8-GalNAcT cells indicates that it contains at least seven repeating LDN units, if the glycan is assumed to be a tetraantennary structure, and up to nine repeating units, if the glycan is assumed to be a bi-antennary structure. The largest N-glycan mass detected in L8-GalNAcT-FucT cells indicates 4–6 repeating LDNF units, depending on the assumed number of antennae. To our knowledge, this is the first reported occurrence of poly-LDN or poly-LDNF glycan structures biosynthesized by cells.

The presence of repeating units in both cell lines is remarkable and demonstrates that the endogenous β1,3-N-acetylgalcosaminyltransferase, or i-extension enzyme(s) (iGn-T), can act on LDN structures in vivo. Several β1,3-N-acetylgalcosaminyltransferases capable of elongating LN units and generating poly-LN structures have been identified, and these include iGn-T (37) and two different enzymes termed β3Gn-T1 and β3Gn-T2 (38). All of these enzymes can act in vitro and in vivo on terminal LN units to generate poly-LN structures. However, none of these studies on recombinant forms of the β1,3-N-acetylgalcosaminyltransferases examined their activity toward acceptors with nonreducing terminal LN rather than terminal LN structures. Several independent β3Gn-T gene families have been described (39), and the role of individual members or even as yet unidentified β3Gn-Ts in modification of terminal LDN versus terminal LN acceptors is not known. At least one of the β1,3-N-acetylgalcosaminyltransferases may act on terminal LDN structures, as shown by studies on a human serum-derived preparation of β1,3-N-acetylgalcosaminyltransferase(s). This preparation demonstrated activity toward acceptors with nonreducing terminal LN rather than terminal LN structures. Several independent β3Gn-T gene families have been described (39), and the role of individual members or even as yet unidentified β3Gn-Ts in modification of terminal LDN versus terminal LN acceptors is not known. At least one of the β1,3-N-acetylgalcosaminyltransferases may act on terminal LDN structures, as shown by studies on a human serum-derived preparation of β1,3-N-acetylgalcosaminyltransferase(s). This preparation demonstrated activity toward acceptors with nonreducing terminal LN rather than terminal LN structures. 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The likely pathways for LDN, poly-LDN, and poly-LDNF synthesis in the newly created cell lines are depicted in Fig. 6 (top), whereas the contrasting biosynthetic pathway for LN, poly-LN, and poly-Le" is depicted in Fig. 6 (bottom). This scheme is also supported by previous studies on enzyme activities in cell-free systems (28). The LN and/or LDN sequences could be generated by the sequential actions of β3GlcNAcT(s) and either β4GalT (Fig. 6, bottom) or β4GalNAcT (Fig. 6, top), respectively. As the chain grows through the action of these two sets of enzymes, the chain is fucosylated, thereby allowing
the production of large sized polyfucosylated structures, such as poly-Le\(^a\) or poly-LDNF.

In most cells, the addition of terminal Gal residues by endogenous GalTs supercedes the activity of endogenous GalNAcTs, which is generally low to undetectable in most mammalian cell lines and tissues (41). Thus, whereas expression of GalNAcT may allow some generation of LDN-containing structures, the stronger expression of GalT promotes the synthesis of predominantly LN-containing glycans. An exception could be the GalNAcT that recognizes pituitary hormones, which might allow a shift to LDN-type glycans in this subset of glycoproteins. In any case, the balance between the LDN versus LN pathways may be strongly dependent on the activity levels of the GalTs versus GalNAcTs. This general idea of a possible dichotomy in biosynthetic pathways in LDN versus LN has been suggested previously in studies on N-glycosylation pathways in invertebrates (42, 43).

Most studies on mammalian glycoproteins have reported that the LDN-type glycans are modified to contain the terminal sequence NeuAc\(^2\)-6GalNAc\(^\beta\)-1-4GlcNAc-R or SO\(_4\)-4GalNAc\(^\beta\)-1-4GlcNAc\(^\beta\)-1-2Man\(^\alpha\)-R, generated by an \(\alpha\)2,6-sialyltransferase or a GalNAc4-ST, respectively (42, 44). Thus, sialylation or sulfation of newly generated LDN structures would probably act as chain terminators and block glycan elongation by \(\beta\)1,3-N-acetylglucosaminyltransferase(s), thus limiting the generation of poly-LDN-type glycans. CHO cells do not express either the \(\alpha\)2,6-sialyltransferase (45) acting on LN acceptors or a GalNAc4-ST acting on terminal GlcNAc acceptors (present study); thus, there are no competitive chain termination events to block poly-LDN synthesis in these cells. Interestingly, the bovine \(\beta\)1,2,6-sialyltransferase has been shown to act on both terminal LN and LDN acceptors (46, 47). We speculate that the recombinant \(\alpha\)-subunit expressed in L8-GalNAcT cells acquires poly-LDN structures, like other common glycoproteins in these cells, causing its apparent \(M_r\) to be anomalously high, whereas co-expression with the recombinant GalNAc4-ST caused a truncation and termination of poly-LDN synthesis, causing a decrease in the apparent \(M_r\) of the recombinant \(\alpha\)-subunit (Fig. 5). Studies are in progress to define all of the N-glycans structures from the \(\alpha\)-subunit expressed in these cells and the precise effects of 4-O-sulfation on LDN and LDNF biosynthesis.

FIG. 6. Alternate N-glycan processing pathways. Proposed pathways for the synthesis of LDN- and LN-based N-glycan structures.
The presence of poly-LN in animal cell glycoproteins is well described and can occur in both N- and O-glycans (48) as well as in glycosphingolipids (49, 50). However, there have so far been no reports of poly-LDN-type structures synthesized by animal cells, except for the structures defined here. There may be several reasons for why these structures have thus far failed to be detected. Structural analyses of any polysaccharide, and especially poly-LDN, can be especially difficult. A common technique to characterize large sized glycans in animal cells is to probe for the presence of poly-LN sequences using the bacterial endo-β-galactosidase from *Bacillus fragilis* (51) or *Escherichia freundii* (52), both of which cleave poly-LN structures at internal Galβ1-4 linkages to release fragments with a reducing Gal residue. However, the actions of these enzymes toward poly-LDN-containing glycans are not well defined. Only one study has reported such an analysis, where it was demonstrated that the semisynthetic glycan Galβ1-4GlcNAcβ1-3GalNAcβ1-4Glc-R, which has an internal LN motif, can be cleaved at the internal GalNAc residue by the *B. fragilis* endo-β-galactosidase, although the glycan was clearly less sensitive to the enzyme compared with a conventional poly-LN structure Galβ1-4GlcNAcβ1-3GalNAcβ1-4Glc-R (40), where cleavage at the internal Gal residue was quantitative. It is now possible, using N-glycans from the cell lines described here to perform detailed studies on the sensitivities of different endo-β-galactosidases to poly-LDN and poly-LDNF structures.

One of the major cell types used in studies on LN-type glycans is the human 293 embryonic kidney cell line (HEK-293), whereas CHO cells are often used as cells expressing only LN and not LDN-type glycans. Both HEK-293 and CHO cells have demonstrable β4GalNAcT activity toward acceptors with terminal, nonreducing GlcNAc (8, 29), although HEK-293 cells have a somewhat higher level of activity (8). However, HEK-293 cells also have much higher levels of β3GalNAcT activity toward the same acceptors, yet surprisingly some recombinant glycoproteins expressed in HEK-293 cells acquire significant amounts of LDN-related structures (8, 53). Similar results were seen for canine kidney Madin-Darby canine kidney cells, where secreted glycoproteins contain appreciable amounts of LDN-related glycans (54). Furthermore, several specific glycoproteins were found to contain glycans with both LN and LDN motifs (7–9, 54–57). Interestingly, in such studies so far the glycans examined lack either poly-LN or poly-LDN-type structures, which suggests that the cells generating those glycoproteins may lack IgN-T activities or that the glycans are not accessible to the IgN-T activities, either through compartmentalization or protein folding. In addition, there is evidence that the mammalian β4GalNAcTs may exhibit preference for certain glycan branches (34, 58) or be glycoprotein-specific, as our study demonstrates, CHO Lec8 cells are a useful system in which to explore the specificity of β4GalNAcT.

Previous studies on recombinant human FucT 9 showed that it acts on terminal LN units to generate a terminal Lewis x type structure and on terminal LDN units to generate the LDNF structure (60). However, the enzyme does not efficiently recognize internal GlcNAc residues within acceptors containing poly-LN motifs, thus indicating that FucT 9 strongly prefers the terminal, nonreducing end (28). Thus, based on this observation, it appeared unlikely that human FucT 9 could participate in the generating of polyfucosylated oligosaccharides. However, it was suggested that human FucT 9 may allow expression of polyfucosylated poly-LN by fucosylating the penultinate GlcNAc residues within growing poly-LN units, where fucosylation precedes the addition of a extending β1–3 GalNAc to the terminal Gal residue (28). No previous studies have examined the specificity of human FucT 9 toward poly-LDN-type acceptors. Our results suggest that FucT 9 efficiently acts on LDN-type structures to allow formation of long chain poly-LDNF. This observation might suggest that FucT 9 fucosylates internal GlcNAc residues at the nonreducing end of growing poly-LDN chains, as predicted for the poly-LN chains (28).

Although there are a few cell lines capable of generating glycans containing LDN and LDNF structures (8, 29, 53, 54, 61), the glycosylation is highly heterogeneous and mixed structures of both LDN and LN are generated. By contrast, L8-GalNAcT and L8-GalNAcT-FucT cells are the first cell lines to predictably and exclusively produce LDN or LDNF structures on their complex N-glycans. The availability of these novel cell lines should greatly enhance the investigation into the biological roles of LDN- and LDNF-based N-glycan structures by providing a valuable tool to produce endogenous or recombinant glycoproteins bearing them. In addition, the cells themselves, fixed or otherwise, could be used as antigens for vaccine to produce anti-LDN/LDNF antibodies either for experimentation or as protection against LDN/LDNF-bearing pathogens (12).

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