LEC18, a Dominant Chinese Hamster Ovary Glycosylation Mutant Synthesizes N-Linked Carbohydrates with a Novel Core Structure*

(Received for publication, July 25, 1995)

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The dominant Chinese hamster ovary cell glycosylation mutant, LEC18, was selected for resistance to pea lectin (Pisum sativum agglutinin [PSA]). Lectin binding studies show that LEC18 cells express altered cell surface carbohydrates with markedly reduced binding to PSA and increased binding to Phaseolus vulgaris agglutinin (DSA) compared with parental cells. Desialylated [3H]Glc-labeled LEC18 cellular glycopeptides that did not bind to concanavalin A-Sepharose exhibited an increased proportion of species that were bound to DSA-agarose. Most of these glycopeptides bound to ricin-agarose and were unique to LEC18 cells. This fraction was purified from ~10^10 cells and shown by 1H NMR spectroscopy and methylation linkage analysis to contain novel N-linked structures. Digestion of these glycopeptides with mixtures of β-o-galactosidases and N-acetyl-β-o-glucosaminidases gave core glycopeptides that, in contrast to cores from parental cells, were mainly not bound to concanavalin A-Sepharose or to PSA-agarose. 1H NMR spectroscopy, matrix-assisted laser desorption ionization/time of flight mass spectrometry, electrospray mass spectrometry, and collision-activated dissociation mass spectrometry showed that the LEC18 core glycopeptides contained a new GlcNAc residue that substitutes the core GlcNAc residue. Methylation linkage analysis of the parent compound provided evidence that the GlcNAc is linked at O-6 to give the following novel, N-linked core structure.

The N-linked carbohydrates of mammalian cells are initially synthesized on dolichol-phosphate, transferred en bloc to an Asn-X-(Ser/Thr) sequon, trimmed by glycosidases, and matured via the sequential action of a series of glycosyltransferases (1). The core region of a mature N-linked structure is the Manα1,6[Manα1,3]Manβ1,4GlcNAcβ1,4GlcNAc, originally synthesized on dolichol. In mammalian cells, only four types of core have been described: the Man9GlcNAc2Asn described above; Man6GlcNAc2(Fuc)Asn, where Fucβ1 is linked O-6 to the Asn-linked GlcNAc; and cores, with or without Fuc, that contain a bisecting GlcNAc (2, 3). By contrast, certain plant glycoproteins have N-linked carbohydrates with Xyl attached at O-2 of the β-1,4-linked Man and/or Fuc attached at O-3 of the Asn-linked GlcNAc (4, 5). A glycoprotein from honeybee has been reported to have two Fuc residues substituting the Asn-linked GlcNAc at O-3 and O-6 (6). No substitutions of the latter types have been reported to date in mammalian glycoproteins (1-3). However, an altered N-linked core might be expected to be the basis of cellular resistance to the toxicity of pea lectin (PSA), because PSA binds specifically to the fucosylated core region of bi- and tri-antennary N-linked carbohydrates (7).

LEC18 Chinese hamster ovary (CHO) cells are rare mutants that were isolated following a selection for resistance to PSA (8). They are 39-fold more resistant to PSA compared with parental cells. In addition, LEC18 cells are 16-fold more resistant to Lens culinaris agglutinin (LCA), a lectin with a very similar binding specificity to PSA (9). The properties of LEC18 CHO cells are dominant in somatic cell hybrids formed with parental cells (8), showing that their lectin resistance arises from a gain-of-function mutation. Other gain-of-function CHO glycosylation mutants include LEC10, LEC11, LEC12, LEC29, and LEC30 (10). Each of these mutants synthesizes cell surface carbohydrates carrying a sugar residue that is not synthesized by parental CHO cells and that confers a distinctive pattern of lectin resistance. In this paper, we show that LEC18 cells also synthesize a species of N-linked carbohydrate that is not present in parental cells, nor in any of the previously described dominant CHO mutants. These carbohydrates have a novel GlcNAc substitution in their core region, which markedly alters the conformation of the trimannosyl core and is likely to be the reason that LEC18 cells are highly resistant to both PSA and LCA.

* This work was supported by National Cancer Institute Grant CA 36434 (to P. S.). Partial support was also obtained from Cancer Core Grant PO 13330. The mass spectral facility at the Laboratory of Macromolecular analysis at Albert Einstein College of Medicine was supported by National Institutes of Health Grant RR09113. The methylation analysis was performed at the Complex Carbohydrates Research Center, supported in part by the National Institutes of Health Resource Center for Biomedical Complex Carbohydrates (NIH Grant 2-P41-RR0351-06). 1H NMR spectroscopy was performed in the NMR core facility of the Cancer Center at Albert Einstein College of Medicine. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: Fuc, fucose; Xyl, xylose; PSA, P. sativum (pea) agglutinin; CHO, Chinese hamster ovary; LCA, L. culinaris agglutinin; ConA, concanavalin A; -PHA, Phaseolus vulgaris leukoagglutinin; RCA1, ricin agglutinin I; RCA1, ricin agglutinin II; MS/MS, collision-activated dissociation mass spectrometry; PBS, phosphate-buffered saline; BSA, bovine serum albumin; GLC-MS, gas-liquid chromatography-mass spectrometry; ES-MS, electrospray mass spectrometry; MALDI-TOF-MS, matrix-assisted laser desorption ionization/time of flight mass spectrometry; DSA, D. stramonium agglutinin.
EXPERIMENTAL PROCEDURES

Materials—\(\text{o-}[6-\text{H}]\text{Gal}(31.5 \text{ Ci/mmol}), \text{o-}[2-\text{H}]\text{Man}(11.6 \text{ Ci/mmol}), \text{o-}[6-\text{H}]\text{Glc}(36.25 \text{ Ci/mmol}), \text{o-}[\text{U-3}^\text{2}]\text{Glc}(290 \text{ mCi/mmol}), \text{and}[6-\text{H}]\text{Fuc}(16.1\text{ Ci/mmol})\) were from Amersham Corp.; ConA-Sepharose and Sephadex G-25 were from Pharmacia Biotech Inc.; L-PHA-agarose, from Boehringer Mannheim; the toxicity of PSA (8), Pro\(\text{Gln}\) (pH 7.4), and the columns were washed with \(\text{NaOH}\).

\(\text{HCl}\), and the column was eluted with 1 \text{mL} Promase in 1 \text{mL} Tris-HCl (pH 7.2), 3 \text{mM} \text{CaCl}_2, at 55°C under toluene. Fresh Promase was added every 24 h for 5 days. After boiling for 10 min, the sample was centrifuged for 2 h at 30,000 \text{rpm} in a type 50 Ti rotor. The supernatant was loaded onto a column of Sephadex G-25 (5 cm × 65 cm). The column was eluted with glass distilled water, and fractions of 10 \text{mL} were assayed with phenyl-sulfuric acid for neutral hexosides (16). Positive fractions were pooled, concentrated by roteovaporation to 5 \text{mL}, and diluted with an equal volume of 2× ConA buffer. Insoluble material was removed by centrifugation at 3000 \text{rpm} for 10 min, and the supernatant was chromatographed on a column of ConA-Sepharose (1.5 cm × 22 cm). Unbound glycopeptides were desalted on a Sephadex G-25 column (5 cm × 65 cm), lyophilized, and adjusted to 200 μL water. DSA-retarded fraction was desalted on Bio-Gel P-2 (1.5 cm × 70 cm), lyophilized, desalted in 1 mL of PBS, and chromatographed on a column of RCA1-agarose (0.5 cm × 20 cm). The column was eluted with 200 μL of PBS folowed by 100 μL of 100 μm lactose in PBS. Pooled fractions were desalted on Bio-Gel P-2. A portion of each glycopeptide fraction was used for monosaccharide analysis and the remainder was used to record \(^1\text{H}\) NMR spectroscopy and was subjected to methylation linkage analysis or treated with exoglycosidases to isolate core glycopeptides as described below.

High Performance Anion-exchange Chromatography with Pulsed Amperometric Detection of Monosaccharides and Glycopeptides—For monosaccharide analysis, glycopeptides (2–5 μg) were hydrolyzed with 2M trifluoroacetic acid or 4M hydrochloric acid (100–200 μL) in Teflon-lined, screw capped Eppendorf tubes (Sarstedt) at 100°C for 4–5 h in a heating block filled with glycerine (Lab-Line Instruments, Inc.). After hydrolysis, samples were evaporated to dryness, resuspended in 50–100 μL of glass distilled water, passed through Centrex (Schleicher and Schuell, Inc.) and analyzed by high performance anion-exchange chromatography with pulsed amperometric detection (17) using a model PAD-2 detector ( Dionex Corp., Sunnyvale, CA) and a CarboPac PA1 (4 mm × 250 mm) pellicular anion-exchange column equipped with a CarboPac guard column. Eluant 1 was 200 μM NaOH, eluant 2 was water, eluant 3 was 500 μM NaOH, and eluant 4 was 100 μM NaOH containing 1 μM NaAc. Fuc, GlcN, Gal, and Man were eluted isocratically with 16 μM NaOH. Unless otherwise mentioned, the flow rate was 0.9 mL/min for 25 min. The following pulse potentials and durations were used: \(E_1 = 0.05 \text{ V} (t_1 = 300 \text{ ms})\); \(E_2 = 0.65 \text{ V} (t_2 = 180 \text{ ms})\); \(E_3 = -0.65 \text{ V} (t_3 = 60 \text{ ms})\). Detection was with 1000 nm full scale. No postcolumn base addition was used as no baseline drift was observed; a Dionex Advanced Computer Interface connected to a Gateway 2000, 4XS-33V computer with Dionex A-150 (release 3.32.00) software was used to collect the data. For ralolabeled sugars, 50 fractions of 0.5 min (0.45 μL) were collected and mixed with 5 μL of Ecolume and radioactivity was counted by liquid scintillation spectrometry. Glycopeptides, N-acetyl- and N-glycolylneuraminic acids were eluted using a gradient of 100 μM NaOH and 100 μM NaOH containing 1 μM NaAc. Fuc, GlcN, Gal, and Man were eluted isocratically with 16 μM NaOH. Unless otherwise mentioned, the flow rate was 0.9 mL/min for 25 min. Following the first peak, the flow rate was increased to 8 mL/min, linearly increasing eluant 4 to 80% over 60 min, and finally, eluant 4 isocratically for another 20 min using either CarboPac PA1 (4 mm × 250 mm) or CarboPac PA100 (4 mm × 250 mm) columns.

\(^1\text{H}\) NMR Spectroscopy at 500 MHz—For \(^1\text{H}\) NMR spectroscopy, glycopeptide samples were desalted 3 times on Bio-Gel P-2 (1.5 cm × 70 cm), passed through Chelex-100, exchanged in D$_2$O (Sigma) 3 times, and resuspended in 99.996% D$_2$O (Cambridge Isotope Laboratories) containing a small amount of acetone (Backer analyzed as internal standard). Spectra were recorded on a 500 MHz Varian Spectromete...
ter at 23, 32, or 42 °C using a sweep width of 4000 Hz, a cycle delay time of 3 s, and 90° pulses. Chemical shifts were determined based on acetone being at 2.225 ppm.

Methylation Linkage Analysis—Glycopeptides (100 μg) were permethylated according to Hakomori (18). The permethylated products were purified using a Sep-Pak C$_8$ cartridge (Waters), hydrolyzed with 2 M trifluoroacetic acid, reduced with sodium borodeuteride, acetylated with pyridine-acetic anhydride, and analyzed by gas-liquid chromatography-mass spectrometry (GLC-MS) as described (19). GLC-MS was performed with a Hewlett Packard 5890A gas liquid chromatograph using a SP-2330 column (Supelco) for analysis of neutral monosaccharide derivatives and a DB-1 column (J&W Scientific) for the analysis of N-acetylactosamine derivatives, coupled to a Hewlett-Packard 5970 MSD mass spectrometer.

Mass Spectrometry—Electrospray-mass spectrometry (ES-MS) and MS/MS analysis of glycopeptides were performed on an API-III triple-quadrupole mass spectrometer (PE-SCIEX, Ontario, Canada) using the SCIEX Ionspray interface with nitrogen as the nebulizer gas, an ion spray voltage of 3300, and the orifice at 35 or 70 V. The sample was infused into the mass spectrometer at 2 μl/min using a Harvard apparatus syringe pump after diluting 1:1 with 50% acetoniitrideH$_2$O containing 0.1% trifluoroacetic acid. Collision-activated dissociation mass spectrometry (MS/MS) was performed using argon as the collision gas at a collision gas thickness of ~1 × 10$^{-6}$ molecules/cm$^2$ and a collision energy of 30–35 eV. Under these collision-activated dissociation conditions, daughter ion spectra of selected parent ions were obtained.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was performed on a Voyager RP Biospectrometry work station (Perspective Biosystems, Framingham, MA), using ω-cyano-4-hydroxycinnamic acid as matrix. A laser power of 190 attenuator (nitrogen laser at 377 nm) was used, and an average of 50 scans were taken.

Enzyme Digestions—J-ack bean β-galactosidase digestion was carried out in 100 μl of sodium citrate buffer (pH 3.5) at 37 °C under toluene for 24–72 h with a total of 1 unit enzyme. Bovine testis β-galactosidase and jack bean α-acetyl-β-o-glucosaminidase digestions were performed in 100 μl of sodium citrate buffer (pH 4.5) at 37 °C under toluene for 1 h with a total of 100 munits enzyme. Pneumoniae β-galactosidase and N-acetyl-β-hexosaminidase digestions were performed in 50–100 μl of sodium citrate buffer (pH 5.0) at 37 °C under toluene for 24–96 h, with a total of 100 munits enzyme. Each reaction was started with 25 munits enzyme, and an additional 25 munits were added at 24-h intervals.

RESULTS

Binding of $^{125}$I-PSA and $^{125}$I-DSA to LEC18 Cells—The increased resistance of LEC18 cells to PSA and LCA (8) is expected to be due to the expression of altered cell surface carbohydrates. As predicted, LEC18 mutants exhibited a marked (2- 4-fold) reduction in $^{125}$I-PSA binding when compared with parental CHO cells over a broad range of PSA concentrations (Fig. 1). In the linear region of the binding curves, the differences in $^{125}$I-PSA binding were ~4–5-fold when the assay was performed at room temperature (data not shown). Two independently isolated LEC18 mutants exhibited a similar reduction in PSA binding. Lec13 CHO mutant cells were used as a negative control, as they synthesize N-linked carbohydrates that lack a core Fuc residue and hence do not bind to PSA (20).

Most CHO glycosylation mutants exhibit an increased sensitivity to and binding of certain lectins (21). Such lectins, once identified, can be useful for affinity purification of novel glycoconjugates that lack a core Fuc residue and hence do not bind to PSA (20).

A Glycopeptide Fraction Unique to LEC18 Cells—The increased binding of $^{125}$I-DSA by LEC18 cells would be consistent with increased affinity or increased synthesis of poly-N-acetyllactosamine chains (23). To identify carbohydrates unique to LEC18 cells, detailed studies with metabolically labeled cellular glycopeptides were carried out by serial lectin affinity chromatography. ConA-Sepharose chromatography (25) of $[^3H]$Glc-labeled glycopeptides from parental and LEC18 CHO cells showed no significant differences in profile. About 85% of the label passed through the column, 5% bound and eluted in the biotinyl fraction, while 10% bound and was eluted in the hybrid and oligomannosyl fraction. The glycopeptides that were not bound to the ConA-Sepharose column should include branched N-glycans with poly-N-acetyllactosamine units that would bind to DSA (22, 23). These glycopeptides were subjected to mild acid hydrolysis to remove sialic acid for 24–96 h with a total of 100 munits enzyme. DSA-agarose (Fig. 2). LEC18 glycopeptides separated into late regions (data not shown).

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tarded (45%) and bound (55%) species, whereas parent glycopeptides eluted in the unbound (35%) or early retarded (65%) fractions, indicating that the glycopeptides derived from LEC18 cells were indeed unique to the mutant.

LEC18 Glycopeptides Have an Altered N-Linked Core Region—To determine whether the glycopeptides unique to LEC18 cells were altered in their core region, as might be expected from the PSA resistance of LEC18 cells, DSA-retarded glycopeptides of both parental and LEC18 cells (Fig. 2) were treated exhaustively with a mixture of bovine testis β-D-galactosidase and Jack bean N-acetyl-β-D-glucosaminidase. The digestion products fractionated on Bio-Gel P-2 into two major glycopeptide pools (Vo in the excluded volume and Ve in the included volume) and two monosaccharide pools (Gal and GlcNAc) (Fig. 3). As the Vo fraction was complex and probably contained some partially digested material, the Ve fraction was chosen for further studies.

Fractionation on ConA-Sepharose and PSA-agarose revealed a significant difference between Ve glycopeptides of LEC18 and parent cells. As would be expected, ~95% of parental CHO Ve glycopeptides did bind to ConA-Sepharose, indicating that they had been converted to trimannosyl cores (Fig. 4). By contrast, ~65% of LEC18 Ve glycopeptides did not bind to ConA-Sepharose (Fig. 4). These results suggested that the core glycopeptides from LEC18 cells have a modified structure. Similarly, for core glycopeptides obtained following exhaustive digestion with a mixture of β-D-galactosidase and N-acetyl-β-D-glucosaminidase from D. pneumoniae(27), a much greater proportion of LEC18 Ve glycopeptides did not bind to ConA-Sepharose, compared with parental glycopeptides.

A trimannosyl, fucosylated core structure would be expected to bind to PSA-agarose (7). This was found to be the case for a preparation of fucosylated core glycopeptides from parental CHO cells (Fig. 4). However, the Ve glycopeptides from LEC18 cells did not bind to PSA-agarose (Fig. 4), despite the presence of almost one full Fuc equivalent (Table I). The monosaccharide compositional analysis of the Ve fraction of LEC18 and parental CHO cells in Table I also showed that neither LEC18 nor parent core glycopeptides had any Gal residues. In addition, parent cores had only approximately two GlcNAc equivalents showing that the glycosidase digestion was complete. However, LEC18 core glycopeptides contained an additional GlcNAc residue that had resisted N-acetyl-β-D-hexosaminidase digestion. This GlcNAc was postulated to be the reason most LEC18 core glycopeptides did not bind to ConA-Sepharose or PSA-agarose (Fig. 4).
New GlcNAc Residue in Core of N-Glycans

**Structural Analysis of LEC18 Glycopeptides By $^1$H NMR Spectroscopy, Methylation Linkage Analysis, and Mass Spectrometry**—The studies on $[^3]$H-labeled glycopeptides suggested that an N-linked glycopeptide fraction unique to LEC18 cells gave rise to a core structure with an additional GlcNAc residue. In order to locate the linkage position, cellular glycopeptides from $\sim 10^{10}$ LEC18 cells were isolated and the DSA-retarded/RCA II-bound fraction (Fig. 2) was prepared. The $^1$H NMR spectrum of these LEC18 glycopeptides is shown in Fig. 5. It is evident that the glycopeptides are a mixture of N-linked structures containing poly-N-acetyllactosamine and Fuc and a small amount of sialic acid. Hydrazinolysis of this preparation revealed multiple species eluting at the position of neutral oligosaccharide standards on Glycoprep 1000 (Oxford GlycoSystems, Oxford, UK). Furthermore, Bio-Gel P-4 chromatography of the released oligosaccharides indicated that they are high molecular weight complex carbohydrates with $\gg 24$ Glc units (data not shown).

The $^1$H NMR spectrum in Fig. 5 is unusually complex in the 4.9–5.2-ppm region, and no resonances corresponding to known $^1$H or $^2$H chemical shifts for core Man residues in previously reported structures were present in the NMR carbohydrate data base (Sugabase, version 1.05; 3). The resonance at 2.028 ppm is typical of -NAc groups from GlcNAc residues in poly-N-acetyllactosamine chains (28). Thus the glycopeptides are a mixture of complex N-glycans containing poly-N-acetyllactosamine chains.

After further acid hydrolysis to remove the small amount of sialic acid, a portion of these glycopeptides was subjected to methylation linkage analysis. From the data in Table II, it is evident that the LEC18 glycopeptides have terminal Gal (3), GlcNAc (1), and Fuc (0.7) residues and substituted Gal, GlcNAc, and Man residues. The substitutions of the Man residues are consistent with a mixture of tri- and/or tetraantennary N-linked carbohydrates. The presence of Gal substituted at O-3 is consistent with the presence of poly-N-acetyllactosamine chains, as is the 4-substituted GlcNAc. The most remarkable finding from this analysis was the presence of a single, unsubstituted GlcNAc residue and of 1.75 molar residues of 4,6-substituted GlcNAc. The only 4,6-substituted GlcNAc in the usual N-linked structure arises from the presence of Fuc at the O-6 of the Asn-linked GlcNAc. In the LEC18 glycopeptides, Fuc linkage accounts for $\sim 0.7$ of the 4,6-linked GlcNAc, leaving one full residue of 4,6-substituted GlcNAc to be accounted. In fact, it will be shown subsequently that this residue is located in the altered core region of LEC18 glycopeptides.

The methylation linkage analysis also showed the presence of O-4 substituted Gal residues (Table II), a substitution not observed previously for N-linked carbohydrates from mammalian cells (1–3). However, the presence of this residue in glycolipids and in fish egg N-glycans has been observed previously (29, 30). Since no direct linkage analysis has previously been reported for CHO-derived poly-N-acetyllactosamin containing N-linked carbohydrates, the O-4-substituted Gal may be a feature of some N-glycans in all Pro CHO cells. Whatever its origin, this residue was susceptible to the exoglycosidases used, because Gal residues were absent after digestion (Table I), indicating that 4-substituted Gal residues are part of the poly-N-acetyllactosamine chains and are linked by $\beta$-glycosyl residues. Furthermore, the methylation linkage analysis showed the presence of small amounts of 6-substituted, 4,6-substituted, and 3,6-substituted Gal residues (Table II). Finally, the

### Table I

| Monosaccharide | $V_n$ core glycopeptides |
|---------------|-------------------------|
|                | LEC18 | Parental |
| Fuc            | 0.8   | 0.7     |
| GlcNAc         | 2.8   | 1.5     |
| Gal            | 0     | 0       |
| Man$^a$        | 3.0   | 3.0     |

$^a$ Normalized to three residues of Man per N-linked carbohydrate moiety.

### Table II

| Glycosyl residue | Molar ratio |
|------------------|-------------|
| Fucp1$\rightarrow$ | 0.72 |
| GlcNAcp1$\rightarrow$ | 1.00 |
| $\rightarrow$4GlcNAcp1$\rightarrow$ | 4.86 |
| $\rightarrow$4GlcNAcp1$\rightarrow$ | 1.75 |
| Galp1$\rightarrow$ | 3.0 |
| $\rightarrow$3Galp1$\rightarrow$ | 1.8 |
| $\rightarrow$4Galp1$\rightarrow$ | 1.0 |
| $\rightarrow$6Galp1$\rightarrow$ | 0.3 |
| $\rightarrow$4,6Galp1$\rightarrow$ | 0.35 |
| $\rightarrow$3,6Galp1$\rightarrow$ | 0.2 |
| $\rightarrow$2Manp1$\rightarrow$ | 0.8 |
| $\rightarrow$2,4Manp1$\rightarrow$ | 1.0 |
| $\rightarrow$2,6Manp1$\rightarrow$ | 1.6 |
| $\rightarrow$3,6Manp1$\rightarrow$ | 1.0 |
analysis showed no evidence for 3,4,6-substituted GlcNAc that would be predicted if the Asn-linked GlcNAc was disubstituted, for 3,4,6-substituted Man that would be predicted for the presence of a bisecting GlcNAc, nor for 2,3,6-substituted Man or for a significant molar proportion of any unusual sugar like Xyl.

Structural Analysis of LEC18 Core Glycopeptides—Because our studies of [3H]Glc-labeled glycopeptides identified the core region as the location of a novel modification in LEC18 N-linked carbohydrates (Fig. 4 and Table I), the unlabeled glycopeptides shown in Fig. 5 were treated exhaustively with a mixture of β-D-galactosidases and N-acetyl-β-D-glucosaminidases from D. pneumoniae, bovine testis, and Jack bean. The Ve fraction was obtained by Bio-Gel P-2 chromatography and shown to elute between Man3GlcNAc2Asn and Man5GlcNAc2Asn standards by high performance anion-exchange chromatography with pulsed amperometric detection (data not shown). Fig. 6 shows the MALDI-TOF-MS of these LEC18 Ve core glycopeptides. The mass spectrum contained one major molecular ion (MH⁺) at 1374.4 atomic mass units, which is the mass expected for a glycopeptide with the composition Man3GlcNAc3(Fuc)Asn. Fig. 6 also shows the ES-MS (lower panel) of LEC18 Ve core glycopeptides, which was recorded at 35 V. Again, only one major MH⁺ ion was observed at 1374.4 atomic mass units.

When ES-MS spectra were recorded at 70 V, a strong MH⁺ ion at 1374.4 atomic mass units along with some fragment ions were observed (Fig. 7). Attempts were made to obtain daughter ions by collision-activated dissociation (MS/MS). The MS/MS spectrum in Fig. 8 shows that the MH⁺ ion at 1374.4 atomic mass units gave daughter ions inter alia at atomic mass units 1228.4, 1171.4, 889.2, and 686.2. The interpretation of these ions is shown in Scheme 1. Briefly, the ions at 1228.4 and 1171.4 atomic mass units were derived from the MH⁺ ion with a loss of a Fuc or GlcNAc residue, respectively. However, the ion at 889.2 atomic mass units corresponds to a loss of three Man residues from MH⁺ and has a composition of GlcNAc₃(Fuc)Asn, confirming the presence of an extra GlcNAc residue in the core region. This ion loses a GlcNAc residue to give the ion at 686.2 atomic mass units.

Based on this information, it was possible to interpret many of the fragment ions observed in the ES-MS spectrum of Fig. 7. Four fragmentation pathways could be deduced (Scheme 2). It is evident that the glycopeptide undergoes fragmentation both from the nonreducing end and from the Asn end to give fragment ions that are interpretable in structural terms. The most critical ions observed were at atomic mass units 889.2, 686.2,
and 483.2 and were composed of \text{GlcNAc}3(Fuc)Asn, \text{GlcNAc}2(Fuc)Asn, and \text{GlcNAc}1(Fuc)Asn, respectively. The latter shows that the Asn-linked GlcNAc is substituted with Fuc. This sequence of ions and the MS/MS data provide strong evidence that the new GlcNAc is attached to the core GlcNAc residue and not to the Asn-linked GlcNAc.

1H NMR spectra of the LEC18 Ve core glycopeptides shown by mass spectrometry in Fig. 6 to contain one major molecular species are shown in Fig. 9. It is apparent that the preparation contained a single glycopeptide species with three GlcNAc residues, three Man residues, and one Fuc residue. Spectra were recorded at 23 and at 42°C. At the higher temperature, the HOD peak shifted to reveal certain resonances obscured at 23°C. When the chemical shifts of H1, H2, -NAc, and Fuc regions were entered into Sugabase version 1.05 (3), no structure was given, consistent with the evidence that the LEC18 core is a new structure. Since only four other fucosylated core structures are known to exist in mammalian \textit{N}-linked carbohydrates (1–3) and since the ES-MS, MS/MS, and GLC-MS data provide strong evidence for the proposals in Schemes 1 and 2, the spectra in Fig. 9 were tentatively assigned (Table III). Several reasons argue that these assignments will prove correct once larger quantities of LEC18 core glycopeptides can be subjected to chemical analysis. Whereas the presence of the bisecting GlcNAc causes H1 and H2 resonances for each core Man residue to shift, only the H3 of the Man1,4- resonance was significantly changed in the LEC18 core; whereas the presence of a bisecting GlcNAc does not alter the H3 or -NAc resonances of the Asn-GlcNAc, both are changed in the LEC18 core. Most interestingly, the H3 and -NAc resonances of the LEC18 core GlcNAc are different from any other core, including those from nonmammalian sources. Importantly, none of the latter cores show the resonance at 5.211 ppm. This is assigned to the new GlcNAc of the LEC18 core because it is very similar to the H3 at 5.144 ppm of the GlcNAc in \textit{N},\textit{N}-diacetylchitobiose-p-nitrophenyl (33) and to the N-acetyl of this GlcNAc, which occurs at 2.012 ppm compared with 2.018 ppm for the novel LEC18 GlcNAc residue. The chitobiose disaccharide was synthesized by a new GlcNAc-GlcNAc-transferase from snail, and the LEC18 GlcNAc, also in GlcNAc-GlcNAc linkage, behaves very similarly, although not identically. The 5.211-ppm resonance at both 23 and 42°C has a \textit{J} value of 5.5 Hz, a value intermediate between the \textit{J} values observed for residues in \textalpha-linkage (\textit{J} = 3–4 Hz; 3) and residues in \textbeta-linkage (\textit{J} = 6.5–8.0 Hz; Ref. 3). The resonance assigned to the novel GlcNAc is not present in an equimolar amount, in the same manner that the Asn-GlcNAc is not. However, there was no evidence for 3,4,6-substituted GlcNAc in the linkage analysis (Table II), and the H3 and -NAc chemical shifts of the Asn-GlcNAc are as expected. Thus the novel GlcNAc residue in LEC18 cannot be linked to the Asn-GlcNAc.

The 1H NMR spectra and chemical shift comparisons with all previously assigned core structures from all sources, along with methylation linkage analysis and the combined MALDI-TOF-MS, ES-MS, and MS/MS data provide strong evidence that the new GlcNAc is linked to the nonfucosylated core GlcNAc residue at O-6. It is not clear whether the configuration of the GlcNAc1,6GlcNAc linkage is \textalpha or \textbeta because the 1H NMR spectrum of core glycopeptides revealed an intermediate \textit{J} value of 5.5 Hz for the new GlcNAc residue. The structure proposed for
the novel core region of LEC18 N-linked carbohydrates is shown in Scheme 3.

**DISCUSSION**

In this paper, a series of experiments on highly purified [3H]Glc-labeled and unlabeled glycopeptides from LEC18 CHO cells have provided evidence for a new N-linked core structure that has not previously been observed in N-glycans from any source. The novel core structure was found on branched, polyactosamine-containing, N-glycans from LEC18 cells that were not found in parent CHO cells and that bound to both DSA and RCAII (Fig. 2). Parent CHO glycopeptides were present in the DSA-retarded fraction, but they were shown to have the expected trimannosyl cores (>95% bound to ConA) after exhaustive digestion with β-D-galactosidases and N-acetyl-β-D-glucosaminidases (Fig. 4 and Table I). By contrast, identically treated LEC18 glycopeptides remained largely unbound to ConA and PSA (Fig. 4). Composition analysis revealed that the LEC18 core glycopeptides had an extra GlcNAc compared with core glycopeptides from parent cells (Table I).

Proof of an extra GlcNAc in LEC18 core glycans was obtained by preparing ~500 μg of the unique LEC18 species that binds to DSA and RCAII (Fig. 2). It was shown by 1H NMR spectroscopy to be a mixture of branched, poly-N-acetylactosamine-containing species with a core fucose residue (Fig. 5). Exhaustive exoglycosidase digestion of this material gave a discreet core preparation of ~45 μg. MALDI-TOF-MS analysis revealed a major MH+ ion at 1374.4 atomic mass units corresponding to Hex5HexNAc5Hex5Asn (or Man2GlcNAc5(Fuc)3Asn). Fragment ions generated during ES-MS revealed the critical ion at 889.2 atomic mass units that corresponded to a species of HexNAc5Hex5Asn (or GlcNAc5(Fuc)3Asn). MS/MS analysis of the 1374.4 atomic mass units ion confirmed the generation of this critical fragment ion (Scheme 1). In addition, fragment ions generated by ES-MS confirmed the results of MS/MS. These ions were generated when the sample was bombardeed at 70 V under conditions normally used to obtain the molecular weight of proteins. Interpretation of the ES-MS data revealed a fragment ion corresponding to breakage at every glycosidic linkage and the occurrence of four fragmentation pathways (Scheme 2). Although no examples in the literature describe fragment ions obtained from glycopeptides using ES-MS (34), our experience shows that it is possible to obtain sequence information on underivatized, native glycopeptides by recording ES-MS using two different conditions (one at 35 V and the
other at 70 V). This is very useful when dealing with small amounts of samples that are difficult to derivatize.

Taken together, the $^1$H NMR and MS data provide proof for the existence of a novel core that is characterized by GlcNAc substitution of the core GlcNAc. The evidence that the new GlcNAc is linked at O-6 of the core GlcNAc was derived from GLC-MS of the parent compound (Table I). However the configuration of the new linkage is uncertain because the coupling constant of the $^1$H for the novel GlcNAc is 5.5 Hz (Fig. 9), a value intermediate between known $\alpha$- and $\beta$-linked residues (3). The combined data provide strong evidence for the core structure showed in Scheme 3.

If the new GlcNAc in the LEC18 core is in $\beta$-linkage, as seems likely based on its similarity to the terminal nonreducing GlcNAc in GlcNAcp1,4GlcNAc-p-nitrophenol (33), it was nevertheless not hydrolyzed by two N-acetyl-$\beta$-d-hexosaminidases. This may be because the new GlcNAc residue is internal and hence crowded and not accessible to the enzymes. An analogous situation exists in the case of ganglioside GD$_{1a}$ and in lipopolysaccharides from Campylobacter jejuni, which mimic the GD$_{1a}$ structure. These molecules contain two, nonreducing, terminal sialic acid residues, one linked to the penultimate Gal, and the other linked to the internal Gal. Known sialidases are able to remove only the sialic acid residue that is linked to the penultimate Gal, but the sialic acid linked to the internal Gal remains resistant (35).

The combined data presented in this paper strongly suggest that the addition of the new GlcNAc residue is the result of the dominant mutation in LEC18 cells. The novel core is in species of LEC18 glycopeptides that are not synthesized by parent CHO cells; the novel core is fucosylated, gives a new NMR spectrum, and hence crowded and not accessible to the enzymes. An analogous situation exists in the case of ganglioside GD$_{1a}$ and lipopolysaccharides from Campylobacter jejuni, which mimic the GD$_{1a}$ structure. These molecules contain two, nonreducing, terminal sialic acid residues, one linked to the penultimate Gal, and the other linked to the internal Gal. Known sialidases are able to remove only the sialic acid residue that is linked to the penultimate Gal, but the sialic acid linked to the internal Gal remains resistant (35).

Acknowledgments—We thank Edward Nieverg for recording mass spectra at the Laboratory of Macromolecular Analysis, Albert Einstein College of Medicine.

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$^2$ M. Bhaumik, R. Kumar, A. Zhang, and P. Stanley, unpublished observations.

$^3$ T. S. Raju, and P. Stanley, unpublished observations.
LEC18, a Dominant Chinese Hamster Ovary Glycosylation Mutant Synthesizes N-Linked Carbohydrates with a Novel Core Structure
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J. Biol. Chem. 1995, 270:30294-30302.
doi: 10.1074/jbc.270.51.30294

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