LEC18 and LEC14 cells are gain-of-function glycosylation mutants isolated from Chinese hamster ovary cells for resistance to pea lectin. Structural studies have shown that LEC18 cells synthesize complex N-glycans with a GlcNAc residue linked at the O-6 position of the core GlcNAc (Raju, T. S., Ray, M. K., and Stanley, P. (1995) J. Biol. Chem. 270, 30294–30302), whereas LEC14 cells synthesize complex N-glycans with a GlcNAc residue linked at the O-2 position of the core β-linked Man residue (Raju, T. S., and Stanley, P. (1996) J. Biol. Chem. 271, 7484–7493). Both modifications are novel and have not been reported in glycoproteins from any other source. We now show that, in both LEC18 and LEC14 cells, GlcNAc transfer is mediated by a distinct N-acetylglucosaminyltransferase (GlcNAc-T) activity. The LEC18 activity, termed GlcNAc-TVIII, transfers GlcNAc to GlcNAcβ1-O-pNP and to a GlcNAc-terminating, biantennary, complex N-glycan, with or without a core fucose. By contrast, the LEC14 transferase, termed GlcNAc-TVII, does not have significant activity with simple acceptors, and transfers GlcNAc preferentially to a GlcNAc-terminating biantennary glycopeptide that contains a core fucose residue. The acceptor specificities and other biochemical properties of GlcNAc-TVII and GlcNAc-TVIII differ from previously characterized GlcNAc-transferases including GlcNAc-TIII, indicating that they represent new members of the mammalian GlcNAc-T group of transferases.

Glycosylation mutants of mammalian cells allow the identification of new molecules involved in complex glycan synthesis by revealing the nature of acceptor substrates, glycosyltransferases, co-factors, and regulatory molecules through mutations that alter, in each case, only one glycosyl transfer reaction. Both loss-of-function and gain-of-function mutants have revealed new aspects of glycosylation in mammals (1, 2).

Gain-of-function glycosylation mutants express an activity that is not detectable in the parental cell. Each of several Chinese hamster ovary (CHO) cell gain-of-function mutants expresses a glycosyltransferase activity that is lacking in parental CHO, and that synthesizes N-glycans with a sugar modification absent from the N-glycans of parent cell glycoproteins. Thus, the LEC10 CHO mutant (3, 4) expresses GlcNAc-TIII (5), and the N-glycans of glycoproteins made in LEC10 cells include a proportion that contain the bisecting GlcNAc (3). LEC11, LEC12, LEC29, and LEC30 CHO mutants each express an α(1,3)fucosyltransferase activity that is not detectable in parental CHO cells, and they synthesize N-glycans with fucose in O-3 linkage to the GlcNAc of lactosamine units (6–8).

LEC14 and LEC18 CHO mutants are gain-of-function mutants that were obtained, after mutagenesis, by selection for resistance to pea lectin (9). They have unique and distinct lectin resistance properties and behave dominantly in somatic cell hybrids formed with parent CHO cells (9). Structural analyses of complex N-glycans from LEC14 and LEC18 revealed that both add a specific sugar residue to N-glycans that is not present on N-glycans from CHO cell glycoproteins (10, 11). In LEC18 cells, a proportion of the complex, poly-lactosamine-containing N-glycans contain an additional GlcNAc in the core region at the O-6 position of the β(1,4)-GlcNAc adjacent to the β-linked core Man residue (Ref. 10; Fig. 1). This O-6-linked GlcNAc residue is absent from similar N-glycans of parental CHO cells and has not been observed on glycoproteins from other sources. In LEC14 cells, a proportion of the complex, poly-lactosamine-containing N-glycans contain an additional GlcNAc linked β(1,2) to the β(1,4)-Man residue of the core (Ref. 11; Fig. 1). CHO cells lack this modification, which has to date been observed only in N-glycans from LEC14 cells.

The novel N-glycan cores, which are synthesized by LEC14 and LEC18 mutants, suggest that each mutant expresses a GlcNAc-T activity that is silent in parental CHO cells, in a manner analogous to the previously described gain-of-function CHO mutants (2). In this paper we show that this is indeed the case. These novel activities, termed GlcNAc-TVIII (LEC14) and GlcNAc-TVIII (LEC18), generate in vitro, the N-glycan core characteristic of LEC14 and LEC18 glycoproteins, respectively.

**EXPERIMENTAL PROCEDURES**

**Materials**

UDP-6-[3H]GlcNAc (6.1 Ci/mmol), concanavalin A (ConA)-Sepharose, and Sephadex G-25 were from Amersham Pharmacia Biotech. Pea lectin (PSA)-agarose was from Vector Laboratories. Bio-Gel P-2 (45–90 mesh), and AG1-X4 resin (200–400 mesh, Cl– form) were from Bio-Rad. N-Acetyl-β-d-glucosaminidases (Diplococcus pneumoniae and bovine kidney), Pronase (Streptomyces griseus), and protease inhibitor mixture tablets were from Boehringer Mannheim or Prozyme, and jack bean N-acetyl-β-d-glucosaminidase was from Oxford GlycoSciences. \( \text{A} (\dagger)\text{-Glc, A} (\dagger)\text{-Gal, A} (\dagger)\text{-Man, A} (\dagger)\text{-Fuc, A} (\dagger)\text{-GlcN, A} (\dagger)\text{-GalN, methyl a-α-mannoside (MM), methyl a-β-glucoside, GlcNAcβ1-O-pNP, Galβ1-O-pNP, Glcβ1-O-pNP, Manβ1-O-pNP, } \)

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‡The abbreviations used are: CHO, Chinese hamster ovary; ConA, concanavalin A agglutinin; PSA, *Pisum sativum* (pea) agglutinin; GlcNAc-T, N-acetylglucosaminyltransferase; GlcNAcβ1-O-pNP, p-nitrophenyl-N-acetyl-β-d-glucosamine; MM, methyl α-α-mannoside; GnGn, bi-antennary N-linked glycopolypeptide terminating with GlcNAc; GnGnFuc, bi-antennary N-linked glycopeptide (GnGn) containing a core fucose residue linked α1,6 to the Asn-linked GlcNAc; HPAEC-PAD,
Oligomannosyl glycopeptides were prepared from chicken egg ovalbumin as described (12); biantennary N-linked glycopeptides (GnGn) with no fucose and terminating with GlcNAc were isolated from human fibroblasts as described (13) and the corresponding glycopeptides (GnGn(Fuc)) were prepared from human fibroblasts. Diacetylchitooligosaccharide and N,N',N'-triacetylchitooligosaccharide were isolated from chitin essentially as described by Rupley et al. (14). Glycopeptides with the structures shown in Fig. 1 were obtained from glycopeptides of LEC14 and LEC18 cells as described (10, 11).

**Preparation of Cell Extracts**

Postnuclear supernatant from LEC18, LEC14, and parent CHO cells was prepared essentially as described (15). Briefly, cells (6 × 10⁶) were washed twice with saline, followed by one wash with homogenizing buffer (10 mM Tris-[HCl], pH 7.4, with 250 mM sucrose). The cells were disrupted by sonication (200 W for 10 sec) and cell debris was removed by centrifugation (13,000 rpm for 30 min at 4°C). Glycerol was added to the supernatant to a final concentration of 20%, before storage at −70°C. For LEC10 cells, extraction after cell death was prepared as described (9) and the corresponding glycopeptides were desalted on a Bio-Gel P-2 column (1.5 cm × 70 cm) and characterized by glycopeptide mapping using a Dionex HPAEC-PAD. Glycopeptide Mapping by Dionex HPAEC-PAD—Product analysis by HPAEC-PAD was performed using a Dionex Bio LC gradient pump and a pulsed amperometric model PAD-2 detector (Dionex Corp., Sunnyvale, CA) as described (17). Briefly, GlcNAc-T-reactions containing GlcNAcβ1-O-pNP acceptor were passed through a Sep-Pak C₁₈ cartridge, and the 50% aqueous methanol eluant was dried using a Savant Speed Vac. Then, 50–100 μl of glass distilled water passed through a Centrex filter (Schleicher & Schuell) and analyzed by HPAEC-PAD using a CarboPac PA-10 (4 mm × 25 mm) pellicular anion-exchange column equipped with a CarboPac guard column. The column was eluted with 15 mM NaOH generated from 15% eluant 1 (100 mM NaOH) and 85% eluant 2 (water) for 25 min, followed by eluant 3 (500 mM NaOH) for 10 min, at a flow rate of 0.92 ml/min. The following eluents were used: E₁ = 0.05 V (t = 180 s); E₂ = 0.65 V (t = 80 s); E₃ = 0.65 V (t = 60 s). Detection was with 1000 nm full scale. A Dionex Advanced Computer Interface connected to a Gateway 2000, 4SX-33V computer with Dionex AI-450 software (release 3.32.00) was used to collect the data. For radioactivity measurement, fractions of 0.5 min (0.45 ml), were collected, mixed with 5 ml of Ecolume, and counted by liquid scintillation spectrometry. For chemical inactivation, LEC18 cell extract (0.5 ml of protein) was incubated in a final volume of 40 μl with ~40–180 mmol of GlcNAc or GlcNAc(Fuc), in the presence of 50 mM Pipes buffer, pH 7.0, protease inhibitors (Boehringer Mannheim tablet) according to manufacturer's instructions, 0.5% Triton X-100, 10 mM MnCl₂, 0.2 mM GlcNAc, and 24 mM of UDP-GlcNAc (~25,000 cpm/mmol) for 2 h at 37°C. Assays lacking acceptor were used to determine incorporation into endogenous acceptors and degradation of donor sugar. After incubation at 37°C for 15–120 min, the reaction was stopped by adding 950 μl of cold water. Reactions containing simple sugars or the glycopeptides, GnGn or GnGn(Fuc) were passed through a 1-ml column of AG1-X4 resin (Cl⁻ form) that was subsequently washed with 3 ml of water. Eluate and washings were combined, mixed with 17 ml of EcoLume, and counted by liquid scintillation spectrometry. For chemical inactivation, LEC18 cell extract (60–100 μg) of protein in 30 μl of extraction buffer/tube in duplicate) were placed in a water bath set at the appropriate temperature for 10 min before being returned to 4°C and then assayed at 37°C under standard conditions with both GlcNAcβ1-O-pNP and glycopeptide acceptor GnGn, and in the absence of acceptor. For chemical inactivation, LEC18 cell extract (~300 μg of protein in 30 μl of extraction buffer/tube in duplicate) were incubated on ice with 15 μl of buffer alone or 15 μl of buffer containing increasing concentrations of chloramine T. After 5 min at 4°C, 15 μl of potassium metabisulfite at the same concentration as the chloramine T was added to the oxidation reaction. Subsequently, treated extracts were assayed under standard conditions with both GlcNAcβ1-O-pNP and GnGn acceptors, and in the absence of acceptor.

**Product Analysis following N-Acetyl-β-D-Glucosaminidase Treatment**

Transferase reactions containing GnGn or GnGn(Fuc) acceptors were passed through a 1-ml column of AG1-X4 resin (Cl⁻ form) that was subsequently washed with 3 ml of water. The combined eluate and washings were concentrated to ~1 ml, desalted on Bio-Gel P-2 (1.5 cm × 70 cm), and redissolved in buffer appropriate for digestion with counted by liquid scintillation spectroscopy. Lectin Affinity Chromatography—Assay tubes, in which GnGn or GnGn(Fuc) were acceptors, were adjusted to 1 ml with cold water and passed through a 1-ml column of AG1-X4 resin (Cl⁻ form) that was subsequently washed with 3 ml of water. Combined eluate and washings were concentrated to ~250 μl, mixed with 2× ConA buffer (0.2 M sodium acetate, 0.02 mM MgCl₂, 0.02 mM CaCl₂, 0.02 mM MnCl₂, 0.04% sodium azide, pH 7.3), and applied to a ConA-Sepharose column (0.5 cm × 20 cm). The column was washed with at least 10 column volumes of ConA buffer before bound glycopeptides were eluted with at least 4 column volumes of ConA buffer containing 10 mM methyl-α-D-glucoside, followed by ConA buffer containing 10 mM MM, and finally 200 mM MM in ConA buffer. Reaction products were also fractionated on PSA-agarose (0.5 cm × 20 cm) and eluted with ConA buffer followed by ConA buffer containing 200 mM MM. Fractions of 1 ml were collected, and a portion was counted by scintillation spectrometry. Pooled products from lectin columns were desalted on Bio-Gel P-2 (1.5 cm × 70 cm) and characterized by glycopeptide mapping using a Dionex HPAEC-PAD.
Two New GlcNAc-T Activities, GlcNAc-TVII and GlcNAc-TVIII

**Fig. 1.** N-Glycan cores of LEC10, LEC14, and LEC18 glycopeptides. The core GlcNAc residue attached to GnGn(Fuc) that is characteristic of LEC10, LEC14, and LEC18 CHO N-glycans is shown in bold. These structures have been previously established by a variety of techniques (3, 10, 11). These and related glycopeptides were used as standards in Figs. 6 and 7.

Different N-acetyl-β-D-glucosaminidases. Jack bean N-acetyl-β-D-glucosaminidase digestion was performed in 50–100 μl of sodium citrate phosphate buffer (pH 4.5) at 37 °C under toluene for 48 h with a total of 50 milliunits of enzyme. D. pneumoniae or bovine kidney N-acetyl-β-D-hexosaminidase digestion was performed in 50–100 μl of sodium citrate phosphate buffer (pH 5.0) at 37 °C under toluene for 25–48 h with a total of 50–100 milliunits of enzyme. Digestion was stopped by heating in a boiling water bath for 5 min, and products were desalted on a Bio-Gel P-2 column (1.5 cm × 70.0 cm) before analysis by HPAEC-PAD as described above for GnGn and GnGn(Fuc) product analysis.

To determine if the LEC18 product from GlcNAcβ1-O-pNP was susceptible to digestion with N-acetyl-β-glucosaminidase, ~3,500 cpm product was digested for 24 h under the conditions described above with N-acetyl-β-glucosaminidase from D. pneumoniae. As controls, N,N′-diacetyctiohexitolose, N,N′,N″-triacetyctiohexitolose, and N,N′,N″,N‴-tetracetyctiohexitolose (100 μg each) were digested under the same conditions with either jack bean or bovine testis or D. pneumoniae N-acetyl-β-glucosaminidase. Chitobiose and chitotriose digests were dried, resuspended in 15 μl of 15% aqueous acetic acid containing 9-aminoxyrene 1,3,5-trisulfonate to which ~5 μl of sodium cyanoborohydride in THF was added, and incubated at 65 °C for 2 h. The derivatization reaction was stopped by adding 0.5 ml of cold water. The mixture was analyzed using a capillary electropherograph (PACE System 5000, Beckman Instruments, Palo Alto, CA) equipped with an argon ion laser induced fluorescence detector and a coated capillary at 20 kV. The digests of LEC18 product from GlcNAcβ1-O-pNP and the chitobioseβ1-O-pNP standard were analyzed by Dionex HPAEC-PAD as described above.

**RESULTS**

**LEC18 Cells Have a GlcNAc-T Activity Undetectable in CHO Cells—**A subset of complex N-glycans in LEC18 glycoproteins carry a GlcNAc linked to the O-6 position of the core GlcNAc residue (Fig. 1). A GlcNAc-T capable of performing this transfer might act on simple monosaccharide acceptors, similar to chitin synthases (19, 20), or to the GlcNAc-T from the snail, Lymnaea stagnalis, that transfers GlcNAc in β1,4-linkage to simple GlcNAc derivatives and to terminal GlcNAc residues of N- and O-glycans (21–23). Therefore, extracts from LEC18 cells were tested for their ability to transfer GlcNAc to simple monosaccharide acceptors.

Initial assays, under conditions similar to those used for the snail GlcNAc-T (21), revealed that, among a variety of simple sugars, GlcNAcβ1-O-pNP was the only significant acceptor of GlcNAc with LEC18 cell extract, and that parent CHO cell extract had no comparable activity. Interestingly, LEC18 extract had no detectable activity with GlcNAcβ1-S-pNP, whereas this acceptor is optimum for the snail GlcNAc-T (21).

The LEC18 GlcNAc-T also did not transfer GlcNAc at significant levels under these assay conditions to free GlcNAc, chitobiose (GlcNAcβ1,4GlcNAc), or chitotriose (GlcNAcβ1,4GlcnGnβ1,4GlcNAc), showing that it also differs from chitin synthase activities (19, 20). Several other simple oligosaccharides were also not acceptors (Table I), and neither UDP-Glc nor UDP-GalNAc could substitute for UDP-GlcNAc as a donor sugar (data not shown).

Conditions for optimal solubilization of the LEC18 GlcNAc-T were tested with 14 different detergents. At 0.5% final concentration, maximum activity was obtained with Triton X-100 followed by Surfactant Amps-PX, Lubrol, and Nonidet P-40. A dose-response curve showed that 0.5% was the optimal concentration, maximum activity was obtained with Triton X-100 and there was no activity in phosphate-buffered saline, pH 7.0. In 20 mM sodium cacodylate buffer, pH 7.0 or 7.5, gave similar good activity, but there was no activity in phosphate-buffered saline, pH 7.0. In 20 mM sodium cacodylate buffer, LEC18 GlcNAc-T activity gave a relatively sharp optimum at 40 mM MnCl2 (21). Like the snail GlcNAc-T, the LEC18 GlcNAc-T requires the presence of ATP (Fig. 2C). In its absence, no activity was observed, and, when microsomal membranes prepared by centrifugation from the postnuclear supernatant were assayed in 0.5% Triton X-100, 98–99% of the LEC18 GlcNAc-T activity was membrane-associated.

A variety of organic buffers at 10 mM and pH 7.0 or 7.5 gave similarly good activity, but there was no activity in phosphate-buffered saline, pH 7.0. In 20 mM sodium cacodylate buffer, LEC18 GlcNAc-T activity gave a relatively sharp optimum at pH 7.0 (Fig. 2A), and had an absolute requirement for Mn2+, with a rather broad optimum between 20 mM and 60 mM (Fig. 2B). By contrast, the snail β1,4GlcNAc-T is strongly inhibited at 40 mM MnCl2 (21). Like the snail GlcNAc-T, the LEC18 GlcNAc-T requires the presence of ATP (Fig. 2C). In the absence of detergent, no activity was obtained, and, when microsomal membranes prepared by centrifugation from the postnuclear supernatant were assayed in 0.5% Triton X-100, 98–99% of the LEC18 GlcNAc-T activity was membrane-associated.

**TABLE I**

| Acceptor   | Specific activity (nmol/mg/h) |
|------------|------------------------------|
| Parent     | LEC18                        |
| GlcNAcβ1-O-pNP | 0.18 | 2.72 |
| GlcNAcβ1-O-pNP | 0.01 | 0.01 |
| GlcNAcβ1-S-pNP | 0.1 | 0.14 |
| GlcNAcβ1-O-benzyl | 0.2 | 0.3 |
| GalNAcβ1-O-pNP | 0.2 | 0.23 |
| GalNAcβ1-O-pNP | 0.01 | 0.01 |
| Galβ1-O-pNP | 0.01 | 0.01 |
| Manβ1-O-pNP | 0.01 | 0.01 |
| Man, GlcNAc, Asn | 0.01 | 0.01 |
| GlcNAc | 0.3 | 0.3 |
| GalNAc | 0.1 | 0.1 |
| Gal | 0 | 0.03 |
| Glc | 0.02 | 0.02 |
| Man | 0.02 | 0.02 |
| Galβ1, Glc | 0.01 | 0.01 |
| GlcNAcβ1,4GlcNAc | 0.05 | 0.05 |
| GlcNAcβ1,4GlcNAcβ1,4GlcNAc | 0.05 | 0.05 |

LEC18 cell extract transfers GlcNAc to GlcNAcβ1-O-pNP. Cell extracts were incubated with ~50 nmol of acceptor as described under “Experimental Procedures.” Duplicates were performed and the results shown are the average of at least two and up to six independent experiments.
Lineweaver-Burk plots, the apparent $K_m$ for UDP-GlcNAc was calculated as 1.1 mM ($V_{\text{max}}$ = 10 nmol/mg/h) and for GlcNAc-$b$-$1-O$-$p$-NP as 1.8 mM ($V_{\text{max}}$ = 20 nmol/mg/h), respectively.

Under the optimal conditions established for LEC18 extract, parent CHO extract routinely produced a small amount of labeled material (Table I). However, fractionation on Bio-Gel P-2 showed that CHO "product" included a substantial amount of unutilized UDP-GlcNAc, as well as a small amount of material that eluted one fraction beyond the product from LEC18 extract (Fig. 4A). When the latter was analyzed on Dionex HPAEC-PAD, it was clear that CHO extract produced no oligosaccharide product, because all label eluting with product on Bio-Gel P-2 was found to elute at the position of free GlcNAc on Dionex HPAEC-PAD (Fig. 4B). By contrast, LEC18 product from Bio-Gel-P2 eluted on Dionex HPAEC-PAD prior to the substrate GlcNAc-$b$-$1-O$-p-NP, and after authentic GlcNAc-$b$-$1$-$4$-$GlcNAc$-$b$-$1-O$-p-NP, consistent with its being the predicted product, GlcNAc-$b$-$1$-$6$-$GlcNAc$-$b$-$1-O$-p-NP, for which no standard was available. Digestion of the LEC18 product with $N$-$\text{acetyl-}$-$b$-$\text{hexosaminidase}$s from $D$. pneumonae failed to release GlcNAc (data not shown). However, this result is not surprising as digestion of chitobiose or chitotriose with $N$-$\text{acetylglucosaminidase}$s from three sources also failed to release GlcNAc, as measured by capillary electrophoresis. It seems that the terminal GlcNAc in such linear oligosaccharides is highly resistant to removal by $b$-hexosaminidases.

LEC18 and LEC14 Cells Have Distinct GlcNAc-Ts That Act on the Biantennary N-Glycan GnGn(Fuc)—Attempts to identify a GlcNAc-T that transferred GlcNAc to simple sugar acceptors in LEC14 extracts failed (data not shown). Therefore, acceptors that could potentially be biosynthetic intermediates were tested. The novel GlcNAc residues found in the core of LEC14

\[ \text{FIG. 2. Optimum pH, Mn}^{2+}, \text{and ATP concentrations for the LEC18 GlcNAc-T.} \]

\[ \text{FIG. 3. Kinetic analysis of the LEC18 GlcNAc-T. Using optimal assay conditions and 1 mM GlcNAc-$b$-$1-O$-p-NP, the UDP-[3H]GlcNAc concentration was varied (A); under the same conditions with 0.25 mM UDP-[3H]GlcNAc, the GlcNAc-$b$-$1-O$-p-NP concentration was varied (B).} \]

\[ \text{FIG. 4. Product analysis for LEC18 GlcNAc-T by Bio-Gel P2 chromatography. LEC18 and CHO extracts were incubated with GlcNAc-$b$-$1-O$-p-NP (10 nmol) under optimal assay conditions for 2 h. At termination, 2 tubes of LEC18 and 10 tubes of CHO were separately combined, concentrated to 2 ml, and desalted, on a Bio-Gel P-2 column eluted in water. UDP-GlcNAc elutes anomalously under these conditions due to an interaction with Bio-Gel-P2 (A). Products (fractions 25–30) were pooled as indicated, concentrated, and resuspended in 100 $\mu$L of water, and 50 $\mu$L was analyzed on Dionex HPAEC-PAD (B) as described under "Experimental Procedures." The elution positions of standard markers, GlcNAc-$b$-$1-O$-p-NP, and GlcNAc-$b$-$1$-$4$-$GlcNAc$-$b$-$1-O$-p-NP are indicated. Under these conditions, free GlcNAc eluted at $\sim$22 min. The parent "product" eluted as free $^3$H-GlcNAc only.} \]
and LEC18 N-glycans (Fig. 1) are somewhat analogous to the bisecting GlcNAc (Fig. 1), in the case of LEC14, and to a fucose that modifies the core GlcNAc (24), in the case of LEC18. GlcNAc-TIII transfers the bisecting GlcNAc to the O-4 position of Man in GnGn (3, 5), and GnGn is also the preferred substrate of core α(1,6)Fuc-T (25) and core β(1,2)xylosyl-T (26). It has previously been established that parent CHO extract does not transfer GlcNAc to GnGn under assay conditions in which GlcNAc-TIII in LEC10 extract transfers the bisecting GlcNAc at a specific activity of 6–10 nmol/mg/h (3, 4). Therefore, the branching transferases, GlcNAc-TVII and GlcNAc-TV, which are certainly active in CHO cells based on structural studies of CHO-derived glycoproteins (for example Ref. 27), and from assays with GnGn and CHO extracts performed under different conditions (15), were not detected under the conditions optimal for the LEC18 GlcNAc-T.

When LEC18 and LEC14 extracts were incubated with GnGn(Fuc) and UDP-[3H]GlcNAc, both extracts gave significantly more transfer than did parent CHO extract (Table II). Mixing experiments showed that there was no inhibitor in parent CHO extracts (Table II). Glycopeptide products of these assays were therefore characterized by lectin affinity chromatography on ConA-Sepharose and PSA-agarose, and by oligomapping on Dionex HPAEC-PAD, both before and after digestion with β-hexosaminidases.

Biantennary glycopeptides like GnGn and GnGn(Fuc) bind to ConA-Sepharose even if they contain a bisecting GlcNAc (3, 4). However, GnGn(Fuc) glycopeptides from LEC14 and LEC18 cells that contain an extra core GlcNAc do not bind to ConA-Sepharose (10, 11). When the products of GlcNAc-T assays with GnGn(Fuc) were chromatographed on ConA-Sepharose, >90% of the counts from parent CHO products bound to ConA (data not shown), suggesting that they arose not by the addition of a branching GlcNAc, but by replacement of a GlcNAc in the acceptor due to the action of GlcNAc-TI or GlcNAc-TII (reviewed in Ref. 28). Approximately 20–30% of LEC14 and LEC18 products also bound to ConA (data not shown), presumably for the same reason. However, the majority of the LEC14 and LEC18 products (70–80%) did not bind to ConA, as expected if they contained the additional core GlcNAc.

The biantennary N-glycan glycopeptides from LEC14 and LEC18, which carry an extra core GlcNAc (Fig. 1), were also shown previously not to bind to PSA-agarose (10, 11). Thus authentic products of a novel GlcNAc-T in LEC14 and LEC18 cells should not bind to PSA-agarose. Consistent with this, a majority of the reaction products from LEC18 and LEC14 extracts did not bind to PSA-agarose (Fig. 5). By contrast, almost all the products from CHO extracts bound to this column.

To further characterize GlcNAc-T assay products, they were analyzed by oligomapping on a Dionex HPAEC-PAD (17) after partial purification by chromatography on Bio-Gel P-2. Structurally characterized glycopeptides shown in Fig. 1, as well as GnGn and GnGn(Fuc), were used to calibrate the column (Fig. 6). All extracts gave rise to a small amount of product eluting at ~21 min. This may be triantennary glycopeptides arising from the action of GlcNAc-TV and/or GlcNAc-TV. However, the major product of each cell extract eluted at a unique position. The major CHO product eluted earliest, at the same position as authentic GnGn(Fuc), providing strong evidence that it had acquired a labeled GlcNAc by hydrolysis and re-addition of a GlcNAc residue; the major product from LEC14 extract eluted at the position of authentic LEC14 glycopeptide at 22 min (Ref. 11; Fig. 1); the LEC18 product interacted most tightly with the column, eluting at ~42 min at the position of authentic LEC18 glycopeptide (Ref. 10; Fig. 1); and LEC10 product containing the bisecting GlcNAc (Fig. 1), eluted at 29 min (Fig. 6). These data clearly show that CHO extract has no activity similar to GlcNAc-TI or LEC14 or LEC18 GlcNAc-Ts. In addition, the major product from LEC10, LEC14 and LEC18 eluted beyond GnGn(Fuc) at the position predicted from the respective glycopeptide standards.

Further characterization of GlcNAc-T products was obtained by digestion with N-acetyl-β-D-hexosaminidases from jack bean, bovine kidney, or D. pneumoniae (Fig. 7). CHO product was converted almost completely to free GlcNAc by hexosaminidase treatment, as predicted if the CHO product was labeled GnGn(Fuc). The LEC10 product was also completely digested to give free GlcNAc, as expected because the bisecting GlcNAc is susceptible to β-hexosaminidases (29, 30). Some of the LEC14 products were hydrolyzed to release GlcNAc, but the majority were resistant to digestion and eluted at the same
position as untreated product. This is exactly what was observed in the N-glycans characteristic of LEC14 glycoproteins (11). Despite exhaustive digestion with jack bean and D. pneumoniae hexosaminidases, the major LEC14 N-glycans with a \(b_{1,2}\)-linked core GlcNAc remained unaffected (11). The combined evidence shows that LEC14 cells express a novel GlcNAc-T activity that is undetectable in CHO cells, LEC10 cells, or LEC18 cells. This new transferase activity will henceforth be called GlcNAc-TVII.

The products of LEC18 extract also behaved as expected (Fig. 7). Hexosaminidase digestion almost completely eliminated the major LEC18 product that eluted at 42 min in Fig. 6. Previous results have shown that LEC18 N-glycans containing a \(\beta_{1,2}\)-linked core GlcNAc remained unaffected (11). The combined evidence shows that LEC14 cells express a novel GlcNAc-T activity that is undetectable in CHO cells, LEC10 cells, or LEC18 cells. This new transferase activity will henceforth be called GlcNAc-TVII.

Two New GlcNAc-T Activities, GlcNAc-TVII and GlcNAc-TVIII

The products of LEC18 extract also behaved as expected (Fig. 7). Hexosaminidase digestion almost completely eliminated the major LEC18 product that eluted at 42 min in Fig. 6. Previous results have shown that LEC18 N-glycans containing the extra core GlcNAc are relatively resistant to hexosaminidase digestion (10), but prolonged digestion removes both arm \(\beta_{1,2}\)-linked GlcNAc residues, although it does not remove the extra core GlcNAc residue, which is highly resistant to digestion (10). Thus, the major product of \(\beta_{1,2}\)-hexosaminidase digestion of LEC18 N-glycans is Man$_3$GlcNAc$_3$(Fuc)$_n$Asn (10). This glycopeptide, previously characterized by mass spectrometry and composition analysis (10), was shown to elute from the glycopeptide mapping column at 16 min, the same position as the major digestion product in Fig. 7. The combined results provide strong evidence that the LEC18 GlcNAc-T indeed synthesizes the novel N-glycan core of LEC18 cells. This represents a GlcNAc-T activity not present in CHO, LEC10, or LEC14 cells that will henceforth be known as GlcNAc-TVIII.

\[ \text{GlcNAc-TVIII Is Responsible For Transferring GlcNAc to GlcNAc}\beta_{1-O-pNP and to the Core of a N-Glycan—Because LEC18 extract transfers GlcNAc to GlcNAc}\beta_{1-O-pNP, the question arises as to whether LEC18 extracts possess two novel GlcNAc-Ts: GlcNAc-TVIII that transfers GlcNAc to the core of \(N\)-glycans and another GlcNAc-T that transfers GlcNAc to... \]
GlcNAc-TVII utilized this acceptor very poorly (Fig. 9, a panel) and suggests that this transferase may not act until after the consistent with the fact that LEC14 glycopeptides with the b1-4GlcNAc linked core GlcNAc were core fucosylated (11). Interestingly, for 5 min on ice, potassium metabisulfite was added, and the extract was assayed for transfer of GlcNAc to GnGn as described under “Experimental Procedures.” Specific activity for the 100% value was 2.1 nmol/mg/h for GlcNAc-TVII and 5.7 nmol/mg/h for GnGn. The results are the average of one or two experiments performed in duplicate. Other experiments gave similar results.

GlcNAc-TVIII transfers GlcNAc to both GlcNAc1-O-pNP and to GnGn. To obtain a heat inactivation profile, LEC18 cell extract was treated for 10 min at increasing temperatures before being assayed under standard conditions for transfer of GlcNAc to GlcNAc1-O-pNP or GnGn as described under “Experimental Procedures.” Specific activity for the 100% value was 2.8 nmol/mg/h for GlcNAc-TVII-O-pNP and 6.1 nmol/mg/h for GnGn. For chemical inactivation, LEC18 cell extract was treated with increasing concentrations of chloramine T for 5 min on ice, potassium metabisulfite was added, and the extract was assayed for transfer of GlcNAc to GlcNAc1-O-pNP or GnGn as described under “Experimental Procedures.” Specific activity at 100% was 2.1 nmol/mg/h for GlcNAc-TVII-O-pNP and 5.7 nmol/mg/h for GnGn. The results are the average of one or two experiments performed in duplicate. Other experiments gave similar results.

The results are the average of one or two experiments performed in duplicate. Other experiments gave similar results.

Glycopeptides containing the core GlcNAc unique to LEC18 glycoproteins were also core fucosylated (10). However, in contrast to GlcNAc-TVII, GlcNAc-TVIII utilized GnGn and GnGn(Fuc) rather equivalently in vitro (Fig. 9).

Under our assay conditions, neither GlcNAc-TVII nor GlcNAc-TVIII transferred GlcNAc to ManβGlcNAc1Asn, the intermediate act on by GlcNAc-TI (see Ref. 28), despite the fact that the sugar residues to which both enzymes transfer are present in ManβGlcNAc1Asn (Table I). This N-glycan intermediate will also not serve as an acceptor for GlcNAc-TVIII (reviewed in Ref. 31). However, GlcNAc-TVII and GlcNAc-TVIII are clearly distinct transferases from GlcNAc-TIII, as became apparent when GnGn and GnGn(Fuc) were compared as acceptors for GlcNAc-TIII. At saturating levels of glycoprotein acceptor, GlcNAc-TVIII transferred GlcNAc preferentially to GnGn(Fuc) compared with GnGn, GlcNAc-TVIII transferred well to GnGn(Fuc) and had good activity with GnGn, whereas GlcNAc-TVIII from LEC10 cells transferred the bisecting GlcNAc preferentially to GnGn (Fig. 10). These results suggest that each transferase recognizes a different aspect of the GnGn(Fuc) core acceptor.

**DISCUSSION**

Molecules that participate in glycosylation reactions responsible for the synthesis of complex glycans are identified from knowledge of glycan structures that act as acceptors in assays for glycosyltransferases, by characterizing the products of new glycosyltransferase genes, and by determining the biochemical basis of mutations that affect individual glycosylation reactions. The advantage of the mutant strategy is that it often leads to the identification of an activity for which existence was not be predicted from structural studies because the activity generates an intermediate never found on a mature glycoconjugate, or because glycoconjugates bearing the modification generated by that activity have never been isolated. This latter situation is the case with LEC14 and LEC18 CHO cells, which we have shown in this paper to express two new GlcNAc-T activities termed GlcNAc-TVII (LEC14) and GlcNAc-TVIII (LEC18).

Previous studies of the N-glycans present on LEC14 and...
LEC18 glycoproteins showed that each of these mutants adds a GlcNAc to the core of N-glycans in a linkage not observed in previously characterized N-glycans (Fig. 1; Refs. 10 and 11). Based on the properties of similar gain-of-function CHO glycosylation mutants (2), the biochemical basis for the LEC14 and LEC18 phenotypes was predicted to be the de novo expression of a glycosyltransferase activity (10, 11). In vitro assays with detergent extracts or microsomal membranes have now shown that GlcNAc-TVII and GlcNAc-TVIII are unique to LEC14 and LEC18 cells, respectively. They appear to be Golgi transferases because of their membrane association and because biantennary glycopeptides that are generated in the medial or trans Golgi are acceptors.

The major product obtained from GnGn(Fuc) by GlcNAc-TVIII behaved exactly as authentic LEC18-derived N-glycans described previously (10); it did not bind to either ConA-Sepharose or PSA-agarose, eluted from CarboPac PA-1 at the same position as the authentic LEC14 glycopeptide shown in Fig. 1, and it was completely resistant to digestion with N-acetyl-β-glucosaminidases (Fig. 7). GlcNAc-TVIII is a GlcNAc-to-Man transferase and catalyzes a reaction most similar to a β(1,2)xylosyltransferase recently purified from plants (26). However, in contrast to the β(1,2)xylosyltransferase, which utilizes GnGn and GnGn(Fuc) equivalently as acceptors, GlcNAc-TVIII strongly prefers GnGn(Fuc).

Two outcomes of this study are of some interest with respect to the properties of mammalian GlcNAc-Ts. The first is that GlcNAc-TI and GlcNAc-TIII are not active under the in vitro conditions optimal for GlcNAc-TVII and GlcNAc-TVIII (Table I), and GlcNAc-TVIV and GlcNAc-TV activities are minimal under these conditions (Fig. 6). The second is the clear difference in acceptor preference of the three GlcNAc-Ts that modify the core of N-glycans. When presented with GnGn versus GnGn(Fuc), GlcNAc-TIII clearly prefers GnGn(Fuc), whereas GlcNAc-TVIII uses both acceptors equivalently (Figs. 9 and 10). These results provide additional evidence of the distinct nature of GlcNAc-TII, GlcNAc-TVII, and GlcNAc-TVIII. In addition, they indicate that the in vivo action of these GlcNAc-Ts may depend on the prior action of other glycosyltransferases, such as α(1,6)fucosyltransferase or branching GlcNAc-Ts. In this context, it is of note that monoclonal antibodies that recognize the α(1,6)fucose residue in an N-glycan core do not bind well to LEC14 or LEC18 glycoproteins (32).

It will now be important to clone the genes that encode GlcNAc-TVII and GlcNAc-TVIII. This should be possible by expression cloning using a cDNA library from LEC14 or LEC18 to complement CHO cells. We have recently shown that expression of GlcNAc-TIII in LEC10 CHO cells corresponds to transcriptional activation of the Mgat3 gene. Northern blot analyses, with probes from the mouse Mgat3 gene that encodes GlcNAc-TIII (33), revealed the predicted ~4.7-kilobase Mgat3 gene transcript in LEC10 cells and no signal from the silent Mgat3 gene of CHO cells. Expression of the α(1,3)Fuc-T in LEC11 cells also corresponds to transcriptional activation of a Chinese hamster FUT gene. Three independent LEC11 mutants have been shown to possess an ~1.8-kilobase transcript that hybridizes to a CHO FUT gene probe, whereas this gene is transcriptionally inactive in parent CHO cells (34). It therefore seems likely that GlcNAc-TVII and GlcNAc-TVIII reflect transcriptional activation of new glycosyltransferase genes. Although it is possible that, like the blood group A, B, and O transferases that represent alleles of a single gene (35), GlcNAc-TVII and/or GlcNAc-TVIII might arise from the alteration of a known GlcNAc-T gene, the properties of genes encoding GlcNAc-Ts make this unlikely. To date, any GlcNAc-T that transfers GlcNAc to a unique substrate (e.g. GlcNAc-TI versus GlcNAc-TII; see Ref. 28), or in a specific linkage, is encoded by a unique gene. There is no significant sequence homology between the genes encoding GlcNAc-TI, GlcNAc-TII, GlcNAc-TIII, GlcNAc-TV, or core 2 GlcNAc-T (reviewed in Ref. 36). Although there is homology between core 2 GlcNAc-T and the GlcNAc-T that creates the I antigen (37), these genes encode transferases that perform essentially identical reactions. By contrast, GlcNAc-TVII (LEC14) and GlcNAc-TVIII (LEC18) perform unique reactions, distinct from all the other GlcNAc-Ts described to date, and therefore are predicted to be encoded by distinct genes.

Cloning of both genes is an essential step to determining biological functions for the GlcNAc residues they add to N-
Two New GlcNAc-T Activities, GlcNAc-TVII and GlcNAc-TVIII

glycans. The reason N-glycans with these GlcNAc residues have not previously been observed is presumably because they are present on a limited number of tissue specific glycoproteins or serum glycoproteins, or present in small amounts, or present only at particular times of development. Cloning the genes that encode GlcNAc-TVII and GlcNAc-TVIII will allow their spatio-temporal expression patterns to be determined and their expression to be altered or ablated in a complex organism such as the mouse.

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