The Lec4A CHO Glycosylation Mutant Arises from Miscompartmentalization of a Golgi Glycosyltransferase

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Abstract. Two CHO glycosylation mutants that were previously shown to lack N-linked carbohydrates with GlcNAcβ1,6Manα1,6 branches, and to belong to the same genetic complementation group, are shown here to differ in the activity of N-acetylglucosaminyltransferase V (GlcNAc-TV) (UDP-GlcNAc:α1,6mannose β-N-acetylglucosaminyltransferase V). One mutant, Lec4, has no detectable GlcNAc-TV activity whereas the other, now termed Lec4A, has activity equivalent to that of parental CHO in detergent cell extracts. However, Lec4A GlcNAc-TV can be distinguished from CHO GlcNAc-TV on the basis of its increased sensitivity to heat inactivation and its altered subcellular compartmentalization. Sucrose density gradient fractionation shows that the major portion of GlcNAc-TV from Lec4A cells cofractionates with membranes of the ER instead of Golgi membranes where GlcNAc-TV is localized in parental CHO cells. Other experiments show that Lec4A GlcNAc-TV is not concentrated in lysosomes, or in a post-Golgi compartment, or at the cell surface. The altered localization in Lec4A cells is specific for GlcNAc-TV because two other Lec4A Golgi transferases cofractionate at the density of Golgi membranes. The combined data suggest that both lec4 and lec4A mutations affect the structural gene for GlcNAc-TV, causing either the loss of GlcNAc-TV activity (lec4) or its miscompartmentalization (lec4A). The identification of the Lec4A defect indicates that appropriate screening of different glycosylation-defective mutants should enable the isolation of other mammalian cell trafficking mutants.

Attempts to identify signals responsible for the compartmentalization of molecules in the secretory pathways of mammalian cells usually involve protein engineering that may result in a molecule which fails to traverse the pathway for nonspecific reasons. Endogenous mutations that give rise to altered proteins that retain function, despite being incorrectly compartmentalized, should therefore provide a valuable source of information on the molecular nature of cellular targeting signals. However, a selection giving rise to such mutants in mammalian cells has not so far been reported. In this paper, we describe a Chinese hamster ovary (CHO) glycosylation mutant, termed Lec4A, which possesses an active, but miscompartmentalized N-acetylglucosaminyltransferase V (GlcNAc-TV; UDP-GlcNAc:α1,6mannose β1,6-N-acetylglucosaminyltransferase V), a transferase normally resident in the medial Golgi complex (9).

The glycosyltransferase GlcNAc-TV adds GlcNAc in β(1, 6)-linkage to the Manα1,6 residue of N-linked carbohydrates to initiate the formation of a GlcNAcβ1,6Manα1,6 branch (6). A BWS147 mutant that lacks this activity has previously been described (6, 31). Two independent CHO glycosylation mutants that seemed likely to possess a similar mutation causing the loss of GlcNAc-TV activity have also been described (25, 27). Like the lymphoma mutant, both CHO mutants fail to synthesize GlcNAcβ1,6Manα1,6-branched carbohydrates on glycoproteins (21, 28) and, as a consequence, are highly resistant to the lectin l-phytohemagglutinin (l-PHA; 27) that binds Gal residues of these carbohydrates (5, 10). The altered phenotypes of the CHO mutants were indistinguishable by many criteria (21, 26-28) and, in addition, both mutants belong to the same complementation group (25) showing that the same gene is affected in each isolate. Therefore, it was expected that both mutants would have the same biochemical defect. However, in this paper we show that assays for GlcNAc-TV activity revealed a dramatic difference between the two mutants. One of the mutants, previously termed Gar'LeC4.2D (21), has no detectable GlcNAc-TV activity. In contrast, the other mutant, previously termed Pro'LeC4.12-2 (21, 28), has GlcNAc-TV activity comparable to that of parental CHO cells in detergent cell extracts. To distinguish between the mutant types, the one lacking GlcNAc-TV activity is referred to as Lec4, while the mutant type possessing GlcNAc-TV activity is now termed Lec4A. Evidence is presented that GlcNAc-TV in Lec4A cells is not able to act in the intact cell because it is not concentrated in Golgi membranes.
Materials and Methods

Cell Lines

The cell lines used in these studies were isolated previously: Pro-5 and Pro Lec4.12-2 (22, 27); Gat Lec4.2D (25); Pro Lec8.3D and Gat Lec8.1C (22). A detailed description of the nomenclature for lectin-resistant CHO cells isolated by this laboratory has been published (22). Because of the findings presented in this paper, the Pro Lec4.12-2 cell line will henceforth be called Pro Lec4.12-2. Cells were cultured in alpha medium (Gibco Laboratories, Grand Island, NY) containing 10% horse serum (Hazelton Laboratories, Lenexa, KS), and 2% fetal calf serum (Flow Laboratories, Inc., McLean, VA) in suspension at 37°C. Tests for mycoplasma contamination using Hoechst 33258 staining (4) were negative.

Selection of New Mutants

Independent mutants of the Lec4 phenotype were selected from a Pro-5 cell population mutagenized with ethylmethanesulfonate (200 μg/ml for 18 h; ~14-d expression period) and pregrown for 2 d in 5 μg/ml Con A (Pharmacia, Uppsala, Sweden), by plating 10^6 cells/100-mm dish in alpha medium containing 10% FCS, 25 μg/ml L-PHA lymphoagglutinin (Vector Laboratories, Inc. Burlingame, CA), 7.5 μg/ml Lens culinaris agglutinin (Vector Laboratories, Inc.), and 10 μg/ml Con A. After 8 d at 37°C in a humidified atmosphere of 5% CO_2, surviving colonies arose at a frequency of ~10^-5. Of 11 colonies picked and tested for lectin resistance against L-PHA and Con A (22), 9 behaved as expected for Lec4 cells (27), and none of those had detectable GlcNAc-TV activity (data not shown). Two clones derived by limiting dilution were shown to exhibit noncomplementation in hybrids formed with Gat Lec4.2D cells. Therefore, they represented independently derived Lec4 isolates. No mutants with the properties of Lec4 cells were obtained from this selection.

The double mutant Lec4A Lec8 was selected from Lec4A cells (Pro Lec4A.12-2) that were pregrown in Con A (5 μg/ml) and 0.5 mg/ml ricin (Vector) for 2 d in suspension culture. For selection, the cells were plated in alpha medium containing 10% FCS and 20 μg/ml wheat germ agglutinin (WGA; Sigma Chemical Co., St. Louis, MO) at 10^5 cells/100-mm dish. After 7 d at 37°C in a 5% CO_2 humidified atmosphere, the medium with WGA was replaced with alpha medium containing 10% FCS and Con A (5 μg/ml) and ricin (0.5 mg/ml). Colonies arising at a frequency of ~10^-3 were picked 4 d later, cultured, and tested for lectin resistance as described (22). Of 12 colonies tested, 7 had Lec4 phenotypes similar to those expected for a Lec4A Lec8 Lec8 phenotype (24). Clones were derived by limiting dilution from two isolates and shown by comparison analysis (22) to be recessive and to exhibit noncomplementation in hybrids formed with Gat Lec8.1C cells.

Complementation Analysis

To identify genotypes belonging to a particular recessive complementation group, hybrids were formed between the appropriate pairs of glycosylation mutant and DMSO as previously described (22). Each mutant in the cross carried a different auxotrophic marker (Pro- or Gat-). All hybrids were pregrown in alpha medium containing 10% FCS and 20 μg/ml Con A (5 μg/ml) and 0.5 mg/ml ricin (Vector) for 2 d in suspension culture. For selection, the cells were plated in alpha medium containing 10% FCS and 20 μg/ml wheat germ agglutinin (WGA; Sigma Chemical Co., St. Louis, MO) at 10^5 cells/100-mm dish. After 7 d at 37°C in a 5% CO_2 humidified atmosphere, the medium with WGA was replaced with alpha medium containing 10% FCS and Con A (5 μg/ml) and ricin (0.5 mg/ml). Colonies arising at a frequency of ~10^-3 were picked 4 d later, cultured, and tested for lectin resistance as described (22). Of 12 colonies tested, 7 had Lec4 phenotypes similar to those expected for a Lec4A Lec8 phenotypes (24). Clones were derived by limiting dilution from two isolates and shown by comparison analysis (22) to be recessive and to exhibit noncomplementation in hybrids formed with Gat Lec8.1C cells.

Preparation of lidoactive Glycopeptide

The glycopeptide GlcNAcβ2Manα1Manβ2(Manα1Manβ1)Glcnacβ2Fucα1Manα1Asn, termed GlnGn(Fuc), was prepared by exhaustive digestion with Pronase (Boehringer Mannheim Biochemicals, Indianapolis, IN) of ~10 g of either human gamma globulin (Miles Scientific Div. Naperville, IL) or porcine thyroglobulin (Sigma Chemical Co.). Pronase glycopeptides which bound to Con A-Sepharose (Pharmacia) were eluted with 200 mM α-methylmannoside, extensively desalted, and applied to a peat lectin-Sepharose column. The specifically bound peak was eluted with 200 mM α-methylmannoside, extensively desalted, and digested with neuraminidase (Sigma Chemical Co.; 0.5 U/50 μmol glycopeptide in 1 ml 50 mM citrate phosphate buffer, pH 5.0, for ~20 h at 37°C) followed by jack bean β-galactosidase (Sigma Chemical Co.; 1.5 U/6 μmol glycopeptide in 1 ml 50 mM citrate phosphate buffer, pH 3.5, for ~20 h at 23°C). Two further additions of β-galactosidase and incubation at room temperature for an additional 2 d, the sample was boiled, desalted extensively, passed through Chelex 100 (Bio-Rad Laboratories, Richmond, CA), repeatedly exchanged with deuterium oxide, and examined by 1H-NMR spectroscopy at 23°C as described (28). The spectra showed that the glycopeptide was authentic GlnGn(Fuc) and contained equimolar amounts of α-linked fucose with no detectable galactose or sialic acid residues.

Glycopeptide GlnGn(Fuc) was conjugated with fresh Bolton-Hunter reagent (Sigma Chemical Co.) by dissolving 2 mg glycopeptide in 1 ml 0.1 M Na borate, pH 8.5, on ice and adding 20 mg Bolton-Hunter reagent. After 2 h, 1 ml distilled water was added and unconjugated reagent removed by desalting on a 1.5 x 40-cm column of BioGel-P2 (Bio-Rad Laboratories). Fractions were monitored by OD at 230 nm and 280 nm and the material eluting in the V_c was pooled and termed GlnGn(Fuc)BH. This was incubated for 3 h at 37°C with 500 μg of trout spleen a-galactosidase (Boehringer Mannheim Biochemicals, Indianapolis, IN) and 25 μg of human placental lactogen. The material was purified on a Sephadex G25 column. Assuming ~90% recovery, GlnGn(Fuc)BH - 125I with a specific activity of ~2 x 10^10 cpm/nmol was obtained. Preparations with much lower specific activities were sometimes obtained and found not to act as good substrates in the GlcNAc-TV assay.

GlcNAc-TV Assays

Exponentially growing cells were washed with saline, and disrupted with 75 μl 1.5% Triton X-100 (Sigma Chemical Co.) in distilled water per 10^7 washed cells. Nuclei were removed by centrifugation at 3,000 rpm for 3 min in an IEC centrifuge and the supernatant was assayed for GlcNAc-TV activity using lidoactive as acceptor as described by Cummings et al. (6), or using a synthetic acceptor as described by Pierce et al. (17).

Lidoactive as acceptor. The reaction mixture included 25 mM 2N-morpholinoethanesulfonic acid (MES; Sigma Chemical Co.) buffer, pH 6.0, 10-20 mM UDP-N-acetylgalcosamine (Sigma Chemical Co.), ~200,000 cpm (~0.1 nmol) GlnGn(Fuc)BH - 125I, 0.74 nmol GlnGn(Fuc)BH, 10-20 mM MnCl_2, and 50-150 μg cell extract protein in a final volume of 40 μl. Incubation was at 37°C for 2-6 h. Reactions were stopped by adding 0.5 ml Con A buffer (1.0 M NaN_3, 0.1 M Na acetate, 10 mM CaCl_2, 10 mM MnCl_2, 10 mM MgCl_2, 0.02% NaN_3, pH 7.0) and freezing. Reaction products were purified by lectin-affinity chromatography on 2-ml columns of Con A-Sepharose followed by a lectin-agarose eluate in each case with Con A buffer. Branch hybrids passed through the Con A-Sepharose which bound unmodified substrate, GlnGn(Fuc)BH - 125I. The product of GlcNAc-TV action (GlnGn[β2][β2]GlcNAc)BH - 125I bound specifically to pea lectin-agarose (6) and was eluted with Con A buffer containing 200 mM α-methylmannoside. Specific activities were calculated after subtraction of radioactivity in the product fraction of a boiled extract control or an extract lacking UDP-GlcNAc (typically 300-600 cpm), and are given as pmol/h per mg protein. Experiments involving internal comparisons of GlcNAc-TV activity were performed with nonlysing preparations of GlnGn(Fuc)BH - 125I to conserve this substrate which was not commercially available.

Synthetic acceptor. The synthetic carbohydrate substrate, GlcNAcβ2Manα1Manα1O-(CH_2)_3COOCH_3 (30), is a specific acceptor of GlcNAc transferred by GlcNAc-TV (11, 16). Using this acceptor oligosaccharide (kindly donated by Dr. O. Hindsgaul, University of Alberta, Canada), detergent extracts were assayed for GlcNAc activity in a 20-μl reaction mixture containing 40 μg acceptor, 32 amol UDP-[3H]-GlcNAc (New England Nuclear, Boston, MA; 20 Ci/mmol) at ~20,000 cpm/nmol, 100 mM MES buffer (pH 6.0), and 50-100 μg cell extract protein. After 5 h at 37°C, the radioabeled product was purified on a SEP-PAK 18 cartridge (Millipore Continental Water Systems, Bedford, MA) as described (17). Specific activities (pmol/h per mg protein) were calculated from the radioactivity transferred to product after subtraction of that obtained from a control extract lacking substrate.

Lectin Affinity Chromatography of Radiolabeled Glycopeptides

Lec8 and Lec4A, Lec8 cells growing in a 10-ml suspension culture were la-
beled in complete medium for ~20 h with 50 µCi D-[6-3H]glucosamine hydrochloride (Amersham Corp.; ~35 Ci/mmol). After washing three times with PBS (pH 7.2), the cells were lysed in 1 mM Tris HCl (pH 7.4) containing 10% NP-40 (Sigma Chemical Co.). The cell extracts were dialyzed against 1 mM Tris HCl (pH 7.4) and 200 mM α-methylmannoside. 1.9-mL fractions were collected and counted after the addition of 18 mL aqueous scintillation fluid in a scintillation counter (Beckman Instruments, Inc., Fullerton, CA).

Preparation of Membrane Fractions

Crude membranes were prepared from washed cells resuspended in 20 mM MES buffer (pH 6.0) containing 1 mM CaCl2 and a mixture of protease inhibitors (Sigma Chemical Co.): soybean trypsin inhibitor (200 µg/mL), benzamidine (5 mM), caproic acid ethyl ester (5 mM), and paramethylmercurisulfonylfluoride (PMSF; 4 mM). After swelling 15 min on ice, the cells were Dounce homogenized (75-150 strokes), centrifuged at 1,600 rpm for 10 min in an IEC centrifuge at 4°C, and the supernatant was spun at 100,000 × g for 30 min at 4°C. The membrane pellet was resuspended in 100 µL 1% Triton X-100 containing 20 mM MES (pH 6.0) with 4 mM PMSF and assayed for GlcNAc-TV activity.

Sucrose density gradients were performed essentially according to Balch et al. (2). Washed cells were resuspended in homogenizing buffer (0.25 M sucrose, 10 mM Tris HCl, pH 7.4) at 6 × 107 cells/mL, allowed to swell on ice 20 min, and homogenized by seven passes using a 0.2459-inch ball-bearing in a steel homogenizer (1). The homogenate was centrifuged at 3,000 rpm for 10 min in an IEC centrifuge at 4°C, and the supernatant was spun at 100,000 × g for 30 min at 4°C. The membrane pellet was resuspended in 100 µL 1% Triton X-100 containing 20 mM MES buffer (pH 6.0) with 4 mM PMSF and assayed for GlcNAc-TV activity.

Protein Determinations

Proteins were determined by a modified Lowry procedure (13), except in Percoll gradient fractions when the Percoll was removed by precipitation, after the addition of NaOH and Triton X-100 to final concentrations of 0.05 N and 0.05%, respectively (14). After centrifugation at 12,000 × g for 15 min in a microfuge, the supernatant was assayed for protein by addition of the Bio-Rad Coomassie blue reagent (Bio-Rad Laboratories) and absorbance read at 595 nm. BSA (Sigma Chemical Co.; fraction V) was used to obtain standard curves by both methods.

Cell Surface Labeling with 125I-WGA

Iodination of WGA was performed on ice by the chloramine T method using 15 µg WGA and 100 µCi 125I (Amersham Corp., 100 mCi/mL) in 50 µL PBS to which 5 µg chloramidine T was added. After 15 min, 10 µL K metabisulfite was added, and free 125I was removed by chromatography on Sephadex G25 to give 125I-WGA of specific activity ~106 cpm/µg protein. To label cell surface glycoproteins, ~105 cpm 125I-WGA were incubated at 4°C with 6 × 107 washed CHO or Lec4A cells in the presence of PBS containing 2% BSA. After 1 h, the cells were washed two times in PBS 2% BSA, and once in homogenization buffer at 4°C before being homogenized and analyzed by sucrose density centrifugation.

Results

Lec4 but Not Lec4A Mutants Lack GlcNAc-TV Activity

Both the previously described CHO mutants that belong to complementation group 4 (25) fail to synthesize β,6-branched, N-linked carbohydrates (21, 28), and are consequently highly resistant to the cytotoxicity of L-PHA (27), and exhibit reduced binding of this lectin (26). To determine if this glycosylation phenotype results from a lack of GlcNAc-TV activity, as observed previously with the lymphoma mutant Pha2.1 (6), extracts from both mutants were assayed for GlcNAc-TV activity. The results in Fig. 1 show that one of the mutants (termed Lec4) had no detectable GlcNAc-
TV activity in detergent extracts. However, the other mutant (now termed Lec4A) had the same level of activity as parental cells. In addition, this activity was completely membrane associated in parental and Lec4A cells (Fig. 1), as expected for a Golgi transferase. No significant stimulation of GlcNAc-TV activity was observed in these crude membrane preparations, but later experiments provide additional evidence for the membrane association of GlcNAc-TV in both Lec4A and parental cells (see Figs. 5 and 6).

The GlcNAc-TV assay using iodinated GnGn(Fuc)BH involves product identification by virtue of the requirement for specific lectin binding by the GlcNAcβ1,6-branched product (see Materials and Methods). However, it was important to show that the Lec4A enzyme also gave the correct product with a synthetic substrate that is not an acceptor for any other GlcNAc-transferase (11). Therefore, parental and Lec4A extracts were compared in the assay developed by Pierce et al. (17) using GlcNAcβ1,2Manç1,6Manβ-O-(CH₂)₅COCH₂ (30) as acceptor. Under theoretically saturating conditions but in the absence of Mn²⁺, the parental extract gave an average activity of 141 pmol/h per mg protein, while the Lec4A extract had an activity of 114 pmol/h per mg protein. Therefore, both parental and Lec4A extracts possessed authentic GlcNAc-TV activity of similar specific activity under these conditions.

The fact that Lec4 and Lec4A cells belong to the same general complementation group (25) was confirmed by analysis of freshly formed hybrids (data not shown). In addition, several independent Lec4 mutants were isolated from Pro-5 CHO cells as described in Materials and Methods and shown to lack GlcNAc-TV activity (data not shown). An independent isolate with partial GlcNAc-TV activity (~30% compared with parental CHO) was uncovered amongst several L-PHA-resistant mutants selected from Gatt 2 CHO cells. The combined data are consistent with the idea that different mutations affecting GlcNAc-TV activity, or its localization, occur at different sites in the structural gene for the enzyme.

### The GlcNAc-TVs of Parental and Lec4A Cells Are Similar

To determine whether parental and Lec4A GlcNAc-TV activities were equivalent and that Lec4 mutants did not possess an inhibitor of GlcNAc-TV, mixing experiments were performed (Table 1).

- In mixed cell extracts, or in hybrids formed between different cell combinations, the observed specific activity of GlcNAc-TV was close to that expected, if all enzyme activities were additive. No evidence for an inhibitor was obtained in either experimental system, and the parental and Lec4A enzymes appeared equivalent.

- To compare the pH, metal ion, and substrate requirements of parental and Lec4A GlcNAc-TV enzymes, it was first necessary to optimize the GlcNAc-TV assay. Highest specific activities were obtained when glycopeptide substrate GnGn(Fuc) was conjugated with Bolton-Hunter reagent, and iodinated with chloramine T as described in Materials and Methods. Unconjugated GnGn(Fuc) did not effectively compete as a substrate since, at a concentration of ~1 mM, no competition with GnGn(Fuc)BH was observed (data not shown). However, conjugated glycopeptide (GnGn(Fuc)BH) was a competitive inhibitor of the iodinated substrate (Fig. 2), indicating a possible role for the hydrophobic Bolton-Hunter moiety in acceptor activity.

### Table 1. Mixing Experiments

| GlcNAc-TV Activity | cpm (× 10⁻³)/h per mg protein |
|--------------------|-----------------------------|
| **Cell extracts**  |                             |
| Parent (Pro⁻)      | 12.7                        |
| (Gat⁻)             | 11.3                        |
| Lec4A              | 9.1                         |
| Lec4               | ≤0.16                       |
| Parent + Lec4A     | 10.7 (10.9)                 |
| Parent + Lec4      | 5.7 (5.7)                   |
| Lec4A + Lec4       | 5.3 (4.7)                   |
| Parent + Parent    | 9.0 (12.0)                  |
| **Hybrids**        |                             |
| Parent × Lec4A     | 10.0                        |
| Parent × Lec4      | 3.8                         |
| Lec4A × Lec4       | 5.3                         |
| Parent × Parent    | 11.4 (11.7)                 |

GlcNAc-TV activity was assayed as described in Materials and Methods under nonsaturating conditions (30-90 pmol GnGn(Fuc)BH⋅·121 and 10-15 mM UDP-GlcNAc). GlcNAc-TV activity of cell extracts was assayed individually and in paired mixtures containing an equal volume of each extract. Specific activities expected if GlcNAc-TV activity were additive are given in parentheses. GlcNAc-TV activity of hybrids formed by cell fusion as described in Materials and Methods were assayed under the conditions described above. The specific activity expected for hybrids obtained by fusing parental cells is based on that obtained for parent cells in the same assay which is given in parentheses.
to fourfold by the presence of Mn²⁺ (Fig. 2). A similar stimulation with Mn²⁺ has been noted for GlcNAc-TV from hen oviduct (Brockhausen, I., A. Grey, J. Carver, O. Hinds- gaul, and H. Schachter, unpublished observations), in con- trast to GlcNAc-TV activity in lymphoma cells (6), and BHK CHO enzyme, provided it has access to the appropriate substrates.

Parental and Lec4A GlcNAc-TVs Are Differentially Sensitive to Heat

Enzymes that contain a mutation might be expected to be more heat sensitive than their wild-type counterparts. In fact, this phenotype enabled the parental and Lec4A GlcNAc-TV activities to be clearly distinguished. Whereas both enzymes were equivalently active over several hours at 37°C, the Lec4A enzyme was largely inactivated by incubation at 45°C (Fig. 3). This suggests that the parental and Lec4A enzymes are structurally distinct, and provides indirect evidence that the Lec4A mutation affects the structural gene for GlcNAc-TV.

Lec4A GlcNAc-TV Is Not Concentrated in Golgi Membranes

Since GlcNAc-TV from Lec4A cells was membrane associated (Fig. 1), but not active in the intact cell (21, 25–28), it seemed likely that it was localized somewhere other than the medial Golgi. To see if it was in a compartment beyond medial Golgi, glycopeptides from a double mutant, Lec4A.Lec8, were exam- ined by lectin-affinity chromatography. The lec8 mutation reduces translocation of UDP-Gal into the Golgi lumen (7), so that N-linked carbohydrates of Lec8 cells terminate in GlcNAc residues, providing an appropriate in vivo substrate for GlcNAc-TV in any compartment beyond medial Golgi. Glucosamine-labeled Pronase glycopeptides from Lec8 and Lec4A.Lec8 were therefore examined by lectin-affinity chromatography. Although Lec8 cells synthesized branched carbohydrates (not bound to Con A-Sepharose) with a β1,6GlcNAc arm (bound to pea lectin–agarose), Lec4A.Lec8 glycopep- tidess contained no species with these properties (Fig. 4). Therefore, the GlcNAc-TV enzyme of Lec4A cells does not appear to be mislocated to trans Golgi, trans Golgi network, or any other compartment beyond the medial Golgi that contains UDP-GlcNAc.

Trypsinization experiments provided evidence that the Lec4A GlcNAc-TV is not exposed at the cell surface. When washed parental and Lec4A cells were treated with trypsin (0.5% wt/vol in citrate saline at 37°C for 10 min), washed
with PBS containing 2% BSA, and assayed for GlcNAc-TV activity, they had slightly higher specific activities compared with mock-treated controls (consistent with loss of surface protein), but there were no significant differences between parental and Lec4A cell extracts (data not shown).

Since GlcNAc-TV did not appear to be in a post-medial Golgi membrane compartment, sucrose density gradients that clearly separate Golgi vesicles from the more dense vesicles of ER and lysosomes (2) were performed. Golgi membranes from parental cell homogenates contained the majority of the GlcNAc-TV activity, as well as another marker of medial Golgi, N-acetylglucosaminyltransferase I (GlcNAc-TI; reference 8), and also β1,4galactosyltransferase (β1,4Gal-T; reference 18), a marker of the trans Golgi compartment (Fig. 5). However, Lec4A Golgi membranes were enriched only for GlcNAc-TI and β1,4Gal-T (Fig. 5). The major portion of the GlcNAc-TV activity in Lec4A homogenates remained with the more dense membrane fractions (III and IV; Fig. 5).

To identify enzyme markers that cofractionated with Lec4A GlcNAc-TV, sucrose gradients similar to those in Fig. 5 were assayed for β1,4Gal-T, GlcNAc-TI, GlcNAc-TV, Dol-α-Man synthetase, cytochrome C reductase, and β-galactosidase. Parallel gradients were performed with cells surface labeled with 125I-WGA at 4°C before homogenization. The profiles in Fig. 6 show that GlcNAc-TV from Lec4A cells was largely concentrated in the more dense fractions with enzyme markers of ER (Dol-α-Man synthetase and cytochrome C reductase) and lysosomes (β-galactosidase), and was well separated from Golgi membranes (GlcNAc-TI and Gal-T) and plasma membrane vesicles (125I-WGA).

To determine whether GlcNAc-TV was mislocalized to lysosomes, cell homogenates were centrifuged in a 27% Percoll gradient designed to separate lysosomes from ER, plasma membrane, and Golgi membranes which cofractionate in the less dense region (14). It is apparent from Fig. 7 that, in this gradient, GlcNAc-TV from Lec4A and parental CHO cells cofractionated near the top of the gradient with the ER enzyme marker cytochrome C reductase, and well removed from the β-hexosaminidase of lysosomes. Therefore, (a) Lec4A GlcNAc-TV is not concentrated in lysosomes, and (b) in two different gradient systems, the major portion cofractionates at a similar density to markers of endoplasmic reticulum.

**Discussion**

An important approach to determining the mechanisms of intracellular trafficking is to identify mutations in the cellular
and membrane fractions separated on 37-m1Percoll gradients as described in Materials and Methods. L-ml fractions were collected and every second fraction assayed for the enzyme activities indicated. Total activities in each fraction were calculated as the product of specific activity and the amount of protein in that fraction. Recoveries ranged from 70 to essentially 100%. Enrichments were four- to sevenfold for specific activities in peak fractions compared with the postnuclear supernatants loaded on the gradients. The bottom of the gradient is on the left (fraction 1).

Based on several lines of evidence, it seems likely that the lec4 and lec4A mutations are in the structural gene for GlcNac-TV. The mutations belong in the same genetic complementation group, but affect GlcNac-TV activity differently. In Lc4 mutants no GlcNac-TV activity is detectable, whereas in Lc4A mutants the enzyme is perfectly functional but is markedly reduced in Golgi membranes. Finally, the Lc4A enzyme is more heat sensitive than GlcNac-TV from parental cells, providing evidence that it is structurally distinct. It seems likely, therefore, that the lec4 mutation affects a region of the transferase important in enzyme activity, while the lec4A mutation affects a distinct region that is not required for transferase activity, but is important for transit from ER or some other pre-Golgi compartment to Golgi. It is interesting that if the Lc4A GlcNac-TV is localized to ER, it is not degraded as might be predicted from recent reports of a degradative pathway for ER proteins that are not properly sorted (12). If the lec4 and lec4A mutations turn out not to affect the structural gene for GlcNac-TV they will, at the very least, identify a new molecule necessary for the localization of this medial Golgi transferase.

One of the intriguing features of the Lc4A phenotype is that it is so far indistinguishable from the Lc4 phenotype with respect to the altered carbohydrates synthesized (21, 28) and the lectin-resistance phenotype to which they give rise (26, 27). Therefore, the only way to find a Lc4A mutant is to screen amongst Lc4A mutants for GlcNac-TV activity. Screens of other glycosylation mutants (23) may also reveal new mutations that result in the mislocalization of glycosyltransferases. Replicate filters of independent isolates with the same lectin-resistance phenotype should allow each colony to be assayed for a specific transferase activity in situ (15), and may be the best way to identify active transferases in the wrong compartment. Such mutants would provide an approach to identifying targeting mechanisms in endogenous molecules of mammalian cells, which is a necessary complement to studies of the targeting of exogenously added, engineered molecules.

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References

1. Balch, W. E., and J. E. Rothman. 1985. Characterization of protein transport between successive compartments of the Golgi apparatus: asymmetric properties of donor and acceptor activities in a cell-free system. Arch.
2. Balch, W. E., W. G. Dunphy, W. A. Braell, and J. E. Rothman. 1984. Reconstitution of the transport of protein between successive compartments of the Golgi measured by the coupled incorporation of N-acetylgalcosamine. Cell. 39:405–416.

3. Chaney, W., and P. Stanley. 1986. Leu1A Chinese hamster ovary cell mutants appear to arise from a structural alteration in N-acetylgalcosaminyltransferase I. J. Biol. Chem. 261:10551–10557.

4. Chen, T. R. 1977. In situ demonstration of mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 stain. Exp. Cell Res. 104:255–262.

5. Cummings, R. D., and S. Kornfeld. 1982. Characterization of the structural determinants required for the high affinity interaction of asparagine-linked oligosaccharides with immobilized Phaseolus vulgaris leukoagglutinating and erythroagglutinating lectins. J. Biol. Chem. 257:11230–11234.

6. Cummings, R. D., I. S. Trowbridge, and S. Kornfeld. 1984. A mouse lymphoma cell line resistant to the leukoagglutinating lectin from Phaseolus vulgaris is deficient in UDP-GlcNAc-α-D-mannoside β1,6 N-acetylgalcosaminyltransferase. J. Biol. Chem. 259:13421-13427.

7. Deutscher, S. L., and C. B. Hirschberg. 1986. Mechanism of galactosylation in the Golgi apparatus: a Chinese hamster ovary cell mutant deficient in translocation of UDP-galactose across Golgi vesicle membrane. J. Biol. Chem. 261:96–100.

8. Dunphy, W. G., R. Brands, and J. E. Rothman. 1985. Attachment of terminal N-acetylgalcosamine to asparagine-linked oligosaccharides occurs in central cisternae of the Golgi stack. Cell. 40:463–472.

9. Goldberg, D. E., and S. Kornfeld. 1983. Evidence for extensive subcellular organization of asparagine-linked oligosaccharide processing and lysosomal enzyme phosphorylation. J. Biol. Chem. 258:3159–3165.

10. Hammarstrom, S., M.-L. Hammarstrom, G. Sundblad, J. Arnarp, and J. Lonngren. 1982. Mitogenic leukoagglutinin from Phaseolus vulgaris binds to a pentasaccharide unit in N-acetyllactosamine-type glycoprotein glycans. Proc. Natl. Acad. Sci. USA. 79:1611–1615.

11. Hindsgaul, O., S. H. Tahir, O. P. Srivasta, and M. Pierce. 1986. The tri saccharide β-D-GlcpNAc(1→2)-α-D-Manp(1→6)-β-D-Manp, as its 8-methoxycarbonyloctyl glycoside, is an acceptor selective for N-acetylglucosaminyltransferase V. Carbohydr. Res. 173:263–272.

12. Lippincott-Schwartz, J., J. S. Bonifacino, L. C. Yuan, and R. D. Klausner. 1988. Degradation from the endoplasmic reticulum: disposing of newly synthesized proteins. Cell. 54:209–220.

13. Markwell, M. A., S. M. Haas, L. L. Bieber, and N. E. Tolbert. 1978. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. 87:206–210.

14. Marsh, M., S. Schmid, H. Kern, E. Harms, P. Male, I. Mellman, and A. Helenius. 1987. Rapid analytical and preparative isolation of functional endosomes by free flow electrophoresis. J. Cell Biol. 104:875–886.

15. Ornstein, P. C., A. Albright, and P. W. Robbins. 1988. Cloning and sequencing of the yeast gene for dolichol phosphate mannos synthase, an essential protein. J. Biol. Chem. 263:17499–17507.

16. Palcic, M. M., L. D. Heerze, M. Pierce, and O. Hindsgaul. 1988. The use of hydrophobic synthetic glycosides as acceptors in glycosyltransferase assays. Glycoconj. J. 5:49–63.

17. Peterson, M., J. Arango, S. H. Tahir, and O. Hindsgaul. 1987. Activity of UDP-GlcNAc:α-mannoside β1,6 N-acetylgalcosaminyltransferase (Gnt V) in cultured cells using a synthetic trisaccharide acceptor. Biochem. Biophys. Res. Commun. 144:679–684.

18. Roth, J., and E. G. Berger. 1982. Immunocytochemical localization of galactosyltransferase in HeLa cells: codistribution with thiamine pyrophosphatase in trans-Golgi cisternae. J. Cell Biol. 93:223–229.

19. Schekman, R. 1985. Protein localization and membrane traffic in yeast. Annu. Rev. Cell Biol. 1:115–143.

20. Stanley, P. 1981. Selection of specific wheat germ agglutinin resistant (Wga®) phenotypes from Chinese hamster ovary cell populations containing numerous lec® genotypes. Mol. Cell. Biol. 1:687–696.

21. Stanley, P. 1982. Carbohydrate heterogeneity of vesicular stomatitis virus G glycoprotein allows localization of the defect in a glycosylation mutant of CHO cells. Arch. Biochem. Biophys. 219:128–139.

22. Stanley, P. 1983. Selection of lectin-resistant mutants of animal cells. Methods Enzymol. 96:157–184.

23. Stanley, P. 1984. Glycosylation mutants of animal cells. Annu. Rev. Genet. 18:523–552.

24. Stanley, P. 1989. Chinese hamster ovary mutants with multiple glycosylation defects for production of glycoproteins with minimal carbohydrate heterogeneity. Mol. Cell. Biol. 9:377–383.

25. Stanley, P., and L. Siminovich. 1977. Complementation between mutants of CHO cells resistant to a variety of plant lectins. Som. Cell Genet. 3:391–405.

26. Stanley, P., and T. Sudo. 1981. Microheterogeneity among carbohydrate structures at the cell surface may be important in recognition phenomena. Cell. 23:763–769.

27. Stanley, P., V. Caillibot, and L. Siminovich. 1975. Selection and characterization of eight phenotypically distinct lines of lectin-resistant Chinese hamster ovary cells. Cell. 6:121–128.

28. Stanley, P., G. Vivona, and P. H. Atkinson. 1984. 1H NMR spectroscopy of carbohydrates from the G glycoprotein of vesicular stomatitis virus grown in parental and Lec4 Chinese hamster ovary cells. J. Cell Biol. 93:223–230.

29. Stoll, J., A. R. Robbins, and S. S. Krag. 1982. Mutant of Chinese hamster ovary cells with altered mannose-6-phosphate receptor activity is unable to synthesize mannosylphosphoryl dolichol. Proc. Natl. Acad. Sci. USA. 79:2296–2300.

30. Tahir, S. H., and O. Hindsgaul. 1986. Substrates for the differentiation of the N-acetylgalactosaminyltransferases: synthesis of βDGlcNAc(1→2)-α-D-Manp(1→6)-β-D-Manp and βDGlcNAc(1→2)-α-D-Manp(1→6)-β-D-Manp(1→3)-βD-Man glycosides. Can. J. Chem. 64:1771–1780.