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 *lec*4 and *lec*4A mutations affect the structural gene for GlcNAc-TV, causing either the loss of GlcNAc-TV activity (*lec*4) or its miscompartmentalization (*lec*4A). 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;[1] UDP-GlcNAc:α1,6mannose β1,6*N*-acetyl-glucosaminyltransferase 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 BW5147 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 Gat$^-$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.

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1. *Abbreviations used in this paper*: GlcNAc-TV, *N*-acetylglucosaminyltransferase V; PHA, phytohemagglutinin; PMSF, paramercurisulfonyl fluoride; WGA, wheat germ agglutinin.

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 (20). 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⁻Lec4A.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 ethylmethaneusulfonate (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 Lec4A 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 ng/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 10^6 cells/100-mm dish. After 7 d at 37°C in 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 ng/ml). Colonies arising at a frequency of ~10^{-5} were picked 4 d later, cultured, and tested for lectin resistance as described (22). Of 12 colonies tested, 7 had Lec^R phenotypes similar to those expected for a Lec4A.Lec8 Lec^R phenotype (24). Clones were derived by limiting dilution from two isolates and shown by complementation 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 mutants using polyethylene glycol and DMSO as previously described (22). Each mutant in the cross carried a different auxotrophic marker (Pro⁻ or Gat⁻) allowing hybrids to be selected in deficient medium (22). Controls for each cross included tests for reversion frequency (~10^{-5}) and spontaneous hybrid formation (<10^{-5}). Typically, 100–300 hybrids were obtained per 2 × 10^5 cells plated after fusion treatment. The hybrids were tested for lectin resistance using L-PHA in crosses involving Lec4 mutants (22, 25) and WGA in crosses involving Lec8 mutants (20, 22). When two cell lines belonged to the same complementation group, hybrids between them were lectin resistant, whereas cell lines belonging to different complementation groups gave hybrids that were not lectin resistant as reported previously (20, 22, 25).

Preparation of Iodinated Acceptor

The glycopeptide GlcNAcβ1,2Manα1,3(GlcNAcβ1,2Manα1,6)Manβ1,4GlcNAcβ1,4(Fucα1,6)GlcNAcβ1,1Asn, termed GnGn(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, desalted, and applied to pea lectin–agarose (Vector Laboratories, Inc.). Those specifically bound were eluted with 200 mM α-methylmannoside, extensively desalted, and digested with neuraminidase (Sigma Chemical Co.; 0.5 U/~6 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). After 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 ^{1}H-NMR spectroscopy at 23°C as described (28). The spectra showed that the glycopeptide was authentic GnGn(Fuc) and contained equimolar amounts of α1,6-linked fucose with no detectable galactose or sialic acid residues.

Glycopeptide GnGn(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 × 40-cm column of BioGel-P2 (BioRad Laboratories). Fractions were monitored by OD at 230 nm and 280 nm and the material eluting in the V_o was pooled and termed GnGn(Fuc)BH. This was iodinated by incubating 93 nmol GnGn(Fuc)BH with 5 μg Chloramine T (Kodak Laboratory and Specialty Chemicals, Rochester, NY) in 150 μl PBS containing 1 mM Ca^{2+} and 1 mM Mg^{2+} (pH 7.4) and 500 μCi $Na^{125}I$ (Amersham Corp., Arlington Heights, IL; 100 mCi/ml) for 30 min on ice. The reaction was stopped with 10 μg K metabisulfite and unreacted ^{125}I was removed by passage through a small G25 Sephadex column. Assuming ~90% recovery, GnGn(Fuc)BH - ^{125}I with a specific activity of ~ 2 × 10^6 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 iodinated acceptor as described by Cummings et al. (6), or using a synthetic acceptor as described by Pierce et al. (17).

Iodinated Acceptor. The reaction mix included 25 mM 2[*N*-morpholino]ethanesulfonic acid (MES; Sigma Chemical Co.) buffer, pH 6.0, 10–20 mM UDP-N-acetylglucosamine (Sigma Chemical Co.), ~200,000 cpm (~0.1 nmol) GnGn(Fuc)BH-^{125}I, 0–74 nmol GnGn(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 NaCl, 0.1 M Na acetate, 10 mM $CaCl_2$, 10 mM $MnCl_2$, 10 mM $MgCl_2$, 0.02% Na azide, pH 7.0) and freezing. Reaction products were purified by lectin-affinity chromatography on 2-ml columns of Con A–Sepharose followed by pea lectin–agarose eluted in each case with Con A buffer (6). Branched carbohydrates passed through the Con A–Sepharose which bound unmodified substrate, GnGn(Fuc)BH-^{125}I. The product of GlcNAc-TV action (GnGn(β1,6)Gn(Fuc)BH-^{125}I) 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 nonsaturating concentrations of GnGn(Fuc)-BH to conserve this substrate which was not commercially available.

Synthetic Acceptor. The synthetic carbohydrate substrate, GlcNAcβ1, 2Manα1,6Man-O-$(CH_2)_8COOCH_3$ (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-TV activity in a 20-μl reaction mix containing 40 μg acceptor, 32 nmol UDP-[6-^{3}H]-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 radiolabeled 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-^{3}H]-glucosamine hydrochloride (Amersham Corp.; ~33 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 three changes of 1 mM Tris HCl (pH 7.4) and an aliquot was exhaustively digested with 1 mg/ml Pronase (Sigma Chemical Co.) at 50°C for 24 h. Additional Pronase was added and the incubation continued for another day. After boiling, the samples were resuspended in Con A buffer and applied to a 1.2-ml column of Con A–Sepharose. Glycopeptides that did not bind to Con A–Sepharose were analyzed on a 20-ml column of pea lectin–agarose eluted with 95 ml Con A buffer, followed by 95 ml Con A buffer containing 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 $CaCl_2$ 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 paramercurisulfonylfluoride (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 1 h 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 6×10^7 cells/ml, allowed to swell on ice 20 min, and homogenized by seven passes using a 0.2459-inch ballbearing 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 mixed with an equal volume of 2.75 M sucrose in 10 mM Tris HCl, pH 7.4, containing 0.1 mM EDTA. After vortexing vigorously, 4 ml of this postnuclear supernatant (~1.6 M in sucrose) was layered over 1 ml 2 M sucrose, and then overlaid successively with 3 ml 1.4 M sucrose, 3 ml 1.2 M sucrose, and 1 ml 0.8 M sucrose. After centrifugation at 40,000 rpm for 3 h at 4°C in an SW41 rotor, the gradient was either fractionated from the bottom into 1-ml fractions or the membranes at each interface were collected by needle.

Percoll gradient centrifugation was based on the method of Marsh et al. (14). Cells were homogenized by four passes through the homogenizer described above in a different homogenization buffer: 0.25 M sucrose in TEA buffer (10 mM triethanolamine, 10 mM acetic acid, and 1 mM EDTA, pH 7.4). After centrifugation at 1,900 rpm for 10 min at 4°C in an IEC centrifuge, 4.4 ml supernatant was layered over 0.5 ml 1 M sucrose in TEA buffer and centrifuged at 33,000 rpm for 35 min at 4°C in an SW50.1 rotor. The microsomes were resuspended in 1 M sucrose and mixed with TEA buffer to give 0.25 M sucrose final concentration. After five strokes in a Dounce homogenizer, large aggregates were removed by centrifugation at 1,900 rpm for 10 min at 4°C in an IEC centrifuge. Supernatant containing ~10 mg protein (assayed rapidly with the Bio-Rad reagent) was mixed with 0.25 M sucrose to give a volume of 25.9 ml. 11.1 ml 90% Percoll (Pharmacia) was added to this, to give a final Percoll concentration of 27%. After gentle mixing, the Percoll with membranes was layered over 4 ml 2.5 M sucrose in TEA in a quick-seal tube (Beckman Instruments Inc.). The Percoll gradient was generated by centrifugation at 16,000 rpm for 2 h at 4°C in a VTi50 rotor (Beckman Instruments Inc.). 1-ml fractions were collected from the bottom at 4°C and assayed for protein and enzyme activities.

Enzyme Assays

GlcNAc-TI was assayed under the optimized conditions described by Chaney and Stanley (3), using as acceptor $Man_5GlcNAc_2Asn$ prepared from ovalbumin and characterized by ^{1}H-NMR spectroscopy, and as donor UDP-[6-^{3}H]-GlcNAc (New England Nuclear; ~20 Ci/mmol), at a specific activity of 10,000 cpm/nmol.

Galactosyl transferase was assayed in 0.05 M MES buffer (pH 5.7) containing 35 nmol UDP-[6-^{3}H]-galactose (New England Nuclear; ~20 Ci/mmol), at a specific activity of 7,167 cpm/nmol, 0.9% Triton X-100, 0.06 M $MnCl_2$, 0.02 M GlcNAc, and 10–20 μl cell extract or gradient fraction in a final volume of 50 μl. After incubation for 2 h at 37°C, the reaction was stopped with 0.5 ml iced water and passed over a 1.5-ml column Dowex-1 (Cl^-form; Bio-Rad Laboratories) equilibrated in water. Unreacted UDP-^{3}H-Gal was bound to the Dowex while Galβ1,4GlcNAc product passed through. Specific activities were calculated after subtraction of a control incubation that contained boiled postnuclear supernatant or lacked GlcNAc. Control experiments showed that the extent of hydrolysis of UDP-^{3}H-Gal was very low during the 2-h incubation (<1%).

Cytochrome C reductase was assayed in a final volume of 1 ml containing 47 μM cytochrome C, 36 mM potassium phosphate buffer (pH 7.4), 28.8 μM nicotinamide, 22 μM potassium cyanide, and 10–100 μl cell extract or gradient fraction. The reaction was initiated by the addition of 74 nmol NADPH (Sigma Chemical Co.) and OD_{550} was read after exactly 10 min at room temperature. Specific activity (nmol/min per mg protein) was calculated from the extinction coefficient of reduced cytochrome C (19.6 cm^{-1} mM^{-1}).

β-Galactosidase activity was assayed in a final volume of 1.0 ml containing 0.5% Triton X-100 in 160 mM citrate buffer (pH. 4.0), 5 mM O-nitrophenyl-β-galactoside (Sigma Chemical Co.), and 10–200 μl cell extract or gradient fraction. After 30 min at room temperature, the reaction was stopped by adding 3 ml 0.2 M Na borate (pH 9.8). Hydrolysed O-nitrophenol was determined from OD_{410} after subtraction of a buffer blank. Specific activities (nmol/min per mg protein) were calculated from the extinction coefficient for O-nitrophenol of 4.6 $cm^{-1}mM^{-1}$.

β-Hexosaminidase activity was assayed in a final volume of 1.4 ml, containing 36 mM Na acetate (pH. 5.0), 0.4% Triton X-100, 0.3 mM *p*-nitrophenol-β-*N*-acetylglucosaminide (Sigma Chemical Co.), and 10–200 μl cell extract or gradient fraction. After 30 min at 37°C, the reaction was stopped by adding 2 ml 0.2 M Na borate (pH 9.8). Released *p*-nitrophenol was determined from OD_{400} using an extinction coefficient of 18 $cm^{-1}mM^{-1}$. Specific activities were calculated as nmol/min per mg protein.

Dol-P-Man synthetase was assayed according to Stoll et al. (29), in a 100-μl reaction mix containing 30 μg dolichol phosphate (Sigma Chemical Co.), 156 pmol GDP-^{14}C-mannose (New England Nuclear; 253.7 mCi/nmol) at a specific activity of 455 cpm/pmol, 2.5 mM $MgCl_2$, 0.015% Triton X-100, 20 mM Tris HCl (pH 7.4), and 10–100 μl cell extract or gradient fraction. After 5 min at 37°C, the reaction was stopped by the addition of 2 ml chloroform/methanol (2:1) and the samples were extracted and counted as described (29).

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 ^{125}I-WGA

Iodination of WGA was performed on ice by the chloramine T method using 15 μg WGA and 100 μCi ^{125}I (Amersham Corp., 100 mCi/ml) in 50 μl PBS to which 5 μg chloramine T was added. After 15 min, 10 μg K metabisulfite was added, and free ^{125}I removed by chromatography on Sephadex G25 to give ^{125}I-WGA of specific activity ~10^8 cpm/μg protein. To label cell surface glycoproteins, ~10^6 cpm ^{125}I-WGA were incubated at 4°C with 6×10^7 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 β1,6-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 Pha^{R}2.1 (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-

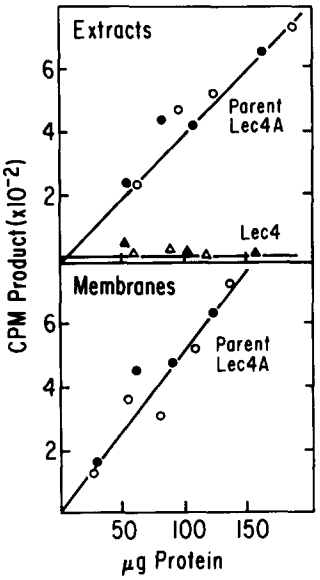

Figure 1. Cell extracts prepared in Triton X-100 from parental (○), two independent Lec4 (△, ▲), and Lec4A (●) cells were assayed for GlcNAc-TV activity as described in Materials and Methods using 55 pmol GnGn(Fuc)BH-^{125}I (1.8 × 10^6 cpm/nmol), 5 mM UDP-GlcNAc, 30 mM $MnCl_2$, and 30 mM sodium cacodylate buffer (pH 6.0) at 37°C for 2 h. Crude cell membranes were also prepared as described in Materials and Methods, solubilized in 20 mM MES (pH 6.0), 1% Triton X-100, and assayed for GlcNAc-TV activity as described above.

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 GlcNAcβ1,2Manα1,6Manβ-O-$(CH_2)_8COOCH_3$ (30) as acceptor. Under theoretically saturating conditions but in the absence of Mn^{2+}, 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 genetic complementation group (25) was confirmed by analysis of freshly formed hybrids (data not shown). In addition, several independent Lec4 mutants were isolated from Pro$^-$5 CHO cells as described in Materials and Methods and shown to lack GlcNAc-TV activity (data not shown). An independent isolate with partial GlcNAc-TV activity (~30% compared with parental CHO) was uncovered amongst several L-PHA-resistant mutants selected from Gat$^-$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.

Table I. 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-^{125}I 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.

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 I). 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-^{125}I 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.

The assay conditions giving optimal activity with parental CHO contained ~0.1 nmol GnGn(Fuc)BH-^{125}I (~2 × 10^6 cpm ^{125}I/nmol), ~1 mM GnGn(Fuc)BH, 20 mM UDP-GlcNAc, 15 mM $MnCl_2$, and 15 mM MES buffer, pH 6.0. Under these conditions, activities of 600–1,000 pmol/h per mg protein were obtained with parental CHO cells (Fig. 2), and Lec4 cells had no detectable activity. Comparisons of parental and Lec4A specific activities over increasing $MnCl_2$ or substrate concentrations and at different pH's revealed no significant differences between them (Fig. 2). The pH optimum for both transferases (compared under nonsaturating conditions) was ~6.0, though the curves differed somewhat in overall shape. Both transferases were active in the absence of Mn^{2+}, but their activity was stimulated two-

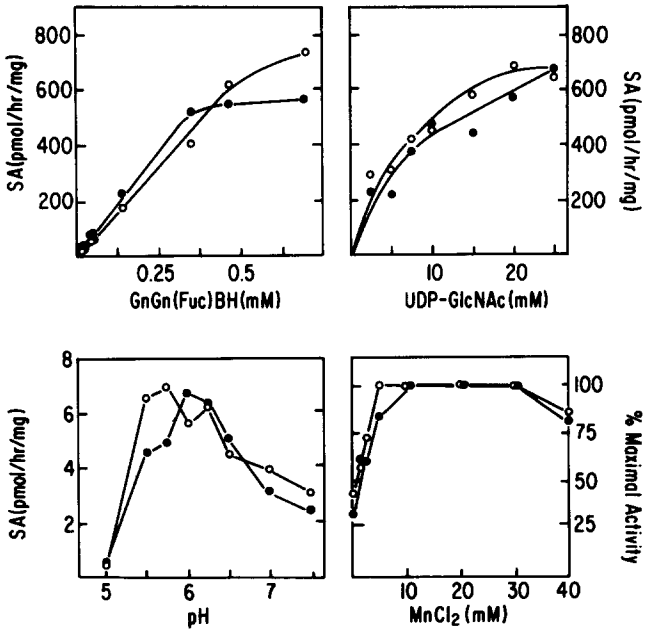

Figure 2. Optimization of the GlcNAc-TV activity of parental (○) and Lec4A(●) CHO cells in 1.1% Triton X-100 containing 30 mM MES (pH 6.0) and either (*a*) GnGn(Fuc)BH acceptor concentration in the presence of 0.16 nmol GnGn(Fuc)BH-^{125}I (1.2 × 10^6 cpm/nmol), 30 mM $MnCl_2$, and 20 mM UDP-GlcNAc; (*b*) UDP-GlcNAc concentration in the presence of 30 mM $MnCl_2$, 13.9 nmol GnGn(Fuc)BH, and 0.1 nmol GnGn(Fuc)BH-^{125}I (1.6 × 10^6 cpm/nmol); or (*c*) $MnCl_2$ concentration in the presence of 0.1 nmol GnGn(Fuc)BH-^{125}I (2 × 10^6 cpm/nmol) and 20 mM UDP-GlcNAc. The pH curves were obtained using 30 mM MES buffers (pH 5.0–6.25) or 30 mM Na cacodylate (pH 6.5–7.5), 20 mM UDP-GlcNAc, 30 mM $MnCl_2$, and 0.15 nmol GnGn(Fuc)BH-^{125}I (1.4 × 10^6 cpm/nmol). Each point represents the average of duplicate assays performed with 10 μl extract (~100–150 μg protein).

to fourfold by the presence of Mn^{2+} (Fig. 2). A similar stimulation with Mn^{2+} has been noted for GlcNAc-TV from hen oviduct (Brockhausen, I., A. Grey, J. Carver, O. Hindsgaul, and H. Schachter, unpublished observations), in contrast to GlcNAc-TV activity in lymphoma cells (6), and BHK cells (17) which were not stimulated by Mn^{2+}. Finally, there were no marked differences in the saturation curves for both substrates (Fig. 2). The apparent K_m's for both transferases were 0.2–0.4 mM for GnGn(Fuc)BH and 15–25 mM for UDP-GlcNAc. The requirement for UDP-GlcNAc was reproducibly about eightfold higher than for GlcNAc-TI in CHO cells (3). The combined data suggest, that in the intact cell, the Lec4A GlcNAc-TV should be as active as the parental 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

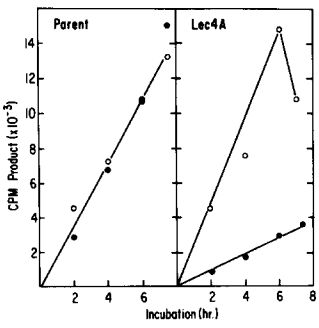

Figure 3. Cell extracts were assayed for GlcNAc-TV activity in replicate incubations including 0.15 nmol GnGn(Fuc)BH-^{125}I (1.3 × 10^6 cpm/nmol), 20 mM UDP-GlcNAc, 15 mM $MnCl_2$, and 25 mM MES buffer (pH 6.0) at either 37° (○) or 45°C (●) for various times. Each point represents the average of three determinations.

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 examined 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 glycopeptides 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

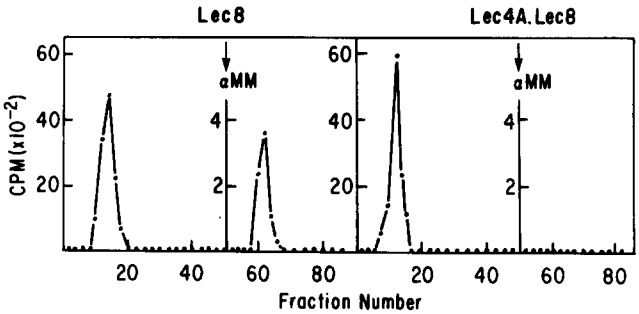

Figure 4. Pronase glycopeptides from Lec8 and Lec4A.Lec8 cells labeled with ^{3}H-glucosamine were fractionated on Con A–Sepharose as described in Materials and Methods (not shown). The branched carbohydrates (not bound to Con A-Sepharose) were applied to pea lectin–agarose and eluted with Con A buffer (shown above). At the arrow the buffer was changed to Con A buffer containing 200 mM α-methylmannoside (αMM). 1.9-ml fractions were collected and the entire aliquot was counted.

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-P-Man synthetase, cytochrome C reductase, and β-galactosidase. Parallel gradients were performed with cells surface labeled with ^{125}I-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-P-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 (^{125}I-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.

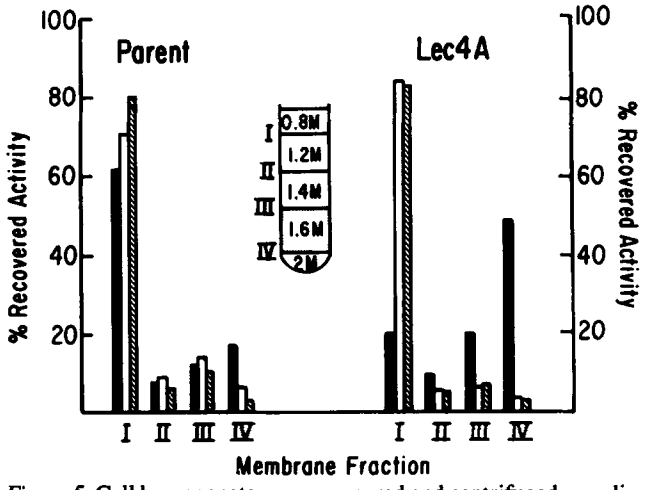

Figure 5. Cell homogenates were prepared and centrifuged on a discontinuous sucrose gradient as described in Materials and Methods. The postnuclear supernatant was in 1.6 M sucrose. The membrane fractions at each interface were collected by needle and assayed after solubilization in Triton X-100 for GlcNAc-TV (■), GlcNAc-TI(□), and Gal-T(■) activities. Approximately 50% of the activities applied to the gradient were recovered. The fold-purification was 10–20-fold for Gal-T and GlcNAc-TI and 25-fold for GlcNAc-TV from parental CHO.

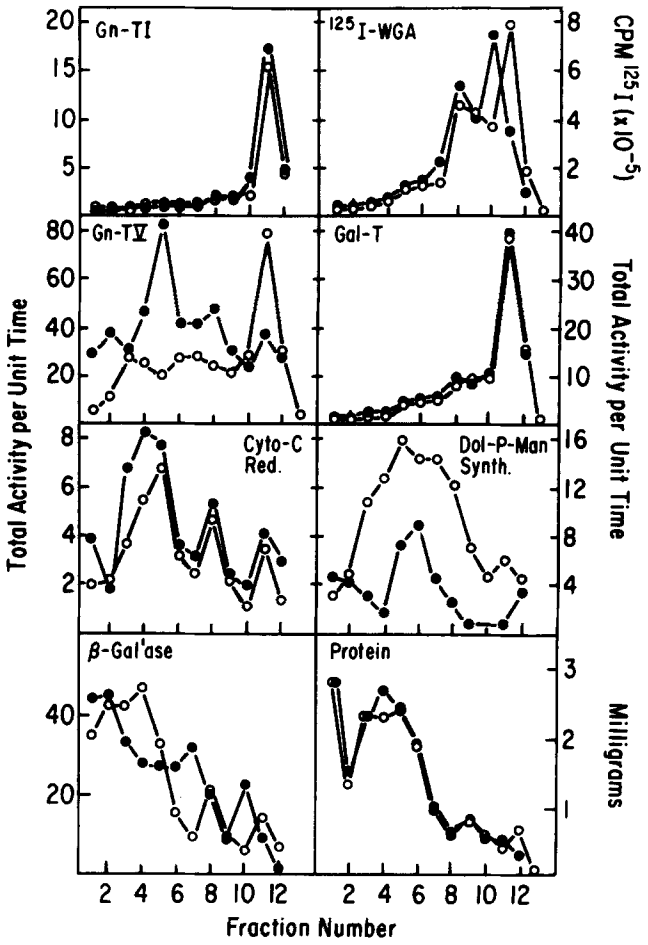

Figure 6. Lec4A(●) and parental (○) cells were homogenized and membrane fractions separated by sucrose gradient centrifugation as described in Materials and Methods. Fractions of ~1 ml were collected and assayed for several enzyme activities and protein. The β-galactosidase and ^{125}I-WGA data were obtained in separate experiments. Values are given as total activity calculated as the product of specific activity and the amount of protein in the fraction. The specific activities of the medial Golgi transferases were enriched 10–15-fold in fraction 11. Recoveries varied from 80 to 100% for GlcNAc-TV(Gn-TV), Dol-P-Man synthetase, Gal-T, and β-galactosidase; and 35 to 50% for GlcNAc-TI(Gn-TI) and cytochrome C reductase in these particular experiments. In a separate experiment there was no significant difference between the profiles of parent and Lec4A Dol-P-Man synthetase activities. The bottom of the gradient is on the left (fraction 1).

Discussion

An important approach to determining the mechanisms of intracellular trafficking is to identify mutations in the cellular

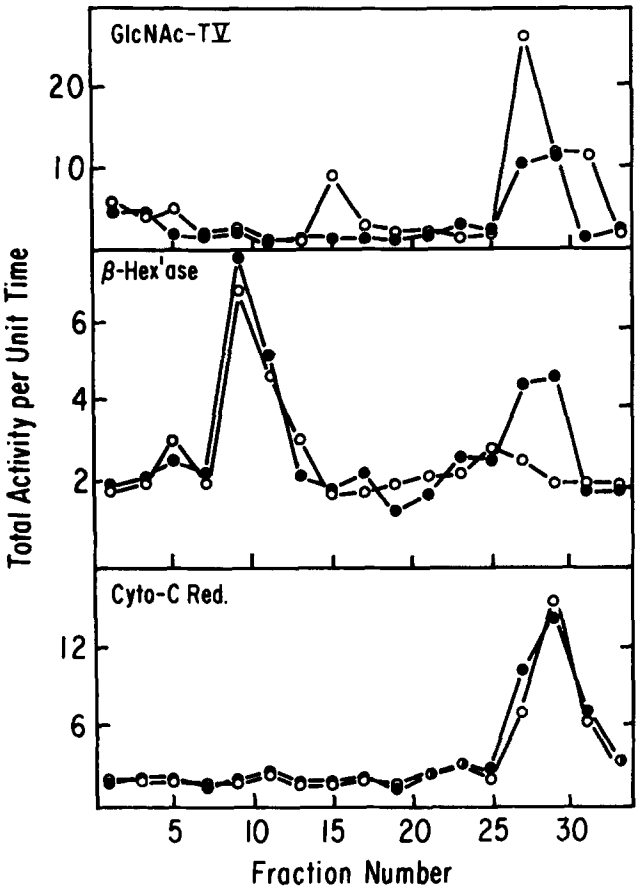

Figure 7. Lec4A(●) and parental CHO cells (○) were homogenized and membrane fractions separated on 37-ml Percoll gradients as described in Materials and Methods. 1-ml fractions were collected and every second fraction assayed for the enzyme activities indicated. Total activities in each fraction were 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).

genome that give rise to altered compartmentalization patterns. Presumably, mutations that affect molecules of the trafficking machinery itself will be conditional-lethal as observed with many of the yeast Sec mutants (19). However, mutations that affect the localization of nonessential molecules, like the glycosyltransferases involved in terminal glycosylation reactions, provide an ideal way to identify targeting signals recognized by the cell. The mutation expressed by Lec4A CHO cells falls into this category.

Cell fractionation on sucrose gradients yielded a membrane fraction from parental CHO cells 10–25-fold enriched for the classical marker of *trans* Golgi, β1,4Gal-T (18) and the medial Golgi transferases GlcNAc-TI (8) and GlcNAc-TV (9). Lec4A cells gave essentially identical gradient profiles, except that their Golgi membranes were enriched only in Gal-T and GlcNAc-TI. The GlcNAc-TV activity of Lec4A cells is clearly not concentrated in Golgi membranes. Studies with the Lec4A.Lec8 double mutant provide evidence that the Lec4A GlcNAc-TV is not localized to other membranes of the secretory pathway between medial Golgi and the cell surface, and trypsinization experiments indicate that it is not exposed at the cell surface. Percoll gradient fractionation showed that it is not enriched in lysosomes. In Lec4A cells, the majority of the GlcNAc-TV activity is found in a dense membrane fraction that cofractionates with markers of the ER. Therefore, the transferase may be localized to the ER of Lec4A mutants or to another, as yet unidentified, compartment of similar density. Unfortunately, it is not possible to test directly where the enzyme is compartmentalized because GlcNAc-TV has not been purified. Therefore, no antibodies or nucleic acid probes are presently available for molecular analyses.

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 Lec4 mutants no GlcNAc-TV activity is detectable, whereas in Lec4A mutants the enzyme is perfectly functional but is markedly reduced in Golgi membranes. Finally, the Lec4A 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 Lec4A 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 Lec4A phenotype is that it is so far indistinguishable from the Lec4 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 Lec4A mutant is to screen amongst Lec4 phenotypes 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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