A Point Mutation Causes Mistargeting of Golgi GlcNAc-TV in the
Lec4A Chinese Hamster Ovary Glycosylation Mutant*

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The Lec4A and Lec4 Chinese hamster ovary glycosylation mutants lack N-linked glycans with GlcNAcβ-(1,6)Manα-(1,6) branches that are initiated by the transf
erase termed GlcNAc-TV. Detergent extracts of Lec4 cells have no detectable GlcNAc-TV activity, but Lec4A extracts have activity equivalent to that of parental Chinese hamster ovary cells. This discrepancy occurs because Lec4A GlcNAc-TV activity co-localizes with membranes of the endoplasmic reticulum (ER) instead of with Golgi membranes (Chaney, W., Sundaram, S., Friedman, N., and Stanley, P. (1989) J. Cell. Biol. 109, 2089–2096). cDNAs from the coding region of the GlcNAc-TV gene have now been isolated from each mutant line. Lec4 Glc
NAc-TV cDNA was found to possess two insertions, the first of which shifts the open reading frame and codes for a truncated transferase missing 585 amino acids from the catalytic domain. By contrast, Lec4A GlcNAc-TV cDNA possesses a single point mutation from T to G, which results in a change from Leu to Arg at position 188. When transfected into Lec4 cells, both cDNAs gave the appropriate phenotype; Lec4 cDNA was unable to restore Glc
NAc-TV activity, whereas Lec4A cDNA converted Lec4 cells to the Lec4A phenotype, with an active GlcNAc-TV mislocalized to ER membranes. Moreover, Lec4A cDNA cured of its mutation restored a functional, Golgi-localized GlcNAc-TV to Lec4 cells. The results demonstrate that a single change in the 740 amino acids of GlcNAc-TV serves to functionally inactivate the transferase in an intact cell by causing it to localize to the ER instead of the Golgi compartment. The mislocalized transferase retains full enzyme activity, showing that it is well folded and stable and suggesting that the L188R mutation either prevents association with exit complexes from the ER or causes retrograde transport from a Golgi compartment.

Glycoproteins and glycolipids have covalently attached glycans that acquire additional sugars as they transit the secretory pathway en route to the plasma membrane. The glycosyltransferases that catalyze each sugar transfer reside in discrete regions of the Golgi apparatus (1, 2). The transferases cloned to date are type II transmembrane proteins or glycoproteins that contain four loosely defined domains: a short, N-terminal, cytoplasmic domain; a transmembrane hydrophobic domain; a helical stem region; and a large, C-terminal catalytic domain (3–5). Experiments with chimeric molecules engineered from cloned glycosyltransferase genes have shown that the targeting of glycosyltransferases to specific Golgi compartments is caused primarily by the transmembrane domain but that other regions of the molecule are also very important for precise localization (reviewed in Ref. 2). In fact, depending on the particular chimeric molecule and host cell, each membrane-adjacent region, as well as the catalytic domain, has been implicated in obtaining maximal targeting to Golgi membranes.

While experiments with chimeric molecules are a necessary first step in identifying protein targeting determinants, they have a propensity to generate artefactual results, because relatively large regions of diverse molecules are interchanged. The site-directed mutagenesis approach is arduous and difficult to interpret if it must be undertaken in the absence of realistic structural information. An alternative is to select for mutant alleles that exhibit mistargeting of a glycosyltransferase due to a mutation in the structural gene for the enzyme. A mutant of this type was previously isolated by this laboratory from Chinese hamster ovary (CHO) cells (6). Lec4A CHO cells behave as if they lack the glycosyltransferase termed GlcNAc-TV, which adds a β(1,6)GlcNAc branch to the core α(1,6)Man residue in complex N-glycans. However, Lec4A cell-free extracts have normal levels of GlcNAc-TV activity. This apparent discrepancy is due to a problem of targeting; GlcNAc-TV activity in Lec4A cells associates with endoplasmic reticulum (ER) membranes rather than Golgi membranes in a sucrose density gradient. This mislocalization is specific for GlcNAc-TV, since other glycosyltransferases in Lec4A cells fractionate appropriately with Golgi membranes. Thus, the Lec4A mutant exhibits the interesting properties of a mammalian trafficking mutant. Since Lec4A cells possess normal levels of GlcNAc-TV activity (which is, however, more sensitive to heat than the wild-type enzyme) and belong to the same complementation group as Lec4 CHO cells that completely lack GlcNAc-TV activity, it was proposed by Chaney et al. (6), that the GlcNAc-TV structural gene is mutated in both mutants so as to either inactivate transferase activity (Lec4) or mislocalize the active enzyme (Lec4A). In this paper we show that this hypothesis was correct. Using the rat GlcNAc-TV gene (7), whose deduced amino acid sequence reveals an unusually large glycosyltransferase

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The abbreviations used are: CHO, Chinese hamster ovary; ER, endoplasmic reticulum; L-PHA, leukoagglutinin from P. vulgaris; sRBC, sheep red blood cells; MES, 2-(N-morpholino)ethanesulfonic acid.

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with type II membrane topology typical of all the known glycosyltransferases, sequences of cDNA products of the homologous GlcNAc-TV gene in CHO, Lec4, and Lec4A cells were obtained. A point mutation was found to be responsible for the mislocalization of GlcNAc-TV in Lec4A cells. The combined results suggest approaches to investigate the trafficking of Golgi glycosyltransferases.

MATERIALS AND METHODS

Cell Lines—CHO mutant cell lines were isolated previously (8), and a detailed description of the nomenclature for lectin-resistant CHO cells has been published (9). Pro-5.Lec4.7B (termed Lec4) and Pro-5.Lec4A.12-2 (termed Lec4A) cells were previously characterized (6, 10, 11). CHO-K1 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in Dulbecco’s modified Eagle’s medium or a medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum or newborn calf serum, glutamine, and nonessential amino acids.

Preparation and Screening of cDNA Libraries from CHO, Lec4, and Lec4A Cell Lines—Total RNA was prepared from the three cell lines by the method of Chomczynski and Sacchi (12). cDNA libraries were generated using a specific oligonucleotide primer and the protocol described in the TimeSaver cDNA synthesis kit (Pharmacia Biotech Inc.). The sequence of the oligonucleotide primer used to generate the wild-type CHO-K1 library was derived from a partial mouse cDNA clone (7) and was 5’-GCTATAGGCAGTCTTTGC. The sequence of the primer used to generate the Lec4 and Lec4A specifically primed cDNA libraries was derived from an oligonucleotide DNA clone that encoded the COOH-terminal region of GlcNAc-TV.7 The sequence of this primer, which spanned the stop codon, was 5’TCTGTTAGACGTCTTTG. cDNAs were ligated into lgt10 and packaged using the Gigapack II gold packaging kit (Stratagene, La Jolla, CA). Two million plaque forming units from each library were plated onto a lawn of C600 hfl bacterial cells. The libraries were screened with a 32P-labeled probe representing the 5’-end of the rat GlcNAc-TV cDNA (probe A described under “DNA Analysis”), which allowed for the rapid isolation of clones representing the entire open reading frame. Radiolabeled probe was generated with the Multiprime DNA labeling system (Amersham Corp.). A clone containing the first coding exon of the GlcNAc-TV gene was isolated from a CHO-K1 genomic DNA library (Clontech, Palo Alto, CA) using probe A (see “DNA Analysis”). Screening and plaque purification of the clones with the longest inserts were performed by standard molecular biology techniques (13). The full-length cDNA clones were subcloned into the pUC vector (Pharmacia) for sequencing. Sequencing was performed on a Single Amino Acid Change Can Mislocalize GlcNAc-TV

Transfection of Lec4 Cells with GlcNAc-TV cDNA—The Lec4 cDNA (3.8 kilobase pairs) and Lec4A cDNA (2.8 kilobase pairs) were excised by EcoRI digestion and subcloned into the EcoRI site of the pcDNA3 mammalian expression vector (Invitrogen, San Diego, CA). This plasmid has the cytomegalovirus promoter for high level expression in mammalian cells and a neomycin resistance gene for the selection of stable transfectants in the presence of G418 (Life Technologies). Plasmid DNA (1 µg/confluent 10-cm dish) was transfected into Lec4 cells by the Polybrene method (14). G418 resistant colonies were tested for their ability to bind the leukoagglutinin from Phaseolus vulgaris (L-PHA; Burrough’s Wellcome, Research Triangle Park, NC) conjugated to sheep red blood cells (sRBC), as described previously (15, 16). CHO cells expressing GlcNAc-TV bind the L-PHA/sRBC conjugate, whereas mutants such as Lec4 and Lec4A that do not express β1,6-branched glycans at the cell surface do not bind this lectin conjugate (15, 16). Individual colonies were picked and cultured in medium containing G418. Independent isolates were characterized for L-PHA/sRBC binding and lectin resistance properties as described previously (8, 9, 15, 16). Transfectants were referred to as Tf:CHO, Tf4, or Tf4A, respectively.

GlcNAc-TV Assay and Sucrose Density Gradient Analysis—GlcNAc-TV activity was measured by a modification of the assay described by Khan et al. (16). Cells were washed and extracted with 1.5% Triton X-100 (Sigma) as described previously (6). Transfet of GlcNAc in β(1,6)-linkage to the artificial and specific substrate βGlcNAc(1,2)-Man(1,6)-βGlc-O-(CH2)7CH3 (Ref. 16; very kindly provided by O. Hindsgaul, University of Alberta) was performed in a volume of 40 µl containing a final concentration of 1.1% Triton X-100, 50,000 cpm of UDP-[6-3H] GlcNAc (20 Ci/mmol; DuPont NEN), 1 mM UDP-GlcNAc, 10 mM MnCl2, 30 mM MES (Sigma), pH 6, 20 nmol of substrate, and 100–1000 µg of cell protein. After 3 h at 37 °C, the reaction was stopped by the addition of 1 ml of cold water, and each sample was centrifuged for 10 min at 15,000 rpm in a microcentrifuge and loaded onto a Sep-Pak C18 cartridge (Millipore Corp., Bedford, MA). The column was washed with 30 ml of water, and product was eluted in 6 ml of 50% ethanol and counted in a scintillation counter.

For separation of membrane fractions by sucrose gradient centrifugation, postnuclear supernatant was prepared from each transfected and centrifuged to density equilibrium in a stepwise sucrose gradient as described previously (6). Fractions (1 ml) were collected continuously by peristaltic pump, or membranes were collected from each sucrose interface and assayed for GlcNAc-TV as described above, as well as for the Golgi-localized transferases, β1,4-Gal-transferase and GlcNAc-TI, and the ER marker enzyme cytochrome c reductase, as described previously (6).

Mutagenesis of Lec4A GlcNAc-TV cDNA—Mutagenesis was performed on the Lec4A cDNA to cure the single T to G mutation observed in this mutant. The pcDNA3-Lec4A GlcNAc-TV cDNA was transformed into the CJ236 bacterial strain, and single strand uracil containing pcDNA-Lec4A was isolated following superfection of helper phage MJ3K07 as described in instructions for the Mutagen-Gene kit (New England Biolabs, Bedford, MA). The synthetic mutagenic oligonucleotide primer had the following sequence: 5’TCAAGTGGAGTGTGAAAT-TGTTGTCCCTCG. This oligonucleotide was phosphorylated with ATP by T4 polynucleotide kinase (Boehringer Mannheim) before use in the mutagenesis reaction. The mutagenesis reaction contained 200 ng of single-stranded uracil-containing PCDNA3-Lec4A mixed with 8 pmol of phosphorylated mutagenic primer. Following the synthesis of double-stranded DNA, the revertant plasmid (pcDNA3-Lec4ARev) was transformed into Escherichia coli DH5α. Mutagenesis was confirmed by DNA sequencing of pcDNA3-Lec4ARev. This plasmid was also transfected into Lec4 cells as described above, and transfectants are referred to as Tf4ARev.

RESULTS

The GlcNAc-TV Gene in Lec4 and Lec4A Cells—Southern analysis of genomic DNA from CHO-K1, Lec4, and Lec4A cells provided no evidence for major rearrangements in the GlcNAc-TV gene in either mutant (Fig. 1). A similar pattern of exon distribution was obtained for each cell line using three probes that spanned the coding region of the rat GlcNAc-TV cDNA. Two of the CHO GlcNAc-TV cDNA sequences were determined (Fig. 2), and the nucleotide sequence of Lec4A was determined after sequencing both strands (2). A complete nucleotide sequence of the pcDNA-Lec4ARev plasmid was obtained from the Hinc II site, so the different bands seen in Fig. 1, A and C, must represent six different exons. However, a single EcoRV site is present at nucleotide 1982 of CHO GlcNAc-TV, so that two of

\(^{6}\) J. Weinstein, S. Sundaram, X. Wang, D. Delgado, R. Basu, and P. Stanley, unpublished observations.
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The bands in Fig. 1B must derive from a single exon. Therefore, probes encompassing approximately the entire open reading frame of rat GlcNAc-TV identify at least 11 exons in the CHO GlcNAc-TV gene. Eleven exons were also reported by Saito et al. (17) for the human GlcNAc-TV homologue. While Northern analysis failed to show detectable levels of a CHO GlcNAc-TV RNA, a transcript of ~7.5 kilobases for GlcNAc-TV has been observed in rat kidney (7, 18), suggesting that more than 11 exons are probably encoded in the GlcNAc-TV gene.

GlcNAc-TV cDNA from Lec4 Cells Codes for a Truncated Protein—cDNA libraries were constructed from CHO-K1 and Lec4 poly(A)+ RNA by specific priming with an oligonucleotide that spans the stop codon of the previously cloned rat GlcNAc-TV cDNA (7). Since GlcNAc-TV mRNA is extremely rare, even specifically primed libraries contained few full-length clones. Two CHO.K1 GlcNAc-TV cDNAs and one Lec4 cDNA were isolated and sequenced (Figs. 2 and 3). A genomic clone representing the first coding exon of the CHO GlcNAc-TV gene was also sequenced. This exon is 383 base pairs long. BESTFIT comparison of the published rat GlcNAc-TV cDNA sequence (7) with CHO-K1 GlcNAc-TV cDNA demonstrated 93% identity in nucleotide sequence and 99% identity at the amino acid level.

The Lec4 GlcNAc-TV cDNA sequence (Fig. 2) was identical to the CHO GlcNAc-TV sequence (Fig. 3), in the 5′-untranslated region and for the first 402 nucleotides from the start codon. At this stage, an 822-base pair insertion interrupted the reading frame, causing it to terminate after an additional 21 amino acid residues. GlcNAc-TV from this mutant gene would be only 155 residues long and not be expected to have any catalytic activity. There is a second insertion in the Lec4 cDNA of 263 base pairs (Fig. 2). Since genomic DNA analysis (Fig. 1) did not show evidence of significant insertions or deletions in the GlcNAc-TV gene of Lec4 cells, the insertions in the cDNA may be due to aberrant splicing resulting in improper excision of certain intronic sequences from the primary transcript. Consistent with this prediction is the fact that the nucleotide sequence at the 5′-site of each insertion does not conform to the consensus predicted for a 5′-splice site in mammalian genes (19). In addition, these insertions appear like introns because the GlcNAc-TV cDNA coding sequence continues uninterrupted after the 3′-end of the inserted sequence (Figs. 2 and 3). Perng et al. (18) previously reported that GlcNAc-TV transcripts in a GlcNAc-TV-deficient mutant of BW5147 cells were ~500 nucleotides larger than in parent BW5147 cells, suggesting a similar mechanism of mutational origin.

GlcNAc-TV cDNA from Lec4A Cells Has a Single Point Mutation—A comparison of the cDNA and deduced amino acid sequence of GlcNAc-TV from CHO and Lec4A cDNAs is shown in Fig. 3. Surprisingly, the Lec4A cDNA differed in sequence from the CHO-K1 cDNA at a single residue. A point mutation from T to G at nucleotide 1147 in the Lec4A cDNA results in a single amino acid change from Leu to Arg at position 188 (boxed in Fig. 3). This is considerably downstream of the signal anchor sequence (amino acid residues 14–30), which plays an essential role in the Golgi localization of glycosyltransferases (2). The lack of any change in the transmembrane region in Lec4A GlcNAc-TV was confirmed by comparison with the GlcNAc-TV genomic DNA sequence encoding the first N-terminal 81 amino acids of the CHO gene.

GlcNAc-TV Gene of Lec4 Cells Contains Mutations—Molecular characterization of the Lec4GlcNAc-TV gene was confirmed by comparison with the GlcNAc-TV genomic DNA sequence encoding the first N-terminal 81 amino acids of the CHO gene. The GlcNAc-TV gene of Lec4 cells, the insertions in the cDNA may be due to aberrant splicing resulting in improper excision of certain intronic sequences from the primary transcript. Consistent with this prediction is the fact that the nucleotide sequence at the 5′-site of each insertion does not conform to the consensus predicted for a 5′-splice site in mammalian genes (19). In addition, these insertions appear like introns because the GlcNAc-TV cDNA coding sequence continues uninterrupted after the 3′-end of the inserted sequence (Figs. 2 and 3). Perng et al. (18) previously reported that GlcNAc-TV transcripts in a GlcNAc-TV-deficient mutant of BW5147 cells were ~500 nucleotides larger than in parent BW5147 cells, suggesting a similar mechanism of mutational origin.

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Fig. 1. Southern analysis of the GlcNAc-TV gene in CHO-K1 (lane 1), Lec4 (lane 2), and Lec4A (lane 3). Genomic DNA was digested with either HindIII (blots A and C) or with EcoRV (blot B). Blots A, B, and C were probed with radiolabeled probes A, B, and C, respectively, which were prepared as described under "Materials and Methods." Differences in intensity of bands were not consistent.

The Single Amino Acid Change in Lec4A GlcNAc-TV Causes Mislocalization and Functional Inactivation of the Transferase—The sequencing results are consistent with predictions based on previous observations (6): (a) Lec4 and Lec4A CHO mutants belong to the same genetic complementation group; (b) Lec4 mutants lack β(1,6)GlcNAc-branched N-glycans and have no detectable GlcNAc-TV activity; (c) Lec4A mutants, by contrast, have GlcNAc-TV activity in detergent extracts, but the transferase is unable to act in intact cells because it is mislocalized. The indication that a point mutation far from the transmembrane domain appeared responsible for the miscompartmentalization of GlcNAc-TV (Fig. 3) was intriguing, and was further investigated to obtain definitive proof.

The cDNAs from CHO-K1, Lec4A, and Lec4 were subcloned into the expression vector pcDNA3 that contains the neomycin resistance gene. In addition, a revertant of the Lec4A cDNA, generated by site-directed mutagenesis to correct the point mutation at amino acid 188 (Fig. 3), was cloned into pcDNA3. Transfectants were obtained following transfer of the cDNAs into Lec4 null mutant cells, by selection with G418, and tested for the following: (a) their ability to bind the lectin, L-PHA (which preferentially recognizes β(1,6)-branched N-glycans) conjugated to sheep red blood cells (L-PHA/sRBC); (b) their sensitivity to L-PHA; and (c) their GlcNAc-TV activity. The results in Table I show that the cDNAs behaved as predicted. Most importantly, the corrected Lec4A cDNA (Tf.4ARev), bound L-PHA/sRBC and acquired sensitivity to the toxicity of L-PHA. In addition, GlcNAc-TV from Tf.4A extracts was temperature-sensitive at 45 °C, whereas GlcNAc-TV from Tf.4ARev had the same activity at 37 and 45 °C (data not shown). This difference between the mutant and wild-type GlcNAc-TV was observed previously in Lec4A versus CHO extracts (6). To confirm that the Lec4A point mutation causes mislocalization of GlcNAc-TV to membranes that fractionate with the ER, postnuclear supernatants from Tf.4A and Tf.4ARev were characterized by sucrose density centrifugation (Fig. 4). The location of Golgi membranes was followed by assaying gradient
fractions for β(1,4)Gal-T and GlcNAc-TI, while ER membranes were localized by assaying cytochrome c (Cyto-c) reductase activity. Previous studies showed that Lec4AGlcNAc-TI is not found in the lysosomal fraction (6). The profiles in Fig. 4 show that while both Tf.4A and Tf.4ARev have similar β(1,4)Gal-T and cytochrome c reductase profiles, they differ markedly, and as predicted, for their GlcNAc-TV profiles. Tf.4A GlcNAc-TV activity associated predominantly with dense membranes enriched in cytochrome c reductase, while Tf.4ARev GlcNAc-TV activity was shifted to the light Golgi membrane fraction. It is noticeable in Fig. 4 that Tf.4ARevGlcNAc-TV was not as tightly localized to Golgi membranes as β(1,4)Gal-T. This was also true when membranes harvested from each interface of the sucrose gradient were assayed (Fig. 5). In these experiments, the medial Golgi transferase, GlcNAc-TI (2) was assayed in addition to the other activities. Approximately 33% of GlcNAc-TV activity in Golgi membranes averaged 70% (ranging from 64 to 82%). Thus, there seems to be a real and consistent difference in the proportion of GlcNAc-TV versus β(1,4)Gal-T and GlcNAc-TI in the Golgi membranes of CHO cells, indicating that GlcNAc-TV may not be entirely confined...
to the Golgi in wild-type CHO cells. Nevertheless, it is clear that a large proportion of GlcNAc-TV activity must be associated with Golgi membranes for the transferase to function in adding a β(1,6)GlcNAc branch to N-glycans, since Lec4A cells and Tf.4A transfectants are as resistant to L-PHA as Lec4 cells that completely lack GlcNAc-TV activity (Table I).

**DISCUSSION**

The finding that a point mutation at amino acid 188 of GlcNAc-TV causes it to localize to ER membranes in a stable, catalytically active form, is an unexpected result. The 740-amino acid GlcNAc-TV has a predicted molecular mass of 85 kDa, which would increase substantially if some of the six potential N-glycosylation sites are utilized in the native protein. Purification of GlcNAc-TV from rat kidney in the presence of protease inhibitors showed a band at 95 kDa in addition to a doublet at 69 and 75 kDa, which is usually observed on SDS-polyacrylamide gels of purified GlcNAc-TV (7). Gu et al. (20) reported bands as low as 60 kDa in purified preparations of GlcNAc-TV, although it is not clear whether these low molecular weight forms retain catalytic activity. They are presumably the result of proteolytic cleavage occurring in the stem region of GlcNAc-TV to produce a soluble, secreted, active form of the enzyme, as has been observed for other glycosyltrans-
ferases (21, 22). The fact that a 69-kDa form of GlcNAc-TV maintains measurable catalytic activity suggests that Glc-NAc-TV can lose 200 amino acid residues from the NH2 terminus without a profound impact on enzymatic function. This implies that position 188, which is mutated in Lec4A GlcNAc-TV, may lie at the boundary between the stem region and the catalytic domain of the transferase. Furthermore, the GlcNAc-TV gene is very conserved in mammalian species, particularly in a long span between amino acid residues 197 and 655 that shows only two and four amino acid residue differences between the CHO transferase and rat and human transferases, respectively (Fig. 6a). This highly conserved region could constitute the catalytic domain, further supporting the possibility that position 188 is just upstream of the catalytic domain.

It will be interesting to see how the L188R change affects the structure of GlcNAc-TV once this has been determined. Meanwhile, it can be predicted from hydrophobicity analyses that a mutation from Leu to Arg would be expected to have a significant effect on GlcNAc-TV conformation. Hydropathy plots performed by the method of Kyte and Doolittle (23) reveal that the region near residue 188 occurs within a relatively small hydrophobic pocket (residues 179–189) that is flanked on either side by 21-amino acid stretches of a relatively hydrophilic nature (8 of 21 residues are charged). Comparison of hydropathy plots of CHO and Lec4A GlcNAc-TV shows a significant shift toward hydrophilicity in the region of the mutation (Fig. 6b). This change is presumably the cause of the mistargeting of GlcNAc-TV activity in Lec4A cells, since curing this single mutation restores proper localization of the active enzyme.

The Lec4A mutant is a novel tool for studying mechanisms of intracellular trafficking between the ER and Golgi, because its mutant GlcNAc-TV, although mistargeted to the ER, retains specific transferase activity. Single mutations in other glycoproteins cause ER retention (Ref. 24 and references therein; Refs. 25–27), but these molecules are often aggregated or rapidly degraded. The unique properties of Lec4A GlcNAc-TV provide an approach to identifying factors involved in transporting GlcNAc-TV (and probably other transferase glycoproteins) to the Golgi, where GlcNAc-TV is predicted (based on its substrate requirements) to reside in the medial compartment.

It will be important to determine the post-translational modifications of Lec4A GlcNAc-TV. A tyrosine residue lying adjacent to the new Arg residue, although not in a known consensus sequence (28, 29), may be a candidate for phosphorylation at residue 187. Also, carbohydrate structural analysis will reveal whether the mutant transferase remains in the ER following synthesis or returns to the ER by retrograde transport. However, the lack of β(1,6)GlcNAc-branched N-glycans in Lec4A cells implies that the mutant enzyme is at best a very transient

| Cells          | Source cDNA | L-PHA/sRBC | L-PHA (D10) | GlcNAc-TV |
|----------------|-------------|------------|-------------|-----------|
| Transfectants  |             |            |             |           |
| Tf.CHO-K1      | CHO-K1      | ++         | 5           | 11.3      |
| Tf.4A          | Lec4A       | –          | >250        | 92        |
| Tf.4ARev (corrected) | Lec4A  | ++         | 5           | 147       |
| Tf.4          | Lec4        | –          | >250        | <0.1      |
| Controls       |             |            |             |           |
| CHO            | None        | ++         | 5           | 51        |
| Lec4           | None        | –          | >250        | 4         |

**Fig. 4.** Sucrose gradient analysis of Tf.4A and Tf.4ARev. Post-nuclear supernatants from ~4 × 10^6 transfectants were mixed with an equal volume of 2.75 M sucrose, layered onto a cushion of 2 M sucrose, and overlaid successively with 1.4 M, 1.2 M, and 0.8 M sucrose. Fractions of 1 ml were collected, and aliquots were assayed for protein and GlcNAc-TV, β(1,4)Gal-T, and cytochrome c (Cyto-c) reductase activities. Total activities were calculated and are presented as the percentage of activity recovered per fraction. Dashed line, Tf.4A; solid line, Tf.4ARev.

**Fig. 5.** GlcNAc-TV is less Golgi-associated than β(1,4)Gal-T. Post-nuclear supernatants from CHO and Lec4A GlcNAc-TV were fractionated by sucrose gradient as described in the legend to Fig. 4. Membranes at the indicated interfaces were harvested and assayed for GlcNAc-TV (solid bars), β(1,4)Gal-T (striped bars), and GlcNAc-TI (open bars). Histograms represent the percentage of recovered activity in each fraction.

**Table I.** Properties of GlcNAc-TV cDNA transfectants

Lec4 mutant CHO cells that have no GlcNAc-TV activity were transfected with cDNAs encoding CHO-K1 and mutant GlcNAc-TV. Transfectants were selected in G418 and tested for their ability to bind L-PHA/sRBC (+), for the concentration of L-PHA that killed 90% of the cells (D10), and for GlcNAc-TV activity. CHO and Lec4 cells were used as controls. The values for GlcNAc-TV activity in transfectants represent the average obtained from 2 assays of 2 independent transfectants.
residents of the medial Golgi. Immunoprecipitation experiments should identify chaperones associated with wild-type and mutant GlcNAc-TV. The fact that the Lee4A cells retain full GlcNAc-TV transferase activity provides an approach to isolating molecules that induce retrograde transport from the medial Golgi. Since, under normal conditions, glycoproteins in Lee4A cells do not carry β(1,6)GlcNAc-branched N-glycans, treatments that induce retrograde trafficking or allow anterograde transport of Lee4A GlcNAc-TV could be detected by screening T.Lee4A transfectants that express several copies of the Lee4A cDNA for reversion to L-PHA binding or sensitivity. Transfectants overexpressing Lee4A GlcNAc-TV would also be useful in determining whether factors causing GlcNAc-TV to be ER-localized can become saturated. Such an overexpressor would also be useful in identifying molecules associated with a mutant GlcNAc-TV molecule.

Clearly, the shift in GlcNAc-TV compartmentalization in Lee4A cells does not cause a concomitant shift in the medial Golgi transferase GlcNAc-TI (Fig. 5), which acts just prior to GlcNAc-TV in the synthesis of complex N-glycans. Biochemical evidence suggests that Golgi glycosyltransferases exist as oligomers that are, in some cases, disulfide-bonded (2, 30). Directed targeting of GlcNAc-TI to the ER by attachment of the cytoplasmic tail of the p33 invariant chain caused it to be localized primarily to the ER and also caused an enrichment of α-mannosidase II (the enzyme that acts just after GlcNAc-TI) in ER membranes (31). The fact that GlcNAc-TI retains tightly Golgi-localized in Lee4A cells (6) and transfectants (Fig. 5) may mean that (a) it does not form a complex with GlcNAc-TV, (b) that such a complex is in minor proportion, or (c) that Lee4A GlcNAc-TV does not encounter the medial Golgi compartment.

Apart from its general usefulness as a marker of a specialized region of the Golgi, GlcNAc-TV is of biological interest since its expression is regulated. Common among the glycosylation changes associated with malignancy and metastasis (32, 33) is an increase in the β(1,6)GlcNAc branching of complex N-glycans, which has been correlated with increased GlcNAc-TV activity (18, 34, 35). Inhibition of this activity is therefore a potential way to prevent metastasis.

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REFERENCES
1. Kornfeld, R., and Kornfeld, S. (1985) Annu. Rev. Biochem. 54, 631–664
2. Gleeson, P. A., Teasdale, R. D., and Burke, J. (1994) Glycogenylactase J. 11, 381–394
3. Paulson, J. C., and Colley, K. J. (1989) J. Biol. Chem. 264, 17615–17618
4. Joziasse, D. H. (1992) Glycobiology 2, 271–277
5. Kofeze, R., and Berger, E. G. (1993) Biochim. Biophys. Acta 1154, 283–325
6. Chaney, W., Sundaram, S., Friedman, N., and Stanley, P. (1989) J. Cell. Biol. 109, 2089–2096
7. Shoreibah, M., Perng, G.-S., Adler, B., Weinstein, J., Basu, R., Cupples, R., Wen, D., Browne, J. K., Buckhaults, P., Freigen, N., and Pierce, M. (1993) J. Biol. Chem. 268, 15381–15385
8. Stanley, P., Callibot, V., and Siminovitch, L. (1975) Cell 6, 121–128
9. Stanley, P. (1983) Methods Enzymol. 96, 157–184
10. Stanley, P., and Sudo, T. (1981) Arch. Biochem. Biophys. 210, 20–28
11. Stanley, P., Vivona, G., and Atkinson, P. (1982) Arch. Biochem. Biophys. 219, 126–139
12. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
13. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
14. Stanley, P., and Sudo, T. (1981) Cell 23, 765–769
15. Stanley, P., and Sudo, T. (1981) Cell 23, 765–769
16. Stanley, P., and Sudo, T. (1981) Cell 23, 765–769
17. Saito, H., Nishikawa, A., Gu, J., Ibara, Y., Ooijima, H., Wada, Y., Sekiya, C., Niiwaki, N., and Taniguchi, N. (1994) Biochim. Biophys. Acta 1194, 318–322
18. Perng, G.-S., Shoreibah, M., Margitich, I., Pierse, M., and Freigen, N. (1994) Glycobiology 4, 867–871
19. Shapir, M. J., and Senapathy, P. (1987) Nucleic Acids Res. 15, 7175–7174
20. Gu, J., Nishikawa, A., Tsurukko, N., Ohno, M., Yamaguchi, N., Kangawa, K., and Taniguchi, N. (1993) J. Biochem. (Tokyo) 113, 614–619
21. Weinstein, J., Lee, E. U., McEntee, K., Lui, P.-H., and Paulson, J. C. (1987) J. Biol. Chem. 262, 17745–17743
22. Colley, K. J., Lee, E. U., Adler, B. Browne, J. K., and Paulson, J. C. (1989) J. Biol. Chem. 264, 17619–17622
23. Kyte, J., and Doollittle, R. F. (1982) J. Mol. Biol. 157, 105–132
24. Sifers, R. N., Brashears-Macatee, S., Kidd, V. J., Muensch, H., and Woo, S. L. C. (1988) *J. Biol. Chem.* **263**, 7330–7335
25. Racchi, M., Watzke, H. H., High, K. A., and Lively, M. O. (1993) *J. Biol. Chem.* **268**, 5735–5740
26. Gardner, A. M., Aviel, S., and Argon, Y. (1993) *J. Biol. Chem.* **268**, 25940–25947
27. Petrolla, M., Chiatayat, D., Peltonen, L., and Jalanko, A. (1994) *Hum. Mol. Genet.* **3**, 2237–2242
28. Pearson, R. B., and Kemp, B. E. (1994) *Methods Enzymol.* **200**, 62–81
29. Songyang, Z, Carraway, K. L., III, Eck, M. J., Harrison, S. C., Feldman, R. A., Mohammad, M., Schlessinger, J., Hubbard, S. R., Smith, D. P., Eng, C., Lorenzo, M. J., Ponder, B. A. J., Mayer, B. J., and Cantley, L. C. (1995) *Nature* **373**, 536–539
30. Ma, J., and Colley, K. J. (1996) *J. Biol. Chem.* **271**, 7758–7766
31. Nilsson, T., Hoe, M. H., Slusarewicz, P., Rabouille, C., Watson, R., Hunte, F., Watzke, G., Berger, E. G., and Warren, G. (1994) *EMBO J.* **13**, 562–574
32. Yamashita, K, Tachibana, Y., Ohkura, T., and Kobata, A. (1995) *J. Biol. Chem.* **260**, 3963–3969
33. Cornil, I., Kerbel, R. S., and Dennis, J. W. (1990) *J. Cell. Biol.* **111**, 773–781
34. Dennis, J. W., Laferte, S., Waghorne, C., Breitman, M. L., and Kerbel, R. S. (1987) *Science* **236**, 582–585
35. Demetriou, M., Nabi, I. R., Coppoloni, M., Dedhar, S., and Dennis, J. W. (1995) *J. Cell. Biol.* **130**, 383–392
36. Kumar, R., and Stanley, P. (1990) *Mol. Cell Biol.* **10**, 3857