# Independent Lec1A CHO Glycosylation Mutants Arise from Point Mutations in *N*-Acetylglucosaminyltransferase I That Reduce Affinity for Both Substrates. Molecular Consequences Based on the Crystal Structure of GlcNAc-TI[†,‡]

Wei Chen,[§] Ulug M. Ünligil,[||] James M. Rini,[||,⊥] and Pamela Stanley*[,§]

*Department of Cell Biology, Albert Einstein College of Medicine, Bronx, New York 10461, Department of Medical Genetics and Microbiology, University of Toronto, Toronto, Ontario, Canada M5S 1A8, and Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada M5S 1A8*

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ABSTRACT: A key enzyme in regulating the maturation of N-linked glycans is UDP-*N*-acetylglucosamine: α-3-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase I (GlcNAc-TI, EC 2.4.1.101). Lec1 CHO cells lack GlcNAc-TI activity and synthesize only the oligomannosyl class of *N*-glycans. By contrast, Lec1A CHO mutants have weak GlcNAc-TI activity due to the reduced affinity of GlcNAc-TI for both the UDP-GlcNAc and $Man_5GlcNAc_2Asn$ substrates. Lec1A CHO mutants synthesize hybrid and complex *N*-glycans, albeit in reduced amounts compared to parental CHO cells. In this paper, we identify two point mutations that gave rise to the Lec1A phenotype in three independent Lec1A CHO mutants. The G634A mutation in Lec1A.2C converts an aspartic acid to an asparagine at amino acid 212, disrupting a conserved DXD motif ($E_{211}DD_{213}$ in all GlcNAc-TIs) that makes critical interactions with bound UDP-GlcNAc and $Mn^{2+}$ ion in rabbit GlcNAc-TI. The C907T mutation in Lec1A.3E and Lec1A.5J converts an arginine conserved in all GlcNAc-TIs to a tryptophan at amino acid 303, altering interactions that are important in stablizing a critical structural element in rabbit GlcNAc-TI. Correction of each mutation by site-directed mutagenesis restored their GlcNAc-TI activity and lectin binding properties to parental levels. The effect of the two amino acid changes on GlcNAc-TI catalysis is discussed in relation to the crystal structure of rabbit GlcNAc-TI complexed with manganese and UDP-GlcNAc.

The glycosyltransferase UDP-*N*-acetylglucosamine: α-3-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase I (GlcNAc-TI,[1] EC 2.4.1.101) is a type II transmembrane protein with the catalytic domain in the C-terminal portion of the protein (*1*, *2*). It is localized in the medial Golgi and initiates complex and hybrid N-linked carbohydrate synthesis (reviewed in ref *3*). The substrate of GlcNAc-TI, $Man_5GlcNAc_2Asn$, accumulates in the absence of GlcNAc-TI, showing that it is a key enzyme regulating cell surface complex and hybrid glycan synthesis in mammals. Chinese hamster ovary (CHO) cell mutants (*4*, *5*) and embryonic stem cells (*6*) that lack GlcNAc-TI activity have doubling times in culture similar to those of wild-type cells, showing that GlcNAc-TI is not required for cell viability or normal cell growth. However, GlcNAc-TI is essential to the viability of mammals since mice lacking functional GlcNAc-TI die at midgestation (*7*, *8*). Mice with only one wild-type allele encoding GlcNAc-TI appear normal, although *Mgat-1*$^{+/-}$ WW6 ES cells contribute very poorly to the organized layer of bronchial epithelium (*6*), identifying a cell type specific defect arising from reduced GlcNAc-TI activity. Introduction of an allele encoding a weak GlcNAc-TI onto a null background should identify additional functions of complex *N*-glycans in development. Such alleles were predicted to occur in Lec1A CHO glycosylation mutants (*9*, *10*).

Lec1A CHO cell mutants were isolated from CHO cell populations using lectins as selective agents (*9*). They were shown by complementation analysis to belong to the Lec1 genetic complementation group but to have a quantitatively distinct lectin resistant phenotype. Whereas Lec1 CHO cells have no GlcNAc-TI activity and cannot make complex carbohydrates (*4*, *11*), Lec1A CHO cells have partial GlcNAc-TI activity and convert some $Man_5GlcNAc_2Asn$ to hybrid and complex *N*-glycans (*9*). Kinetic studies (*10*) showed that GlcNAc-TI in Lec1A.2C cells has an altered optimum pH and increased apparent $K_m$ values for both

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[‡] GenBank accession numbers: AF343963 for Pro$^-$5 CHO *Mgat-1*, AF343964 for Pro$^-$ Lec1A.2C *Mgat-1*, and AF343965 for Gat$^-$ Lec1A.3E and Pro$^-$ Lec1A.5J *Mgat-1*. Protein Data Bank coordinates: 1FOA for the UDP-GlcNAc−$Mn^{2+}$-bound form that was used in the analysis, 1FO9 for the unliganded form, and 1FO8 for the C123-methylmercury-bound derivative form.

* To whom correspondence should be addressed. Phone: (718) 430-3346. Fax: (718) 430-8574. E-mail: stanley@aecom.yu.edu.

[§] Albert Einstein College of Medicine.

[||] Department of Medical Genetics and Microbiology, University of Toronto.

[⊥] Department of Biochemistry, University of Toronto.

[1] Abbreviations: GlcNAc-TI, UDP-*N*-acetylglucosamine:α-3-D-mannoside β-1,2-*N*-acetylglucosaminyltransferase I; CHO, Chinese hamster ovary; FITC, fluorescein isothiocyanate; L-PHA, *Phaseolus vulgaris* leucoagglutinin; WGA, wheat germ agglutinin; ConA, concanavalin A; MFI, mean fluorescence index; PBS, phosphate-buffered saline; BSA, bovine serum albumin; DEPC, diethyl pyrocarbonate; RT-PCR, reverse transcription polymerase chain reaction.

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Table 1: Properties of Lec1A Compared to Those of CHO and Lec1 Cells[a]

| cells | relative GlcNAc-TI activity (%) | WGA resistance | L-PHA resistance | apparent $K_m$ for $M_5$ (mM) | apparent $K_m$ for UDP-GlcNAc (mM) | optimum pH |
|---|---|---|---|---|---|---|
| CHO | 100 | − | − | 0.26 | 0.17 | 6.25 |
| Lec1A | 2 | $R_9$ | $R_{300}$ | 5.43 | 7.56 | 7.5 |
| Lec1 | 0 | $R_{30}$ | $R_{1000}$ | NA | NA | NA |

[a] NA, not applicable because Lec1 has no detectable GlcNAc-TI activity. $R_9$ represents a 9-fold resistantance compared to CHO cells. $M_5$ is the $Man_5GlcNAc_2Asn$ acceptor for the GlcNAc-TI assay. Data taken from refs *9* and *10*.

substrates, $Man_5GlcNAc_2Asn$ and UDP-GlcNAc, as summarized in Table 1. Although Lec1A isolates have no detectable GlcNAc-TI activity in cell-free extracts assayed under conditions in which GlcNAc-TI is detected in CHO cell extracts (CHO condition), their GlcNAc-TI activity is readily detected at increased substrate concentrations and at pH 7.5 (Lec1A assay condition). These studies provided evidence that Lec1A cells contain a mutant GlcNAc-TI that is structurally altered. In this paper, we identify two point mutations in GlcNAc-TI that give rise to the Lec1A phenotype. The predicted effect of these mutations on GlcNAc-TI structure is discussed in terms of the crystal structure of rabbit GlcNAc-TI complexed with manganese and UDP-GlcNAc (*12*).

## EXPERIMENTAL PROCEDURES

*Materials.* FITC-conjugated lectins wheat germ agglutinin (WGA) and *Phaseolus vulgaris* leucoagglutinin (L-PHA) were purchased from Vector Laboratories, Inc. (Burlingame, CA). G418 was from Gemini Bio-Product, Inc. (Calabasas, CA). Concanavalin A (Con A)-Sepharose was from Pharmacia (Uppsala, Sweden). UDP-*N*-acetyl-D-[6-$^3$H]glucosamine (UDP-[$^3$H]GlcNAc) (25 $\mu$Ci/mmol), UDP-[1-$^3$H]galactose (UDP-[$^3$H]Gal) (25 $\mu$Ci/mmol), and [$\alpha$-$^{32}$P]dCTP (6000 Ci/mmol) were from DuPont NEN Products (Boston, MA). TRIzol reagent, superscript II reverse transcriptase, fetal calf serum, and oligonucleotide primers were from Life Technologies (Grand Island, NY). *Taq* polymerase was from Perkin-Elmer. *Pfu* polymerase and the QuikChange site-directed mutagenesis kit were from Stratagene (La Jolla, CA). Protease inhibitor tablets, Rnase H, Dnase I, and the Rnase inhibitor were from Boehringer Mannheim (Indianapolis, IN). The QIAquick DNA agarose gel extraction kit was from Qiagen (Valencia, CA). Bovine serum albumin (BSA), Polybrene, and Nonidet P-40 were from Sigma-Aldrich Co. (St. Louis, MO). $Man_5GlcNAc_2Asn$ was prepared as described previously (*10*).

*Cell Culture.* Parental CHO cells (Pro$^-$5), Lec1 mutant Pro$^-$Lec1.3C cells, and Lec1A mutant (Pro$^-$Lec1A.2C, Pro$^-$Lec1A.2A, Gat$^-$Lec1A.3E, and Pro$^-$Lec1A.5J) cells isolated previously (*9*) were cultured in suspension at 37 °C in $\alpha$ medium containing 10% fetal calf serum. Transfectants carrying cDNA constructs in pcDNA3.1(+) (Invitrogen, Carlsbad, CA) were grown in the same medium containing G418 at 1.0 mg/mL active weight.

*DNA Preparations.* Genomic DNA was prepared using the QIA amp genomic kit from Qiagen. CHO cells (5−10 $\times$ $10^6$) were washed with PBS once, suspended in 200 $\mu$L of PBS, and treated with 0.5 mg/mL proteinase K at 70 °C. Column purification was performed as recommended by the manufacturer. Genomic DNA was eluted in 10 mM Tris (pH 8.0) and stored at 4 °C.

Plasmid DNA was amplified in bacterial host cells and isolated using standard Qiagen Plasmid Mini or Midi Kits following the protocol recommended by the manufacturer.

*RNA Preparation.* Total RNA from $10^7$ cells was prepared using 1 mL of TRIzol reagent as suggested by the manufacturer. After chloroform extraction, RNA was precipitated by 2-propanol, washed with 70% ethyl alcohol, and resuspended in diethyl pyrocarbonate (DEPC)-treated water. After treatment with 0.5 unit of Dnase I at 37 °C for 30 min and extraction with phenol and chloroform, the RNA was precipitated with 2-propanol, washed with 70% ethyl alcohol, and resuspended in DEPC-treated water.

*Northern Analysis.* Total RNA (10 $\mu$g) was electrophoresed in a 1.2% formaldehyde−agarose gel, transferred to Hybond nylon membrane (Amersham, Piscotaway, NJ), and UV cross-linked to the membrane in a Stratalinker. The membrane was prehybridized at 60 °C in prehybridization buffer [0.05 M piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), 0.1 M NaCl, 0.05 M $Na_2PO_4$, 1 mM EDTA, 5% sodium dodecyl sulfate (SDS), and 0.06 mg/mL herring sperm DNA denatured at 100 °C for 5 min, chilled on ice], for at least 3 h, and hybridized with a 575 bp *Pst*I fragment from the coding region of mouse *Mgat-1* cDNA (*19*) in the same buffer. The probe was labeled with [$\alpha$-$^{32}$P]dCTP using the Prime-It RmT dCTP-labeling kit (Stratagene) to a specific activity of $\sim$$10^9$ cpm/$\mu$g and purified on a G-50 minispin column (Worthington, Lakehood, NJ). After hybridization at 60 °C for $\sim$24 h, the membrane was finally washed in 0.2$\times$ SSC [1$\times$ SSC is 150 mM NaCl and 15 mM sodium citrate (pH 7.0)] and a 0.1% SDS solution at 37 °C for 20 min and at 55 °C for 30 min, before being exposed to Kodak X-OMAT X-ray film at −80 °C.

*Reverse Transcription.* Total RNA (5−10 $\mu$g) was used to obtain first-strand cDNA using dATP, dCTP, dGTP, and dTTP (0.2 mM each), 10 mM dithiothreitol (DTT), 20 pmol of oligo-dT primer, 1 unit of the Rnase inhibitor, first-strand buffer (Life Technologies), and 5 units Superscript II reverse transcriptase. After the RNA and oligo-dT primer had been heated at 70 °C for 10 min and immediately chilled on ice, dNTPs, DTT, Superscript II reverse transcriptase, and first-strand buffer were added and the reaction mixtures were incubated at 42 °C for 50 min. After incubation at 70 °C for 15 min, 0.5 unit of Rnase H (Dnase free) was added and the incubation continued at 37 °C for 30 min before storage at −20 °C. cDNA products (5 $\mu$L) were used for amplification of target genes by PCR.

*Polymerase Chain Reaction.* cDNAs encoding GlcNAc-TI were isolated by PCR amplification using cDNA products of various cell lines as a template. Genomic DNA was also

PCR amplified under the same conditions. Reaction mixtures contained 2.0 mM $MgCl_2$, 5 units of *Taq* polymerase, 0.5 unit of *pfu* polymerase, 6 $\mu$M primers (final concentration), and 0.2 mM dNTPs in PCR buffer (Perkin-Elmer PCR buffer system with 1.5 mM $MgCl_2$) in a final volume of 50 $\mu$L. Cycle conditions were as follows: 94 °C for 1 min, 40 cycles of 1 min at 94 °C, 1 min at 58 °C, and 1 min at 72 °C, and a final cycle of 15 min at 72 °C. Primers were designed according to mouse, rat, rabbit, and human 5′ and 3′ untranslated regions spanning the coding region of the *Mgat-1* gene. The forward primer (5′ CCAAGCTTCCTC-CCCKGYGGGGGCCAGG 3′) contained a *Hin*dIII site at the 5′ end, and the reverse primer (5′ GGCTCGAGCCCA-GRARGGAMAGGCAGGWGCT 3′) contained an *Xho*I site at the 5′ end. PCR products were recovered using a QIAquick gel extraction kit. PCR products were sequenced either directly or after subcloning into the mammalian expression vector pcDNA3.1(+) (Invitrogen).

*Sequence Analysis.* At least two clones were sequenced in the vector in both directions for each cDNA. All mutations found in a cDNA were confirmed by sequencing PCR or cloned products from the corresponding genomic DNA in both directions. Sequencing was performed by the Sequencing Facility at the Albert Einstein College of Medicine.

*Site-Directed Mutagenesis.* Site-directed mutagenesis was performed on cDNA clones in a pcDNA3.1(+) vector using the QuikChange site-directed mutagenesis kit and *pfu* DNA polymerase. The primers used to correct D212N in Lec1A.2C were forward (5′ GCTGTGGTAGTGGAGGACGATCTG-GAGG 3′) and reverse (5′ CCTCCAGGTCCTCCACTAC-CACAGC 3′) spanning nucleotides 619−646 of the CHO *Mgat-1* gene coding sequence. The primers used for correcting R303W in Lec1A.3E and Lec1A.5J were forward (5′ CAGCGGAAGGGGCGGGCCTGTATTC 3′) and reverse (5′ GAATACAGGCCCGCCCCTTCCGCTG 3′) spanning nucleotides 895−920 of the CHO *Mgat-1* gene coding sequence. Cycling conditions were as follows: 95 °C for 2 min and 18 cycles at 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 12 min. Corrected sequences were confirmed by sequencing both strands.

*Transfection of cDNA Constructs into Lec1 Cells.* cDNAs cloned into the pcDNA3.1(+) vector were transfected into Lec1 cells using the Polybrene method as described previously (*13*). Briefly, $10^5$ cells were seeded in a 100 mm tissue culture dish 1 day before transfection. For transfection, 2 $\mu$g of DNA was mixed with 3 mL of α medium containing 10% fetal bovine serum and 30 $\mu$g (3 $\mu$L) of Polybrene and added to the cells. After 6 h at 37 °C in a $CO_2$ incubator, cells were treated with 30% dimethyl sulfoxide (DMSO) in α medium for 4 min, and α medium containing 10% fetal bovine serum was added. Stable transfectants were selected for resistance to G418 (1 mg/mL active weight) added the next day. Single colonies were expanded and tested for their ability to bind L-PHA and wheat germ agglutinin, and for GlcNAc-TI activity.

*Flow Cytometry Analysis.* Cells ($2-4 \times 10^5$) were washed once with 3 mL of a cold PBS/2% BSA mixture and resuspended in 100 $\mu$L of a PBS/2% BSA mixture with a final concentration of 10 $\mu$g/mL WGA-FITC or L-PHA-FITC on ice for 30 min. After being washed once with 3 mL of a cold PBS/2% BSA mixture, the cells were resuspended in PBS and subjected to flow cytometry using a Becton-Dickenson (San Jose, CA) immunocytometry system.

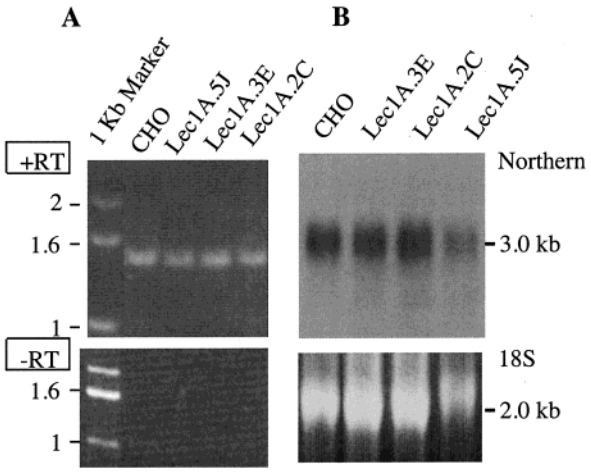


FIGURE 1: *Mgat-1* transcripts in Lec1A.2C, Lec1A.3E, and Lec1A.5J cells. (A) RT-PCR analysis of CHO and Lec1A *Mgat-1* coding regions was performed as described in Experimental Procedures. The lower panel shows that no products were obtained if reverse transcriptase was omitted from the reaction mixtures. (B) Northern blot analysis of *Mgat-1* transcripts from CHO and Lec1A cells. Total RNA (10 $\mu$g) from CHO, Lec1A.2C, Lec1A.3E, and Lec1A.5J cells was fractionated on a 1.2% agarose−formaldehyde gel, transferred to membrane, and hybridized to a *Mgat-1* coding region probe of 575 bp as described in Experimental Procedures. The lower panel shows ethidium bromide staining of 18S RNA.

*GlcNAc-TI Assay.* Postnuclear cell extracts were prepared in 1.5% Nonidet P-40 in saline in the presence of protease inhibitors. GlcNAc-TI activity was assayed under the CHO condition as follows. Fifty to one hundred micrograms of cell extract was incubated at 37 °C for 2 h in the presence of 0.83 mM $Man_5GlcNAc_2Asn$, 62.5 mM 2-(*N*-morpholino)-ethanesulfonate (MES) (pH 6.25), 25 mM $MnCl_2$, and 0.75 mM UDP-[$^3$H]GlcNAc (∼6000 cpm/nmol) in a final volume of 40 $\mu$L. For the Lec1A assay condition, reactions were carried out in 62.5 mM MES buffer (pH 7.5), 4.6 mM $Man_5GlcNAc_2Asn$, and 15 mM UDP-[$^3$H]GlcNAc (1300 cpm/nmol) with all other reagents unchanged. Reactions were stopped by adding 0.5 mL of Con A buffer (0.1 M sodium acetate, 1.0 M NaCl, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM $MnCl_2$, and 0.02% sodium azide); the solutions were spun in a microfuge for 5 min, and the supernatant was fractionated on a 1 mL column of Con A-Sepharose. The product of GlcNAc-TI action, $GlcNAcMan_5GlcNAc_2Asn$, binds to Con A, while UDP-[$^3$H]GlcNAc and [$^3$H]GlcNAc do not. After being washed, bound products were eluted with 200 mM α-methyl mannoside in Con A buffer. Activities were determined as the difference in the extent of $^3$H incorporation into Con A-bound product between samples with and without the exogenous acceptor $Man_5GlcNAc_2Asn$.

## RESULTS

*Mgat-1 Transcripts in Lec1A.2C, Lec1A.3E, and Lec1A.5J Cells.* The independent Lec1A mutants, Lec1A.2C and Lec1A.5J, were isolated from Pro$^-$5 CHO cells, and Lec1A.3E was isolated from Gat$^-$ 2 CHO cells (*9*). Northern analysis showed that all three mutants express *Mgat-1* transcripts of a similar size and in amounts similar to those of parent CHO cells (Figure 1). To obtain GlcNAc-TI cDNAs, RT-PCR was performed with total RNA prepared from CHO, Lec1A.2C,

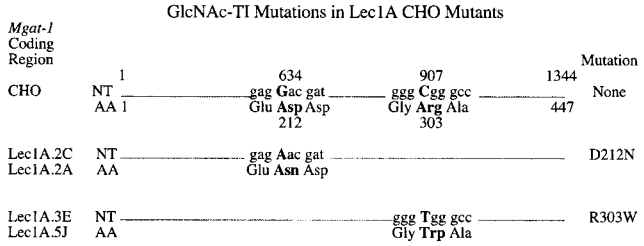


FIGURE 2: GlcNAc-TI mutations in Lec1A CHO mutants. RT-PCR products encoding the *Mgat-1* gene coding region were generated from total RNA of CHO and Lec1A mutants and sequenced. Nucleotide sequence from residue 1 to 1344 of the *Mgat-1* gene coding region is designated by a line, and nucleotides (NT) and amino acids (AA) identified as Lec1A mutations are in bold. The sequence obtained from CHO is identical to that reported by Puthalakath et al. (GenBank accession number U65791) except for one difference; C1340 in U65791 is A1340 in CHO sequences. A1340 is likely to be correct as it is conserved in a mutant CHO *Mgat-1* sequence (U69792), in Pro$^{-}$5 CHO cells, and in the four Lec1A sequences.

Lec1A.3E, and Lec1A.5J cells. Primers were designed from mouse, human, rat, and rabbit 5′ and 3′ untranslated sequences of the *Mgat-1* gene, and a 1.4 kb product that includes the entire coding region of *Mgat-1* was amplified. RT-PCR products from CHO and each Lec1A mutant were similar in size, and PCR performed without RT gave no product, excluding the possibility of genomic DNA contamination (Figure 1).

*Identification of Point Mutations in Lec1A.2C, Lec1A.5J, and Lec1A.3E.* The 1.4 kb RT-PCR products from CHO, Lec1A.2C, Lec1A.3E, and Lec1A.5J were cloned into the pcDNA3.1(+) vector and sequenced. The sequences that were obtained were identical to that reported previously by Puthalakath et al. (*14*) (GenBank accession number U65791) except for one difference; C1340 in the U65791 sequence is A1340 in the Pro$^{-}$5 CHO sequence. This changes the last amino acid of U65791 from Thr447 to Asn447. Asn as the last amino acid is likely to be correct as it is conserved in the mouse, rat, and human sequences, a mutant CHO *Mgat-1* sequence (U69792) (*14*), and the four Lec1A sequences reported here.

The coding sequence of *Mgat-1* cDNAs from Lec1A.2C, Lec1A.5J, and Lec1A.3E was sequenced and compared to the parental *Mgat-1* sequence. Only one nucleotide difference was found between Lec1A.2C and CHO; A634 in the Lec1A.2C sequence is G634 in the CHO sequence. This change results in an amino acid substitution at position 212 in the lumenal domain of GlcNAc-TI from Asp in CHO to Asn in Lec1A.2C (D212N; Figure 2). To rule out the possibility that this change was introduced during RT-PCR or cloning, the same PCR was performed on genomic DNA from Lec1A.2C cells. PCR products were sequenced directly, and cloned products were also sequenced. The genomic DNA sequence data confirmed the single-nucleotide change G634A in Lec1A.2C. In addition, the same nucleotide difference (G634A) was found in genomic DNA from a separate clone (Lec1A.2A) derived from the same colony as Lec1A.2C.

Lec1A.3E and Lec1A.5J are two independent isolates and might be expected to have different *Mgat-1* gene mutations. Lec1A.5J was isolated from Pro$^{-}$5 cells, and Lec1A.3E was isolated from Gat$^{-}$ 2 CHO cells (*9*). Interestingly, however, they were both found to have the same point mutation (C907 to T907). This changes amino acid 303 from Arg in CHO cells to Trp in each Lec1A mutant (R303W; Figure 2). This same point mutation and no other changes were found in *Mgat-1* gene coding sequences determined from PCR products obtained from genomic DNA of both mutants.

Table 2: GlcNAc-TI Activity of Lec1 Transfectants Expressing a CHO, Lec1A.2C, or Corrected *Mgat-1* cDNA[a]

| | specific activity [nmol h$^{-1}$ (mg of protein)$^{-1}$] | |
|---|---|---|
| cell extract | CHO condition | Lec1A condition |
| controls | | |
| CHO | 6.3 | 26.0 |
| Lec1A.2C | 0.16 | 39.0 |
| Lec1 transfectants | | |
| Tf.CHO.1 | 64.0 | 169.0 |
| Tf.CHO.2 | 31.9 | ND |
| Tf.1A.2C.1 | 0.46 | 8.0 |
| Tf.1A.2C.2 | 0.18 | 8.2 |
| Tf.1A.2C.3 | 0.8 | 46.0 |
| REV.2C.1 | 45.0 | ND |
| REV.2C.2 | 44.0 | ND |
| REV.2C.3 | 38.9 | ND |
| REV.2C.4 | 14.2 | ND |
| REV.2C.5 | 27.7 | ND |

[a] Cell extracts were assayed for GlcNAc-TI activity under CHO or Lec1A assay conditions as described in Experimental Procedures. Transfectants expressing a CHO or Lec1A *Mgat-1* cDNA are designated Tf. Transfectants expressing a cDNA corrected by site-directed mutagenesis is designated REV. ND, not determined.

*Lec1A Phenotypes Arise from Point Mutations in the Mgat-1 Gene Coding Sequence.* To prove that the point mutations identified above are responsible for the Lec1A glycosylation phenotype, site-directed mutagenesis was performed. Mutant and CHO *Mgat-1* coding regions were cloned into the pcDNA3.1(+) vector and transfected into Lec1 CHO cells, which have no GlcNAc-TI activity (*4*, *10*, *15*). Stable transfectants were assayed for binding of the lectins L-PHA and WGA by flow cytometry analysis. Lec1 cells expressing a transfected Lec1A.2C *Mgat-1* cDNA had a similar mean fluorescence index (MFI) for L-PHA and for WGA to Lec1A control cells (Figure 3). The MFI of parental CHO cells was ∼500 for WGA and ∼140 for L-PHA. Whereas Lec1A cells and Lec1 cells expressing a Lec1A *Mgat-1* cDNA bound less WGA and less L-PHA than CHO cells (MFI for each lectin ∼50% lower), Lec1 cells bound even less of both lectins (MFI ∼20% of CHO for WGA and only background binding for L-PHA; Figures 3 and 4).

Following site-directed mutagenesis, a corrected Lec1A.2C *Mgat-1* cDNA construct expressed in Lec1 cells restored the binding of L-PHA and of WGA to the same level as in CHO cells (Figure 4), consistent with an improved ability to make hybrid and complex *N*-glycans on cell surface glycoproteins. The GlcNAc-TI activities of transfectants were also measured under both the CHO and Lec1A conditions described in Experimental Procedures. When CHO *Mgat-1* cDNA was overexpressed in Lec1 cells, the GlcNAc-TI activity in cell extracts was 5−10-fold greater than that in extracts from CHO cells transfected with the vector alone (Table 2). However, despite this high GlcNAc-TI activity, the lectin binding ability of Lec1 cells expressing a CHO *Mgat-1* cDNA was not significantly increased compared to that of CHO cells.

Lec1 transfectants expressing a Lec1A.2C cDNA had undetectable GlcNAc-TI activity under the CHO assay condition (similar to Lec1A controls), while they had

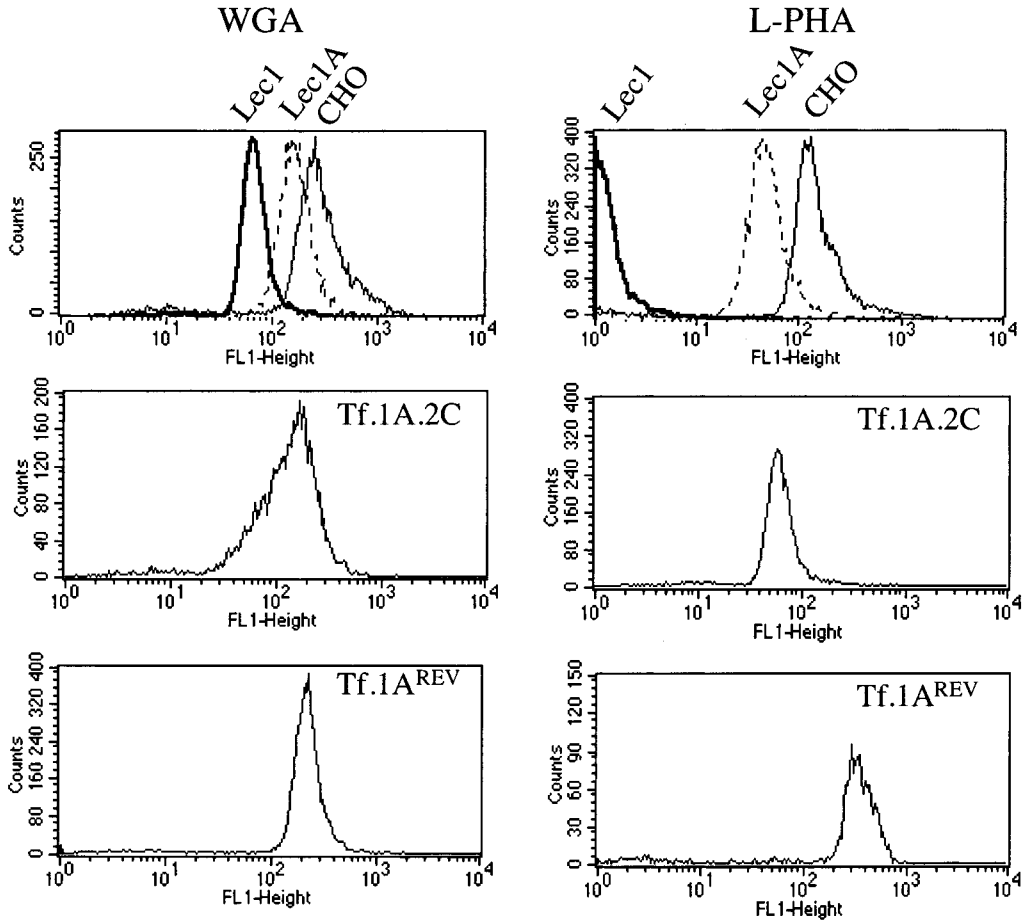


FIGURE 3: Flow cytometry of L-PHA and WGA binding to CHO, Lec1, Lec1A, and transfectants. Cells were incubated with FITC-conjugated L-PHA or FITC-WGA at 4 °C for 30 min, washed, and analyzed by flow cytometry as described in Experimental Procedures. The top panel shows the flow cytometry profile of CHO, Lec1, and Lec1A cells. The middle panel shows binding to Lec1 cells stably expressing a *Mgat-1* cDNA from Lec1A.2C cells (Tf.1A.2C). The bottom panel shows binding to Lec1 cells stably expressing a corrected Lec1A.2C *Mgat-1* cDNA (Tf.1A$^{REV}$).

GlcNAc-TI activity as high as that of CHO cells under the Lec1A assay condition. Therefore, the Lec1A.2C cDNA converted Lec1 cells to a Lec1A phenotype. The revertants that overexpressed corrected Lec1A.2C *Mgat-1* cDNA constructs also had high (3−8-fold) GlcNAc-TI activity compared to CHO cells (Table 2). The combined data show that the G634A mutation is responsible for the Lec1A.2C phenotype.

The only change in the *Mgat-1* gene coding sequence of Lec1A.3E and Lec1A.5J is C907T, converting arginine 303 to tryptophan. Site-directed mutagenesis to correct *Mgat-1* cDNAs from Lec1A.3E and Lec1A.5J was performed. Lec1 transfectants expressing uncorrected Lec1A.3E or Lec1A.5J cDNAs were found to have no detectable GlcNAc-TI activity under the CHO assay condition, but activity comparable to that of CHO under the Lec1A assay condition. Uncorrected Lec1A.3E or Lec1A.5J *Mgat-1* cDNAs converted Lec1 cells to the Lec1A phenotype. Lec1 cells overexpressing corrected Lec1A.3E or Lec1A.5J *Mgat-1* cDNAs had the same ability as CHO cells to bind WGA and L-PHA (Figure 4), and they had 4−7-fold more GlcNAc-TI activity than CHO cells (Table 3).

*Asp212 and Arg303 Contribute Significantly to Wild-Type GlcNAc-TI Activity.* Asp at position 212 and Arg at position 303 are conserved in GlcNAc-TI from all known species, including humans (*1*, *17*), rats (*18*), rabbits (*2*), mice (*19*, *20*), golden hamsters (*16*), *Drosophila* (GenBank accession number AF251495), and *Caenorhabditis elegans* (*21*). These two amino acids are even conserved in two recently cloned plant genes that encode GlcNAc-TI in *Nicotiana tabacum* (*22*) and *Arabidopsis thaliana* (*23*). The conservation from plants through animals suggests that Asp212 and Arg303 contribute significantly to wild-type GlcNAc-TI activity. To assess the role played by these residues, we have examined the X-ray crystal structure of the $Mn^{2+}$−UDP-GlcNAc complex of the catalytic fragment of rabbit GlcNAc-TI (*12*). The amino acid sequence of this fragment is 94% identical to the same region of Chinese hamster GlcNAc-TI.

D212 is the central residue of a consensus sequence element (DXD motif) loosely conserved among a number of glycosyltransferase families (*24*−*26*). Although the central residue itself is not well conserved, all GlcNAc-TIs possess aspartic acid at this position. The motif ($E_{211}DD_{213}$ in all GlcNAc-TIs) has been implicated in metal binding and catalysis and, as shown by the rabbit GlcNAc-TI structure, forms the first three residues ($i$ to $i + 2$) of a canonical type I $\beta$-turn (Figure 5A). Moreover, the side chains of the motif are found to make key interactions with the bound UDP-GlcNAc and $Mn^{2+}$ ion. With regard to D212, the direct hydrogen bond with the ribose moiety of the nucleotide sugar and the salt bridge with R117 (also absolutely conserved among GlcNAc-TIs) are most notable (Figure 5A). The latter

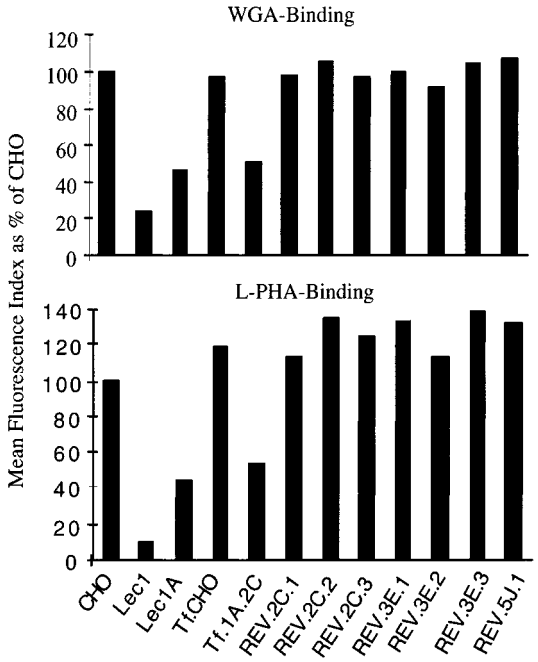


FIGURE 4: Correction of Lec1A mutations by site-directed mutagenesis. Control cells (CHO, Lec1, and Lec1A.2C), Lec1 cells expressing a *Mgat-1* cDNA from CHO (Tf.CHO), or Lec1A.2C (Tf.1A.2C). Lec1A *Mgat-1* cDNAs corrected by site-directed mutagenesis were from Lec1A.2C cDNA (REV.2C.1−3), Lec1A.3E cDNA (REV.3E.1−3), or Lec1A.5J cDNA (REV.5J) expressed in Lec1 cells. Cells were incubated with FITC-conjugated L-PHA or WGA before fluorescence cytometry analysis. The MFI was determined from each profile and compared to that of CHO cells (100%). The CHO MFI was ∼500 for WGA and ∼140 for L-PHA.

Table 3: GlcNAc-TI Activity of Lec1 Transfectants Expressing CHO, Lec1A.3E, Lec1A.5J, or Corrected *Mgat-1* cDNA[a]

| | specific activity [nmol $h^{-1}$ (mg of protein)$^{-1}$] | |
|---|---|---|
| cell extract | CHO condition | Lec1A condition |
| controls | | |
| CHO | 7.0 | 9.5 |
| Lec1A.3E | 0.46 | 3.3 |
| Lec1A.5J | 0.21 | 10.3 |
| Lec1 transfectants | | |
| Tf.1A.3E | 0.26 | 12.2 |
| Tf.1A.5J | 0.08 | 5.8 |
| REV.3E.1 | 42.8 | ND |
| REV.3E.2 | 20.4 | ND |
| REV.5J.1 | 23.3 | ND |

[a] Cell extracts were assayed for GlcNAc-TI activity under CHO or Lec1A assay conditions as described in Experimental Procedures. Transfectants expressing a CHO or Lec1A *Mgat-1* cDNA are designated Tf. Transfectants expressing a cDNA corrected by site-directed mutagenesis are designated REV. ND, not determined.

interaction is likely of considerable importance as R117 not only forms a salt bridge with the α-phosphate of UDP-GlcNAc but also, through interaction with the main chain carbonyl oxygen atom of K319, serves to assist in the stabilization of a 13-residue loop (residues 318−330) which is structured upon UDP-GlcNAc binding (*12*). The loss of the negative charge on the side chain of the D212N mutant would conceivably perturb the position of both D212 and R117. Because of the direct interactions that these residues make with the nucleotide sugar, this perturbation presumably accounts for the observed increase in $K_m$ for UDP-GlcNAc (*10*). Since R117 also participates in the UDP-GlcNAc-dependent loop structuring required for acceptor binding, perturbation of these residues could also account for the observed increase in $K_m$ for the oligosaccharide acceptor (*10*).

Although R303 (absolutely conserved among all GlcNAc-TIs) is quite distant from the active site of GlcNAc-TI, it appears to play a role in the stabilization of the α5−α6 helix−loop−helix motif, a structural element involved in substrate binding and catalysis. As shown in Figure 5B, R303 makes a salt bridge interaction with E280 and a hydrogen bond with the main chain carbonyl oxygen atom of residue K301. These interactions form the core of a network of hydrogen bonds, involving R303, K301, E280, Q299, K284, and a water molecule, which serves to bridge α5 and α6 (residues 280−303 are identical in rabbit and hamster GlcNAc-TI). Disruption of the salt bridge in the R303W mutant would be expected to perturb and/or destabilize the conformation of the helix−turn−helix motif and, as a result, affect the position of W290 and D291, residues located at the tip of the loop. The former makes a hydrogen bond to O4 of the GlcNAc moiety of the nucleotide sugar, while the latter serves as the base in GlcNAc-TI-mediated catalysis (Figure 5B and ref *12*). Since F289 is also involved in UDP-GlcNAc binding, and several of the residues on this helix−turn−helix motif are expected to be important in acceptor binding (*12*), increased $K_m$s for both the donor and acceptor would be consistent with this mutation (*10*).

## DISCUSSION

Point mutations that inactivate mammalian GlcNAc-TI catalytic activity, Cys123 → Arg and Gly320 → Asp, have previously been described (*14*, *16*). In this paper, we identify two mutations that alter the kinetic properties of GlcNAc-TI and give rise to Lec1A CHO glycosylation mutants. Lec1A mutants were initially characterized as lectin-resistant CHO cells that are less resistant to plant lectins than Lec1 CHO cells but exhibit a qualitatively similar pattern of lectin resistance (*9*). They belong to the same genetic complementation group as Lec1 and thus are affected in the same gene. Biochemical analysis revealed that GlcNAc-TI from Lec1A cells has altered kinetic properties (see Table 1) consistent with a missense mutation. Herein, we identify two point mutations from three independent Lec1A isolates that severely compromise GlcNAc-TI activity. In Lec1A.2C and Lec1A.2A, the G634A mutation replaces Asp at residue 212 with Asn; in Lec1A.3E and Lec1A.5J, the C907T mutation replaces Arg303 with Trp, confirming the prediction that Lec1A CHO mutants arise from a structural alteration in GlcNAc-TI (*10*).

The Lec1A.2C and Lec1A.2A mutants are subclones of a single colony, and as expected, they have the same mutation (G634A). However, Lec1A.3E was isolated from $Gat^{-}2$ CHO cells, while Lec1A.5J was isolated from $Pro^{-}5$ CHO cells and might be expected to have arisen from different mutations. Nevertheless, the same point mutation (C907T) is present in both isolates. The possibility of contamination was excluded by confirming the presence of the identical point mutation in genomic DNA from original stocks of the Lec1A.3E and Lec1A.5J cell lines. This site may be a hot spot for mutation in the *Mgat-1* gene. In each case, correction of the point mutation by site-directed mutagenesis resulted in a cDNA that conferred wild-type glycosylation properties

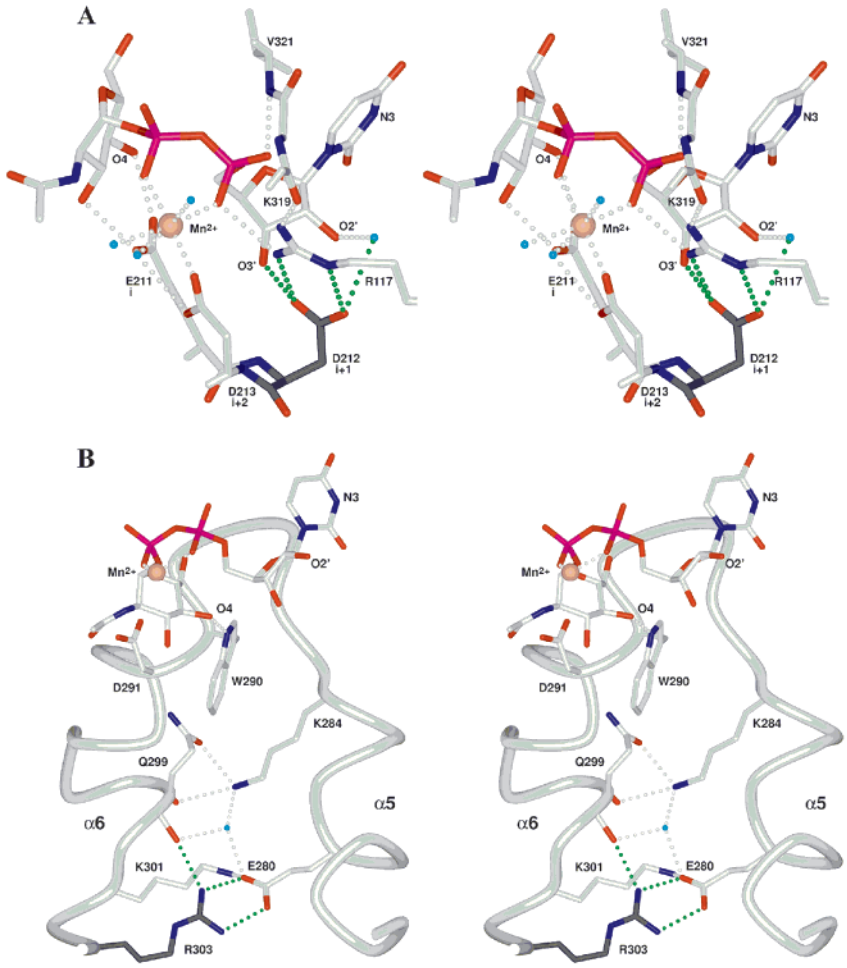


FIGURE 5: Stereo backbone-and-stick models of rabbit GlcNAc-TI showing residues D212 and R303 and selected interactions. Carbon, oxygen, nitrogen, sulfur, and phosphorus are white, red, blue, yellow, and purple, respectively; water molecules are cyan, and the $Mn^{2+}$ ion is salmon. N3, O2′/O3′, and O4 label atoms on the uracil, ribose, and GlcNAc moieties of the nucleotide sugar, respectively. Hydrogen bonds and $Mn^{2+}$ ion interactions are shown as small dots connecting the interacting atoms. (A) Residue D212 is shown in dark gray. Direct hydrogen bonds to D212 are shown as green dots. The letters and numerals, *i* to *i* + 2, label the first three residues of a canonical type I $\beta$-turn. (B) Selected salt bridge and hydrogen bonding interactions within the α5−α6 helix−turn−helix structural element. Residue R303 is shown in dark gray, and its direct interactions are shown as green dots.

and high levels of GlcNAc-TI activity to Lec1 cells. Interestingly, despite the high levels of overexpression, *Mgat-1* cDNAs carrying a *lec1A* mutation generated only a Lec1A phenotype, and GlcNAc-TI activity remained low in cell-free extracts assayed under the CHO assay condition. Therefore, weak GlcNAc-TI activity was not dosage-dependent and could not be increased by overexpression. It is also of interest that overexpression of wild-type *Mgat-1* cDNAs in Lec1 cells, resulting in GlcNAc-TI specific activities of up to 169 nmol $h^{-1}$ (mg of protein)$^{-1}$ in vitro (Table 2), did not induce a level of L-PHA or WGA binding that was significantly higher than that in CHO cells in which GlcNAc-TI activities were 30−40-fold lower. This suggests that there is a limit to the glycoprotein substrates on which GlcNAc-TI acts in CHO cells.

The positions of the Lec1A mutations in GlcNAc-TI identify two conserved residues that are important for optimal GlcNAc-TI catalytic activity (Figure 5A,B). Exactly how residue N212 or W303 alters the structure of GlcNAc-TI must await crystallization of the mutant proteins. The characterization of these mutants has, however, revealed an efficient approach to determining structure−function relationships for GlcNAc-TI. A lectin selection strategy that is specific for the Lec1 phenotype has been devised (*27*), and identifying Lec1A mutants, or even selecting directly for them, should not be difficult since the *Mgat-1* gene mutates at a high frequency (~$10^{-3}$ after mutagenesis). By sequencing a large panel of Lec1A mutants, we can identify many missense mutations that weaken GlcNAc-TI. These mutations in conserved GlcNAc-TI residues may be used to generate mutant mice with hypomorphic *Mgat-1* alleles that will reveal functions of complex or hybrid *N*-glycans in development.

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