Glycosylation Defect in Lec1 Chinese Hamster Ovary Mutant Is Due to a Point Mutation in N-Acetylglucosaminyltransferase I Gene*

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The Lec1 Chinese hamster ovary (CHO) mutant is a leuco-phytohemagglutinin resistant cell line unable to synthesize complex and hybrid N-glycans due to the lack of N-acetylglucosaminyltransferase I (GnTI) activity. Here we have identified the lec1 mutation. Using specific antibodies to GnTI we demonstrate that Lec1 cells synthesize an inactive GnTI protein identical in size to the wild-type CHO enzyme. We have cloned and sequenced the gene coding GnTI from parental CHO and Lec1 mutant cells. Comparison of GnTI sequences detected three mutations within the luminal domain of Lec1 GnTI, each resulting in an amino acid substitution. The effect of each mutation on enzyme activity was analyzed by site-directed mutagenesis of wild-type rabbit GnTI and transient expression in COS cells. One of the three mutations (Cys123 → Arg123) resulted in complete loss of activity, whereas the other two mutations had no apparent effect on enzyme activity. This conclusion was confirmed by expression of GnTI mutants in the GnTI null background of Saccharomyces cerevisiae. Both Lec1 GnTI and the GnTI mutant (Cys123 → Arg123) are correctly localized to the Golgi apparatus, indicating that the inactive GnTI molecules are sufficiently well folded for efficient transport from the endoplasmic reticulum. These results demonstrate that the lec1 mutation is a point mutation and that Cys123 is a critical residue for GnTI activity.

Cytoxic lectins have been used to isolate a variety of lectin-resistant cell lines, many of which exhibit altered cell-surface carbohydrates as a result of defects in oligosaccharide biosynthesis (1, 2). A number of glycosylation mutants have been characterized which have alterations in a specific glycosylation activity, however, in most cases it is unknown whether the mutation affects a structural or a regulatory gene (3). Glycosylation defective mutants have been instrumental in the elucidation of glycosylation pathways and in the identification of endogenous oligosaccharide substrates (4, 5). They have also been successfully employed for the cloning of glycosyltransferase genes by complementation (6) and as tools for the study of intra-Golgi protein transport in cell-free transport assays (7, 8). However, the genetic basis of the mutant phenotypes have not been identified.

UDP-GlcNac:α-d-mannoside-β1,2-N-acetylglucosaminyltransferase I (GnTI)1 (EC 2.4.1.101) is a type II integral membrane protein, localized to medial-Golgi cisternae, which catalyzes the first step in the conversion of high mannose N-glycans into complex and hybrid structures (4, 9). Complex N-glycans are critical for the viability of the developing embryo, as mice lacking a functional GnTI gene die before birth (10, 11). However, complex N-glycans are not essential for viability of cells cultured in vitro as a number of mutants have been isolated which lack GnTI activity (12–16). One such mutant is the leuco-phytohemagglutinin (L-PHA) resistant Lecl Chinese hamster ovary (CHO) cell (13). Due to the lack of GnTI, Lec1 cells are unable to synthesize complex and hybrid N-glycans and accumulate oligosaccharides bearing Man3GlcN3C-Asn structures (17). The block in complex N-glycan synthesis accounts for the reduction in L-PHA binding sites. Lec1 cells had been shown to produce a GnTI mRNA transcript of similar size as parental CHO cells (6), indicating that the lec1 mutation may be a point mutation or small deletion. However, the nature of the mutation responsible for the loss of GnTI enzyme activity has not been identified and, furthermore, it is not known if Lec1 cells synthesize a GnTI protein.

We have previously described the production of polyclonal antibodies to rabbit GnTI which cross-react with hamster GnTI (18). Here we used these antibodies to identify a Golgi-localized GnTI protein in Lec1 cells which is identical in size to the wild-type hamster GnTI. Furthermore, we have identified the lec1 mutation as a point mutation in the GnTI gene and demonstrated that Cys123 is a critical residue for GnTI activity. The results reported here illustrate the potential of glycosyltransferase defective cell mutants to identify critical residues associated with enzyme activity.

EXPERIMENTAL PROCEDURES

Cell Culture—Cells were maintained in exponential growth as monolayers in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 0.1% (w/v) streptomycin (complete Dulbecco’s modified Eagle’s medium). Parental CHO (Pro-5) (CRL 1781) and the Lec1 CHO mutant (CRL 1735) (12) were obtained from the American Type Culture Collection and grown in Dulbecco’s modified Eagle’s medium supplemented with 40 μg/ml proline.

Antibodies—Mouse and sheep anti-rabbit GnTI antibodies were produced as described (18). The sheep anti-GnTI antibodies were affinity-purified and used in Western blots.

1 The abbreviations used are: GnTI, UDP-GlcNac:α-d-mannoside-β1,2-N-acetylglucosaminyltransferase I; CHO, Chinese hamster ovary; L-PHA, leuco-phytohemagglutinin; PCR, polymerase chain reaction; FITC, fluorescein isothiocyanate; Manα, octyl, Manα1–6[Manα1–3]Manβ-ocyt; PBS, phosphate-buffered saline; PIPES, 1,4-piperazinediethanesulfonic acid.
purified on a glutathione S-transferase-GnTI fusion protein matrix, as described (19).

Transfections—COS-7 cell monolayers were transfected essentially as described previously (18), using the mammalian expression vector pCI-neo (Promega), and immunofluorescence was performed 48 h after transfection. Lecl cells were transfected with pSVT-rabbit GnTI (d and h) and incubated with mouse anti-rabbit GnTI antibodies followed by FITC-conjugated anti-mouse Ig (a-d) or stained directly with FITC-conjugated L-PHA (e-h). Bar, 15 μm (a-d); 37 μm (e-h).

Metabolic Labeling of Cultured Cells and Immunoprecipitation—Subconfluent cell monolayers were metabolically labeled with [35S]methionine/cysteine (Express [35S]protein labeling mixture, DuPont NEN), extracted, and immunoprecipitated with affinity-purified sheep anti-rabbit GnTI antibodies as described (18). Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions followed by fluorography.

Immunoblotting—Cell extracts were diluted in reducing SDS electrophoresis sample buffer and separated by SDS-polyacrylamide gel electrophoresis. The separated proteins were electrophoretically transferred to nitrocellulose and immunoblotting carried out by the method previously described (20), using 5% non-fat milk in PBS containing 0.05% Tween 20 as the blocking solution.

Immunofluorescence—Cells, grown either on 12-well glass microscope slides or on coverslips, were fixed, permeabilized, and stained with mouse anti-rabbit GnTI antibodies, as described previously (18). Alternatively, cells were fixed in 3% paraformaldehyde for 15 min, free aldehyde groups quenched in 50 mM NH4Cl in PBS, and the cells permeabilized with 50 μM PIPES, pH 6.8, containing 0.05% saponin, 5 mM EGTA, and 1 mM MgCl2 for 5 min. Monolayers were washed with 0.05% saponin/PBS, and incubated with 5% fetal calf serum in 0.05% saponin/PBS (fetal calf serum/saponin/PBS) for 60 min to reduce non-specific binding. Monolayers were then incubated in affinity-purified sheep anti-rabbit GnTI antibody (3 μg/ml), diluted in fetal calf serum saponin/PBS, for 1 h and washed in saponin/PBS, and incubated with fluorescein isothiocyanate (FITC)-conjugated donkey anti-sheep/goat immunoglobulin (Silenus, Australia) for 30 min. After washing in saponin/PBS, slides were mounted in moviol and examined with an Axiophot microscope (Zeiss).

GnTI Enzyme Assay—Cells were extracted in 50 mM Tris maleate buffer, pH 6.9, containing 1% Triton X-100, 150 mM NaCl, 50 μM/ml phenylmethylsulfonyl fluoride, and the protease inhibitor mixture, Complete 334 (Boehringer Mannheim), for 30 min on ice, and the nuclei removed by centrifugation at 10,000 × g for 5 min. The protein concentration of the extracts were determined by BCA protein assay, and aliquots were assayed for GnTI activity with either 0.1–0.5 mM Manα1–3Manβ1–4GlcNAc or 2 mM ovalbumin as an acceptor, as described (21). After incubation at 37°C for 60 min, radioactive oligosaccharides were collected (16) or radioactive Manα1–4GlcNAc products purified by chromatography on Sep-Pak C18 reverse-phase cartridges (22).

Isolation and Sequence Analysis of Parental CHO and Lecl GnTI Clones—Genomic and cDNA clones of GnTI were isolated from parental CHO and Lecl cells. Genomic clones of GnTI were obtained by PCR amplification of genomic DNA using primers based on the DNA sequence of human, rat, mouse, and rabbit GnTI and are as follows: 5′-CCGAATTCGAGGTCTGAAAGAGGAG3′ and 5′-GGCAATTCTAA(T/A)TTCA(A/G)CTAGGATCATA-3′. The primers include an EcoRI cloning site. Oligonucleotide primers were used at 0.2 pmol/μl in the presence of 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin, 0.2 μM of each of the four deoxynucleotide triphosphates, 2.5 units of Taq polymerase (Life Technologies, Inc/BRL), and 0.016 units of cloned Pfu DNA polymerase (Stratagene). Cycle conditions were as follows: 1 cycle of 5 min at 94°C, 1.5 min at 55°C and 2 min at 72°C; 30 cycles of 1.5 min at 94°C, 1.5 min at 55°C and 2 min at 72°C; final cycle of 1.5 min at 94°C, 1.5 min at 55°C, and 7 min at 72°C. A 1.5-kilobase PCR product, confirmed as the GnTI gene by Southern blot analysis using [32P]-labeled rabbit GnTI cDNA as a probe, was isolated from agarose gels, digested with EcoRI, and subcloned into the EcoRI site of pBluescript K5+ (Stratagene).

cDNA clones were also isolated by PCR amplification of cDNA generated from RNA. RNA isolation and reverse transcription were performed essentially as described (23). Briefly, 3–4 μg of total RNA was reverse transcribed using 5 units of avian myeloblastosis virus reverse transcriptase and 50 μM dNTPs in a 20 μl reaction for 60 min at 37°C. The cDNA product was used as template for PCR using the above primers to generate a 1.3-kilobase product which was cloned into the EcoRI site of pBluescript K5+. The nucleotide sequence of cDNA clones was determined by the dyeodeoxy method, using a DEAZA reagent sequencing kit (Promega) in conjunction with T7 DNA polymerase (Pharmacia) or by automated sequencing using a Prism dye-deoxy terminator cycle kit and a 373A DNA sequencer (Applied Biosystems).

Site-directed Mutagenesis—cDNA encoding rabbit GnTI (24) was cloned into the EcoRI site of M13mp18, and oligonucleotide-mediated mutagenesis using single stranded template was carried out as described (25) using the following oligonucleotides: C123-R, 5′-GGCTGTGCACACCGCGGCGCGAGCG-3′; Q339-R, 5′-AGGGTAGAACGCGGGCTGGTT-TCAGC-3′; K401-R, 5′-ACACCCAGGGCGCGGCGAAAGC-3′. Point mutants were identified by hybridization using [32P]-labeled oligonucleotides and mutations were confirmed by DNA sequence analysis.

Yeast Transformation and Analysis—Rabbit GnTI cDNA and mutant sequences were cloned into the BamHI site of the 2-micron based episomal vector, pRS28 kindly supplied by Dr. R. Devenish, Department of Biochemistry, Monash University) by blunt end ligation. Saccharomyces cerevisiae BJ 5462 (MATa ura3–52 trpl leu2Δ 1 his3Δ2 200 pep4::HIS3 prf1Δ 1.6R can1 GAL) were transformed with recombinant plasmids by electroporation and transformants selected by leucine prototrophy as described (25).

The expression of heterologous genes was induced by the addition of galactose to the medium as follows: S. cerevisiae transformants were grown overnight in 15 ml of YEPD medium (0.5% yeast extract, 1% peptone, and 1% dextrose) at 30°C with vigorous shaking. Cells were harvested, washed in sterile water, and resuspended in 0.5% yeast extract, 1% peptone, and 2% galactose and incubated for a further 24 h. Cells were then harvested by centrifugation at 2000 × g, washed once with 50 mM Tris maleate buffer, pH 6.9, containing 150 mM NaCl and re suspended in 500 μl of the same buffer containing 50 μg/ml phenylmethylsulfonyl fluoride and the protease inhibitor mixture, Complete 334 (Boehringer Mannheim). Aliquots (200 μl) were mixed with an equal volume of acid-washed glass beads (average diameter 500 μm, Sigma) and lysed using a homemade bead-beater (six pulses of 30 s each with 30 s cooling on ice between pulses). Following lysis, Tris X 100 was added to a final concentration of 1% and the lysate incubated on ice for 45 min. Cell extracts were centrifuged in a microcentrifuge at 4°C and supernatants assayed immediately for GnTI activity.

RESULTS

Lecl Cells Express a Protein Which Reacts with Anti-GnTI Antibodies—We have previously generated a polyclonal antibody to recombinant rabbit GnTI that reacts with the native protein. It was noted that these antibodies reacted not only with rabbit GnTI but also with hamster GnTI, whereas no detectable cross-reactivity was observed with GnTI from other species tested (18). These antibodies were therefore used to determine if GnTI protein is present in Lecl cfoO cells, a glycosylation mutant with no detectable GnTI activity.

Parental CHO and Lecl cells were analyzed by indirect immunofluorescence using anti-GnTI antibodies. Staining was observed in the perinuclear region in both parental CHO and
Lec1 cells, a staining pattern characteristic of Golgi localization (Fig. 1, a and b). This indicates that Lec1 cells express a GnTI protein which is correctly localized to the Golgi apparatus.

To determine the size of the GnTI protein in Lec1 cells, GnTI was immunoprecipitated from metabolically-labeled parental CHO and Lec1 cells using affinity-purified sheep anti-GnTI antibodies (Fig. 2). The immunoreactive GnTI component of 48 kDa synthesized by Lec1 cells is identical in size to that synthesized by the parental cell line.

Expression of Rabbit GnTI in Lec1 Cells—Lec1 cells were transfected with rabbit GnTI cDNA, as described under “Experimental Procedures.” Rabbit GnTI-transfected Lec1 cells showed strong juxtanuclear staining with the anti-GnTI antibodies (Fig. 1d); the staining pattern indicates that rabbit GnTI and Lec1 GnTI are co-localized. Rabbit GnTI-transfected Lec1 cells had substantial levels of GnTI activity and regained the ability of the wild-type parent CHO cell lines to bind to FITC-conjugated L-PHA (Fig. 1, e-h) and wheat germ agglutinin (not shown). This indicates that the inability of Lec1 cells to synthesize complex N-glycans is due solely to the lack of GnTI enzyme activity, confirming the results of Kumar and Stanley (26).

Identification of Point Mutations in Lec1—The above results indicate that the genetic lesion in Lec1 cells is likely to be a point mutation(s) within the coding region of GnTI resulting in amino acid substitution(s). To identify the putative mutation(s), the GnTI genes from the Lec1 mutant, and parental CHO cells, were cloned and sequenced. The coding region of the GnTI gene resides in a single exon in human and mouse (27–29) and, as it seemed likely that the gene structure of hamster GnTI would be similar, genomic clones of parental and Lec1 CHO cells were cloned and sequenced. The coding region of the GnTI gene resides in a single exon in human and mouse (27–29), and it seemed likely that the gene structure of hamster GnTI would be similar, genomic clones of parental and Lec1 CHO cells were isolated by PCR using redundant 5' and 3' primers as described under “Experimental Procedures.”

The Lec1 GnTI gene has an open reading frame encoding a polypeptide of 447 amino acids with a predicted molecular mass of 51.7 kDa, consistent with the estimated size of Lec1 GnTI by SDS-polyacrylamide gel electrophoresis (Fig. 2). Comparison between the parental CHO and Lec1 GnTI DNA sequences identified three point mutations in the Lec1 sequence. These point mutations are located in the luminal (catalytic) domain of the enzyme and all result in amino acid substitutions (Fig. 3). The mutations are Cys$^{122}$ → Arg$^{122}$ (C123R), Glu$^{339}$ → Arg$^{339}$ (Q339R), and Lys$^{401}$ → Arg$^{401}$ (K401R) (Fig. 3). In addition to genomic clones, cDNA clones were also isolated from Lec1 and parental CHO cell lines by reverse transcriptase-PCR. The GnTI genomic and cDNA clones from each cell line had identical sequences, indicating the sequence differences between parental and Lec1 GnTI were not due to PCR introduced errors.

Wild-type GnTI is very efficiently localized to the Golgi apparatus at very high expression levels (18). To determine if the Lec1 GnTI behaves in a similar manner, the intracellular localization of Lec1 GnTI was analyzed in COS-7 cells, a transient mammalian expression system which characteristically displays high expression levels. Using sheep anti-GnTI antibodies, Lec1 GnTI was found predominantly localized to a juxtanuclear location in transfected COS cells (Fig. 4). A very similar staining pattern was also obtained for wild-type rabbit GnTI (Fig. 4), a pattern typical of the Golgi apparatus of COS cells. Therefore, even at high expression levels, Lec1 GnTI protein is effectively localized to the Golgi apparatus.

To determine whether one or more of the identified Lec1 point mutations were responsible for the loss of GnTI activity, each mutation was analyzed individually by site-directed mutagenesis of rabbit GnTI. Three rabbit GnTI mutants were generated, namely C123R, Q339R, and K401R. These mutant proteins were all localized to the Golgi apparatus in transfected COS cells, with a staining pattern similar to wild-type rabbit GnTI and to Lec1 GnTI (Fig. 4), indicating that these mutations have no effect on transport and Golgi localization.

COS cells were transfected with each of the mutants, and with wild-type rabbit GnTI, and the level of heterologous gene expression determined by immunoblotting using anti-rabbit GnTI antibodies. Similar amounts of rabbit GnTI protein were detected in extracts from each transfection (Fig. 5). The total GnTI activity of detergent extracts of transfected COS cells was determined using ovalbumin as acceptor. Wild-type GnTI transfected COS cells showed a 3-fold increase in GnTI activity compared with untransfected COS cells (Table I). COS cells transfected with GnTI mutants Q339R and K401R also showed an approximately 3-fold increase in GnTI activity over endogenous levels, indicating that these two mutants were as active as the wild-type sequence. On the other hand, COS cells transfected with the mutant GnTI C123R showed no increase in GnTI activity over endogenous levels (Table I), even though the amount of mutant GnTI protein in the transfected cells was similar to wild-type rabbit GnTI. In four separate transfection experiments, GnTI C123R transfected COS cells had the same GnTI activity as untransfected COS cells.

The above results indicate that the GnTI C123R mutant is

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**Fig. 2.** Immunoprecipitation of metabolically labeled CHO and Lec1 cells with anti-GnTI antibodies. Parental CHO and Lec1 cells were labeled with [35S]methionine/cysteine, extracted in 1% Triton X-100 and immunoprecipitated with affinity-purified sheep anti-rabbit GnTI antibodies conjugated L-PHA (Fig. 1, e-h) and wheat germ agglutinin (not shown). This indicates that the inability of Lec1 cells to synthesize complex N-glycans is due solely to the lack of GnTI enzyme activity, confirming the results of Kumar and Stanley (26).

**Fig. 3.** DNA sequence comparison of parental CHO and Lec1 GnTI identifies three point mutations in Lec1 GnTI. The positions of the three point mutations in the nucleotide sequence of Lec1 GnTI are shown, together with the predicted sequence of the encoded polypeptide. The mutations are in **bold** and **boxed**.
correctly localized to the Golgi apparatus but is inactive. Nonetheless, it is possible that the mutant protein with this single amino acid substitution has a low level of activity which may be difficult to detect over the background level of endogenous GnTI in COS cells. To exclude this possibility, wild-type and mutant GnTI cDNAs were expressed in transformed S. cerevisiae cells and had a similar activity as wild-type GnTI (data not shown), again indicating that these two amino acid substitutions do not affect enzyme activity. Extracts of the recombinant yeast expressing the C123R GnTI mutant, on the other hand, showed no GnTI activity, confirming that the C123R mutant yeast expressing the C123R GnTI mutant, on the other hand, showed no GnTI activity, confirming that the C123R mutant GnTI was active (Fig. 7), with a specific activity of 0.24 nmol/mg/h.

**FIG. 6. Detection of recombinant GnTI in extracts of transformed S. cerevisiae by immunoblotting.** The expression of GnTI cDNAs in transformed S. cerevisiae cells was induced by the addition of galactose to the medium, as described under "Experimental Procedures." Untransformed (UT) cells and cells transformed with wild-type rabbit GnTI (wt GnTI) and C123R, Q339R, or K401R GnTI mutants were lysed in buffer containing 1% Triton X-100 and aliquots containing equal amounts of protein were analyzed by immunoblotting as described in the legend of Fig. 5. The arrow indicates the position of the 45-kDa GnTI protein.

**FIG. 5. Immunoblotting of extracts of COS cells transfected with GnTI constructs.** COS cells, either untransformed (UT) or transfected with pCI-neo containing the cDNA coding wild-type rabbit GnTI (wt GnTI), Lec1 GnTI (Lec1), or rabbit GnTI mutants C123R, Q339R, or K401R, as indicated. 7 h after transfection, cells were fixed in 3% paraformaldehyde, permeabilized in 0.05% saponin, and stained using affinity-purified sheep anti-rabbit GnTI antibodies followed by FITC-conjugated secondary antibody. Untransformed COS-7 cells, or COS-7 cells transfected with either wild-type rabbit GnTI or GnTI mutants, were extracted in buffer containing 1% Triton X-100 and assayed for GnTI activity using ovalbumin as acceptor.

**TABLE I**

| Cell transfecion | Acceptor | [14C]GlcNAc product | GnTI activity (dpm nmol/mg/h) |
|------------------|----------|----------------------|-------------------------------|
| Untransfected    | +        | 6,778                | 2.7                           |
| COS-7            | −        | 834                  |                               |
| Wild-type rabbit | +        | 16,761               | 7.3                           |
| GnTI             | −        | 692                  |                               |
| GnTI C123-R      | +        | 6,485                | 2.7                           |
| GnTI Q339-R      | +        | 16,446               | 7.1                           |
| GnTI K401-R      | +        | 18,665               | 8.2                           |

**DISCUSSION**

The Lec1 CHO mutant was first described 20 years ago (12). Although Lec1 cells have been used extensively in biochemical, genetic, and functional studies of mammalian glycoproteins, the genetic basis of this mutant, or any other lectin-resistant cell line with a defect in glycoprotein biosynthesis, has not been identified. Here we have shown that the Lec1 cells produce an inactive GnTI of the same size as active wild-type CHO enzyme which is correctly localized to the Golgi apparatus. Further-
more, we have identified the lec1 mutation as a single point mutation in the open reading frame of the GnTI gene resulting in substitution of Cys\textsubscript{123} with Arg\textsubscript{123}. Our results indicate that Cys\textsubscript{123} is a critical residue for GnTI activity. The demonstration that the lec1 mutation affects the primary structure of GnTI illustrates the potential of lectin-resistant mutants for the identification of key residues involved in glycosyltransferase activity.

Sequence comparison of the GnTI gene from parental CHO and Lec1 identified three point mutations in the catalytic domain of Lec1 GnTI. Although all three mutations result in amino acid substitutions, two of these mutations, namely Gln\textsubscript{339} and Arg\textsubscript{401}, have no apparent affect on catalytic activity. This result is not surprising in view of the fact that Gln\textsubscript{339} and Arg\textsubscript{401} are not conserved in GnTI sequences across species. In fact, Arg residues are found at each of these positions, for example, Arg\textsubscript{339} is found in chicken GnTI (30, 31) and Arg\textsubscript{401} in frog GnTI.\textsuperscript{2} In contrast, Cys\textsubscript{123} is conserved in human (6), chicken (30, 31), frog,\textsuperscript{2} mouse (28), rabbit (24), and rat (32) GnTI sequences. (In the Caenorhabditis elegans GnTI homologue (GenBank accession number Z46381), Cys\textsubscript{123} residue is substituted with a histidine, however, it is not clear whether this C. elegans GnTI homologue encodes an active enzyme.)

Previous studies have demonstrated that β1,4-galactosyltransferase and α2,6-sialyltransferase can be expressed in S. cerevisiae as active enzymes (33, 34) and here we demonstrate that wild-type GnTI can also be expressed as an active enzyme. The GnTI C123R mutant showed no activity in the GnTI null background of yeast, directly demonstrating that a Cys\textsubscript{123} to Arg\textsubscript{123} substitution results in loss of GnTI activity. There are five cysteine residues in the luminal domain of GnTI and, as the disulfide bonds have not yet been mapped it is unknown whether Cys\textsubscript{123} forms a disulfide bond with one of the other Cys residues or exists as a free sulfhydryl group.

A possible explanation for the lack of enzyme activity of Lec1 GnTI is that the C123R mutation causes a drastic change in the folding of the protein. However, this does not appear to be the case as the Lec1 GnTI protein is localized to the Golgi in CHO cells and also in transient COS cells, where the level of Lec1 GnTI expression is greatly increased. Furthermore, the C123R mutant protein is efficiently localized to the Golgi apparatus of transected COS cells. As misfolded proteins accumulate in the endoplasmic reticulum and are blocked for forward transport, this result indicates that inactive GnTI is sufficiently well folded for normal exit from the endoplasmic reticulum. This data suggests that the C123R mutation involves a subtle structural alteration. For example, Cys\textsuperscript{139} may be a critical residue for the enzyme active site or it may be involved in a disulfide bond which holds the enzyme in a precise conformation that is required for catalytic activity. It is interesting to note that three of the five cysteine residues in β1,4-galactosyltransferase are important in enzymatic activity; Cys\textsuperscript{129} and Cys\textsuperscript{264} are likely to form a disulfide bond and Cys\textsuperscript{445} has been implicated in the binding of UDP-galactose (35).

Very little is known about the nature of the active site of the N-acetylgalcosaminyltransferases. The characterization of Lec1 GnTI now clearly provides a very useful mutant protein for structural comparison with the native active GnTI and the identification of substrate binding sites. Analysis of other GnTI defective cell mutants, such as Rix\textsuperscript{114}14 baby hamster kidney cell mutant (16), is also likely to be also instructive.

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