**Medial Golgi but Not Late Golgi Glycosyltransferases Exist as High Molecular Weight Complexes**

**ROLE OF LUMINAL DOMAIN IN COMPLEX FORMATION AND LOCALIZATION**

(Received for publication, August 17, 1999, and in revised form, December 23, 1999)

Andrew S. Opat‡, Fiona Houghton, and Paul A. Gleeson§

From the Department of Pathology and Immunology, Monash University Medical School, Melbourne, Victoria 3181, Australia

To investigate the organization of Golgi glycosyltransferases and their mechanism of localization, we have compared the properties of a number of medial and late acting Golgi enzymes. The **medial** Golgi enzymes, N-acetylgalcosaminyltransferase I and II (GnTI and GnTII) required high salt for solubilization and migrated as high molecular weight complexes on sucrose density gradients. In contrast, the late acting Golgi enzymes, β1,4-galactosyltransferase and α1,2-fucosyltransferase, were readily solubilized in low salt and migrated as monomers/dimers by sucrose density gradient centrifugation. Analysis of membrane-bound GnTI chimeras indicates that the formation of high molecular weight complexes does not require the transmembrane domain and cytoplasmic tail sequences of GnTI. Furthermore, a soluble form of GnTI, containing the stem region and catalytic domain, accumulated in the Golgi prior to secretion, in contrast to β1,4-galactosyltransferase. Soluble GnTI, which also associated with high molecular weight complexes, was comparable with membrane-bound GnTI in its ability to glycosylate newly synthesized glycoproteins in vivo. Mutation of charged residues within the stem region of GnTI, known to be important for “kin recognition”, had no effect on the efficiency of Golgi localization, the inclusion into high molecular weight complexes, nor functional activity in vivo. The differences in behavior between the **medial** and late acting Golgi enzymes may contribute to their differential localization and their ability to glycosylate efficiently in the correct Golgi subcompartment.

The Golgi apparatus is a highly complex and dynamic organelle consisting of a series of flattened cisternae associated with numerous vesicles and membrane tubules. The Golgi is central to the secretory pathway, since it plays important roles in the maturation and sorting of newly synthesized secretory and membrane proteins and also in the recycling of proteins and lipids to the endoplasmic reticulum (1, 2). In addition, the Golgi has a fundamental role in the biosynthesis of the glycan chains of glycoproteins, proteoglycans, and glycolipids in eukaryotic cells. Glycosylation occurs in a highly regulated manner as the newly synthesized molecules move from the cis to the **trans** side of the Golgi stack (3). The synthesis of carbohydrate chains of glycoconjugates in mammalian cells is likely to require more than 200 different glycosyltransferase enzymes distributed throughout the Golgi stack (4–6). To understand the control of glycan biosynthesis in vivo requires an appreciation of the organization of glycosyltransferases within the membranes of the individual compartments.

All Golgi glycosyltransferases cloned to date are **N** out of **C** in (type II) membrane proteins containing a short N-terminal cytoplasmic domain, a single hydrophobic membrane-spanning domain, and a large carboxyl-terminal catalytic domain situated in the lumen of the Golgi apparatus (4–6). The catalytic domain is linked to the transmembrane domain by a loosely defined “stem” region that may play a role in positioning the catalytic domain away from the lipid bilayer, facilitating access to the substrates. A number of the enzymes involved in the synthesis of complex N-glycans have been precisely localized and show distinct but overlapping distributions that are consistent with their order in the glycoprotein biosynthetic pathway (7).

The mechanisms that determine the steady state distribution of Golgi glycosyltransferases are not well understood. Analysis of glycosyltransferase chimeras from transfected eukaryotic cells has demonstrated that the transmembrane domain of glycosyltransferases plays a critical role in Golgi localization; in addition, in a number of cases contributions from the luminal domain and cytoplasmic tail have also been detected, suggesting that there may be multiple signals involved in the specific localization of these Golgi enzymes (for reviews, see Refs. 8 and 9). Although these studies have been informative, in most cases it remains unclear whether the Golgi-localized glycosyltransferases chimeras are targeted correctly so that they can function appropriately in vivo. A number of models for the Golgi retention of glycosyltransferases have been proposed including oligomerization, lipid-mediated sorting, and intra-Golgi retrograde transport (10, 11). These models are not mutually exclusive, although they need to be considered in the light of the current evidence for cisternal maturation (12, 13) and the ability of green fluorescent protein (GFP)§-tagged resident Golgi glycosyltransferases to diffuse rapidly and freely in Golgi membranes (14), which indicates that Golgi targeting

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* The abbreviations used are: GFP, green fluorescent protein; GnTI, β1,2-N-acetylgalcosaminyltransferase I (EC 2.4.1.101); sGnTI, soluble GnTI; GnTIII, β1,2-N-acetylgalcosaminyltransferase II (EC 2.4.1.143); Gal-TI, β1,4-galactosyltransferase (EC 2.4.1.38); HT, blood group H α1,2-fucosyltransferase (EC 2.4.1.69); CHO, Chinese hamster ovary; FCS, fetal calf serum; L-PHA, leucyl-phthoysagglutinin; TR, transferrin receptor; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; ER, endoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis; FITC, fluorescein isothiocyanate; bp, base pair; MES, 4-morpholineethanesulfonic acid.
GnTImyc—A full-length GnTI cDNA was amplified by PCR from human GnTI genomic DNA (19), with primers incorporating a c-myc epitope (18). The sense primer (5'-CGGAGTTCGTCGTTAGGAAAGCG-3') included an EcoRI restriction site, and the antisense primer (5'-TCGAGCTCGAGGATCTGAGCTGTC-3') included a BamHI restriction site and the c-myc epitope (underlined). The 1391-bp PCR product was cloned into the EcoRI/BamHI sites of pBluescript KS+ (Stratagene), and the EcoRI/BamHI fragment of this construct was cloned into EcoRI/XbaI-digested pCI-neo (Promega).

GnTImyc—Full-length GnTI cDNA was amplified by PCR from full-length GnTI cDNA (20) with the sense primer 5'-TCGCCATGAGGTGGCAGCTTAAAGC-3' (c-myc epitope underlined). The resulting 1398-bp PCR product was cloned into the EcoRI/BamHI sites of pBluescript KS+, and the EcoRI/XbaI fragment of this construct cloned into EcoRI/XbaI-digested pCI-neo (Promega).

NSS-GnTImyc—A full-length HT cDNA was amplified by PCR from human HT cDNA (21) using the sense primer 5'-CCGAGTTCGTCGTTAGGAAAGCG-3' and antisense primer 5'-TCGCCATGAGGTGGCAGCTTAAAGC-3' (c-myc epitope underlined). The resulting 1398-bp PCR product was cloned into the EcoRI/BamHI sites of pBluescript KS+, and the EcoRI/XbaI fragment from this plasmid was cloned into EcoRI/XbaI-digested pCI-neo (Promega).

NSS-TfR/GnTImyc—The TfR/GnTI hybrid, TTG, was excised from pSVT-3 (7) and subcloned into the EcoRI/XbaI-digested pCI-neo (Promega).

**Cell Culture and Antibodies**

Cells were maintained at 37 °C as monolayers in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 40 µg/ml proline, 100 units/ml penicillin, and 0.1% streptomycin. Transfected cells were maintained in the above medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 40 µg/ml proline, 100 units/ml penicillin, and 0.1% streptomycin. Transfected cells were maintained in the above medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 40 µg/ml proline, 100 units/ml penicillin, and 0.1% streptomycin. Transfected cells were maintained in the above medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 40 µg/ml proline, 100 units/ml penicillin, and 0.1% streptomycin. Transfected cells were maintained in the above medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 40 µg/ml proline, 100 units/ml penicillin, and 0.1% streptomycin. Transfected cells were maintained in the above medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 40 µg/ml proline, 100 units/ml penicillin, and 0.1% streptomycin. Transfected cells were maintained in the above medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 40 µg/ml proline, 100 units/ml penicillin, and 0.1% streptomycin. Transfected cells were maintained in the above medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 40 µg/ml proline, 100 units/ml penicillin, and 0.1% streptomycin. Transfected cells were maintained in the above medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 40 µg/ml proline, 100 units/ml penicillin, and 0.1% streptomycin. Transfected cells were maintained in the above medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 40 µg/ml proline, 100 units/ml penicillin, and 0.1% streptomycin. Transfected cells were maintained in the above medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 40 µg/ml proline, 100 units/ml penicillin, and 0.1% streptomycin. Transfected cells were maintained in the above medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 40 µg/ml proline, 100 units/ml penicillin, and 0.1% streptomycin.
three amino acid substitutions (underlined) and also eliminates a SacII site by conservative substitution. The flanking sense primer was based on the signal/anchor sequence of the human transferrin receptor (TfR) (5′-CCGAAATTCACCTAGTGTC-3′), and the antisense flanking primer was 5′-CAGTGGACGTGTACAATAGCCCAAGTAGC-3′. The 1239-bp fragment was then blunt end-ligated into the Tisense primer used for full-length GnTImyc as above. The resulting 9 pbSHT (24), which positions the GnTI luminal sequence immediately 3′ of the hemagglutinin signal peptide sequence. A similar approach was used to construct mutated NSS-sGnTImyc using NSS-TfR/GnTImyc of the hemagglutinin signal peptide sequence. A similar approach was used to construct mutated NSS-sGnTImyc using NSS-TfR/GnTImyc of the hemagglutinin signal peptide sequence.

\[ \text{PCR product was cloned into pEGFP-C1 (CLONTECH, Palo Alto, CA), which had been linearized by digestion with NheI, and the 3′-recycled ends were filled in with the Klenow fragment of DNA polymerase I.} \]

\[ \text{sGnTImyc—A truncated GnTImyc cDNA, lacking the first 44 amino acids of GnTII, was amplified by PCR from human GnTII genomic DNA using the sense primer 5′-GGACGATTTGAATAGCCTCCACCCGGGA-3′ and the antisense primer used for full-length GnTImyc as above. The resulting 1239-bp fragment was then blunt end-ligated into the Smal site of pSHT (24), which positions the GnTI luminal sequence immediately 3′ of the hemagglutinin signal peptide sequence. A similar approach was used to construct mutated NSS-sGnTImyc using NSS-TfR/GnTImyc cDNA as template.} \]

\[ \text{Gal-T1 constructs in pSVTgpt and pSHT were as described (17).} \]

\[ \text{CHO or CHO Lec1 cells (25) were transfected using Superfect (Qiagen) or FuGene-6 (Roche Molecular Biochemicals) in accordance with the manufacturer’s instructions. Constructs in pSVTgpt or pSHT were co-transfected with pCI-neo. Constructs in pCI-neo were transfected alone. Cells were selected in 800 \mu M G418 and screened by indirect immunofluorescence. Chloroform was used for transfection of populations by limiting dilution.} \]

\[ \text{CHO or CHO Lec1 cells expressing sGnTImyc were incubated for 6 h in serum-free Dulbecco’s modified Eagle’s medium. Following incubation, the culture medium was collected and centrifuged for 15 min at 15,000 g to remove cellular debris. An aliquot (600 \mu l) of supernatant was layered on a 4–20% sucrose gradient containing 50 mM MES (pH 6.5), 1% Triton X-100, and 100 mM NaCl. The gradient was centrifuged and analyzed as above.} \]

\[ \text{Triton X-114 Phase Separation} \]

\[ \text{Triton X-114 extraction was performed as described by Bordier (31). Cells were extracted in 0.5% Triton X-114 containing 250 mM NaCl and Complete protease inhibitors for 45 min on ice, and the extracts were centrifuged for 15 min at 15,000 \times g.} \]

\[ \text{Cells were harvested by scraping in PBS, extracted in MNT containing both 100 and 250 mM NaCl for 1 h on ice, and then centrifuged for 15 min at 15,000 \times g. The supernatant was then further centrifuged for 1 h at 100,000 \times g.} \]

\[ \text{Cells were harvested by scraping in PBS and then lysed in MNT (50 mM MES (pH 6.5) containing 1% Triton X-100, Complete protease inhibitors (Roche Molecular Biochemicals), and 0–250 mM NaCl for 1 h on ice, and the extracts were centrifuged for 15 min at 15,000 \times g. The supernatant was further centrifuged for 1 h at 100,000 \times g.} \]

\[ \text{The low and high speed pellets and the final supernatant were made to the same volume in SDS sample buffer and analyzed by immunoblotting as described below.} \]

\[ \text{Cells were harvested by scraping in PBS and then lysed in MNT (50 mM MES (pH 6.5) containing 1% Triton X-100, Complete protease inhibitors (Roche Molecular Biochemicals), and 0–250 mM NaCl for 1 h on ice, and the extracts were centrifuged for 15 min at 15,000 \times g. The supernatant was further centrifuged for 1 h at 100,000 \times g.} \]

\[ \text{The low and high speed pellets and the final supernatant were made to the same volume in SDS sample buffer and analyzed by immunoblotting as described below.} \]

\[ \text{Glycosyltransferase Assays} \]

\[ \text{Clonal CHO Lec1 cells transfected with GnTImyc were harvested by scraping in Tris-buffered saline and extracted in MNT containing between 50 and 250 mM NaCl for 1 h at 4 °C. Extracts were either left on ice or centrifuged at 100,000 \times g for 1 h at 4 °C.} \]

\[ \text{GnTI activity was assayed as described by Vischer and Hughes (27) using 200 \mu g of extract protein in 100-\mu l reactions, with ovalbumin as acceptor. GnTII was assayed as described (28) using the synthetic acceptor Manα1–6[GalNACβ1,2Manα1–3]Manβ1–octyl (29).} \]
HT is responsible for the synthesis of blood group H determi-
nant (21, 37). For detection of bovine Gal-T1 in transfected
cells, a rabbit polyclonal antibody was used (17). To be able to
detect GnTI, GnTII, and HT in transfected cells, we have
tagged these three glycosyltransferases with a myc epitope at
the C terminus. All three myc-tagged enzymes were shown to
be active in transfected cells (data not shown); therefore, the
addition of the myc epitope does not appear to perturb the
folding of these enzymes.

Stable transfected CHO cell clones expressing each of the
four glycosyltransferases were obtained. For GnTI constructs
throughout this study, CHO Lec1 cells were transfected, since
this Lec mutant is devoid of GnTI activity (25, 38) and therefore
provided an assay to assess the in vivo function of the GnTI
constructs. Staining of GnTI-, GnTII-, or HT-transfected cells
with the myc-specific monoclonal antibody, 9E10, showed in
each case a perinuclear staining pattern, characteristic of the
Golgi apparatus (Fig. 2). In each case, the staining pattern
remained unchanged after a 4-h treatment with cycloheximide.
Furthermore, treatment of transfected cells with brefeldin A
resulted in a diffuse cytoplasmic staining pattern typical of the
ER (not shown). Together, these results confirm that these
three myc-tagged enzymes are actively retained in the Golgi
apparatus. Staining of bovine Gal-T1 transfected CHO cells
with affinity-purified anti-bovine Gal-T1 antibodies also showed
a perinuclear staining pattern similar to the other
enzymes (Fig. 2).

**Medial Golgi glycosyltransferases form high molecular
weight complexes**—To determine if glycosyltransferases may be
associated with complexes in Golgi membranes of mammalian
cells, Triton X-100 extracts containing a range of NaCl concen-
trations were analyzed by immunoblotting. Extracts were cen-
trifuged at 15,000 \(\times g\) to remove any insoluble material, and
then the supernatant was collected and centrifuged at
100,000 \(\times g\) to determine if high molecular weight complexes
were present. After extraction in 50 mM NaCl/Triton X-100, the
majority of myc-tagged GnTI protein was found in the insoluble
pellet of a low speed centrifugation (15,000 \(\times g\)) (Fig. 3A). The
minor fraction of GnTI found in the supernatant of a low speed
centrifugation was pelleted at high speed (100,000 \(\times g\)), indi-
cating that this soluble protein existed as a high molecular
weight complex. Myc-tagged GnTI was detected as two compo-
ents by immunoblotting with molecular masses of 49.5 and 53
kDa. This heterogeneity is due to glycosylation, since human
GnTI has previously been shown to be O-glycosylated (39), and
treatment of cell extracts with neuraminidase prior to immu-
noblotting resulted in collapse of the higher molecular weight
to a similar size as the lower species (not shown). Extraction of
myc-tagged GnTI-transfected CHO Lec1 cells with Triton
X-100 containing 100 mM NaCl resulted in greater than 50% of
GnTI protein remaining in the supernatant after a 15,000 \(\times g\)
centrifugation. At this salt concentration, approximately equiv-
alent amounts of GnTI were found in the low speed pellet, the
high speed pellet, and in the final supernatant. The amount of
material remaining in the 100,000 \(\times g\) supernatant increased
with increasing salt concentration, and at 250 mM NaCl the
majority of GnTI protein was detected in the 100,000 \(\times g\)
supernatant (Fig. 3A). Slusarewicz et al. (40) have reported
that GnTI from rat liver Golgi membrane preparations also
requires 100 mM NaCl/Triton X-100 for solubilization (i.e.
15,000 \(\times g\) supernatant), indicating that GnTI in transfected
CHO cell behaves in a similar fashion to endogenous GnTI.

It is possible that the pelleted material in the low salt ex-
tractions represented aggregated inactive enzyme. To investi-
gate whether this was the case, we assayed extracts for GnTI
activity before and after 100,000 \(\times g\) centrifugation. Triton
X-100 extracts containing 50, 150, and 250 mM NaCl prior to
centrifugation, all showed similar GnTI activity (Fig. 3B). Cen-
trifugation of the 50 mM NaCl extract at 100,000 \(\times g\) resulted
in the loss of the majority of GnTI activity from the superna-
tant (Fig. 3B), demonstrating that the pelleted GnTI repre-
sents active enzyme. In contrast, and consistent with the im-
munoblotting data, only a small proportion of GnTI activity
was removed from Triton X-100 extracts containing 250 mM
NaCl after a 100,000 \(\times g\) centrifugation.

Myc-tagged GnTI was detected as a single component of
\(\sim 50\) kDa by immunoblotting with monoclonal antibody 9E10.
GnTI behaved in a similar manner to GnTI after extraction
with Triton X-100 (Fig. 3A). At 50 mM NaCl, all the GnTI
protein was detected in the pellets of the 15,000 \(\times g\) and
100,000 \(\times g\) centrifugations. As for GnTI, with increasing salt
concentration an increasing percentage of the GnTI protein
was recovered in the final 100,000 \(\times g\) supernatant.

In stark contrast to the behavior of the two medial Golgi
enzymes, the two late acting Galg enzymes were detected
almost exclusively in the 100,000 \(\times g\) supernatant of low (50
mM) NaCl extractions. Increasing the salt concentrations had
little effect on the distribution of either Gal-T1 or HT proteins
in the fractions. By immunoblotting, myc-tagged HT was de-
tected as two components with molecular masses of 47 and 52
kDa. The difference in size between the two components is due
to heterogeneous

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**Fig. 2. Intracellular localization of glycosyltransferases in sta-
ble CHO cell clones.** CHO Lec1 cells stably expressing either GnTImyc, HTmyc, or Gal-T1 were
fixed in paraformaldehyde, permeabilized with Triton X-100, and
stained with monoclonal antibody 9E10 or anti-Gal-T1 antibodies as
described under “Experimental Procedures,” and confocal images were
collected. Bar, 20 \(\mu m\).
does not bear O- or N-glycans) (22) showed that rabbit GnTImyc exhibited a similar distribution in the low speed pellet, high speed pellet, and supernatant to that of human GnTImyc (not shown).

A pulse-chase study was carried out to determine the kinetics of high molecular weight complex formation. CHO Lec1 cells expressing myc-tagged GnTI were pulse-labeled for 15 min with [35S]Cys/Met and then chased for either 0 or 2 h. Cell extracts in 100 mM NaCl/Triton X-100 were centrifuged at 15,000 \( \times g \) to remove insoluble material. Half the supernatant (total soluble fraction) was then centrifuged at 100,000 \( \times g \). The low speed pellet (L), the high speed pellet (H), and the final supernatant (S) were adjusted to the same volume with SDS sample buffer and analyzed by immunoblotting. Myc-tagged GnTII, GnTII, and HT were detected with monoclonal antibody 9E10, and Gal-T1 was detected with affinity-purified rabbit anti-bovine Gal-T1 antibodies. Sizes indicate relative molecular mass compared with Bio-Rad low molecular weight protein standards.

Analysis of Glycosyltransferase Complexes by Sucrose Gradients—To further investigate the size and heterogeneity of the glycosyltransferase complexes, Triton X-100 extracts were analyzed on sucrose gradients. Cell extractions were performed using NaCl at 100 mM or higher, since the majority of the glycosyltransferase proteins were soluble under these conditions (i.e. remained in the 15,000 \( \times g \) supernatant). The 15,000 \( \times g \) supernatants from Triton X-100 extractions were separated on 5–20% sucrose gradients, and fractions were collected and immunoblotted for glycosyltransferase protein with either 9E10 monoclonal antibody or anti-bovine Gal-T1 antibody. Myc-tagged GnTImyc was detected in two distinct fractions throughout the sucrose gradient (Fig. 4A). Under the same conditions, myc-tagged GnTII was also resolved into two fractions, one with a molecular mass of \(-150 \text{ kDa}\) and a second at the bottom of the gradient. In contrast, both the late acting Golgi enzymes migrated as a single peak in the sucrose gradient (Fig. 4A).
Medial Golgi Enzymes and Complex Formation

The Formation of High Molecular Weight Complexes Is Independent of GnTI Transmembrane and Cytoplasmic Sequences—To determine whether the sequences of the transmembrane domain and/or short cytoplasmic tail of GnTI were required for the inclusion of GnTI into high molecular weight complexes, a construct was generated containing the myc-tagged luminal domain of GnTI fused with the transmembrane domain and truncated cytoplasmic tail of the transferrin receptor (TfR/GnTI(myc)) (Fig. 1). The truncated cytoplasmic tail of this construct excludes the internalization motif of the native transferrin receptor molecule. CHO Lec1 clones expressing myc-tagged TfR/GnTI were obtained, and the chimeric GnTI protein was found to be predominantly localized to the Golgi apparatus in transfected cells, as assessed by indirect immunofluorescence (Fig. 5A). As for full-length GnTI, TfR/GnTI was relocated to an ER-type staining pattern after treatment with brefeldin A and remained in the Golgi region after a 4-h treatment with cycloheximide (not shown), indicating that TfR/GnTI is efficiently Golgi-localized. Analysis of TfR/GnTI-transfected CHO Lec1 cells by flow cytometry revealed low levels of the myc-tagged chimeric protein on the cell surface, whereas myc-tagged full-length GnTI was absent from the cell surface of transfected Lec1 cells (not shown). The presence of low levels of TfR/GnTI at the cell surface indicates that the chimeric protein is not as efficiently Golgi-localized as full-length GnTI, suggesting that both the transmembrane and luminal domains play a role in Golgi retention, consistent with our previous studies of the nontagged TfR/GnTI chimera (22). To determine whether the TfR/GnTI chimeric protein is functionally active within Golgi membranes of CHO Lec1 cells, the synthesis of complex N-glycans was assessed by cell surface binding of the lectin L-PHA, which binds to galactose-containing complex N-glycans (42). Flow cytometric analysis showed that, whereas untransfected CHO Lec1 cells did not bind FITC-L-PHA, TfR/GnTI transfected CHO Lec1 cells bound FITC-L-PHA at a similar level to parental CHO cells (Fig. 5B). Therefore, the TfR/GnTI chimeric protein is functionally active within Golgi membranes.

Analysis of Triton X-100 extracts of TfR/GnTI-expressing CHO cells demonstrated that myc-tagged TfR/GnTI behaved in a similar manner to full-length GnTI (Fig. 7A). In the absence of NaCl or in the presence of 50 mM NaCl, all of the TfR/GnTI protein was recovered in pellets from the 15,000 × g or 100,000 × g centrifugations, indicating that TfR/GnTI was extracted as high molecular weight complexes. In extracts containing 100 mM NaCl, all of the fusion protein sedimented at 100,000 × g. However, in 250 mM NaCl extracts, a substantial proportion of the TfR/GnTI protein was found in the final supernatant (Fig. 7A). Results from sucrose gradients of Triton X-100 extracts of TfR/GnTI-transfected cells were in agreement with these findings (Fig. 8). Almost all of the myc-tagged TfR/GnTI in 100 mM NaCl extracts was recovered at the bottom of the gradient, whereas in 250 mM NaCl extracts TfR/GnTI was distributed throughout the gradient as well as at the bottom of the gradient, indicative of heterogeneous high molecular weight complexes (Fig. 8). Thus, formation of complexes containing GnTI appears to be independent of the GnTI transmembrane domain and cytoplasmic tail sequences.

It is unlikely that the TfR sequence of the TfR/GnTI protein is influencing complex formation in the above analysis. However, to directly ascertain the behavior of this TfR sequence on solubilization, a fusion protein was generated consisting of GFP attached to the C terminus of the transmembrane domain and truncated cytoplasmic tail of the transferrin receptor (TfR/GFP). As expected, TfR/GFP-transfected CHO cells showed high levels of the membrane-bound GFP fusion protein at the
with L-PHA-FITC for 30 min at 4 °C, washed, and then analyzed by immunoblotting using the monoclonal antibody 9E10. Pulse-chase analysis of sGnTI. Cells expressing sGnTImyc were pulse-labeled with [35S]methionine/cysteine for 15 min and then chased for 0, 30, 60, or 120 min. The medium was collected, and the cells were harvested and lysed. Both the culture medium (M) and cell lysate (C) were immunoprecipitated with 9E10, and the immunoprecipitates were analyzed by SDS-PAGE and fluorography.

A Soluble Form of GnTI Is Retarded within the Golgi Apparatus—The above data indicates that the luminal domain of GnTI may be involved in the formation of high molecular weight complexes via protein-protein interactions. Such interactions may facilitate the localization of chimeric molecules within the medial Golgi. The question then arises whether soluble medial Golgi enzymes may behave in a different manner from soluble Gal-T1, which we have previously demonstrated to be rapidly secreted from transfected cells (17). Therefore, we decided to investigate whether the luminal domain of GnTI, as a soluble protein, could be retained within the Golgi as a result of interaction with other components.

The luminal domain of GnTI, containing both the stem region and catalytic domain, was fused to the cleavable signal peptide derived from hemagglutinin (Fig. 1). CHO Lec1 cells were transfected with this construct, and clonal cell lines were established. Surprisingly, the immunofluorescence staining of CHO cells expressing myc-tagged sGnTI indicates that, at steady state, the majority of intracellular material was Golgi-localized, since a strong perinuclear staining pattern was observed (Fig. 5A). In contrast, CHO cells expressing a soluble form of Gal-T1 showed only a reticular intracellular staining pattern by immunofluorescence (Fig. 5A). Since soluble Gal-T1 is secreted from CHO cells (not shown), these results indicate that soluble Gal-T1 does not accumulate within the Golgi during secretion, consistent with our previous findings in transfected COS cells (17).

To confirm that the GnTI product was a soluble protein, transfected CHO cells were extracted in Triton X-114/250 mM NaCl, and the aqueous and detergent phases were analyzed for the myc-tagged sGnTI protein by immunoblotting (Fig. 6A). The majority of the sGnTI protein was recovered in the aqueous phase with a molecular mass of approximately 45 kDa, consistent with the removal of the hydrophobic signal peptide and the production of a soluble protein (Fig. 6A). As expected, membrane-bound full-length GnTI was recovered in the detergent phase of a Triton X-114 extraction (Fig. 6A).

To determine whether the intracellular sGnTI was secreted, transfected CHO Lec1 cells were treated with cycloheximide and analyzed over a 4-h period by immunofluorescence. In contrast to membrane-bound full-length GnTI, after a 4-h cycloheximide treatment, the majority of the intracellular myc-tagged sGnTI had disappeared (not shown). Pulse-chase studies were then carried out to determine the kinetics of secretion (Fig. 6B). Two major bands were detected by immunoprecipitation after a 30-min chase, a component of ~44 kDa and a slightly higher molecular mass species, which is probably O-glycosylated. After a 30-min chase period, the majority of sGnTI protein was cell-associated. After a 60-min chase, >50% of the sGnTI protein was found cell-associated, and the remainder was in the medium. Whereas the secreted sGnTI protein was exclusively the higher molecular weight component, very little of this higher molecular component was cell-associated, indicating that once the sGnTI was fully glycosylated it was rapidly transported out of the Golgi. By 2 h of chase, almost all of the sGnTI was found in the medium, and very little remained cell-associated (Fig. 6B). Collectively, these results in-
were extracted in 50 mM MES (pH 6.5) and 1% Triton X-100 containing B.

Cells expressing the soluble proteins sGnTI

m and NSS-sGnTI were extracted in 50 mM MES (pH 6.5) and 1% Triton X-100 containing either 0, 50, 100, or 250 mM NaCl. Extracts were centrifuged at 15,000 × g, and then the supernatant was spun at 100,000 × g. The low speed pellet (L), the high speed pellet (H), and the final supernatant (S) were adjusted to the same volume with SDS sample buffer and analyzed by immunoblotting. GnTI proteins were detected with monoclonal antibody 9E10, and Tir/GFP was detected with anti-GFP antibodies.

Fig. 7. Triton X-100 solubilization of Lec1 cells expressing non-mutated and mutated Tir/GnTI and sGnTI proteins. CHO Lec1 cells expressing the membrane-bound proteins Tir/GnTI or NSS-Tir/GnTI and CHO cells expressing Tir/GFP (A) and CHO Lec1 cells expressing the soluble proteins sGnTI

m and NSS-sGnTI (B) were extracted in 50 mM MES (pH 6.5) and 1% Triton X-100 containing either 0, 50, 100, or 250 mM NaCl. Extracts were centrifuged at 15,000 × g, and then the supernatant was spun at 100,000 × g. The low speed pellet (L), the high speed pellet (H), and the final supernatant (S) were adjusted to the same volume with SDS sample buffer and analyzed by immunoblotting. GnTI proteins were detected with monoclonal antibody 9E10, and Tir/GFP was detected with anti-GFP antibodies.

Indicate that sGnTI may accumulate briefly within the Golgi apparatus before subsequent transport to the cell surface and secretion.

Analysis of extracts of sGnTI-transfected CHO Lec1 cells showed that the sGnTI protein was active in vitro. To determine if sGnTI was functionally active in vivo within the Golgi apparatus, we investigated whether sGnTI could restore the ability of CHO Lec1 cells to synthesize branched complex N-glycans. Flow cytometric analysis showed that sGnTI-transfected CHO Lec1 cells bound FITC-L-PHA at a similar level to either parental CHO cells or Lec1 cells transfected with full-length GnTI (Fig. 5B). The GnTI activity associated with sGnTI-transfected CHO Lec1 cells was about 7.3 nmol/mg protein/h compared with 11.1 nmol/mg protein/h from wild-type GnTI-transfected CHO Lec1 cells. The efficient production of branched complex N-glycans by the sGnTI-transfected cells suggests that the soluble enzyme is able to act at the correct location within the glycosylation pathway.

Analysis of extracts of sGnTI-transfected CHO cells showed that intracellular sGnTI was also associated with NaCl-de-}

pendent complexes. In Triton X-100 extracts containing 50 mM NaCl, the majority of intracellular sGnTI sediments at 100,000 × g, and at 100 mM NaCl the protein was equally distributed between the 100,000 × g supernatant and pellet (Fig. 7B). Sucrose gradient analysis of 100 mM extracts showed that about 50% of the material migrated as a monomer and the remainder as high molecular weight complexes throughout the gradient including material found at the bottom of the gradient (Fig. 8). Comparison of sGnTI with either full-length GnTI or Tir/GnTI under these extraction conditions shows that a greater proportion of sGnTI exits as a monomer, suggesting that sGnTI is dissociated from the complexes more readily than membrane-bound GnTI. In Triton X-100 extracts containing 250 mM NaCl, sGnTI migrated as a broad peak with a molecular mass range of approximately 43–660 kDa (not shown). In contrast to intracellular sGnTI, soluble GnTI secreted into the medium migrated exclusively as a monomer on sucrose gradients (Fig. 8).

Mutations in the Stem Region of GnTI, Which Are Required for Kin Recognition, Do Not Affect Golgi Localization nor the Formation of High Molecular Weight Complexes—The Golgi localization of Tir/GnTI and sGnTI and their inclusion in high molecular weight complexes suggests that the luminal domain of GnTI is involved in protein-protein interactions. Nilsson et al. (16) have previously suggested that different Golgi enzymes of the same compartment may be able to interact with each other to form hetero-oligomeric structures (kin oligomers). This conclusion was based on a strategy involving the attachment of an ER retention signal onto Golgi enzymes; these investigators showed that the ER relocation of one enzyme resulted in the accumulation of other Golgi proteins in the ER. Using this kin recognition assay, where interactions occur within the ER compartment, Nilsson et al. (43) identified a region within the stem of GnTI that was required for kin recognition. In particular, when three charged residues within the stem sequence, namely Asp77, Arg83, and Arg85 were mutated to the uncharged residues Asn, Ser, and Ser, respectively, kin recognition was essentially abolished.

To determine if Asp77, Arg83, and Arg85 of the stem region of GnTI are also relevant to Golgi localization of GnTI and to the formation of the GnTI-containing complexes observed here, we have mutated these charged stem residues in Tir/GnTI and sGnTI transmembrane constructs (Fig. 1). Since the GnTI transmembrane domain is absent in these constructs, if the charged stem residues are critical for the ability of the luminal domain of GnTI to contribute to Golgi retention, then these mutations should drastically alter the intracellular steady state distribution of these proteins. However, both myc-tagged NSS-Tir/GnTI and NSS-sGnTI were located predominantly in the Golgi region of stably transfected CHO Lec1 cells (Fig. 5A), and, moreover, the transfected Lec1 cells bound FITC-L-PHA at a
similar level to parental CHO cells, demonstrating that these charged stem residues had little apparent effect on Golgi localization (Fig. 5B). Furthermore, analysis of extracts of transfected Lec1 cells showed that the mutated GnTI proteins behaved in a similar manner to non-mutated proteins (Figs. 7 and 8). Sucrose gradient analysis of 100 mM extracts showed that NSS-TIR/GnTI displayed a similar distribution throughout the gradient as TIR/GnTI. Likewise, NSS-sGnTI also showed a similar distribution throughout the gradient as sGnTI, suggesting that regions of the luminal domain other than the charged stem residues are responsible for Golgi localization and the formation of high molecular weight complexes.

**DISCUSSION**

The synthesis of complex N-glycans requires the sequential action of a number of membrane-bound glycosyltransferases in a highly controlled fashion. To date, however, very little is known about the organization of these enzymes within Golgi membranes or the underlying mechanisms responsible for the differential localization of the various enzymes to different Golgi regions. Here we have compared the characteristics of two medial Golgi enzymes with two late acting Golgi enzymes and found the following differences. First, the two medial Golgi glycosyltransferases are solubilized only in high salt, in contrast to the late acting Golgi enzymes, which are readily solubilized in low salt; second, analysis of detergent extracts showed that the two medial Golgi glycosyltransferases exist as high molecular weight complexes, whereas the late acting Golgi enzymes are present as monomers and dimers; third, formation of GnTI high molecular weight complexes does not require the transmembrane and cytoplasmic sequences of GnTI; and fourth, in contrast to Gal-T1, soluble GnTI containing the catalytic domain and stem region accumulates in the Golgi prior to secretion and is included in high molecular weight complexes. These findings indicate that the two medial Golgi enzymes exist as high molecular weight complexes within Golgi membranes, whereas Gal-T1 and HT do not. We propose that these observed differences between GnTI/GnTII and Gal-T1/HT reflect different characteristics between enzymes found in different Golgi compartments.

The extraction of both GnTI and GnTII from rat liver is known to be salt-dependent (28, 40). In this report, we have demonstrated that these enzymes behaved in a similar manner in transfected cell lines, indicating that this is an inherent property of these proteins. In low concentrations of salt, a large proportion of GnTI and GnTII from transfected CHO cells was insoluble. At higher salt concentrations (100 mM), the enzymes were largely soluble; nonetheless, a significant proportion of both GnTI and GnTII was found to exist in high molecular weight complexes that could be pelleted at 100,000 × g. These complexes are very large (>1000 kDa) and are independent of cholesterol-rich membrane rafts. These results contrast with both Gal-T1 and HT that were solubilized in low salt and remained in the supernatant of a high speed spin. Slusarewicz et al. (40) have previously shown that the medial Golgi enzymes, GtTI and α-mannosidase II from rat liver membranes interact with a detergent-insoluble intercellular matrix in the absence of NaCl; the interaction of GtTI with the insoluble matrix was fully dissociated with 50–100 mM NaCl (40). We concur that GtTI is extracted from Golgi membranes in a NaCl-dependent manner but also observe that GtTI is solubilized as high molecular weight complexes, which dissociate in a salt-dependent manner, a behavior shared by GtTII. The extraction of the medial Golgi glycosyltransferases as high molecular weight complexes is consistent with reports on the purification of GtTII that detected a high Mr form of GtTII (28).

Our data strongly suggest that the luminal domain of GtTI is important for inclusion into the higher molecular weight complexes. First, TIR/GnTI displayed very similar properties to full-length GtTI and was present in high molecular weight complexes; second, intracellular soluble GtTI containing the stem region and catalytic domain was included in a high molecular weight complex, whereas the soluble GtTI secreted into the medium exists as a monomer. Furthermore, soluble GtTI accumulated in the Golgi region prior to secretion as demonstrated by the intracellular localization of soluble GtTI in transfected CHO cells and by pulse-chase experiments. myc-tagged sGnTI, which accumulates within the Golgi at steady state, could be chased out by treatment with cycloheximide, indicating that the Golgi-localized molecules are not long term residents. It is possible therefore that the retardation of soluble GtTI within the Golgi is due to the inclusion into high molecular weight membrane complexes.

Soluble GtTI was able to glycosylate efficiently in vivo as demonstrated by the ability of this soluble enzyme to rescue the glycosylation defect of CHO Lec1 cells. The ability of soluble intracellular GtTI to form high molecular complexes may be important in its ability to coordinate the synthesis of complex N-glycans in Lec1 cells. The relative abilities of membrane-bound and soluble forms of other Golgi glycosyltransferases to glycosylate newly synthesized proteins have been compared, and differences between enzymes have been noted (44, 45). A possible reason for these differences could be that the enzymes that glycosylate efficiently in vivo have sequences in their luminal domains that lead to the formation of protein complexes and retention of the soluble forms. Thus, the inclusion of the medial Golgi enzymes into high molecular weight complexes may be functionally relevant. Munro (15) has reported the presence of multicomponent complexes with glycosyltransferase activity in the cis Golgi of yeast. It is possible that the GtTI and GtTII complexes detected here may also represent multienzyme complexes. However, the nature of these complexes remains obscure at this stage.

Warren and co-workers (16) have previously proposed that resident Golgi enzymes may interact to form large heterooligomeric structures, termed kin oligomers. This suggestion was based on the finding that the addition of an ER retention motif to GtTI not only causes GtTI to localize to the ER but also partially retains another medial Golgi enzyme, namely α-mannosidase II, within the ER. The interaction between GtTI and α-mannosidase within ER membranes has been shown to be mediated by interaction through their luminal domains (43, 46). However, since the interactions between these medial Golgi proteins were detected within the ER, and not the Golgi compartment, the biological relevance of these oligomers is not clear. The detection of high molecular weight complexes of the two medial Golgi enzymes, GtTI and GtTII, in this study is indeed consistent with these earlier suggestions and, furthermore, directly demonstrates that medial enzymes exist as complexes within Golgi membranes. Nonetheless, whereas kin recognition required the presence of charged residues in the stem region of GtTI (43), here we have shown that these residues were not important for the ability of GtTI chimeric molecules to be either localized within the Golgi, included into high molecular weight complexes, or functionally active in vivo. Thus, the interactions observed here do not depend on these charged residues. What is the reason for this difference? The most likely explanation is that Nilsson et al. (43) assessed the importance of the charged residues in chimeric molecules containing the stem of GtTI in the absence of the GtTI luminal catalytic domain; i.e. the mutated chimeric molecules contained only the stem region of GtTI. The forma-
tion of high molecular weight complexes involving medial Golgi enzymes may involve multiple contacts between the protein molecules of the complex, and mutating the charged residues of the GnTI stem should not interfere with contacts with other regions of the luminal domain. Details of the structure of the medial Golgi enzymes will be most important in identifying potential interactive surfaces. Using the kin recognition assay, Munro (46) could not detect a specific interaction between the two trans-Golgi enzymes α2,6-sialyltransferase and GaIT. This finding is also consistent with our observations that the two late-Golgi enzymes, HT and GaIT exist as monomers or dimers within the Golgi and strongly indicates that late Golgi enzymes exist in a different physical state from medial Golgi enzymes.

What is the relationship between the high molecular weight complex formation of the medial Golgi enzymes and Golgi localization? It is likely that inclusion into the high molecular weight complexes accounts for the Golgi localization of TfR/GnTI stem should not interfere with contacts with other molecules of the complex, and mutating the charged residues of enzymes may involve multiple contacts between the protein.

Acknowledgments—We thank Drs. Hans Paulsen and Folkert Reck for the generous gift of Mano1–6GlcNAcβ1,2Mano1–3Manβ-octyl, Professor Mauro Sandrin for the HT cDNA, and Dr. Rohan Teasdale for helpful comments on the manuscript.

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