Steady-state localization of a *medial*-Golgi glycosyltransferase involves transit through the *trans*-Golgi network

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The steady-state localization of *medial*-Golgi enzymes is likely to involve retrograde transport pathways; however, the trafficking of these resident enzymes through the Golgi stack is unclear. To investigate if the *medial*-Golgi enzyme β -1,2-N-acetylglucosaminyltransferase I (GlcNAc-TI) is transported to the late Golgi, a modified GlcNAc-TI bearing an N-glycan site on the C-terminus was constructed. The modified GlcNAc-TI was demonstrated to be functionally active *in vivo*, and was localized to the Golgi stack of transfected cells. In stable Chinese-hamster ovary (CHO) cell clones, the N-glycosylated GlcNAc-TI carried sialylated complex N-glycan chains. Pulse-chase studies showed that the majority of GlcNAc-TI was sialylated within 60 min of synthesis. Treatment of transfected CHO cells with Brefeldin A

resulted in the glycosylated GlcNAc-TI bearing endo- β -N-acetylglucosaminidase H resistant chains; however, the sialylation of glycosylated GlcNAc-TI was dramatically reduced. These data imply that, in CHO cells, newly synthesized GlcNAc-TI is transported rapidly through the Golgi stack to the *trans*-Golgi network, suggesting that GlcNAc-TI continuously recycles from the late Golgi. Furthermore, this data suggests that retrograde transport pathways play an important role in establishing the asymmetric distribution of GlcNAc-TI within the Golgi stack.

Key words: Brefeldin A, Golgi localization, membrane transport, retrograde transport.

INTRODUCTION

The mechanism for maintaining an asymmetric distribution of Golgi-resident glycosyltransferases, in spite of the considerable transport of soluble and membrane proteins moving through the Golgi en route to other destinations, has been intensely debated in recent years. It is clear that the transmembrane domain of the Golgi glycosyltransferases provides a dominant localization signal and, in addition, in a number of cases there are contributions from the luminal domain (reviewed in [1–3]). However, the mechanism that results in the asymmetric distribution of glycosyltransferases across the Golgi stack remains poorly understood. Factors which are likely to be important in the localization of resident Golgi glycosyltransferases include oligomerization (kin recognition) and lipid-mediated sorting (bilayer thickness hypothesis); either feature may mediate the retention of the enzymes within Golgi cisternal membranes and/or regulate their inclusion into retrograde transport vesicles [3,4].

Integral to understanding the basis of steady-state distribution is the mechanism for vectorial movement across the Golgi stack. Anterograde vesicular transport and cisternal maturation are two alternative models of intra-Golgi transport [5–10]. A fundamental difference between the cisternal maturation and anterograde vesicular transport models of protein transport across the stack is the differential movement of resident and cargo proteins. The anterograde vesicular transport model predicts that cargo molecules will move forward in transport vesicles, while resident proteins are specifically retained. The cisternal maturation/retrograde transport model predicts that cargo molecules will move through the stack passively as the cisternae move forward, while resident proteins will be recycled

by retrograde transport to establish differential concentrations across the stack. The two models are not mutually exclusive and may be occurring simultaneously [9,11,12]. Inherent in the cisternal maturation model is the dependence of recycling to localize resident Golgi proteins. Indeed, there is increasing evidence that resident Golgi enzymes (such as glycosyltransferases) have the ability to recycle from the Golgi to the endoplasmic reticulum (ER) [10,13].

At steady state β -1,2-N-acetylglucosaminyltransferase I (GlcNAc-TI; EC 2.4.1.101) is concentrated within the *medial*-cisternae [14–16]. Using a Chinese-hamster ovary (CHO) mutant cell line, in which O-glycosylation can be blocked in a reversible manner, Hoe et al. [17] have demonstrated that GlcNAc-TI can undergo retrograde transport to the early Golgi apparatus and/or ER. Furthermore, GlcNAc-TI has been detected in coatomer protein-1 ('COPI')-dependent transport intermediates that can fuse with the *cis*-cisternae in *in vitro* assays [18]. However, these experiments have not addressed the question of whether GlcNAc-TI is transported beyond the *medial*-Golgi. This issue is important in ascertaining the relative balance of forward and backward transport to establish the steady-state distribution of *medial*-Golgi glycosyltransferases.

Human GlcNAc-TI is O-glycosylated, but lacks N-glycans [17,19]. O-glycans do not provide an appropriate handle for tracing proteins through the late Golgi, as the sialyltransferases responsible for addition of sialic acid to O-glycans are localized throughout the Golgi stack [20,21]. On the other hand, sialylation of N-glycans occurs in the late Golgi/*trans*-Golgi network (TGN) [22,23]. In particular, in CHO cells, the bulk of the sialyltransferase activity responsible for sialylation of N-glycans is located in the TGN and, furthermore, is segregated from the enzymes of the Golgi stack upon Brefeldin A (BFA) treatment

Abbreviations used: GlcNAc-TI, β -1,2-N-acetylglucosaminyltransferase I; CHO, Chinese-hamster ovary; L-PHA, leuco-phytohaemagglutinin; BFA, Brefeldin A; TGN, *trans*-Golgi network; ER, endoplasmic reticulum; DMEM, Dulbecco's modified Eagle's medium; PNGase F, peptide N-glycosidase F; Endo H, endo- β -N-acetylglucosaminidase H.

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[24,25]. The location of the sialyltransferase activity almost exclusively to the TGN in CHO cells may be due to the fact that this cell line synthesizes N-glycans bearing only terminal α -2,3 sialic acid linkages as a functional gene encoding α -2,6 sialyltransferase (*ST6Gal I*) is lacking in CHO cells [26,27].

In the present study we have inserted an N-glycosylation site into GlcNAc-TI and determined the extent of sialylation of the GlcNAc-TI neo-glycoprotein in stable CHO cells. The GlcNAc-TI neo-glycoprotein was found to be sialylated rapidly, and sialylation was inhibited after treatment with BFA. These results indicate that addition of sialic acid to the GlcNAc-TI neo-glycoprotein occurs predominantly in the TGN. Therefore GlcNAc-TI does not appear to be actively retained for long periods within the Golgi stack, suggesting a critical role for Golgi recycling pathways in establishing the *cis-trans* gradient of GlcNAc-TI.

EXPERIMENTAL

Cell culture and antibodies

Cells were maintained in a humidified 10% CO₂ atmosphere at 37 °C as monolayers in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml penicillin and 0.1% streptomycin (complete DMEM). CHO and CHO Lec 1 [28] cells were maintained in the above medium supplemented with 40 µg/ml proline. Transfected cells were maintained in the above medium with 400 µg/ml G418 (Life Technologies). Myc epitope-tagged proteins were detected with 9E10 monoclonal antibody [29]. Rabbit anti- α -mannosidase II antibodies were provided by K. Moreman [30]. Affinity-purified human anti-p230 antibodies were obtained as described in [31]. FITC-conjugates were from Silenus Laboratories (Melbourne, Australia) and Texas Red conjugates were from Molecular Probes. Horseradish peroxidase-conjugated anti-mouse Ig was obtained from DAKO Corporation (Carpinteria, CA, U.S.A.).

Addition of an N-glycosylation site to the human GlcNAc-TI sequence

An N-glycosylation site was engineered into the human GlcNAc-TI sequence by addition of 23 amino acids to the C-terminus (SVNATYTDIEMNPEQKLISEEDL, N-glycosylation site is underlined; Myc epitope is in italics). Due to the length of the sequence added, the construct was generated in two steps by PCR, using conditions previously described in [32]. The first PCR used pGEM-GlcNAc-TI [33] as template, the sense primer, 5'-GGAAGATCTCAAGAACGATGACC-3', which hybridized to the human GlcNAc-TI sequence at +920 and incorporates a *Bgl*II site, and the antisense primer, 5'-CGCGATCCGTT-CATCTCTATATCGGTGTAGGTAGCGTTGACGGAATTCCAGCTAGGATCATA-3', which hybridizes to the 5'-end of the GlcNAc-TI cDNA open reading frame and tagged it with an N-glycosylation site and the hydrophilic linker sequence (-ATYTDIEMN-). The product of this PCR was then purified from an agarose gel and used in a second PCR with the above sense primer and the second antisense primer, 5'-AGCTCT-AGATTACAGGTCTTCTTCAGAGATCAGTTTCTGTTCCGGGTTTCATCTCTATATCGGT-3', which hybridized to the linker sequence and added the Myc epitope tag. The PCR product was cloned into pCIneo-GlcNAc-TI-Myc, from which the corresponding fragment had been excised by digestion with *Bgl*II and *Xba*I (the *Bgl*II site in the pCIneo vector had been removed). Cloned PCR products were tested for fidelity by

automated sequencing (Applied Biosystems). The recombinant construct containing the N-glycan site is designated GlcNAc-TI-Ng-Myc.

Transfections

CHO or CHO Lec1 cells were transfected using Superfect (Qiagen) or Fugene-6 (Roche Molecular Biochemicals), as described in [32].

Immunofluorescence and lectin staining

Cells were grown on 12-well glass microscope slides, or glass coverslips in 12-well plates. Cells were rinsed in PBS, then fixed with 4% paraformaldehyde, then rinsed three times in PBS and blocked with 50 mM NH₄Cl. Paraformaldehyde-fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min, then rinsed three times in PBS. Monolayers were incubated with primary antibody and bound antibodies then detected with either FITC or Texas Red-conjugated secondary antibodies, as described in [34]. For lectin staining, monolayers were incubated with 10 µg/ml of FITC-labelled leuco-phytohaemagglutinin (L-PHA; Sigma), as described in [35]. BFA treatment was carried out as described in [34]. Images were acquired using a Bio-Rad MRC 1024 confocal imaging system.

Immunoblotting

Cell extracts were separated by SDS/PAGE and electrophoretically transferred to nitrocellulose or PVDF membranes. Membranes were blocked for 1 h in 5% (w/v) non-fat skimmed milk in PBS and incubated for 1 h with 9E10 hybridoma supernatant diluted in PBS containing 3% (w/v) non-fat skimmed milk, washed with PBS containing 0.05% (v/v) Tween 20, and then incubated with peroxidase-conjugated anti-mouse Ig. Bound antibodies were detected by enhanced chemiluminescence (NEN, Boston, MA, U.S.A.), as described in [32,36].

Immunoprecipitation

Cells were radiolabelled with [L-³⁵S]methionine/cysteine (TRAN³⁵S-LABEL™; ICN), as described in [36], and extracted in 20 mM Tris/HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 1% Nonidet P40 and 1% sodium deoxycholate, containing Complete® protease inhibitors (Roche Molecular Biochemicals), for 45 min on ice. For pulse-chase experiments, the labelled medium was removed after the pulse time, and the cells were rinsed with warmed complete DMEM, then incubated in complete DMEM. For BFA treatment, subconfluent monolayers of GlcNAc-TI-Ng-Myc-transfected CHO cells were incubated in cysteine/methionine-free DMEM for 45 min at 37 °C, containing 5 µg/ml BFA (Calbiochem), prior to labelling in the presence of BFA. Lysates were centrifuged at 15000 *g* for 15 min and supernatants incubated with 2 µg purified monoclonal antibody 9E10 for 1 h at 4 °C with rotation. Immune complexes were collected by the incubation with Protein G-Sepharose, and washed for 10 min in 1 ml of NETGEL (50 mM Tris/HCl, pH 7.4, 5 mM EDTA, 0.05% Nonidet P40 and 0.25% gelatin) containing 500 mM NaCl, then NETGEL containing 150 mM NaCl and 0.1% SDS, then in 10 mM Tris/HCl, pH 8.0. Immunoprecipitates were then treated with glycosidases (as described below), or analysed by SDS/PAGE followed by fluorography.

Glycosidase treatments

Cells were lysed, or immunoprecipitates were separated from beads by boiling for 10 min in 0.5% SDS containing 1% β -

mercaptoethanol, and insoluble cellular material or Sepharose 4B beads removed by centrifugation at 15000 *g*. Nonidet P40 was added to the supernatants to a final concentration of 1%, and treated as follows:

Peptide N-glycosidase F (PNGase F) digestion

Na₂HPO₄/NaH₂PO₄ buffer (pH 7.5) was added to lysates to a final concentration of 50 mM. PNGase F (500 units; New England Biolabs) was added to 20 μ l reactions and the sample incubated for 1 h at 37 °C.

Endo- β -N-acetylglucosaminidase H (Endo H)

Sodium acetate buffer (pH 6.0) was added to lysates to a final concentration of 50 mM. Endo H (5 m-units) (Boehringer Mannheim) was added to 20 μ l reactions and samples incubated for 16 h at 37 °C.

Neuraminidase

Sodium acetate buffer (pH 6.0) was added to lysates to a final concentration of 50 mM, and CaCl₂ to 8 mM. Neuraminidase (5 mU, *Vibrio cholerae*; Calbiochem) was added to 20 μ l reactions and the samples incubated for 2 h at 37 °C.

Incubations were also carried out in the absence of enzyme. Following the incubations, samples were diluted with SDS/PAGE electrophoresis sample buffer, boiled for 3 min, and analysed by SDS/PAGE.

RESULTS

Construction of epitope-tagged GlcNAc-TI with an N-glycosylation site

At steady state GlcNAc-TI is concentrated within the *medial*-cisternae of the Golgi apparatus [14–16]. It is possible that the localization of GlcNAc-TI may be mediated, in part, by retrieval via retrograde transport pathway(s) from the late Golgi. To address this issue we have examined whether GlcNAc-TI can passage beyond the *medial*-cisternae to the TGN. To monitor the trafficking of GlcNAc-TI throughout the Golgi apparatus, the approach adopted was to engineer an N-glycosylation site into

Figure 1 Addition of N-glycosylation site to human GlcNAc-TI

(A) Construction of human GlcNAc-TI with a glycosylation site. The GlcNAc-TI-Ng-Myc construct is shown. Amino acid 445 is the C-terminal amino acid of human GlcNAc-TI sequence. The glycan acceptor site is marked with an asterisk. The Myc sequence is underlined. (B) Detection of complex N-glycans in CHO Lec1 cells transiently transfected with GlcNAc-TI-Ng-Myc. CHO Lec1 cells were transfected with GlcNAc-TI-Ng-Myc, fixed in 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100. Dual staining for the Myc epitope and complex N-glycans (L-PHA) was carried out as follows: monolayers were incubated with 9E10 hybridoma supernatant and bound antibodies detected with goat anti-mouse Ig Texas Red conjugate. For L-PHA staining, monolayers were incubated with 10 μ g/ml FITC-PHA. Untransfected cells (asterisk) showed no staining with either monoclonal antibody 9E10 or L-PHA. Bar, 20 μ m.

Figure 2 Intracellular localization of GlcNAc-TI-Ng-Myc in stably transfected C17 cells

Monolayers of C17 cells were either (A) untreated, (B) incubated for 4 h with 0.5 mM cycloheximide or (C) incubated with 1 μ g/ml BFA, then fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained for GlcNAc-TI-Ng-Myc with 9E10 monoclonal antibody and FITC-labelled anti-mouse Ig. Bar, 20 μ m.

Figure 3 Co-localization of α -mannosidase II and GlcNAc-TI-Ng-Myc in stably transfected C17 cells

C17 monolayers were fixed, permeabilized, and stained for p230, GlcNAc-TI-Ng-Myc, or α -mannosidase II (ManII) as indicated, with affinity-purified human anti-p230 antibody (p230), monoclonal antibody 9E10, and anti-rabbit α -mannosidase II antibodies respectively. Bound antibody was detected with either FITC-labelled anti-human Ig F(ab')₂ fragment, FITC-labelled sheep anti-mouse Ig or Texas Red-labelled goat anti-mouse Ig, or FITC-labelled sheep anti-rabbit Ig. Confocal images were collected with identical iris settings. Superimposed images reveals regions of co-localization. Similar results were obtained irrespective of the order of the primary antibodies. Control incubations demonstrated no cross-reactivity between the anti-Ig conjugates or between the anti-Ig conjugates and the irrelevant primary antibodies. Bar, 20 μ m.

GlcNAc-TI (wild-type enzyme lacks N-glycans) and determine the extent of sialylation of the GlcNAc-TI neo-glycoprotein. Human GlcNAc-TI contains at least one O-glycan, but no N-glycans [17,19]. An N-glycan site (-Asn-Ala-Thr-) was added to the lumenally orientated, C-terminus of GlcNAc-TI followed by a Myc epitope tag to allow detection of the modified GlcNAc-TI protein in transfected cells. To reduce the possibility of an N-linked oligosaccharide interfering with the binding of Myc epitope 9E10 monoclonal antibody, a short (eight amino acid) linker sequence was inserted between the N-glycosylation site and the Myc epitope tag (Figure 1A). The modified GlcNAc-TI is referred to as GlcNAc-TI-Ng-Myc.

To assess the functional integrity of GlcNAc-TI-Ng-Myc, CHO Lec1 cells (a mutant cell line devoid of GlcNAc-TI activity) were transiently transfected with GlcNAc-TI-Ng-Myc and the synthesis of complex N-glycans assessed by L-PHA binding. Transfected Lec1 cells, which were positive for the GlcNAc-TI-Ng-Myc product by 9E10 staining, also stained strongly with FITC-L-PHA, whereas untransfected Lec1 cells showed no staining with this lectin (Figure 1B). This result clearly demonstrates that GlcNAc-TI-Ng-Myc is functionally active *in vivo* and rescues the defect of the Lec1 mutant.

Stable CHO clones transfected with the GlcNAc-TI-Ng-Myc construct were generated and screened by immunofluorescence using the anti-Myc monoclonal antibody, 9E10. Clone 17 (C17) was used for further analysis. Immunofluorescence of C17 revealed a juxtanuclear staining pattern characteristic of the Golgi apparatus (Figure 2A). Furthermore, GlcNAc-TI-Ng-Myc remained Golgi-localized after 4 h treatment with cycloheximide (Figure 2B), and relocated to a reticular staining pattern, indicative of the ER following treatment with BFA (Figure 2C), indicating that GlcNAc-TI-Ng-Myc is actively retained in the Golgi apparatus.

To more precisely determine the distribution of the GlcNAc-TI-Ng-Myc within the Golgi, dual labelling was performed with the Golgi-resident proteins, α -mannosidase II and p230. α -Mannosidase II is a medial-Golgi enzyme [37] and p230 a trans-Golgi network peripheral membrane protein [36]. Dual staining for α -mannosidase II and p230 showed distinct staining patterns by confocal microscopy, with only a low level of co-localization (Figure 3). This result shows that residents of the Golgi stack and the TGN could be resolved by confocal microscopy. Dual staining for GlcNAc-TI-Ng-Myc and α -mannosidase II showed extensive co-localization of the two proteins (Figure 3), whereas dual

C17 cells were pulse-labelled for 15 min with [³⁵S]cysteine/methionine and chased for up to 4 h as described in the Experimental section. Cells were extracted in lysis buffer and immunoprecipitated with monoclonal antibody 9E10. Immunoprecipitates were treated with glycosidases, as indicated, and analysed by SDS/PAGE and fluorography.

The presence of two GlcNAc-TI-Ng-Myc products after PNGase F treatment is likely to be due to partial O-glycosylation, as the larger product from the PNGase F digestion was susceptible to neuraminidase (Figure 4C). Human GlcNAc-TI has previously been shown to be partially O-glycosylated [17]. Thus the immunoblotting data indicate that GlcNAc-TI-Ng-Myc is completely N-glycosylated and partially O-glycosylated. At steady state the majority of the N-glycans of GlcNAc-TI-Ng-Myc are sialylated.

The kinetics of GlcNAc-TI-Ng-Myc glycosylation was also examined by pulse-chase studies. C17 cells were labelled for 15 min with [³⁵S]cysteine/methionine, then chased for up to 4 h in medium. For each time point, lysates were immunoprecipitated with 9E10 and the immunoprecipitates treated with Endo H and neuraminidase (Figure 5). After the 15 min pulse-labelling, a single component of 51 kDa was detected. Endo H treatment reduced the size of the component by approx. 2 kDa, whereas treatment with neuraminidase had no affect. This indicates that within 15 min of synthesis, GlcNAc-TI-Ng-Myc carries high mannose glycans, as expected for a newly synthesized glycoprotein. After a 30 min chase, approx. 30 % of the GlcNAc-TI-Ng-Myc protein was converted into the 60 kDa component. This 60 kDa component is resistant to Endo H and susceptible to neuraminidase. After 60 min chase, the majority of GlcNAc-TI-Ng-Myc is neuraminidase sensitive and Endo H resistant, and within 4 h it is all neuraminidase sensitive and Endo H resistant. Therefore the addition of sialic acid to GlcNAc-TI-Ng-Myc occurs quite rapidly after arrival within the Golgi.

Treatment of cells with BFA results in the impairment of protein transport, and the relocation of Golgi enzymes and membranes to the ER by preventing the assembly of the COPI coat on membranes [38]. In CHO cells BFA affects TGN proteins differently from proteins of the Golgi stack. Whereas the membranes of the Golgi stack relocate to the ER following treatment with BFA, TGN membranes fuse with the endosomes [24,39]. In particular, the sialyltransferase activity responsible for sialylation of N-glycans in CHO cells is separated from newly synthesized proteins in BFA-treated cells, and is localized to the mixed TGN/endosome compartment [24,25]. This

(A, C) Analysis of glycans of GlcNAc-TI-Ng-Myc products. C17 cells were lysed in 0.5% SDS containing 1% β -mercaptoethanol, boiled, Nonidet P40 added to 1%, and then treated with PNGase, Endo H or neuraminidase, as described in the Experimental section. **(B)** C17 cells were incubated for the indicated time in 0.5 mM cycloheximide, harvested, and lysed in 1% Triton. All samples were analysed by immunoblotting with monoclonal antibody 9E10 followed by peroxidase-conjugated anti-mouse Ig. Bound antibodies were detected by chemiluminescence.

Characterization of the glycosylation of GlcNAc-TI-Ng-Myc

Lysates of C17 were analysed to examine if the potential N-glycosylation site of GlcNAc-TI-Ng-Myc was utilized. Immunoblotting of the GlcNAc-TI-Ng-Myc-transfected cells with 9E10 detected two components of 51 kDa and 60 kDa (Figure 4A). PNGase F treatment of cell lysates prior to immunoblotting demonstrated that the larger 60 kDa molecular mass component was susceptible to PNGase F digestion and therefore was N-glycosylated. However, as two components were observed after PNGase F digestion, it was not clear from this analysis whether the lower 51 kDa component carried N-glycans. On the other hand, digestion of lysates of the GlcNAc-TI-Ng-Myc transfectants with Endo H (Figure 4A) revealed that the 60 kDa component was resistant to Endo H, whereas the smaller 51 kDa component was susceptible and reduced in size by approx. 2 kDa. These results indicate that the 60 kDa component bears complex N-glycans, whereas the smaller 51 kDa component carries high mannose N-glycans. To determine the relationship between the 51 kDa and 60 kDa components, cells were treated with cycloheximide for up to 9 h. After 1 h treatment

Figure 6 BFA inhibits the synthesis of sialylated complex N-glycans of GlcNAc-TI-Ng-Myc

C17 cells were incubated for 45 min with or without 5 μ g/ml BFA. Cells were labelled for 30 min with [35 S]cysteine/methionine and chased for 3 h, in the presence or absence of BFA, as indicated. Cells were extracted in lysis buffer and immunoprecipitated with monoclonal antibody 9E10. Immunoprecipitates were treated with glycosidases, as indicated, and analysed by SDS/PAGE and fluorography.

pharmacologic-induced distinction between the Golgi stack and the TGN by BFA was used to further examine whether sialylation of GlcNAc-TI-Ng-Myc occurs within the TGN. Cells were treated for 30 min with BFA to relocate Golgi stack enzymes to the ER and to block further protein secretion from this organelle. Cells were then pulse-labelled for 15 min, and chased for 3 h in medium containing BFA. GlcNAc-TI-Ng-Myc was immunoprecipitated from lysates of these cells and the immunoprecipitates treated with glycosidases (Figure 6). GlcNAc-TI-Ng-Myc from BFA-treated cells migrated with a lower molecular mass on SDS/PAGE than GlcNAc-TI-Ng-Myc from mock-treated cells. The difference in size is due to decreased sialylation of GlcNAc-TI-Ng-Myc from BFA-treated cells, as GlcNAc-TI-Ng-Myc from treated and untreated cells was of similar size following digestion with neuraminidase. Furthermore, this difference in sialic acid addition is due to differences in sialylation of the N-glycan, as GlcNAc-TI-Ng-Myc from both treated and untreated cells was of similar size following digestion with PNGase F. The decreased sialylation of glycoproteins in BFA-treated CHO cells has been shown to be caused not by the failure of sialyltransferase to function in the BFA-induced ER-Golgi compartment, but by its failure to relocate to the compartment [24]. Indeed, in BFA-treated cells GlcNAc-TI-Ng-Myc did receive some sialic acid during the 3 h chase period, albeit less than in untreated cells. Addition of 0.5 mM cycloheximide during the chase period reduced, but did not eliminate, the partial sialylation of GlcNAc-TI-Ng-Myc (results not shown). This low level of residual sialylation may therefore be due to either newly synthesized sialyltransferase in the ER-Golgi compartment during the initial BFA treatment and pulse period, or alternatively, to low levels of sialyltransferase activity which were present in the Golgi stack. Significantly, Golgi-glycosylation in these BFA-treated cells was not perturbed, as all the GlcNAc-TI-Ng-Myc became Endo H-resistant within the chase period (Figure 6). Taken together, these results indicate that GlcNAc-TI-Ng-Myc receives post-translational modifications carried out in the TGN.

DISCUSSION

Current models of Golgi trafficking involving cisternal progression predict that all Golgi residents move down the stack to the late Golgi. Indeed, *cis*-Golgi residents of yeast cells have been shown to acquire modifications of the late Golgi [40,41]. However, very little is known about the movement of *medial*-Golgi-resident enzymes. The trafficking of *medial*-Golgi enzymes is important in understanding precisely how the asymmetric

gradients of glycosyltransferases are maintained throughout the Golgi stack. Here we have generated human GlcNAc-TI bearing an N-glycosylation site and assessed the extent of sialylation of the N-linked oligosaccharide. It has been previously shown [24] that the bulk of the sialyltransferase activity responsible for addition of sialic acid to N-glycans in CHO cells is associated with the TGN. The distinct localization of the N-glycan sialyltransferase activity to the TGN in CHO cells may be due to the fact that this cell line has only α 2,3 sialyltransferase activity, as a functional gene encoding α 2,6 sialyltransferase (*ST6Gal I*) is lacking in this cell line [26,27]. Thus this cell line is particularly suitable for the present study.

The modified GlcNAc-TI construct behaved in an identical manner to wild-type GlcNAc-TI. The epitope-tagged, N-glycosylated GlcNAc-TI was functionally active in Lec1 cells, and was located within the Golgi stack at steady state with very little co-localization with the TGN marker p230. Furthermore, the neo-glycoprotein was stable, with very little turnover detected over a 9 h period. Therefore the addition of an N-glycosylation site at the C-terminus of GlcNAc-TI did not appear to modify the behaviour of the enzyme. Significantly, the N-glycans of GlcNAc-TI-Ng-Myc were converted into sialylated complex N-glycans almost completely within 60 min of synthesis. The acquisition of Endo H-resistant N-glycan chains occurred in parallel to the addition of sialic acid, indicating sialylation proceeds rapidly after the processing events of the *medial*-Golgi. BFA treatment dramatically reduced the synthesis of the sialylated form of GlcNAc-TI-Ng-Myc; only approx. 30% of the GlcNAc-TI-Ng-Myc was sialylated after 180 min of synthesis in the presence of BFA, compared with almost complete sialylation within 60 min of synthesis in the absence of BFA (compare Figures 5 and 6). These data strongly support the conclusion that sialylation occurred predominantly in the TGN. On the other hand, glycans of GlcNAc-TI-Ng-Myc from BFA-treated cells were Endo H-resistant, demonstrating that the Golgi enzymes can function within the mixed ER/Golgi system, and consistent with previous observations [24,25].

Our data on the sialylation rates of GlcNAc-TI-Ng-Myc shows that GlcNAc-TI is not actively retained for long periods within the Golgi stack, but rather moves rapidly down the stack to the TGN. The rate of sialylation of GlcNAc-TI is comparable with transport rates of cargo molecules; however, it does not distinguish between the vesicular or cisternal progression modes of anterograde transport [9].

The sialylation of GlcNAc-TI-Ng-Myc, together with the steady-state localization within the Golgi stack, indicates that this enzyme recycles from the TGN. Retrograde pathways have been described from the TGN *in vivo*, and indeed Golgi-resident proteins have been shown to cycle through the ER [13]. However, the rate of retrograde transport to the ER is comparatively slow. Furthermore, using a CHO mutant cell line in which O-glycosylation can be blocked in a reversible manner, Hoe et al. [17] have demonstrated that GlcNAc-TI undergoes retrograde transport from the Golgi stack to the *cis*-Golgi network and/or ER with a half-time of several hours. Given the rapid rate of sialylation of GlcNAc-TI-Ng-Myc, together with the fact that the enzyme does not accumulate in the TGN, it seems unlikely that direct retrograde transport from the TGN to the *cis*-Golgi or ER could account for the recycling of this protein. Rather, our data indicates that the bulk of the GlcNAc-TI-Ng-Myc is likely to be recycled from the TGN by intra-Golgi retrograde transport.

A *medial*-Golgi-resident membrane protein of rat cells, namely MG160, has also been shown to be sialylated in the distal Golgi region rapidly (within 1 h) after exit from the ER [42,43]. MG160 may be involved in the trafficking and processing of

endogenous fibroblast growth factors [44] and, therefore, it could be argued that MG160 is selectively recycled as part of its function. However, in view of the similar sialylation kinetics of MG160 and GlcNAc-TI, it is likely that the trafficking of *medial*-Golgi membrane proteins to the TGN is a common characteristic of all residents of the *medial*-Golgi.

How might the steady-state distribution of GlcNAc-TI-Ng-Myc to the *medial*-Golgi be established? The rapid rate of sialylation of GlcNAc-TI-Ng-Myc strongly suggests that specific features of retrograde transport from the TGN are a critical component in establishing the gradient of GlcNAc-TI activity throughout the Golgi stack. It is known that COPI vesicles bud from all levels of the pathway and that COPI is required for intra-Golgi transport *in vitro* [11]. However, our understanding of intra-Golgi retrograde recycling remains rudimentary. Using *in vitro* assays, *medial*-Golgi-resident enzymes have been found to be concentrated into COPI vesicles [45] and, furthermore, transport intermediates containing resident Golgi enzymes, including GlcNAc-TI, have been shown to fuse preferentially with the *cis*-Golgi compartment [18]. But retrieval to the *cis*-Golgi cisternae would not explain how GlcNAc-TI concentrates within the *medial*-Golgi. One possibility is that there is selective retrograde transport of GlcNAc-TI from the TGN to the *medial*-Golgi. However, it is unlikely that there is sufficient machinery available, e.g. sets of cognate soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor (SNARE) molecules, to account for specific intra-Golgi transport pathways at every level of the stack [8,9]. Another possibility is that there is competition between *medial*-Golgi enzymes such as GlcNAc-TI and the *cis*- and *trans*-residents for packaging into vesicles, which could then account for their asymmetric distribution across the stack [46]. A further possibility is that *medial*-Golgi enzymes are efficiently recycled from the TGN in a common pathway back as far as the *medial*-Golgi cisternae, but then are largely excluded from further retrograde transport. Resolving these issues will provide an insight into how *medial*-Golgi enzymes are topographically located within the Golgi stack to regulate the highly ordered process of glycosylation.

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REFERENCES

- Colley, K. J. (1997) Golgi localization of glycosyltransferases: more questions than answers. *Glycobiology* **7**, 1–13
- Gleeson, P. A. (1998) Targeting of proteins to the Golgi apparatus. *Histochem. Cell Biol.* **109**, 517–532
- Munro, S. (1998) Localisation of proteins to the Golgi apparatus. *Trends Cell Biol.* **8**, 11–15
- Füllekrug, J. and Nilsson, T. (1998) Protein sorting in the Golgi complex. *Biochim. Biophys. Acta* **1404**, 77–84
- Bonfanti, L., Mironov, Jr, A. A., Martinez-Menarguez, J. A., Martella, O., Fusella, A., Baldassarre, M., Buccione, R., Geuze, H. J., Mironov, A. A. and Luini, A. (1998) Procollagen traverses the Golgi stack without leaving the lumen of cisternae: evidence for cisternal maturation. *Cell (Cambridge, Mass.)* **95**, 993–1003
- Glick, B. S. and Malhotra, V. (1998) The curious status of the Golgi apparatus. *Cell (Cambridge, Mass.)* **95**, 883–889
- Griffiths, G. (2000) Gut thoughts on the Golgi complex. *Traffic* **1**, 738–745
- Pelham, H. R. B. (1998) Getting through the Golgi complex. *Trends Cell Biol.* **8**, 45–49
- Pelham, H. R. and Rothman, J. E. (2000) The debate about transport in the Golgi – two sides of the same coin? *Cell (Cambridge, Mass.)* **102**, 713–719
- Storrie, B., Pepperkok, R. and Nilsson, T. (2000) Breaking the COPI monopoly on Golgi recycling. *Trends Cell Biol.* **10**, 385–391
- Orci, L., Stannnes, M., Ravazzola, M., Amherdt, M., Perrelet, A., Sollner, T. H. and Rothman, J. E. (1997) Bidirectional transport by distinct populations of copI-coated vesicles. *Cell (Cambridge, Mass.)* **90**, 335–349
- Orci, L., Ravazzola, M., Volchuk, A., Engel, T., Gmachl, M., Amherdt, M., Perrelet, A., Sollner, T. H. and Rothman, J. E. (2000) Anterograde flow of cargo across the golgi stack potentially mediated via bidirectional “percolating” COPI vesicles. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 10400–10405
- Storrie, B., White, J., Rottger, S., Stelzer, E. H., Saganuma, T. and Nilsson, T. (1998) Recycling of Golgi-resident glycosyltransferases through the ER reveals a novel pathway and provides an explanation for nocodazole-induced Golgi scattering. *J. Cell Biol.* **143**, 1505–1521
- Burke, J., Pettitt, J. M., Humphris, D. and Gleeson, P. A. (1994) Medial-Golgi retention of N-acetylglucosaminyltransferase I. Contribution from all domains of the enzyme. *J. Biol. Chem.* **269**, 12049–12059
- Dunphy, W. G., Brands, R. and Rothman, J. E. (1985) Attachment of terminal N-acetylglucosamine to asparagine-linked oligosaccharides occurs in central cisternae of the Golgi stack. *Cell (Cambridge, Mass.)* **40**, 463–472
- Rabouille, C., Hui, N., Hunte, F., Kieckbusch, R., Berger, E. G., Warren, G. and Nilsson, T. (1995) Mapping the distribution of Golgi enzymes involved in the construction of complex oligosaccharides. *J. Cell Sci.* **108**, 1617–1627
- Hoe, M. H., Slusarewicz, P., Misteli, T., Watson, R. and Warren, G. (1995) Evidence for recycling of the resident medial/trans Golgi enzyme, N-acetylglucosaminyltransferase I, in IdID cells. *J. Biol. Chem.* **270**, 25057–25063
- Love, H. D., Lin, C.-C., Short, C. S. and Osterman, J. (1998) Isolation of functional Golgi-derived vesicles with a possible role in retrograde transport. *J. Cell Biol.* **140**, 541–551
- Kumar, R., Yang, J., Larsen, R. D. and Stanley, P. (1990) Cloning and expression of N-acetylglucosaminyltransferase I, the medial Golgi transferase that initiates complex N-linked carbohydrate formation. *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9948–9952
- Locker, J. K., Griffiths, G., Horzinek, M. C. and Rottier, P. J. (1992) O-glycosylation of the coronavirus M protein. Differential localization of sialyltransferases in N- and O-linked glycosylation. *J. Biol. Chem.* **267**, 14094–14101
- Whitehouse, C., Burchell, J., Gschmeissner, S., Brockhausen, I., Lloyd, K. O. and Taylor-Papadimitriou, J. (1997) A transfected sialyltransferase that is elevated in breast cancer and localizes to the medial/trans-Golgi apparatus inhibits the development of core-2-based O-glycans. *J. Cell Biol.* **137**, 1229–1241
- Locker, J. K., Griffiths, G., Horzinek, M. C. and Rottier, P. J. M. (1992) O-Glycosylation of the coronavirus-M protein-differential localization of sialyltransferases in N-linked and O-linked glycosylation. *J. Biol. Chem.* **267**, 14094–14101
- Roth, J., Taatjes, D. J., Lucocq, J. M., Weinstein, J. and Paulson, J. C. (1985) Demonstration of an extensive trans-tubular network continuous with the Golgi apparatus stack that may function in glycosylation. *Cell (Cambridge, Mass.)* **43**, 287–295
- Chege, N. W. and Pfeffer, S. R. (1990) Compartmentation of the Golgi complex: brefeldin-A distinguishes trans-Golgi cisternae from the trans-Golgi network. *J. Cell Biol.* **111**, 893–899
- Doms, R. W., Russ, G. and Yewdell, J. W. (1989) Brefeldin A redistributes resident and itinerant Golgi proteins to the endoplasmic reticulum. *J. Cell Biol.* **109**, 61–72
- Bragonzi, A., Distefano, G., Buckberry, L. D., Acerbis, G., Foglieni, C., Lamotte, D., Campi, G., Marc, A., Soria, M. R., Jenkins, N. and Monaco, L. (2000) A new Chinese hamster ovary cell line expressing α 2,6-sialyltransferase used as universal host for the production of human-like sialylated recombinant glycoproteins. *Biochim. Biophys. Acta* **1474**, 273–282
- Lee, E. U., Roth, J. and Paulson, J. C. (1989) Alteration of terminal glycosylation sequences on N-linked oligosaccharides of Chinese hamster ovary cells by expression of β -galactoside α 2,6-sialyltransferase. *J. Biol. Chem.* **264**, 13848–13855
- Stanley, P., Narasimhan, S., Siminovich, L. and Schachter, H. (1975) Chinese hamster ovary cells selected for resistance to the cytotoxicity of phytohaemagglutinin are deficient in a UDP-N-acetylglucosamine-glycoprotein N-acetylglucosaminyltransferase activity. *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3323–3327
- Evan, G. I., Lewis, G. K., Ramsay, G. and Bishop, J. M. (1985) Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol. Cell Biol.* **5**, 3610–3616
- Moremen, K. W., Touster, O. and Robbins, P. W. (1991) Novel purification of the catalytic domain of Golgi α -mannosidase II. Characterization and comparison with the intact enzyme. *J. Biol. Chem.* **266**, 16876–16885
- Kooy, J., Toh, B. H., Pettitt, J. M., Erlich, R. and Gleeson, P. A. (1992) Human autoantibodies as reagents to conserved Golgi components. Characterization of a peripheral, 230-kDa compartment-specific Golgi protein. *J. Biol. Chem.* **267**, 20255–20263
- Opat, A. S., Houghton, F. and Gleeson, P. A. (2000) Medial Golgi but not late Golgi glycosyltransferases exist as high molecular weight complexes. Role of luminal domain in complex formation and localization. *J. Biol. Chem.* **275**, 11836–11845

- 33 Hull, E., Sarkar, M., Spruijt, M. P. N., Höppener, J. W. M., Dunn, R. and Schachter, H. (1991) Organisation and localisation to chromosome 5 of the human UDP-N-acetylglucosamine: α -3-D-mannoside β -1,2-N-acetylglucosaminyltransferase I gene. *Biochem. Biophys. Res. Commun.* **176**, 608–615
- 34 Kjer-Nielsen, L., van Vliet, C., Erlich, R., Toh, B. H. and Gleeson, P. A. (1999) The Golgi-targeting sequence of the peripheral membrane protein p230. *J. Cell Sci.* **112**, 1645–1654
- 35 Opat, A. S., Puthalakath, H., Burke, J. and Gleeson, P. A. (1998) Genetic defect in N-acetylglucosaminyltransferase I gene of a ricin-resistant baby hamster kidney mutant. *Biochem. J.* **336**, 593–598
- 36 Gleeson, P. A., Anderson, T. J., Stow, J. L., Griffiths, G., Toh, B. H. and Matheson, F. (1996) p230 is associated with vesicles budding from the trans-Golgi network. *J. Cell Sci.* **109**, 2811–2821
- 37 Novikoff, P. M., Tulsiani, D. R. P., Touster, O. and Novikoff, A. B. (1983) Immunocytochemical localisation of alpha-D-mannosidase in the Golgi apparatus of rat liver. *Proc. Natl. Acad. Sci. U.S.A.* **80**, 4364–4368
- 38 Chardin, P. and McCormick, F. (1999) Brefeldin A: the advantage of being uncompetitive. *Cell (Cambridge, Mass.)* **97**, 153–155
- 39 Wood, S. A., Park, J. E. and Brown, W. J. (1991) Brefeldin A causes a microtubule-mediated fusion of the trans-Golgi network and early endosomes. *Cell (Cambridge, Mass.)* **67**, 591–600
- 40 Harris, S. L. and Waters, M. G. (1996) Localization of a yeast early Golgi mannosyltransferase, Och1p, involves retrograde transport. *J. Cell Biol.* **132**, 985–998
- 41 Wooding, S. and Pelham, H. R. (1998) The dynamics of golgi protein traffic visualized in living yeast cells. *Mol. Biol. Cell.* **9**, 2667–2680
- 42 Gonatas, J. O., Mezitis, S. G., Stieber, A., Fleischer, B. and Gonatas, N. K. (1989) MG-160. A novel sialoglycoprotein of the medial cisternae of the Golgi apparatus. *J. Biol. Chem.* **264**, 646–653
- 42a Erratum (1989) *J. Biol. Chem.* **264**, 4264
- 43 Johnson, P. A., Stieber, A. and Gonatas, N. K. (1994) A hypothesis on the traffic of MG160, a medial Golgi sialoglycoprotein, from the trans-Golgi network to the Golgi cisternae. *J. Cell Sci.* **107**, 529–537
- 44 Gonatas, J. O., Mourelatos, Z., Stieber, A., Lane, W. S., Brosius, J. and Gonatas, N. K. (1995) MG-160, a membrane sialoglycoprotein of the medial cisternae of the rat Golgi apparatus, binds basic fibroblast growth factor and exhibits a high level of sequence identity to a chicken fibroblast growth factor receptor. *J. Cell Sci.* **108**, 457–467
- 45 Lanoix, J., Ouwendijk, J., Lin, C.-C., Stark, A., Love, H. D., Ostermann, J. and Nilsson, T. (1999) GTP hydrolysis by arf-1 mediates sorting and concentration of Golgi resident enzymes into functional COP I vesicles. *EMBO J.* **18**, 4935–4948
- 46 Glick, B. S., Elston, T. and Oster, G. (1997) A cisternal maturation mechanism can explain the asymmetry of the Golgi stack. *FEBS Lett.* **414**, 177–181

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