Glycoconjugate Journal (1996) **13:** 213–223

Cloned β1,4*N*-acetylgalactosaminyltransferase: subcellular localization and formation of disulfide bonded species*

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Received 29 June 1995, revised 13 October 1995

Cloned human β1,4*N*-acetylgalactosaminyltransferase (GalNAcT) catalyses the synthesis of the glycosphingolipids GM2, GD2, and gangliotriosylceramide. To determine the subcellular location of this enzyme and whether it exists in intermolecular disulfide bonded species, we stably transfected Chinese hamster ovary (CHO) cells with three myc epitope-tagged forms of the GalNAcT gene: the native enzyme; the lumenal domain of GalNAcT fused to the cytoplasmic and transmembrane domains of *N*-acetylglucosaminyltransferase I (GNT); and the transmembrane and lumenal domains of GalNAcT fused to the cytoplasmic domain of the Iip33 form of human invariant chain in order to retain the enzyme in the endoplasmic reticulum (ER). Immunoelectron microscopic analysis with anti-myc revealed that GalNAcT/myc was present throughout the Golgi stack, the GNT/GalNAcT/myc form was restricted primarily to the medial Golgi cisternae, and the Iip33/GalNAcT/myc form was restricted to the ER. Cells transfected with each of the three constructs contained high levels of GM2 synthase activity *in vitro*, but only the GalNAcT/myc form and the GNT/GalNAcT/myc forms were able to synthesize the GM2 product *in vivo*. The enzyme produced by all three constructs was present in the transfected cells in a disulfide bonded form having a molecular size consistent with that of a homodimer or higher aggregate.

Keywords: glycosyltransferase, Golgi, epitope tagging, glycosphingolipid

Abbreviations: GSL, glycosphingolipid(s); CHO, Chinese hamster ovary; GSL structures: GM2, GalNAcβ1,4(NeuAcα2,3)Galβ1,4GlcCer; GD2, GalNAcβ1,4(NeuAcα2,8NeuAcα2,3)Galβ1,4GlcCer; GM3, NeuAcα2,3Galβ1,4GlcCer; Gg_3, GalNAcβ1,4Galβ1,4GlcCer; LacCer, Galβ1,4GlcCer; GlcCer, glucosylceramide; PBS-BSA, phosphate buffered saline pH 7.4 containing 1% bovine serum albumin; GalNAcT, *N*-acetylgalactosaminyltransferase; GNT, *N*-acetylglucosaminyltransferase I; Iip33, p33 form of human invariant chain; HPTLC, high performance thin layer chromatography; PCR, polymerase chain reaction; and BFA, Brefeldin A.

Introduction

The glycosyltransferases that catalyse the biosynthesis of glycoprotein and glycosphingolipid (GSL) sugar chains are resident membrane proteins of the endoplasmic reticulum (ER) and the Golgi apparatus. The mechanisms responsible for residence of particular enzymes in specific subcompartments of the secretory pathway are the subject of current intense investigation [1]. The formation of intermolecular disulfide bonded species is an important feature of Golgi retention, resulting in either glycosyltransferase homodimers or association with other cellular components [2].

Among the glycosyltransferases that have been cloned to date, only a few are specific for GSL synthesis. One of these enzymes, β1,4*N*-acetylgalactosaminyltransferase (GalNAcT) [3] catalyses the synthesis of gangliosides GM2 and GD2 and the neutral GSL gangliotriosylcer-

*This paper is dedicated to Professor Sen-itiroh Hakomori on the occasion of his 65th birthday.
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amide [4–6]. To date no GSL specific glycosyltransferase has been immunolocalized at the EM level. In this study we report the subcellular location of GalNAcT, the effect of GalNAcT location on GSL synthesis *in vivo*, and intermolecular disulfide bond formation of this enzyme.

Materials and methods

Chimeric constructs

cDNAs encoding human GalNAcT [3], murine *N*-acetylglucosaminyltransferase I (GNT) [7], and Iip33 [8] were kindly provided by Drs K. Furukawa (Nagasaki University, Japan), P. Stanley (Albert Einstein, New York), and M. Jackson (Scripps Research Institute, La Jolla, CA), respectively. To fuse the myc epitope [9] to the carboxy terminus of GalNAcT (Fig. 1), the pM2T1-1 plasmid containing the full length cDNA encoding GalNAcT in the pCDM8 vector was used as template in a polymerase chain reaction (PCR). The amplified sequence consisted of the C-terminal fragment of GalNAcT from the *Eco* R1 restriction site at position 1444 bp to the final amino acid residue followed by an additional Pro residue and the myc epitope. PCR primers (Bio-synthesis, Lewisville, TX) were as follows (bases encoding the myc epitope recognized by the 9E10 mAb [9] are in italics): GalNAcT sense: 5′CGCCTCAGCCGCGTGGCTCATCTGGAATTC-TTC GalNAcT antisense: 5′GATCCACCGTGTGTTA*CA-GGTCTTCTTCAGAGATCAGTTTCTGTTC*CGGCTGGGA-GGTCATGCACTGCAGCCG.

The antisense primer contained a *Dra* III restriction site to allow subcloning of the PCR product into pM2T1-1 which was opened with *Eco* R1 and *Dra* III enzymes. The GalNAcT/myc construct was then subcloned into the pCDNA3 vector (Invitrogen) between *Hind* III and *Not* I restriction sites.

To prepare the GNT/GalNAcT/myc construct, the cDNA fragment encoding the cytoplasmic and transmembrane domains of GalNAcT/myc was replaced with the sequence encoding the cytoplasmic, transmembrane, and first twelve amino acid residues of the lumenal domain of GNT (Fig. 1). This sequence of GNT has been shown to be effective for conferring Golgi retention [10]. The GNT sequence was amplified by PCR using the following primers: GNT sense: 5′GCTAAAGCTTGCCG-CCAGGATGCTGAAGAAG GNT antisense: 5′TCAGCC-CGGGAGACTGAGGGTGGCCTGCC.

The PCR product, which contained a Kozak consensus sequence for proper initiation of translation [11], was digested with *Hind* III and *Xma* I and cloned into the pCDM8 plasmid containing the GalNAcT/myc construct using the same enzymes.

The cDNA construct containing the cytoplasmic domain of Iip33 fused to the transmembrane and lumenal domains of GalNAcT/myc (Fig. 1) was prepared as follows. First, PCR was used to introduce a *Sac* II restriction site by modifying the codon for the sixth amino acid residue of the GalNAcT/myc construct in pCDM8, which resulted in converting Arg to Gly. PCR primers, encoding restriction sites for *Hind* III, *Sac* II, and *Xma* I and GalNAcT/myc amino acid residues 5–27, were: *Sac* II sense: 5′ACTGAAGCTTCCGCGGGGCCCT-GTGCGCTCTGGTC *Sac* II antisense: 5′GCGTCCCGG-GTGCTCGCGTA.

The PCR product was subcloned into GalNAcT/myc in pCDM8 using *Hind* III and *Xma* I. A *Hind* III-*Sac* II fragment encoding the first 46 N-terminal amino acid residues of Iip33 [8] was cut out and purified from the plasmid containing the full length cDNA encoding Iip33 invariant chain in the pCMUIV vector. This fragment was then subcloned into the modified GalNAcT/myc plasmid at the *Hind* III and *Sac* II sites. All clones were

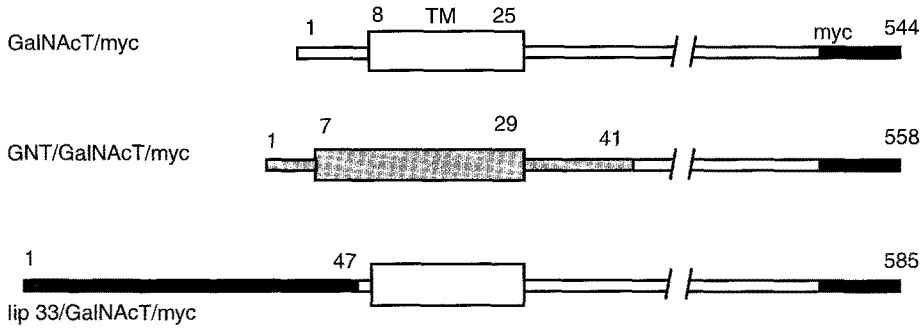

Figure 1. GalNAcT and hybrid proteins. GalNAcT/myc: the sequence encoding the ten amino acid myc epitope recognized by the 9E10 MoAb [9] was fused to the carboxy terminal sequence of GalNAcT. GNT/GalNAcT/myc: the sequence for the cytoplasmic and transmembrane domains plus the first 12 lumenal amino acids of *N*-acetylglucosaminyltransferase I (GNT) were fused to the lumenal domain of GalNAcT/myc. Iip33/GalNAcT/myc: the sequence for the cytoplasmic domain of the Iip33 form of human invariant chain was fused to the transmembrane and lumenal domains of GalNAcT/myc. TM, transmembrane domain.

confirmed by restriction enzyme digestion and sequencing at the splice sites.

Antibodies

Monoclonal IgM anti-GM2 10–11 [12] was a gift from Dr K.O. Lloyd. Monoclonal IgM anti-Gg_3 2D4 was prepared as described previously [13]. Monoclonal IgG1 anti-myc 9E10 [9] and IgG3 anti-GD3 R24 [14] were obtained from the American Type Culture Collection. Anti-A transferase WKH1 [15] was a gift from Dr S. Hakomori.

Cell culture and transfection

Wild type Chinese hamster ovary (CHO) cells were the gift of J. Baenziger (Washington University) and were grown in α-minimal essential medium containing 10% (v/v) fetal calf serum plus glutamine at 37 °C. Transfected cells were maintained in complete medium containing 0.4 mg ml^{-1} of active geneticin (G418; GIBCO, Grand Island, NY). Cells were transfected by the calcium phosphate precipitation method [16] with 20 μg of the GalNAcT/myc construct in pCDNA3. In the case of GNT/GalNAcT/myc and Iip33/GalNAcT/myc in pCDM8, 20 μg of each was co-transfected with 2 μg pRSVneo (American Type Culture Collection). G418-resistant cells were screened for GM2 and Gg_3 expression with specific antibodies by flow cytometry. Cells were removed from monolayer culture with trypsin-EDTA and incubated for 30 min at 4 °C with either monoclonal IgM anti-GM2 10–11 [12] or monoclonal IgM anti-Gg_3 2D4 [13] in PBS-BSA (phosphate buffered saline pH 7.4, containing 1% BSA). The cells were then washed in cold PBS-BSA and incubated for 30 min at 4 °C in PBS-BSA containing fluorescein-conjugated goat anti-mouse Ig. After washing, the cells were resuspended in PBS-BSA, and analysed on an Epics Elite flow cytometer (Coulter, Hialeah, FL).

Indirect immunofluorescence

Cells were grown on ethanol sterilized cover slips for 48 h, rinsed three times in PBS pH 7.4 at 4 °C, and then fixed and permeabilized in MeOH at –20 °C for 10 min. Alternatively, cells were fixed for 15 min at room temperature in freshly prepared 2% p-formaldehyde (EM Science) in PBS pH 7.4, rinsed in serum free medium, and permeabilized at room temperature for 20 min in 0.1% Triton X-100 in PBS. Both methods of fixation and permeabilization produced identical staining patterns with anti-myc 9E10 MoAb. Cells were incubated in succession, each step for 45 min at room temperature with intervening washing, with: PBS-BSA; primary antibody (either MoAb 9E10 anti-myc, anti-GD3 R24, or anti-A transferase WKH1 [15]); and secondary antibody; FITC-goat anti-mouse immunoglobulin (Cappel, Durham, NC). Cover slips were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA), sealed with nail polish, and examined with an Optiphot fluorescence microscope (Nikon).

Immunoelectron microscopy

Cell monolayers were rinsed in serum free medium, fixed in freshly prepared 0.5% glutaraldehyde (Electron Microscopy Sciences, Ft. Washington, PA) in PBS for 30 min at room temperature, rinsed in PBS, and harvested by scraping with a rubber policeman. After centrifugation, the cells were resuspended in 500 μl of 6% gelatin (porcine skin, approximately 300 bloom; Sigma) in PBS. The cells were centrifuged into the gelatin and the tube transferred to ice to solidify the gelatin. The solidified gelatin containing the cell pellet was transferred into 3% paraformaldehyde in PBS for 30 min at room temperature to cross-link the gelatin and improve cryosectioning performance. The paraformaldehyde was aspirated away and replaced with 0.05 M NH_4Cl in PBS for 15 min to amidate free aldehyde groups. The cell pellets in gelatin were cut into small pieces and separated for subsequent processing.

For low temperature dehydration and embedding in Lowicryl K4M, the gelatin pieces containing the cells were processed essentially as previously described [17]. Briefly, after overnight storage at 4 °C, the gelatin pieces were dehydrated in a graded series of ethanol (down to −35 °C) and embedded in Lowicryl K4M at −35 °C. Ultrathin sections of Lowicryl K4M embedded cells were cut with a diamond knife and retrieved onto parlodion/carbon coated nickel grids. Ultrathin cryosections were cut at –110 °C with a cryo diamond knife on a Reichert-Jung ultramicrotome with an FC4 cryoattachment. Sections were retrieved with a wire loop containing a droplet of PBS containing 2 M sucrose and 0.75% gelatin, placed onto a parlodion/carbon coated nickel grid, and stored section side down on solidified 2% gelatin.

Sections were immunolabelled as described previously [18]. Briefly, grids were floated section side down on drops of PBS containing 0.5% ovalbumin for 15–20 min at room temperature, followed by transfer to droplets of anti-myc 9E10 undiluted culture supernatant for 15–20 h at 4 °C. After rinses with PBS, the grids were incubated for 30 min at room temperature on droplets of affinity-purified rabbit-anti mouse IgG (10 μg ml^{-1}; Jackson ImmunoResearch, West Chester, PA). Following rinses with PBS, the grids were floated on drops of protein A-gold for 1 h at room temperature. Colloidal gold particles with an average diameter of 10 nm were prepared according to the tannic acid/citrate method [19] and complexed with protein A according to standard protocols [20]. The protein A-gold complex was diluted with PBS containing 1% bovine serum albumin, 0.075% Triton X-100 and 0.075% Tween 20, to yield an optical density of 0.06 at 525 nm. Following rinses with PBS and distilled water, the sections were contrasted with 3%

aqueous uranyl acetate (5 min) and lead citrate (45 s). All grids were subsequently examined in a Zeiss EM 10 or JEOL JEM1210 electron microscope operating at 60 kV.

GalNAcT enzyme assay

Confluent cell monolayers were rinsed with ice-cold PBS, harvested with a rubber policeman, pelleted by centrifugation, and resuspended in a small volume of lysis buffer consisting of 20 mM Tris-HCl, pH 7.4, 0.65 M sucrose, 10 mM $MgCl_2$, 2% Zwittergent 3-14 (Calbiochem, LaJolla, CA). After 5 min incubation on ice, insoluble material was pelleted by centrifugation at 1100 × **g**, 10 min, 4 °C. For enzyme assay, an aliquot of ganglioside GM3 (15 nmol) dissolved in chloroform:methanol (2:1) was mixed in a test tube with Zwittergent 3–14 (390 nmol), dried on a rotary evaporator, and suspended in 35 µl of assay mixture consisting of 0.2 m Hepes, pH 7.6, 3.6 mM $MnCl_2$, 0.21 mM UDP-[^{3}H] GalNAc (DuPont-New England Nuclear; sp act, 35 mCi mmol^{-1}) plus cell extract (up to 90 µg protein) in a final volume of 70 µl. Following incubation at 37 °C for 1 h, 2 µl of each reaction was applied to a water-wettable C_{18} reverse-phase HPTLC plate (Merck RP-18W) [21], dried for 30 min, developed with water for 10 min, and scanned for GM2 product at the origin using a linear analyser (EG & G Berthold, Salem, MA). Enzyme activity was quantitated as previously described [4]. Reactions were brought to 0.5 ml in $CHCl_3$:MeOH:H_2O (2:43:55) and the GSL product purified on a Bond Elut C18 column (Analytichem, CA) as described elsewhere [22]. GSL product which eluted with MeOH followed by $CHCl_3$:MeOH (1:1, v/v) was quantitated by scintillation counting. Protein concentration was determined by the BCA method [23] (Pierce Chemical Co.).

Metabolic labelling

Confluent cell monolayers were labelled for 4 h at 37 °C in medium containing 25 µCi ml^{-1} [9,10-^{3}H]palmitic acid (30 Ci mmol^{-1}; DuPont-New England Nuclear), rinsed with ice-cold saline, harvested by scraping with a rubber policeman, and stored at –80 °C. Cell pellets were extracted twice on ice with 30 ml of $CHCl_3$:MeOH (2:1, v/v) followed by $CHCl_3$:MeOH (1:2) twice using an Omni Mixer (Omni International, Norwalk, CT). GSL were purified by the acetylation procedure [24] and analysed on high performance TLC plates (Merck) in solvent A ($CHCl_3$:MeOH:0.25% KCl, 50:40:10, v/v). Radiolabelled TLC bands were visualized by autoradiography with fluorographic enhancement (EN^{3}HANCE spray; DuPont-New England Nuclear).

Western blotting

Cell extracts were prepared as described above for the GalNAcT enzyme assay. After SDS-PAGE on a 6.5% stacking, 10% separating gel, proteins were transferred to PVDF membranes (Millipore, Marlborough, MA) in an electroblotting apparatus at 33 V overnight. Blots were blocked in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20 (TTBS) with 5% nonfat dry milk for 1 h at room temperature. Blots were washed twice in TTBS and then incubated with primary antibodies diluted in TTBS for 1 h at room temperature. The culture supernatant of anti-myc 9E10 hybridoma cells was used at a 1:10 dilution. Blots were washed four times for 5 min each in TTBS and then incubated with horseradish peroxidase-coupled sheep anti-mouse Ig at a dilution of 1:5000 in TTBS for 1 h at room temperature. Blots were washed three times in TTBS. The bands were visualized using the ECL detection system (Amersham Corp.).

Results

cDNA constructs and cell transfections

Cloned GalNAcT was tagged with the myc epitope [9] at the carboxy terminus (Fig. 1) in order to detect the enzyme by immunofluorescence, immunoelectron microscopy, and Western blotting. To direct GalNAcT to defined regions of the secretory pathway, two constructs were made. First, the lumenal domain of GM2 synthase was fused to the transmembrane domain and flanking regions of *N*-acetylglucosaminyltransferase I (GNT) which is a known medial Golgi resident [25, 26]. Second, the transmembrane and lumenal domains of GM2 synthase were fused to the cytoplasmic domain of the Iip33 form of human invariant chain [8]. This Iip33 cytoplasmic domain has been used previously to retain other Golgi glycosyltransferases in the ER [27].

Each chimeric cDNA was stably transfected into CHO cells as described in Materials and methods. Transfected cells were analysed for cell surface expression of ganglioside GM2 by flow cytometry. Following transfection with GalNAcT/myc, approximately 8.8% of G418-resistant cells were positive for GM2 whereas untransfected cells were negative for GM2 expression as we previously described [4]. Following a single round of sterile sorting, 66.6% of the cells were GM2 positive. From this population clone C5, which was uniformly positive for GM2 expression (Fig. 2, top left panel), was isolated by limiting dilution. Following transfection with the GNT/GalNAcT/myc construct, 6% of G418-resistant cells were positive for GM2. A single sterile sort increased the GM2 positive population to 70%, from which a clone (B5) that was uniformly positive for GM2 (Fig. 2, middle panel) was isolated by limiting dilution. Following transfection with the Iip33/GalNAcT/myc construct, GM2 cell surface expression on the G418-resistant cells was negative (data not shown). To select cells expressing the transfected enzyme, cell clones were derived by limiting dilution and screened for GM2

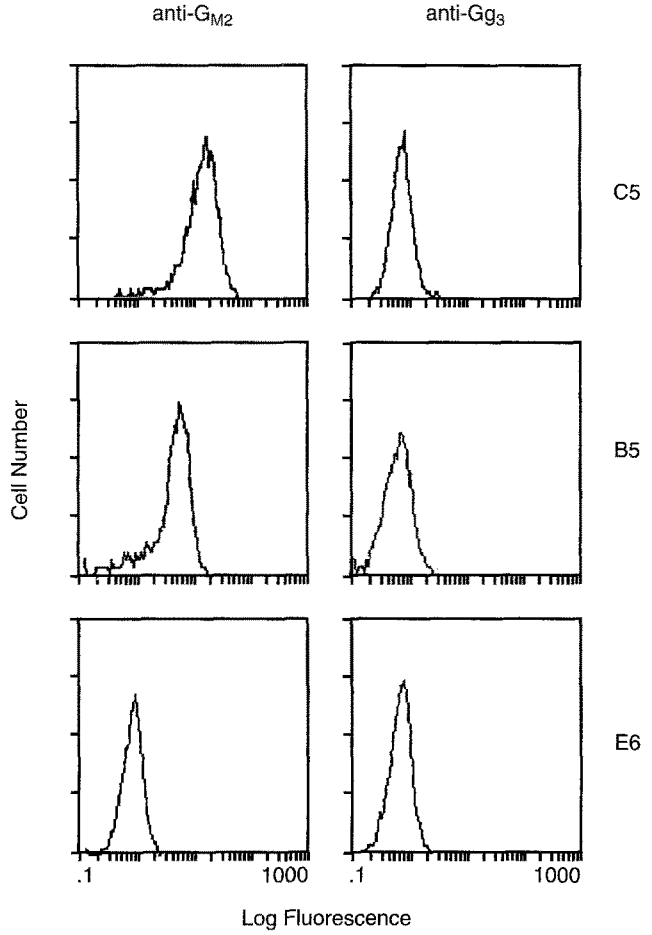

Figure 2. Flow cytometric analysis of GM2 and Gg_3 expression on CHO cells transfected with cloned GalNAcT/myc, GNT/GalNAcT/myc, or Iip33/GalNAcT/myc. Cells were incubated with anti-GM2 antibody 10–11 (left panels) or anti-Gg_3 antibody 2D4 (right panels), followed by staining with FITC-conjugated goat anti-mouse Ig. Top panels, clone C5 of CHO cells transfected with GalNAcT/myc. Middle panels, clone B5 of CHO cells transfected with GNT/GalNAcT/myc. Bottom panels, clone E6 of CHO cells transfected with Iip33/GalNAcT/myc. Relative fluorescence is in arbitrary logarithmic units. Background staining in the first decade was obtained for untransfected CHO cells stained with either anti-GM2 or anti-Gg_3 as well as for primary antibody control staining of transfected clones (data not shown).

synthase activity *in vitro*. Clone E6 had the highest GalNAcT activity *in vitro* among nine clones tested but was negative for GM2 expression (Fig. 2, lower left panel).

Immunolocalization of transfected GalNAcT/myc

Cells transfected with the three forms of GalNAcT/myc were fixed, permeabilized, stained with anti-myc MoAb 9E10, and observed by indirect immunofluorescence microscopy. Anti-myc staining of cells transfected with GNT/GalNAcT/myc (clone B5, Fig. 3, middle left panel) produced an intensely labelled cluster of punctate, perinuclear structures characteristic of the Golgi apparatus. Whereas this pattern was also seen in some cells transfected with GalNAcT/myc (clone C5, Fig. 3, top left panel), in the majority of C5 cells this cluster appeared as a small circle or half moon located over the nucleus. We previously observed this pattern when we stained the trans Golgi elements of fixed CHO cells with NBD-ceramide [28]. This half moon staining pattern of anti-myc was most pronounced in the C5 cells that were shorter, less elongated, and had a smaller area of cytoplasm around the nucleus. Therefore, differences in Golgi staining pattern may reflect an effect of cell shape on Golgi structure.

Anti-myc staining of cells transfected with Iip33/GalNAcT/myc (clone E6, Fig. 3, lower left panel) heavily labelled the nuclear membrane and a reticular cytoplasmic network characteristic of the endoplasmic reticulum.

Immunoelectron microscopy of GalNAcT/myc transfected clone C5 cells revealed strong staining of anti-myc throughout all cisternae of the Golgi stack (Fig. 4C). In contrast staining of cells transfected with GNT/GalNAcT/myc (clone B5, Fig. 4A) was restricted mainly to the medial cisternae, consistent with the medial Golgi location of intact GNT [25, 26]. Staining of cells transfected with Iip33/GalNAcT/myc (clone E6, Fig. 4B) heavily labelled the nuclear membrane and the endoplasmic reticulum, but the Golgi apparatus was not labelled (data not shown). Cryosectioning produced the same distribution patterns as shown here for Lowicryl ultrathin sections.

Assay of GalNAcT activity in vitro and in vivo

Cell clones transfected with the three constructs were assayed for GalNAcT activity *in vitro* as described in Materials and methods. The specific activity (expressed as nmol GM2 produced per mg protein per h) of the extract from GalNAcT/myc transfected clone C5 was 7.8 (mean for triplicate analyses), GNT/GalNAcT/myc transfected clone B5 was 4.6, and Iip33/GalNAcT/myc transfected clone E6 was 4.2. Whereas these *in vitro* activities of clones B5 and E6 were essentially the same and C5 was higher, in striking contrast the patterns of GSL synthesized *in vivo* by clones C5 and B5 were qualitatively similar while the pattern of clone E6 differed markedly. Cell monolayers were labelled with the metabolic precursor [^{3}H] palmitate. Clone C5, transfected with GalNAcT/myc, contained a major GlcCer doublet, a strong doublet co-migrating with ganglioside GM2, and weaker doublets co-migrating with GM3, LacCer, and Gg_3 (Fig. 5, lane 1). Similarly, clone B5, transfected with the GNT/GalNAcT/myc construct, contained a heavy GlcCer doublet, strong doublets co-migrating with LacCer, GM3, and GM2, and a weak doublet in the Gg_3 region (Fig. 5, lane 2). Clone E6, transfected with the Iip33/GalNAcT/myc construct, was indistinguishable from untransfected CHO cells [4], with strong GlcCer and GM3 doublets and a weak LacCer

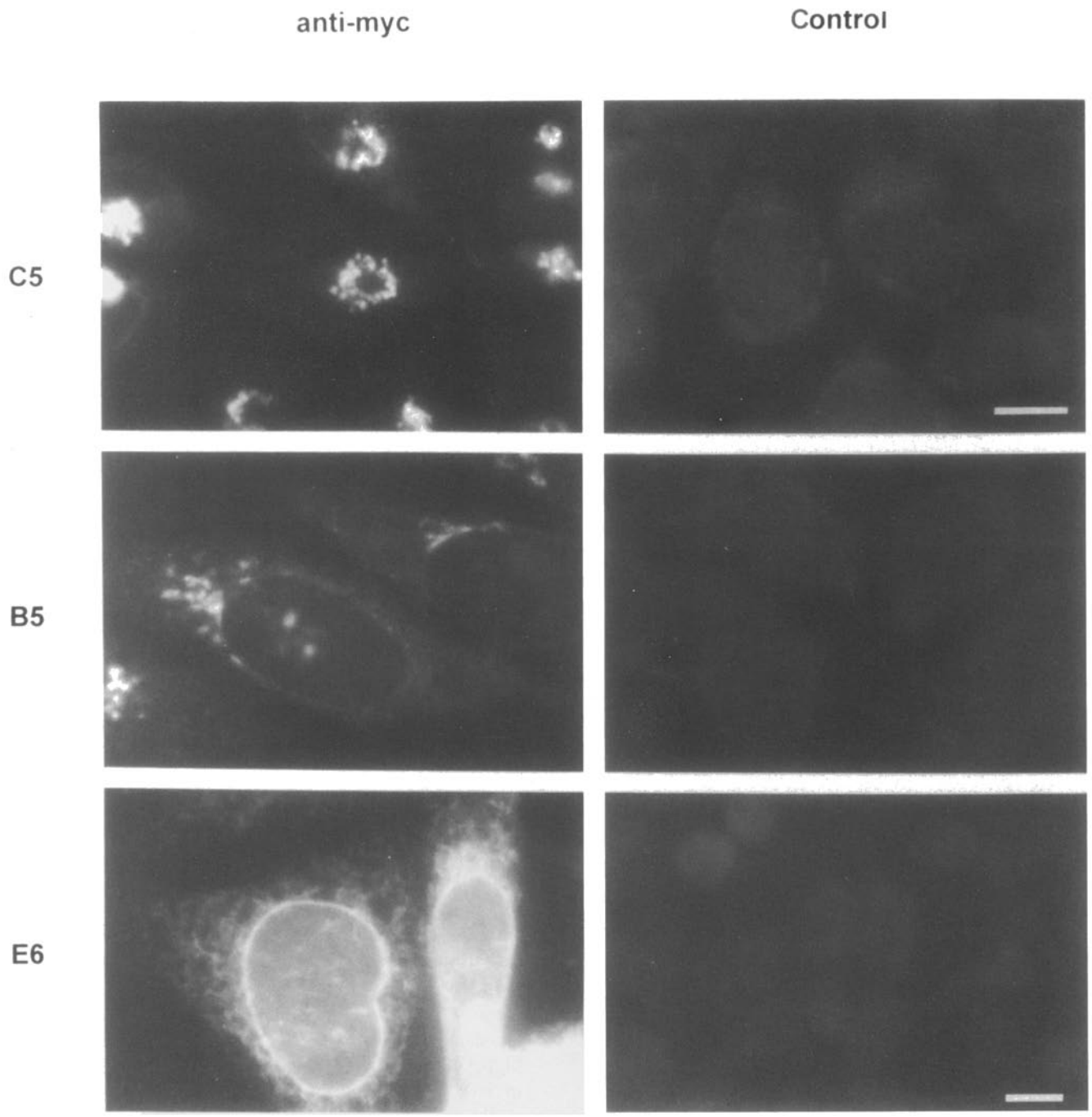

Figure 3. Immunofluorescence analysis of myc epitope expression in transfected cells. Cells were fixed and permeabilized with cold MeOH and then stained with either anti-myc 9E10 (left panels) or control antibodies (right panels) followed by FITC-conjugated goat anti-mouse Ig. Top panels, clone C5 of CHO cells transfected with GalNAcT/myc. Middle panels, clone B5 of CHO cells transfected with GNT/GalNAcT/myc. Lower panels, clone E6 of CHO cells transfected with Iip33/GalNAcT/myc. The control antibody was R24 anti-GD3 for C5 and B5 cells and WKH1 anti-A transferase for E6 cells. Scale bar = 10 μm for all panels except lower right where the scale bar = 20 μm.

doublet but no visible GM2 or Gg_3 species (Fig. 5, lane 3). Thus, the *in vivo* labelling pattern agreed with the anti-GM2 flow cytometry results (Fig. 2) which indicated that cells transfected with either Golgi form of the enzyme, GalNAcT/myc or the GNT/GalNAcT/myc constructs, were capable of synthesizing GM2 *in vivo* whereas cells transfected with the Iip33/GalNAcT/myc did not produce GM2 *in vivo*.

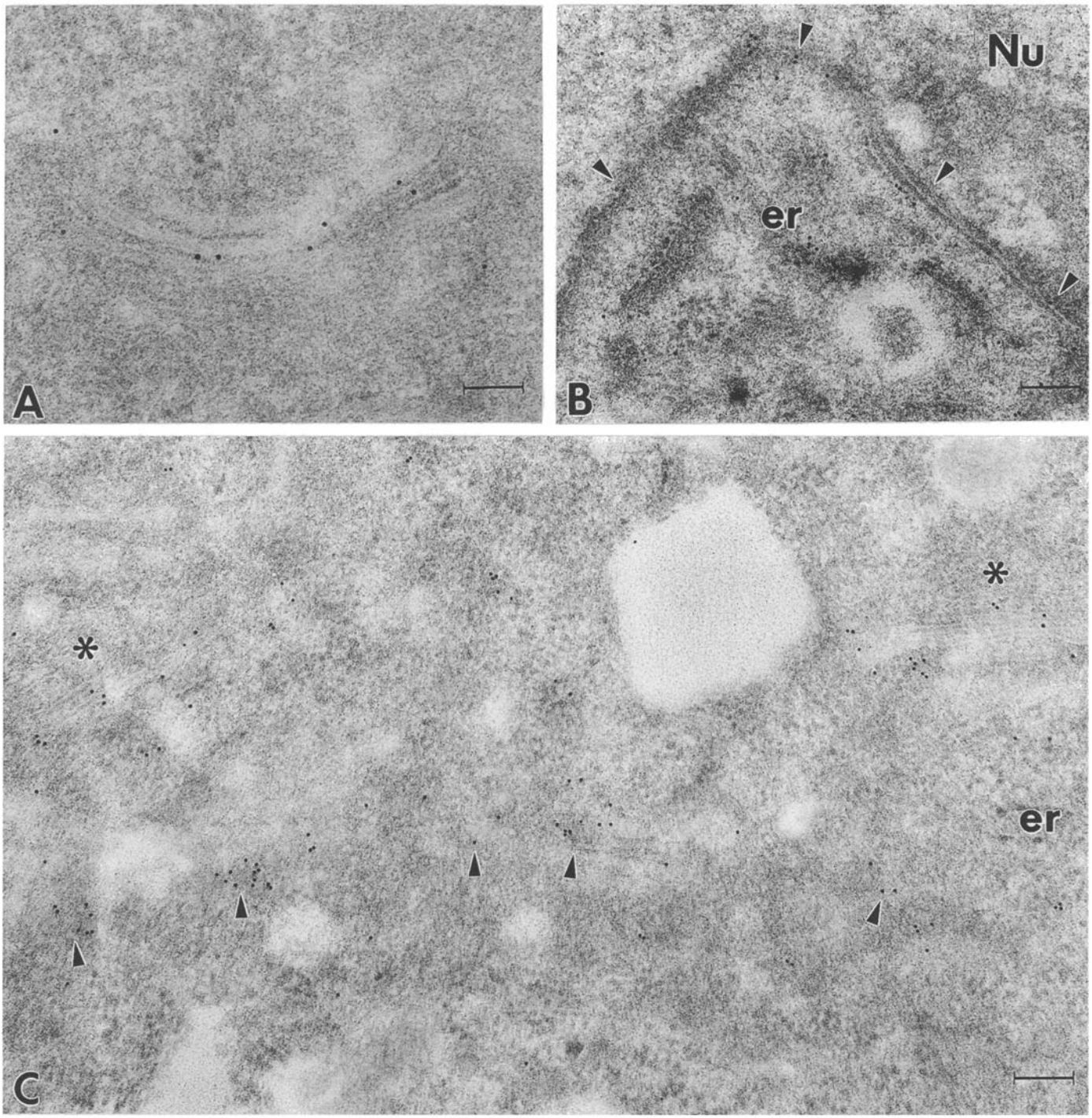

Figure 4. Immunoelectron microscopic localization of myc epitope tagged GalNAcT in transfected cells. Sections were labelled with 9E10 anti-myc followed by protein A-gold (10 nm particle size). All three micrographs are from cells embedded at low temperature in Lowicryl K4M. A, GNT/GalNAcT/myc (clone B5) transfected cells. Gold particle label is restricted to the middle cisternae of the Golgi apparatus; there are at least two unlabelled cisternae visible on each side of the labelled cisternae. B, Iip33/GalNAcT/myc (clone E6) transfected cells, labelling is restricted to the outer membrane of the nuclear envelope (arrowheads) and to the rough endoplasmic reticulum. Note that the nucleus (Nu) is devoid of gold particle label. C, GalNAcT/myc (clone C5) transfected cells, gold particle label is widely distributed throughout the Golgi apparatus cisternal stack (asterisks) and within cisternal portions emanating through the cytoplasm (arrowheads). er, rough endoplasmic reticulum. Magnification × 95 000 (A); × 65 000 (B); × 58 000 (C). Bar = 0.10 μm (A); 0.15 μm (B); 0.17 μm (C).

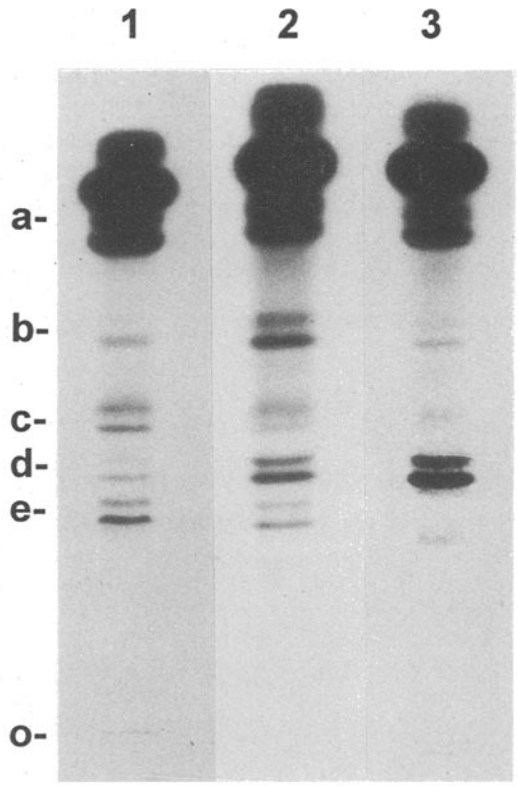

Figure 5. Glycosphingolipid patterns of CHO cells after stable transfection with three GalNAcT constructs. Cells were labelled with [^{3}H] palmitate, harvested, extracted and GSL purified and analysed by high performance thin layer chromatography (HPTLC) in solvent A. The plate was sprayed with EN^{3}HANCE and exposed to X-ray film. Lane 1, clone C5 of CHO cells transfected with GalNAcT/myc; lane 2, clone B5 of CHO cells transfected with GNT/GalNAcT/myc; and lane 3, clone E6 of CHO cells transfected with Iip33/GalNAcT/myc. GSL standards: a, GlcCer; b, LacCer; c, Gg_3; d, GM3; and e, GM2.

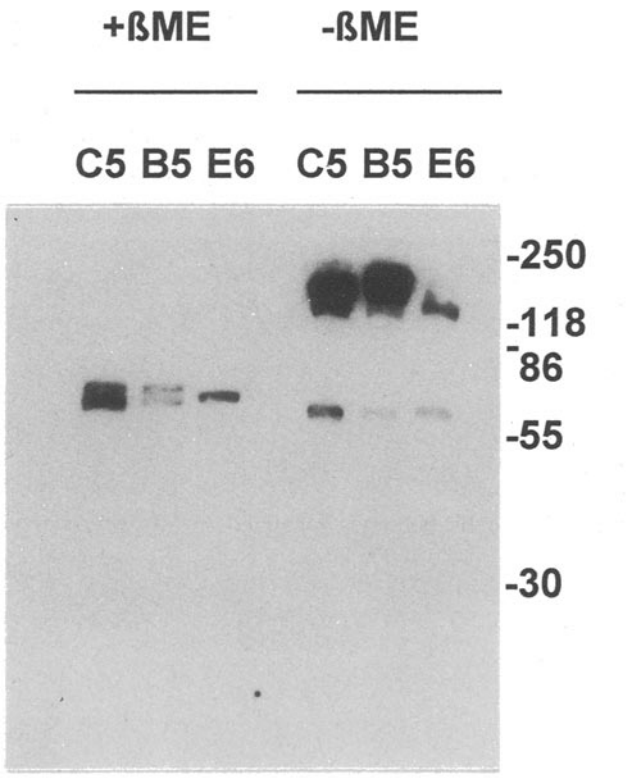

Figure 6. Western blotting with anti-myc to characterize the molecular forms of GalNAcT/myc present in transfected cells. Cells were extracted with detergent, the extracts fractionated by SDS-PAGE, Western blots stained with anti-myc 9E10, and bands visualized by the ECL technique. Three lanes on the left, 5% β-mercaptoethanol (β-ME) in the SDS-PAGE sample buffer; three lanes on right, no reducing agent in the sample buffer. Cell extracts were from: clone C5 of CHO cells transfected with GalNacT/myc; clone B5 of CHO cells transfected with GNT/GalNAcT/myc; and clone E6 of CHO cells transfected with Iip33/GalNAcT/myc.

Western blotting of cellular extacts

Extracts from cells transfected with the three forms of GalNAcT/myc were analyzed by Western blotting with anti-myc. In the presence of reducing agent, the major anti-myc reactive species present in GalNAcT/myc transfected clone C5 (Fig. 6, left lane) and GNT/GalNAcT/myc transfected clone B5 (Fig. 6, second lane from the left) migrated as doublets with the lower band having an apparent molecular weight of 67 000. This value is consistent with the molecular weight of 58 881 predicted from the cloned cDNA sequence of GalNAcT and the presence of three potential sites for N-linked glycosylation [3]. No bands were detected when untransfected CHO extracts were Western blotted with anti-myc (data not shown). Iip33/GalNAcT/myc transfected clone E6 produced a single anti-myc band in the presence of reducing agent (Fig. 6, third lane) which migrated slightly slower than the lower band in C5 and B5 cells. Preliminary results suggest that the upper band is a sialylated form of the lower band (E. Jaskiewicz, unpublished observation). In the absence of reducing agent in the SDS-PAGE sample buffer, cells transfected with all three constructs produced major, broad bands migrating above 118 kDa (Fig. 6, right three lanes) plus a minor band co-migrating with the lower band obtained in the presence of reducing agent in clones C5 and B5. These findings suggest that GalNAcT forms a disulfide bonded species the size of which is consistent with it being at least a homodimer. Intermolecular disulfide bonds must be forming with Cys residues in the lumenal domain of GalNAcT as GNT has no Cys in the cytoplasmic or transmembrane domains [7].

Discussion

The ceramide backbone of sphingolipids is synthesized in the ER (reviewed in [29]). The next step for synthesis of

most GSL, namely GlcCer synthesis, occurs on the cytosolic surface of Golgi membranes [30]. One possibility for the subsequent steps of GSL synthesis is that maturation of oligosaccharide chains occurs as the GSL proceed through successive Golgi cisternae. This model implies that the glycosyltransferases responsible for the subsequent steps of GSL glycosylation might be compartmentalized in the order in which they act. Support for compartmental separation of GSL specific glycosyltransferases has come from subcellular fractionation studies [31–33], transport inhibitor studies ([28] and reviewed in [29]), and analysis of the kinetics of GSL synthesis [34]. The present report describes the first immunoelectron microscopic localization of a GSL specific glycosyltransferase. The results of the present study indicate that GalNAcT tagged with the myc epitope and transfected into CHO cells becomes localized in all cisternae of the Golgi stack (Fig. 4C). These findings do not support the hypothesis that GSL glycosyltransferases are restricted to specific Golgi subcompartments. Previous studies with the transport inhibitor Brefeldin A (BFA) indicated that GalNAcT could not act on its GSL substrates in the presence of the drug [28, 35]. One explanation for these findings was that GalNAcT was located beyond the point of the BFA block, perhaps in the TGN, and, therefore, could not act on its substrates which remained in a redistributed Golgi-ER mixed system in the presence of the drug. The present immunoelectron microscopic localization results do not support this explanation and suggest that BFA blocks GalNAcT action *in vivo* by another mechanism.

Several issues must be addressed concerning the use of cells transfected with epitope-tagged glycosyltransferases for enzyme localization studies. First, cell type and species differences could lead to anomalous results when expressing a human gene in hamster CHO cells [36]. In fact glycosyltransferases have been localized to different Golgi compartments of two cell types within the same tissue [37]. Therefore, our results on the location of GalNAcT in CHO cells may not apply to all other cell types. Second, the myc epitope could alter GalNAcT function or its Golgi retention. We positioned the ten amino acid myc epitope [9] at the C terminus of GalNAcT, as far as possible from probable retention signals in the transmembrane domain and flanking regions. Nilsson *et al.* [38] found that a similar tagging of GNT had no apparent adverse effect on enzyme activity, Golgi retention, or structure of the Golgi apparatus itself. Third, overexpression of certain glycosyltransferases following transient transfection resulted in those enzymes being localized not only to the Golgi but to the ER as well [39]. In contrast, a recent report indicates that following stable expression, several enzymes that were highly overexpressed remained localized to the same compartments as the endogenous enzyme [40]. In the present study, transfected GalNAcT is expressed at levels at least four-to-eight-fold higher than that which we previously found in untransfected mouse lymphoma cells [4] which express this enzyme endogenously. However, there is no evidence by immunofluorescence or immuno EM for retention of either GalNAcT/myc or GNT/GalNAcT/myc in the ER (Figs 3 and 4). In fact the latter construct was localized primarily to the medial Golgi cisternae, in agreement with previous reports about the location of endogenous GNT in untransfected cells [25]. Also, we have recently obtained additional clones of GalNAcT/myc transfected cells which contain approximately eight-fold less GalNAcT activity than clone C5; immunofluorescence staining with anti-myc of these cells produced the identical pattern as we reported here for clone C5 (R. Bassi *et al.*, unpublished observation). We conclude that the data we have obtained from overexpressed, myc tagged GalNAcT provides a valid indication of the location of this enzyme at least in CHO cells.

The GSL pattern of CHO cells transfected with GalNAcT/myc and GNT/GalNAcT/myc (Fig. 5) is similar to that which we previously reported for CHO cells stably transfected with GalNAcT [4] in that GM2 was the major complex GSL product produced. In contrast, no GM2 or Gg_3 was produced in cells transfected with Iip33/GalNAcT/myc even though the *in vitro* GalNAcT activity for these cells was the same as in GNT/GalNAcT/myc transfected cells. The immunolocalization results showed that the Iip33/GalNAcT/myc product was restricted to the ER (Figs 3 and 4). Therefore, these results indicate that GM2 and Gg_3 products cannot be made if GalNAcT is located in the ER. The inability of this enzyme to function in the ER could be due to the absence of UDP-GalNAc in the ER or to other conditions in the ER being inadequate for enzyme function. Alternatively, GalNAcT located in the ER may not have access to its GSL substrates, LacCer and GM3, which are synthesized in the Golgi. In this regard it is interesting to consider whether the double arginine motif of the cytoplasmic domain of Iip33 [41] is a signal for retention in the ER or retrieval from the Golgi compartment to the ER. Whereas the KK motif of type I membrane proteins has been shown to be a retrieval signal [42], no direct evidence has been presented that the RR motif of type II proteins is such a retrieval signal [41]. If Iip33/GalNAcT/myc could recycle into the Golgi, it would seem more likely to come into contact with its GSL substrates than if the enzyme was retained in the ER. Thus, the absence of GM2 and Gg_3 products *in vivo* supports the idea that the enzyme remains in the ER and is not retrieved from a forward compartment.

Several Golgi enzymes have been shown to be homodimers, bound together through their catalytic domains (reviewed in [2]). Exceptions to this rule include

heparan sulfate *N*-deacetylase/*N*-sulfotransferase which is a monomer [43] and β-1,4-galactosyltransferase which forms dimers and large oligomers which are dependent on residues in the transmembrane domain [44]. Our results demonstrate several points about GalNAcT. First, nearly all of this enzyme exists as a disulfide bonded species (Fig. 6), the size of which is consistent with it being a dimer or larger aggregate. Second, the GNT/GalNAcT/myc construct, in which the GalNAcT cytoplasmic and transmembrane domains were replaced by the corresponding domains from GNT, exists as a disulfide bonded species. Unlike GalNAcT which has two Cys residues in the transmembrane domain [3], GNT has no Cys residues in either its cytoplasmic or transmembrane domains [7]. Therefore, the disulfide bonded species produced by the GNT/GalNAcT/myc construct must be forming via Cys residues in the GalNAcT lumenal domain. Finally, the Iip33/GalNAcT/myc form, which is restricted to the ER (Figs 3 and 4), also exists as a disulfide bonded form (Fig. 6). This suggests the possibility that the formation of intermolecular disulfide bonded species which include GalNAcT may be initiated in the ER. However, since the Iip33 cytoplasmic domain contains two cysteine residues [45, 46], we cannot rule out the possibility that these intermolecular species are the result of disulfide bonding between Iip33 cytoplasmic domain cysteine residues.

Acknowledgements

We thank Drs K. Furukawa, P. Stanley, and M. Jackson for plasmids, K.O. Lloyd for antibody 10-11, and S. Peiper and C. Worth for flow cytometry.

This work was supported by NIH grants GM42698 (W.W.Y.), GM46488 (D.J.T.), and DK44332 (D.S.D.); American Cancer Society Faculty Research Award FRA-358 (W.W.Y.) and Junior Faculty Research Award JFRA-552 (D.S.D.), and a Summer Teacher Fellowship from the American Society for Cell Biology (G.E.Z.).

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