GA2/GM2/GD2 synthase localizes to the *trans*-Golgi network of CHO-K1 cells

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UDP-GalNAc:lactosylceramide/GM3/GD3 β-1,4-*N*-acetylgalactosaminyltransferase (GalNAc-T) transforms its acceptors into the gangliosides GA2, GM2 and GD2. It is well established that it is a Golgi-located glycosyltransferase, but its sub-Golgi localization is still unclear. We addressed this question in Chinese hamster ovary K1 cell clones stably transfected with a c-*myc*-tagged version of GalNAc-T which express the enzyme at different levels of activity. In these cell clones we examined the effect of brefeldin A (BFA) on the synthesis of glycolipids (in metabolic-labelling experiments) and on the sub-Golgi localization of the GalNAc-T (by immunocytochemistry). We found that in cell clones expressing moderate levels of activity, GalNAc-T immunoreactivity behaved as the *trans*-Golgi network (TGN) marker mannose-6-P receptor (M6PR) both in BFA-treated and untreated cells, and that BFA completely blocked the synthesis of GM2, GM1 and GD1a. On the other hand, in cell clones expressing high levels of activity and treated with BFA, most GalNAc-T immunoreactivity redistributed to the endoplasmic reticulum, as did the *medial*-Golgi marker mannosidase II, and the synthesis of GM2, GM1 and GD1a was not completely blocked. These results indicate that GalNAc-T is a TGN-located enzyme and that the mechanism that localizes it to this compartment involves steps that, when saturated, lead to its mislocalization to the *cis*-, *medial*- or *trans*-Golgi. Changes of Golgi membrane properties by modification of local glycolipid composition due to the activity of the expressed enzyme were not the main cause of mislocalization, since it persists when glycolipid synthesis is inhibited with D,L-*threo*-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol-HCl.

Key words: Golgi complex, glycolipid glycosyltransferases, Golgi retention, brefeldin A.

INTRODUCTION

Ganglioside glycosylation is carried out by the sequential transfer of sugar moieties from the corresponding sugar nucleotide donor to the proper glycolipid acceptor by glycosyltransferases bound to membranes of the Golgi complex [2,3]. Simple gangliosides GM3, GD3 and GT3, the precursors of a-, b- and c- series gangliosides respectively, are synthesized by the addition of 1, 2 or 3 molecules of sialic acid to lactosylceramide (LacCer). Complex gangliosides are formed by the addition of GalNAc to simple gangliosides, followed by the addition of Gal and further additions of *N*-acetylneuraminic acid (NeuAc) residues [4]. Thus UDP-GalNAc:lactosylceramide/GM3/GD3 β-1,4-*N*-acetylgalactosaminyltransferase (GalNAc-T) is a key enzyme that controls the balance between expression of simple and complex gangliosides at the cell surface [5].

Information on the mechanisms underlying retention of glycolipid glycosyltransferases in the Golgi complex and on their localization in the different sub-Golgi compartments is still scarce. Drugs affecting intra-Golgi transport [6–8] or distal Golgi function [9–11] block the synthesis of GalNAc-bearing gangliosides [12] but not that of simple gangliosides (GM3,GD3), in both neural and non-neural cells. These results indicate that GalNAc-T does not act in proximal (*cis*-, *medial*- and *trans*-) Golgi compartments that sustain the synthesis of simple gangliosides. On the other hand, results of *in vitro* labelling of endogenous glycolipid acceptors bound to the Golgi membranes indicate that the activities for synthesis of simple gangliosides extend also to the distal [*trans*-Golgi network (TGN)] Golgi, and seem to depend on the provision of glucosylceramide (GlcCer) from the proximal Golgi, via vesicular transport [13]. Results of subfractionation of the Golgi complex by centrifugation procedures also point to an enrichment [14,15], or to an almost complete segregation [16], of GalNAc-T to the distal Golgi. However, direct examination of the sub-Golgi location of this enzyme by immunoelectron microscopy in Chinese hamster ovary (CHO) cells transfected with a c-*myc*-tagged form of GalNAc-T revealed its presence also in the proximal Golgi [17].

In this work, we examined the sub-Golgi location of a c-*myc*-tagged form of GalNAc-T in stably transfected clones of CHO-K1 cells expressing different levels of enzyme protein and activity. Results of immunocytochemical, enzymic and metabolic-labelling studies indicate that GalNAc-T localizes to the TGN and that the mechanism that localizes it to this compartment involves steps that when saturated lead to its mislocalization to the *cis*-, *medial*- or *trans*-Golgi. No evidence was found indicating that mislocalization was due to changes in the glycolipid composition of the membranes caused by the overexpressed enzyme activity.

Abbreviations used: CHO, Chinese hamster ovary; ER, endoplasmic reticulum; GalNAc-T, UDP-GalNAc:lactosylceramide/GM3/GD3 β-1,4-*N*-acetylgalactosaminyltransferase; BFA, brefeldin A; HPTLC, high-performance TLC; GlcCer, glucosylceramide; LacCer, lactosylceramide; NeuAc, *N*-acetylneuraminic acid; ManII, mannosidase II; M6PR, mannose 6-phosphate receptor; Sial-T4, CMP-NeuAc:GA1/GM1/GD1b/GT1c sialyltransferase; Gal-T2, UDP-Gal:GA2/GM2/GD2 galactosyltransferase; PPPP, D,L-*threo*-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol-HCl; TGN, *trans*-Golgi network; gangliosides are named according to [1].

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MATERIALS AND METHODS

c-*myc* GalNAc-T expression plasmid

The 3′-end of the human GalNAc-T cDNA [18] subcloned into the *Xho*I cloning site of the eukaryotic expression vector pCIneo (Promega) was amplified by PCR using the following primers: sense 5′-GCCTTCAGGCAGCTTCTGGTCAG-3′, which hybridizes upstream of the internal *Eco*RI site of GalNAc-T; antisense, 5′-GCTCTAGACTGGGAGGTCATGCAC-3′. The PCR product obtained, a fragment of 1.1 kb with the GalNAc-T stop codon replaced by an arginine codon and with an *Xba*I site added downstream, was digested with *Eco*RI and *Xba*I and the released 0.2 kb fragment was isolated and purified by phenol/chloroform (1:1, v/v). The 0.2 kb fragment was subcloned into pCIneo GalNAc-T digested with *Eco*RI and *Xba*I (6.9 kb) and the resulting construct was opened with *Xba*I. A double-stranded oligonucleotide coding for ten amino acids of human c-*myc* [19] followed by a stop codon (underlined) was generated with the oligonucleotides 5′-CTAGAGAACAGAAACTCATCTCTGAAGAGGATCTGTGAT-3′ and 5′-CTAGATCACAGATCCTCTTCAGAGATGAGTTTCTGTTCT-3′. The double-stranded oligonucleotide was phosphorylated and subcloned into the *Xba*I site of the pCIneo GalNAc-T construct.

Cell culture and transfection

CHO-K1 cells were plated on 100 mm plastic dishes and grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal calf serum, 100 μg/ml penicillin and 100 μg/ml streptomycin. At about 70% confluence, cells were transfected with 8 μg/dish of c-*myc* GalNAc-T cDNA using cationic liposomes (Lipofectamine, Life Technology) according to the manufacturer's instructions. At confluence, cells were dispersed, plated on 100 mm culture dishes and grown in complete minimal medium plus 1 mg/ml geneticin (G-418, Calbiochem). This procedure was repeated four times.

Cell panning and cloning

G-418-resistant cells (8×10^6) were plated on to culture dishes treated with 1.0 μg/ml sterile cholera toxin in PBS overnight at 4 °C and washed twice with 10 ml of sterile PBS. Cells were left to stand for 1 h and non-adherent cells were washed out with sterile PBS. Adhered cells were cultured in the same dish with complete minimal medium plus 1 mg/ml G-418 up to confluence. After two additional runs of panning on cholera toxin-coated dishes, about 80% of cells were positive for c-*myc* immunostaining. c-*myc* GalNAc-T-positive cells were cloned by limiting dilution using cloning cylinders (Sigma).

Determination of GalNAc-T activity

Homogenates of transfected cells in cold 0.25 M sucrose/PBS were centrifuged at 14000 ***g*** for 10 min to obtain a membrane fraction which was used as the enzyme source. The incubation system contained, in a final volume of 20 μl, 400 μM GM3, 100 μM UDP-[^{3}H]GalNAc (200000 c.p.m.), 20 mM $MnCl_2$, 100 mM sodium cacodylate, pH 7.2, 3 mM cytidine 5′-diphosphocholine, 20 μg of membrane fraction protein and 20 μg of Triton CF-54/Tween 80 (2:1, w/w). After 60 min of incubation at 37 °C the reaction was stopped with 1 ml of 5% (w/v) trichloroacetic acid/0.5% (w/v) phosphotungstic acid. The precipitate was pelleted at 10000 ***g*** for 10 min and washed twice with trichloroacetic acid/phosphotungstic acid. Lipids were extracted from the pellet with chloroform/methanol (2:1, v/v), the solvent was evaporated into a vial and the residue was taken in 0.25% (w/v) SDS for radioactivity determination by liquid-scintillation counting.

Metabolic labelling

Cells at 1 day in culture [$(2–3) \times 10^5$ cells/35 mm dish] were labelled with 40 μCi/ml [^{3}H]galactose for 5 h. After washing twice with PBS, cells were scrapped off, pelleted by centrifugation and the pellet was extracted with chloroform/methanol (2:1, v/v). The lipid extract was supplemented with 15 nmol of total bovine brain gangliosides, taken in 0.2 M KOH in methanol and saponified for 3 h at 37 °C. The extract was then passed through a C_{18} column (Sep-Pak Cartridge, Waters, Millipore). The column was washed with 10 ml of methanol/water (1:1, v/v) and the lipid extract was eluted with 0.5 ml of methanol and 2.5 ml of chloroform/methanol/water (60:30:4.5, by vol.). The eluate was subjected to high-performance TLC (HPTLC plates, Merck, Darmstadt, Germany) using chloroform/methanol/0.25% $CaCl_2$ (60:36:8, by vol.) as solvent. Routinely, 15000 c.p.m. was spotted on each lane. Radioactive gangliosides were visualized by fluorography after dipping the plate in 0.4% melted 2,5-diphenyloxazole in 2-methylnaphthalene and exposure to a Kodak Biomax MS radiographic film at −70 °C, usually for 7 days.

Immunocytochemistry

Cells grown on coverslips were fixed for 7 min in methanol at −20 °C. After blocking in 3% (w/v) BSA for 1 h, cells were incubated for 1 h at 37 °C with a 1:2 dilution of mouse anti-c-*myc* monoclonal antibody (CRL 1729 hybridoma supernatant, from ATCC) or with a 1:200 dilution of rabbit anti-mannosidase II antibody (ManII, from K. Moremen, University of Georgia, U.S.A.) or with a 1:200 dilution of rabbit anti-mannose 6-P receptor antibody (M6PR, a gift from Dr. A. Cáceres, INIMEC, Córdoba, Argentina). Secondary antibodies were rhodamine-conjugated donkey anti-mouse IgG antibody (1:1000) and FITC-conjugated donkey anti-rabbit IgG antibody (1:1000). Specimens were mounted with Fluorsave (Calbiochem) and observed in a Zeiss Axioplan fluorescence microscope equipped with a 63×, 1.4 NA oil immersion objective and with a Princeton Instrument Micromax camera controlled with Metamorph imaging software (Universal Imaging Corporation, West Chester, PA, U.S.A.).

Western blots

Eighty micrograms of protein from membrane fractions prepared from the different transfected cell clones was taken in Laemmli sample buffer [20] and subjected to SDS/10%-PAGE under non-reducing and reducing (20% 2-mercaptoethanol) conditions. Proteins were electrophoretically transferred to nitrocellulose membrane for 1 h at 300 mA [21]. For immunoblotting, non-specific binding sites were blocked with 5% (w/v) non-fat dry milk for 1 h at room temperature and washed twice with 0.05% (v/v) Tween in 50 mM Tris/HCl, pH 7.4/200 mM NaCl (TTBS). A 1:2 dilution of c-*myc* hybridoma supernatant (CRL1729) was used, followed by rabbit anti-mouse IgG (1:10000, Sigma) and then by horseradish peroxidase–Protein A (1:15000, Sigma). All incubations were carried out in 0.25% polyvinylpyrrolidone/0.25% BSA/TTBS for 1 h at room temperature, followed by three 15 min washes with TTBS. The

bands were visualized with an enhanced chemiluminescence detection kit (ECL, Amersham) and Kodak BioMax MS X-ray film.

D,L-*threo*-1-Phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol-HCl (PPPP) treatment of cells

Inhibition of glycolipid synthesis with PPPP (Matreya Inc., Pleasant Gap, PA, U.S.A.) was carried out as follows: cell clones in culture were treated for different periods of time with 2 μM PPPP added to the culture medium from a recently prepared 600 μM stock solution. Inhibition of glycolipid synthesis was monitored by pulsing PPPP-treated and control (PPPP-untreated) cells with 40 μCi/ml of [^{3}H]Gal during the last 6 h of culture. To monitor the effect of PPPP on GalNAc-T sub-Golgi localization, treated and control cells were examined for GalNAc-T immunolocalization in the absence and in the presence of brefeldin A (BFA) added 30 min before fixation, as indicated above.

RESULTS

Isolation of cell clones

CHO-K1 cells only express the ganglioside GM3, but acquire the ability to synthesize complex gangliosides (GM2, GM1, GD1a) upon transfection with GalNAc-T cDNA due to the constitutive presence of UDP-Gal:GA2/GM2/GD2 galactosyltransferase (Gal-T2) and CMP-NeuAc:GA1/GM1/GD1b/GT1c sialyltransferase (Sial-T4) [12]. CHO-K1 cells were stably transfected with cDNA encoding a c-*myc*-tagged GalNAc-T. The cell-surface expression of GM1 by transfected cells allowed the enrichment of stable transfectants by panning on *Vibrio cholera* toxin-coated dishes. Subsequent cloning of GM1-expressing cells by limiting dilution led to the isolation of seven clones that bore different levels of GalNAc-T activity, ranging between 0.3 and 3.0 nmol sugar transferred $\cdot h^{-1} \cdot$ mg of protein^{-1}. (Figure 1). Differences in enzyme activity among the different clones were in close agreement with GalNAc-T protein expression levels. The Western blot analysis (Figure 2A) clearly shows that the intensity of the GalNAc-T protein band at 69.5 kDa in clones 3 and 7 is higher (2.2-fold and 8.5-fold respectively, by densitometric analysis) than in clone 1. When run on SDS/PAGE under non-reducing conditions, most of the enzyme appears at a higher molecular mass (approx. 140 kDa) in all clones analysed (Figure 2B). It has been shown that this enzyme exists in the form of a homodimer in transfected CHO cells [22].

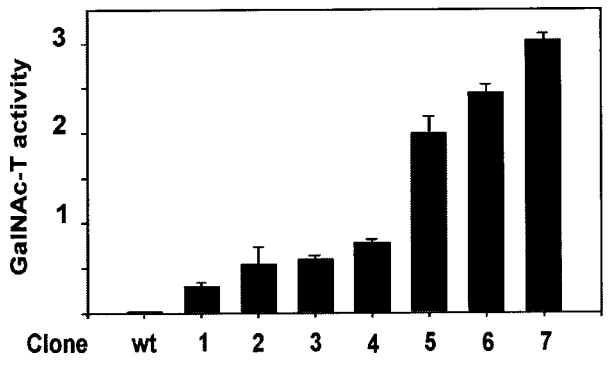

Figure 1 GalNAc-T activity of stably transfected *c-myc* GalNAc-T clones

GalNAc-T activity towards exogenous GM3 was determined as described in the Materials and methods section. Activity is expressed in nmol of GalNAc transferred $\cdot h^{-1} \cdot$ mg of protein^{-1}. wt, mock-transfected CHO-K1 cells resistant to G-418. Results are means $\pm$ S.D. of three independent experiments each performed in triplicate.

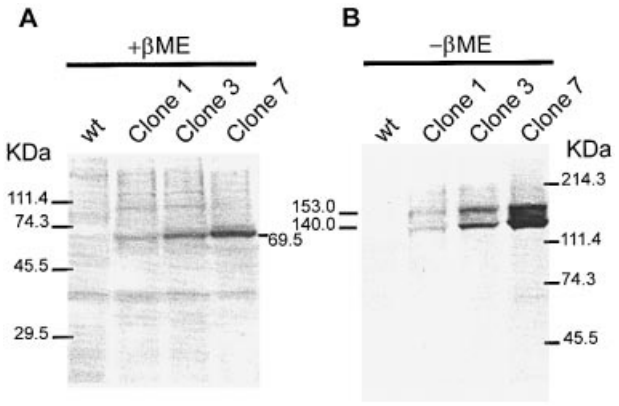

Figure 2 Western blot analysis of clones with low (clone 1), moderate (clone 3) and high (clone 7) GalNAc-T activity levels

Membrane proteins were separated by SDS/PAGE in the presence (**A**) and in the absence (**B**) of 20% 2-mercaptoethanol (βME), electrotransferred, and blotted with anti-c-*myc* antibody as indicated in the Materials and methods section. (**A**) SDS/10%-PAGE; (**B**) SDS/7.5%-PAGE. The positions of molecular-mass markers are indicated.

Immunocytochemical localization of GalNAc-T

In all the clones analysed, GalNAc-T localized to the well-defined, compact immunoreactive area of the Golgi complex defined by the *medial*-Golgi marker ManII [23], as is shown in Figures 3(A) and 3(B) for clone 3. GalNAc-T immunoreactivity was also present in areas immunoreactive for the TGN/endosomal marker M6PR [24], which showed a dotted, vesicular pattern overlying a more dense, defined zone (Figures 4A and 4B). No immunoreactivity was observed at the cell surface in any of these clones. Treatment of cells with BFA was used as a tool to examine the sub-Golgi localization of the enzyme. Owing to its effect of inhibiting the assembly of cytosolic coat proteins on target membranes, BFA inhibits vesicle formation [25,26] and leads to redistribution of proximal (*cis*-, *medial*- and *trans*-) Golgi membranes into the endoplasmic reticulum (ER), and of distal (TGN) membranes to the endosomal membrane system [27].

In clones expressing low or moderate levels of GalNAc-T activity (clones 1–3), BFA did not essentially modify the pattern of GalNAc-T immunolocalization, which remained condensed in the Golgi complex area (Figure 3D). This was in contrast with the effect of BFA on the immunoreactivity pattern of the *medial*-Golgi ManII, which appeared reticular, indicative of its redistribution into the ER (Figure 3C). Conversely, GalNAc-T followed the redistribution pattern of M6PR immunoreactivity, which in BFA-treated cells appeared condensed in a well-defined juxtanuclear region, providing evidence of the fusion between TGN structures and the endosomal system induced by BFA (Figures 4C and 4D). These results indicate that GalNAc-T localizes to the TGN of these cells. The response of GalNAc-T to BFA treatment was different in clones expressing high levels of activity (clones 5–7), in which most GalNAc-T immunoreactivity redistributed into the ER and was partly condensed in fused TGN/endosomal structures (Figures 5A and 5B).

A statistical analysis of the immunocytochemical data shown in Figures 3, 4 and 5 is given in Table 1. The mean integrated absorbance ratios of fluorescence intensity of GalNAc-T, ManII

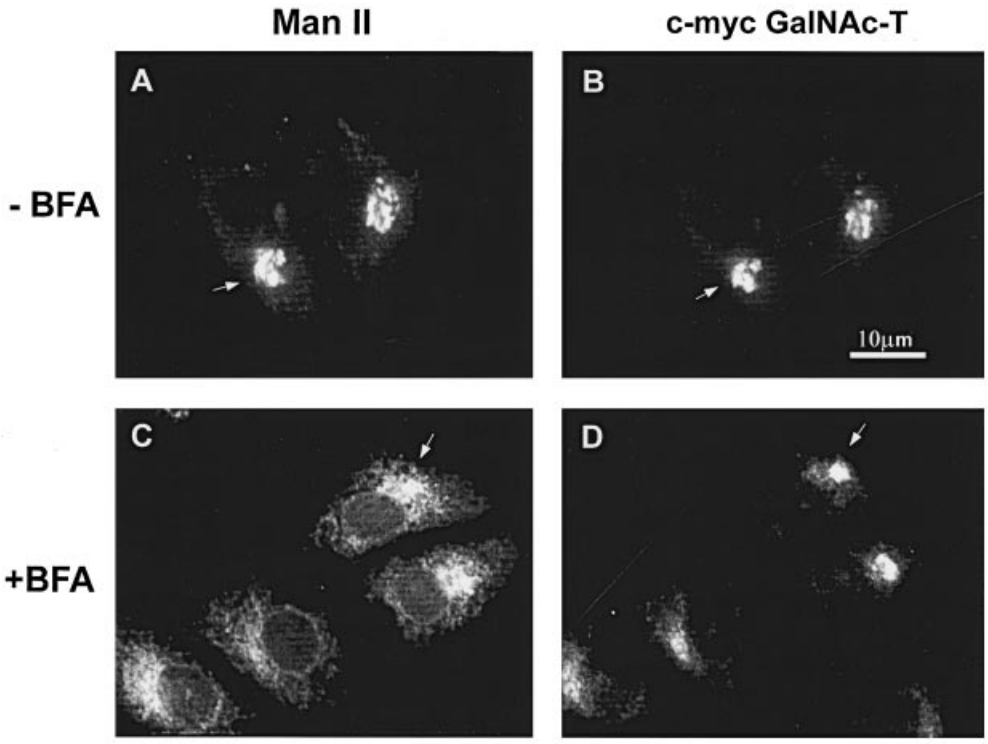

Figure 3 Effect of BFA on the immunolocalization of ManII and *c-myc* GalNAc-T

Stably transfected CHO-K1 cells (clone 3) were fixed, permeabilized with cold methanol and doubly immunostained for ManII (**A** and **C**) and c-*myc* GalNAc-T (**B** and **D**). In (**C** and **D**), cells were treated with 1 μg/ml BFA for 30 min at 37 °C before fixation. For other details, see the Materials and methods section. Arrows indicate localization of ManII and c-*myc* GalNAc-T.

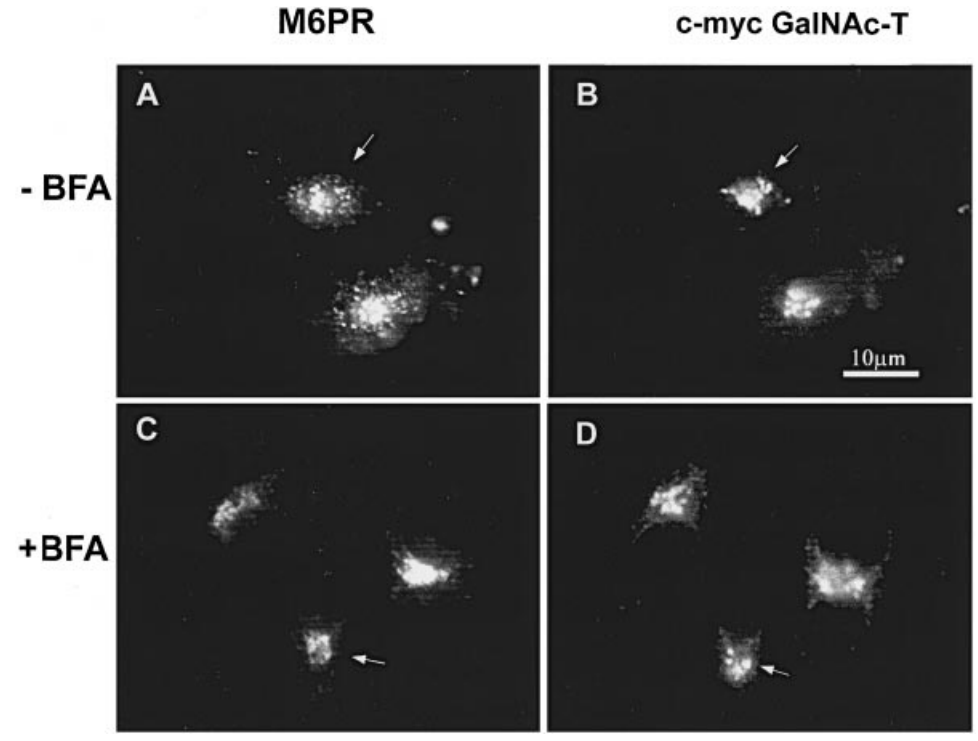

Figure 4 Effect of BFA on the immunolocalization of M6PR and c-*myc* GalNAc-T in cell clone 3

Cells were fixed and permeabilized with cold methanol and doubly immunostained for M6PR (**A** and **C**) and c-*myc* GalNAc-T (**B** and **D**). In (**C** and **D**), cells were treated with 1 μg/ml BFA for 30 min at 37 °C before fixation. For other details, see the Materials and methods section. Arrows indicate localization of M6PR and c-*myc* GalNAc-T.

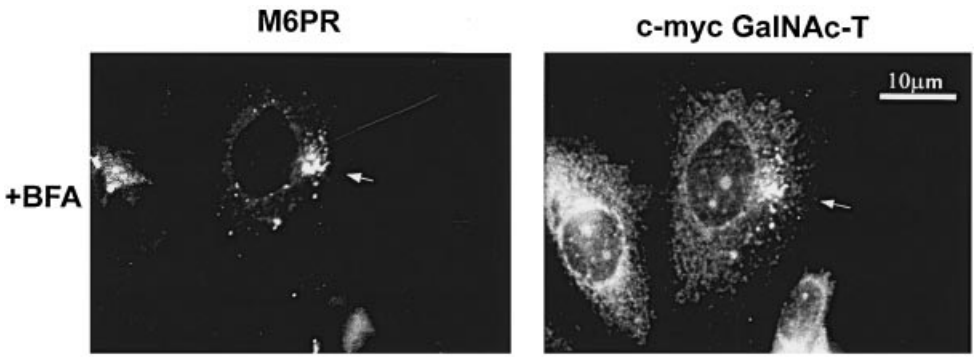

Figure 5 Effect of BFA on the immunolocalization of M6PR and c-*myc* GalNAc-T in cell clone 7

Cells treated with 1 μg/ml BFA for 30 min at 37 °C were processed for M6PR and c-*myc* GalNAc-T immunocytochemistry as indicated in the legends of Figures 3 and 4. Arrows indicate localization of M6PR and c-*myc* GalNAc-T.

Table 1 Effect of BFA on the whole cell:Golgi complex fluorescence ratio for ManII, M6PR and c-*myc* GalNAc-T

Cells immunostained as indicated in Figures 3, 4 and 5 were analysed for the ratio of cell:Golgi region fluorescence intensity using the Metamorph 3.0 Software. Integrated absorbance (IA) = Σ[Gv × Log(Max Gv)/(Gv)]. IA ratio = IA whole cell/IA Golgi complex. (Gv = grey value, referring to the grey colour intensity value of the sample). Values are means ± S.D. for $n = 20$.

	Integrated absorbance ratio					
	ManII		M6PR		c-*myc* GalNAc-T	
	− BFA	+ BFA	− BFA	+ BFA	− BFA	+ BFA
CHO-K1(wt)	10.7 ± 4.8	74.8 ± 18.1	13.8 ± 4.8	11.4 ± 6.0	–	–
Clone 3	8.1 ± 4.0	56.7 ± 22.3	13.5 ± 4.44	10.7 ± 5.7	8.2 ± 1.8	12.2 ± 5.1
Clone 7	8.4 ± 4.1	48.3 ± 20.0	12.8 ± 3.8	9.9 ± 5.5	8.3 ± 2.0	44.2 ± 10.4

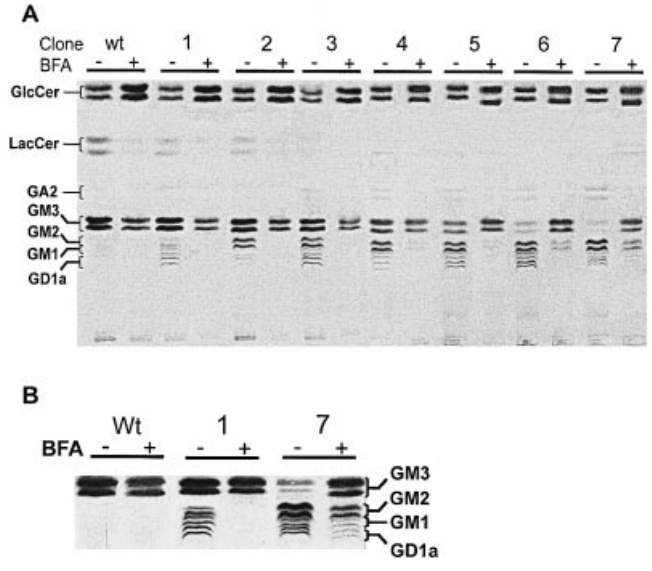

Figure 6 Effect of BFA on metabolic labelling of glycolipids of stably transfected c-*myc* GalNAc-T clones

(**A**) Cell clones 1–7 were metabolically labelled for 5 h in the presence or absence of 1 μg/ml BFA added 30 min before the addition of [^{3}H]Gal. Lipid extracts were prepared, purified, chromatographed and subjected to fluorography as described in the Materials and methods section. (**B**) The pattern of labelling of the indicated clones after longer exposure times is shown.

and M6PR in the whole cell relative to the Golgi regions were similar for clones 3 and 7. BFA led to an increase in this ratio for ManII, but practically did not change it for M6PR in any of the clones. However, the same ratio increased nearly 4-fold for GalNAc-T in clone 7 compared with clone 3. Taken together, results in Figures 3 and 4 and Table 1 suggest that in clone 7 (and also in clones 5 and 6, not shown), but not in clone 3, a fraction of GalNAc-T localizes in the proximal (*cis*-, *medial*- or *trans*-) Golgi compartment.

Radioactive pattern of gangliosides synthesized by different clones

The observation that different clones respond to BFA with different patterns of redistribution of GalNAc-T immunoreactivity prompted us to examine these clones with regard to their sensitivity to BFA in metabolic-labelling experiments. Clones were labelled from [^{3}H]Gal for 5 h in the absence or presence of BFA and the radioactive lipids were isolated, separated by HPTLC and subjected to fluorography. The radioactive-labelling pattern of the mock-transfected cells was dominated by GlcCer and GM3, with a small amount of LacCer and the absence of complex gangliosides (Figure 6A, wt). Lipid extracts from GalNAc-T-transfected clones showed the expected GM2 radioactive band, low amounts of GA2 and, due to the presence of constitutive Gal-T2 and Sial-T4 activities in these cells [12], they also showed radioactive GM1 and GD1a. It was

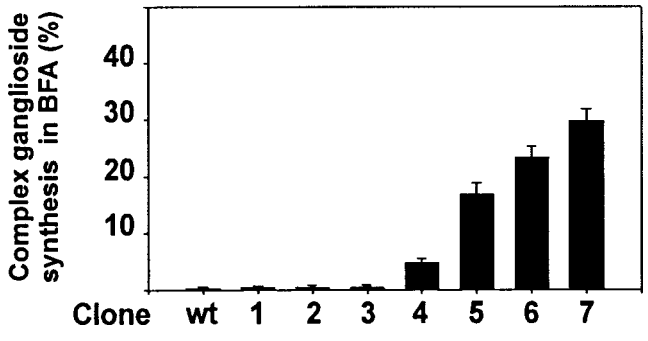

Figure 7 Percentage synthesis of complex gangliosides in the presence of BFA in the different cell clones

Values were obtained by densitometric scanning of the autoradiographic film shown in Figure 6. The percentage of complex ganglioside synthesis was calculated from the ratio (GM2 + GM1 + GD1a)/(GM3 + GM2 + GM1 + GD1a + GA2). Results are means ± S.D. of two independent experiments.

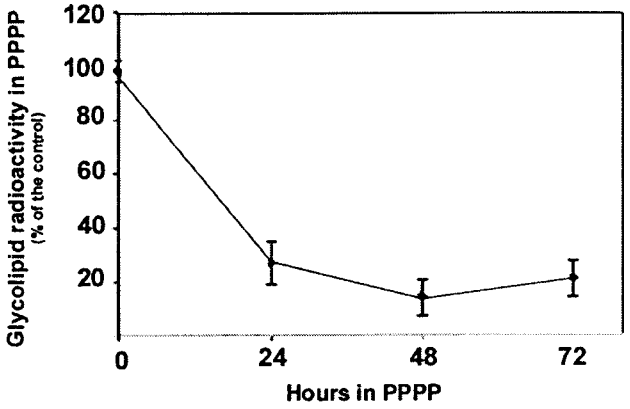

Figure 8 Inhibition of glycolipid synthesis by PPPP

Clone 7 cells were cultured in complete medium without (control cells) or with the addition of 2 μM PPPP during the indicated periods of time. Six hours before harvesting the cells were metabolically labelled from [^{3}H]Gal added to the culture medium (40 μCi/ml). Lipids were extracted and purified and radioactivity was determined as indicated in the Materials and methods section. Values are mean ± S.D. of two independent experiments.

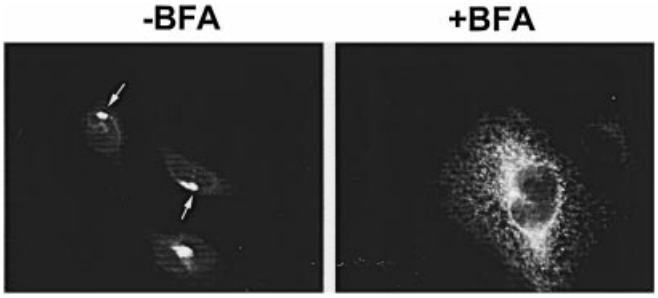

Figure 9 Immunolocalization of c-*myc* GalNAc-T in cells treated with PPPP

Clone 7 cells treated for 72 h with 2 μM PPPP were incubated in the presence or absence of 1 μg/ml BFA for 30 min before fixation and processed for immunofluorescence as described in the Materials and methods section.

evident from the radioactive patterns that, in the different clones, between 10% and 90% of GM3 was consumed to be converted into more complex gangliosides, in correlation with their GalNAc-T activity shown in Figure 1.

Figure 6(A) also shows the pattern of radioactive glycolipids synthesized by the different clones in the presence of BFA. BFA inhibited the synthesis of complex gangliosides and led to a consistent accumulation of GlcCer radioactivity [12]. It was clear, however, that a variable capacity for synthesis of complex gangliosides (GM2, GM1 and GD1a) remained in clones with high-efficiency of conversion of GM3 into complex gangliosides (Figure 6B), as is illustrated in Figure 7. The synthesis of complex gangliosides was completely inhibited by BFA in clones consuming up to 50% of GM3 for conversion into complex gangliosides (clones 1, 2 and 3), whereas the inhibition was incomplete in clones consuming more than this percentage (clones 4, 5, 6 and 7). These results are in line with those in Figures 3, 4 and 5, and indicate that most of the synthesis of GM2 occurred in the distal Golgi (TGN) in clones expressing moderate levels of GalNAc-T. In cell clones expressing higher levels of this enzyme, about 30% of GM2 synthesis persisted in the presence of BFA, indicating the presence of GalNAc-T in the proximal Golgi subcompartments (*cis*-, *medial*- or *trans*-Golgi) of these cells.

Effect of inhibiting glycolipid synthesis on GalNAc-T sub-Golgi localization

The results shown in Figure 6 indicate that the ganglioside composition of the membranes was different in the different cell clones. It was considered a possibility that the presence of GalNAc-T in the proximal Golgi in cell clones expressing high levels of activity was due to local changes in the glycolipid composition of the Golgi membranes; the decrease in GM3 or increase in complex gangliosides shown in Figure 6 may have reduced the GalNAc-T retention capacity of the distal Golgi or increased it in the proximal Golgi, thus causing its mislocalization to proximal Golgi compartments, as has been reported for the M protein of the bronchitis virus [28]. To examine this possibility, glycolipid synthesis was inhibited with PPPP, a potent inhibitor of ceramide glucosyltransferase and hence of synthesis of GlcCer and of more complex glycolipids [29]. Exposure of clone 7 cells to 2 μM PPPP in the culture medium for 1–3 days led to a gradual decrease in [^{3}H]Gal incorporation into glycolipids, reaching about 15% of that in controls after 72 h (Figure 8). However, the immunocytochemical behaviour of GalNAc-T immunoreactivity, in the absence and in the presence of BFA, was indistinguishable between PPPP-treated and control cells (Figure 9). The simplest interpretation of this result is that a local change in glycolipid composition of Golgi membranes is not the main cause of GalNAc-T mislocalization to the proximal Golgi compartment.

DISCUSSION

We have analysed the sub-Golgi location of GalNAc-T in CHO-K1 cell clones stably transfected with a tagged version of the enzyme that expresses different levels of activity. In all these clones, GalNAc-T localized in the Golgi complex, providing evidence that Golgi retention is efficient, at least in the expression-range examined. However, from results with BFA it was evident that the sub-Golgi location of GalNAc-T depended on the level of its expression. The pattern of GalNAc-T immunostaining after BFA treatment of clones expressing moderate levels of activity (clones 1–3) followed the pattern of immunostaining of the endogenous TGN marker M6PR, and differed from that of

the endogenous *medial*-Golgi marker ManII. The simplest interpretation of these results is that in these cell clones GalNAc-T was concentrated in the distal (TGN) part of the Golgi complex. Consistent with this, in the same clones BFA produced a complete block in the synthesis of the complex gangliosides GM2, GM1 and GD1a, as occurs in other cell systems that naturally express GalNAc-T activity [6–8].

In clones expressing higher levels of GalNAc-T (clones 5–7), the pattern of GalNAc-T immunostaining in BFA was more similar to that of the *medial*-Golgi marker ManII than that of the TGN marker M6PR (Figure 5), indicating a proximal Golgi location of most of the enzyme. Since proximal Golgi immunostaining was observed at 30 min of BFA treatment, the possibility was disregarded that this was due to accumulation of GalNAc-T recently synthesized in the ER and in transit to the Golgi complex. Considering that the activity for synthesis of GM2, and even of GM1 and GD1a, persists in these clones in the presence of BFA, a plausible interpretation is that a fraction of GalNAc-T was functionally coupled to enzymes for synthesis of simple gangliosides in the proximal Golgi compartment, allowing their progression to more complex gangliosides *in vivo*. Again, possible accumulation of enzyme in transit from the ER was disregarded because cells metabolically labelled with [^{3}H]Gal in the presence of BFA and cycloheximide (100 μg/ml) showed the same radioactive glycolipid pattern (results not shown).

BFA blocks the TGN incorporation of [^{35}S]sulphate into GalCer [30] and the TGN synthesis of Gb3 and nLc4 [31]. In addition, BFA partially inhibits reactions of the *trans*-Golgi and TGN [32] that lead to terminal glycosylation of N-linked glycoprotein oligosaccharides, such as sialylation and galactosylation [33]. Concerning gangliosides, in almost all cell lines so far examined, BFA uncoupled ganglioside synthesis beyond GM3, GD3 and GT3, suggesting that the concentration of endogenous GalNAc-T in the TGN membranes of these cells would be below its level of saturation, with no GalNAc-T in the proximal Golgi. A survey of the literature allows the calculation that with few exceptions, GalNAc-T activity was lower than 0.1 fmol·h^{-1}·cell^{-1} in different cell lines and tissues [17,34–36], a value that approximates the activity at the threshold found in this work for mislocalization of GalNAc-T to the proximal Golgi ($\cong$ 0.08 fmol·h^{-1}·cell^{-1}).

Overexpression of Golgi-resident type II membrane proteins results in patterns of mislocalization that depend on each protein and probably also on the cell type. Golgi retention of a glycoprotein α2,6-sialyltransferase cannot be saturated, even at expression levels about 100-fold higher than normal [32]. On the contrary, human galactosyltransferase is found located in the distal Golgi when overexpressed 2–4-fold in L cells, or in the *medial*-Golgi or the ER when overexpressed in COS-7 cells [37]. On the other hand, overexpression of GPP130, a *cis*/*medial*-Golgi protein, leads to its partial mislocalization to endocytotic vesicles [38]. In the case of GalNAc-T, in no clone have we observed localization of the enzyme to post-Golgi structures. Rather, results show that at high levels of expression the enzyme mislocalizes to proximal Golgi compartments, which is consistent with the wide distribution of GalNAc-T throughout the Golgi stacks observed by immunoelectronmicroscopy in CHO cells overexpressing this activity [17].

Although the mechanisms of retention of glycosyltransferases in particular sub-Golgi compartments are still under investigation, the relevance of interactions with other transferases or with the Golgi membrane by means of their transmembrane domain has been recognized ([39] and references cited therein). In the present case, changes in the Golgi membrane properties, as a result of either the increase in complex gangliosides or decrease in GM3 due to the activity of the overexpressed enzyme, seems not to be the main cause of its mislocalization, since it persists upon inhibition of glycolipid synthesis with PPPP. In any case, mislocalization to proximal Golgi compartments of overexpressed GalNAc-T not only imposes caution on the use of the transfection approach to study the topology of Golgi membrane proteins, but also indicates that some steps of the retention mechanism may be saturated when the threshold of protein loading is reached.

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