Ganglioside glycosyltransferases organize as multienzyme complexes that localize in different sub-Golgi compartments. Here we studied whether in CHO-K1 cells lacking CMP-NeuAc:GM3 sialyltransferase (SialT2), the sub-Golgi localization of UDP-Gal:glucosylceramide $\beta$1,4-galactosyltransferase (GalT1) and CMP-NeuAclactosylceramide sialyltransferase (SialT1) complex is affected when SialT2, another member of this complex, is coexpressed. GalT1 and SialT1 sub-Golgi localization was determined by studying the effect of brefeldin A (BFA) and monensin on the synthesis of glycolipids and on the sub-Golgi localization of GalT1$_{1,52}$-CFP (cyan fluorescent protein) and SialT1$_{1,54}$-YFP (yellow fluorescent protein) chimeras by single cell fluorescence microscopy and by isopycnic subfractionation. We found that BFA, and also monensin, impair the synthesis of glycolipids beyond GM3 ganglioside in wild type (WT) cells but beyond GlcCer in SialT2$^\ast$ cells. Although BFA redistributed GalT1-CFP and SialT1-YFP to the endoplasmic reticulum in WT cells, a fraction of these chimeras remained associated with a distal Golgi compartment, enriched in trans Golgi network, and recycling endosome markers in SialT2$^\ast$ cells. In BFA-treated cells, the percentage of GalT1-CFP and SialT1-YFP associated with Golgi-like membrane fractions separated by isopycnic subfractionation was higher in SialT2$^\ast$ cells than in WT cells. These effects were reverted by knocking down the expression of SialT2 with specific siRNA. Results indicate that sub-Golgi localization of glycosyltransferase complexes may change according to the relative levels of the expression of participating enzymes and reveal a capacity of the organelle to adapt the topology of the glycolipid synthesis machinery to functional states of the cell.

Glycolipid oligosaccharides are built up in the Golgi apparatus by a complex membrane-bound machinery formed by glycosyltransferases, sugar nucleotide transporters, and ceramide-bound acceptors. With the exception of the first sugar transfer step, catalyzed by ceramide glucosyltransferase, sugar transfer steps are carried out in the lumen of the Golgi cisternae by the successive addition of monosaccharide units catalyzed by glycosyltransferases of relatively wide acceptor specificity (for review, see Ref. 1) (see Fig. 1C). Biochemical and pharmacological studies map these activities to elements of the trans Golgi and trans Golgi network (TGN) with activities for synthesis of complex gangliosides more concentrated toward the TGN (2–4).

The N-terminal domain of glycolipid glycosyltransferases, comprising the cytoplasmic tail, the transmembrane domain, and a few amino acids of a stem region, is sufficient for Golgi retention (5, 6), whereas the cytoplasmic tail is relevant in the determination of proximal or distal Golgi localization (7, 8). Glycolipid glycosyltransferases form multienzyme complexes (6, 9). It has been suggested that glycosyltransferase oligomers (10) may be excluded from entering the micro-vesicles exiting the Golgi by virtue of their size, thus helping retention of these enzymes in the Golgi (6).

At least two complexes of ganglioside glycosyltransferases have been described in Chinese hamster ovary (CHO)-K1 cells, one formed by GalT1, SialT1, and SialT2 and another by GalT2 and GalNAcT, of more distal Golgi location (Ref. 11) (Fig. 1C, scheme), raising the question of how changes in the relative mass contribution of partners may affect their topological distribution along the Golgi apparatus. In this work, we address this issue in wild type (WT) CHO-K1 cells, which lack the expression of SialT2, and in CHO-K1 cell transfectants that stably express SialT2 (SialT2$^\ast$ cells). Information was gathered from the effect of pharmacological agents that affect Golgi

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*This work was supported in part by grants from the Howard Hughes Medical Institute (to H. J. F. M.), Secretaría de Ciencia y Tecnología, Universidad Nacional de Córdoba (to J. L. D. and H. J. F. M.), CONICET (to J. L. D. and H. J. F. M.), Fundación Antorchas (to J. L. D.), and Agencia Nacional de Promoción Científica y Tecnológica (to J. L. D. and H. J. F. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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5 The abbreviations used are: TGN, trans Golgi network; BFA, brefeldin A; CFP, cyan fluorescent protein; GFP, green fluorescent protein; YFP, yellow fluorescent protein; ER, endoplasmic reticulum; HA, nonapeptide of influenza virus hemagglutinin; GalNAcT, UDP-GalNAc-lactosylceramide/GM3/GD3 $\beta$1,4-N-acetyl-lactosaminyltransferase; GalT1, UDP-Gal:galactosylceramide $\beta$1,4-galactosyltransferase; GalT2, UDP-Gal:Ga2/GM2/GD2 $\beta$1,3-galactosyltransferase; P4, d,l-threo-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol-HCl; SialT1, CMP-NeuAclactosylceramide sialyltransferase; SialT2, CMP-NeuAc:GM3 sialyltransferase; GlcCer, glucosylceramide; LacCer, lactosylceramide; WT, wild type; CHO, Chinese hamster ovary; siRNA, small interfering RNA.
SialT2 Expression Modifies GalT1 and SialT1 Localization

organization or function on the synthesis of glycolipids and on the intracellular localization of chimeras of the N-terminal domains of GalT1 and SialT1 and GFP derivatives by single cell fluorescence microscopy and by isopycnic subfractionation. It was concluded from these experiments that the presence of SialT2 promotes changes in the pattern of glycolipid synthesis that exceed the expected one from its enzymatic activity. These changes were accompanied by changes in the sub-Golgi localization of GalT1 and SialT1. Results indicate that the sub-Golgi localization of glycosylating enzymes that form part of multienzyme complexes is influenced by the relative contribution of the partners, suggesting a still unexplored control level of glycolipid synthesis in the Golgi complex.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—CHO-K1 cells (ATCC, Manassas, VA) (WT) and a clone of CHO-K1 cells that stably expresses chicken SialT2-2HA (SialT2+ cells) (12) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 µg/ml streptomycin, and 100 µg/ml penicillin. At about 70% confluence, cells were transfected with 1 µg/ml indicated cDNA using cationic liposomes following the manufacturer’s instructions (Lipofectamine, Invitrogen). Plasmid pEGFP-Rab11a wild type was kindly provided by M. Colombo (Universidad Nacional de Cuyo, Mendoza, Argentina).

Molecular Constructs—Vectors containing the cDNA coding for the N-terminal domains of SialT1 (amino acids 1–54) and GalT1 (amino acids 1–52) fused to the C terminus of CFP and YFP were generated by PCR amplification of the indicated region and subcloning the PCR products into the corresponding plasmid coding the different fluorescent proteins (pECFP/YFP, Clontech).

Glycolipid Pattern Analyses—Cells in culture (3 × 105 cells per 35-mm dish) were treated with brefeldin A (BFA) or monensin (3 µg/ml or 5 µM, respectively), and after 30 min, they were metabolically labeled with 2 µCi/ml D-[U-14C]galactose (329.5 mCi/mmol; PerkinElmer Life Sciences) during 7 h. After washing with cold phosphate-buffered saline, cells were scraped from the plate, and lipids were extracted, purified, and chromatographed on high performance TLC plates (Merck) as described (13).

Inhibition of Glycolipid Synthesis with 4,4'-thio-1-Phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol-HCl (P4)—Inhibition of glycolipid synthesis with P4 (Matreya, Pleasant Gap, PA) was performed essentially as described (14). SialT2+ CHO-K1 cells in culture were treated for 4 days with 1.2 µM P4 added to the culture medium. Inhibition of glycolipid synthesis was monitored by immunodetection of GD3 ganglioside.

Determination of Ganglioside Glycosyltransferase Activities—Transferase activities were determined essentially as described (15). Homogenates of CHO-K1 cells and SialT2+ cells were used as an enzyme source for the determination of GalT1 and SialT1 activities. The activity of GalT1 was determined in an incubation system that contained, in a final volume of 30 µl, 100 µM GlcCer, 500 µM UDP-[14C]Gal (450,000 cpm), 20 mM cytidine 5’-diphosphocholine (as competitor of pyrophosphatases), 10 mM MnCl2, 10 mM MgCl2, 100 µg of Triton X-100, 64 mM sodium cacodylate-HCl buffer (pH 7.2), and cell extract (100 µg of protein). The activity of SialT1 was determined in an incubation system that contained, in a final volume of 30 µl, 400 µM LacCer, 100 µM CMP-[-3H]NeuAc (250,000 cpm), 20 mM MnCl2, 1 mM MgCl2, 20 µg of Triton CF54/Tween 80 (2:1 w/w), 100 mM sodium cacodylate-HCl buffer (pH 6.5), and cell extract (40 µg of protein). Incubations were performed at 37 ºC for 90 min. Samples without exogenous acceptor were used to correct the incorporation into endogenous acceptors. Reactions were stopped with 1 ml of 5% (w/v) trichloroacetic acid/0.5% phosphotungstic acid, and the radioactivity incorporated into lipids was determined as described (16).

Density Gradient Separation and Western Blotting—CHO-K1 cells expressing SialT2, SialT1, and/or GalT1 were homogenized in 10 mM Tris-HCl, pH 7.2, in 0.25 M sucrose. A postnuclear supernatant fraction (1,000 × g, 10 min) was collected and separated by centrifugation using Optima™ TLX ultracentrifuge (Beckman) with a swinging bucket rotor at 200,000 × g for 4 h at a 3–25% continuous gradient of OptiPrep (Nycomed Pharma AS, Oslo, Norway). Twelve fractions of 200 µl were collected from bottom to top, and proteins were precipitated with trichloroacetic acid, taken in sample buffer (17), and subjected to SDS-PAGE. For immunoblotting, the following antibodies were used: mouse monoclonal anti-α-mannosidase II (1:400, from K. Moremen), rabbit polyclonal anti-Bip (1:300, Abcam Inc., Cambridge, MA), mouse monoclonal anti-GM130 (1:200, BD Biosciences), mouse monoclonal antibody anti-TGN38 (1:100, Abcam Inc.), and mouse monoclonal antibody anti-GFP (1:1,000, Roche Applied Science) followed by goat anti-mouse or rabbit biotin-labeled antibodies (1:15,000, Molecular Probes, Eugene, OR) and streptavidin horseradish peroxidase-conjugate (1:30,000, Amersham Biosciences, Buckinghamshire, UK). All incubations were carried out in Tween 20 in TBS for 1 h at room temperature followed by three 10 min-washes with Tween 20 in TBS. Blots were processed using the ECL Plus Western blotting detection system (Amersham Biosciences) and exposed to Hyperfilm MP autoradiography film (Amersham Biosciences). Bands were quantified by densitometry with Scion Image 1 software (National Institutes of Health (NIH), Bethesda, MD).

Immunoprecipitation—The postnuclear fraction of SialT2+ cells co-transfected with SialT2-FLAG and GalT1-CFP was isopycnically separated as described before. Gradient fractions of low density (fractions 9–12) were pooled and treated for 3 h on a rotating wheel at 4 ºC with lysis buffer (50 mM HCl-Tris, pH 7.2, 1% Triton X-100, 150 mM NaCl, 3 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 3 µg/ml aprotinin, 2 µM pepstatin, 1 mM EDTA, 0.05% sodium azide) and 25 µl of protein A-Sepharose (CL-4B, Amersham Biosciences) cross-linked with anti-HA polyclonal antibody (previously washed with lysis buffer). Immunocomplexes were pelleted by centrifugation at 2,500 × g for 20 s and then washed twice with lysis buffer and once with phosphate buffer for immunoblotting. The antibodies used for the immunoblotting were: monoclonal anti-HA (1:6,000), anti-FLAG (M2, Sigma, 1:5000), anti-GFP (1:1,000).

Fluorescence Microscopy—Cells grown on coverslips were fixed for 7 min in methanol at −20 ºC. Fixed cells were incu-
bated with the following specific antibodies, monoclonal mouse anti-GM130 (1:200, BD Biosciences), polyclonal rabbit anti-HA (1:1,000), or hybridoma (ATCC number HB-8445) supernatant containing R24 antibody, and then incubated with the appropriate fluorescent secondary antibodies. Coverslips were mounted with FluorSave (Calbiochem, EMD Biosciences Inc.) and observed in a LSM5 Pascal laser scanning confocal microscope equipped with an argon/helium/neon laser (Zeiss, Jena, Germany), a ×100 Plan-Apochromat oil immersion objective, and appropriate filters for CFP, YFP, rhodamine, and FITC.

siRNA Experiments—For siRNA treatments, double-stranded Stealth™ RNA (Invitrogen) oligonucleotides were designed against the sequence of full-length SialT2 using the BLOCK-it™ RNA interference designer (Invitrogen). Four oligonucleotides were tested, and all of them worked, although all the experiments were performed using the one with the following sequence: 5′-GCTGTTGACTGGAAATCCCGATATA-3′. The cells were incubated in OptiMEM (Invitrogen) with 80 nM these siRNA duplexes and Lipofectamine™ 2000 transfection reagent (Invitrogen) according to the manufacturer’s instructions for 24 h. BLOCK-it™ fluorescent oligonucleotides (Invitrogen) was used as a control nonspecific oligonucleotide since its sequence is not homologous to any known gene and it has a proven correlation of transfection efficiency with Stealth™ RNA interference and traditional siRNA molecules. For single cell fluorescence microscopy experiments, the specific siRNA was co-transfected with the fluorescent nonspecific one (in a 5:1 ratio) to identify the cells that received the siRNA. GaIT1-CFP or SialT1-CFP were also co-transfected with the siRNA.

RESULTS

The Pattern of Glycolipids of SialT2+ Cells Shows Unexpected Modifications—WT CHO-K1 cells lack SialT2 (12) and GalNAcT (18) activities, which results in a very simple glycolipid pattern, dominated by GlcCer and GM3 (Fig. 1D). The glycolipid composition of SialT2+ cells, which show GD3 immunoexpression at the cell surface (Fig. 1A) and co-localization of SialT2 with the Golgi marker GM130 (Fig. 1B), is shown in Fig. 1D. The pattern is characterized by the absence of GM3, whose synthesis seems to be efficiently coupled for total conversion into the more complex derivatives GD3 and GT3 by the activity of GalT1, we co-transfected the N-terminal domain of GalT1 and machinery in SialT2+ cells that left a fraction of the pool of synthesized LacCer uncoupled from GM3 synthesis. We have no clear explanation for the increased activity of GaIT1 and SialT2 in SialT2+ cells. CHO-K1 cells secrete soluble, catalytically active forms of transferases as a result of proteolysis in the Golgi (20). It is possible that changes in the sub-Golgi localization or in the association state of the transferases made this process less effective, thus increasing the pool of intracellularly active enzymes.

BFA and Monensin Disclose Differences in the Topological Organization of Glycolipid Synthesis in WT and SialT2+ Cells—To investigate the possibility that SialT2 expression modified the topological relationships of transfer steps for synthesis of GlcCer, LacCer, and higher order derivatives in Golgi membranes, the glycolipid pattern was determined in cells treated with BFA known to dissect the Golgi into proximal and distal compartments (19, 20) and with monensin, which affect distal Golgi function (21). BFA blocks the synthesis of gangliosides beyond GM3 (22–24), and monensin inhibits the synthesis of complex gangliosides (25–27).

Both BFA (Fig. 1D) and monensin (Fig. 1E) affected partially the pattern of glycolipids in WT cells with accumulation of GlcCer and about 50% decrease GM3, indicating that a fraction of GM3 is synthesized in distal Golgi compartments. Different from WT cells, SialT2+ cells show that transfer steps down GlcCer blocked or impaired by BFA (Fig. 1D) and monensin (Fig. 1E), respectively, suggesting a modification of the organization of the synthesis in the Golgi complex of these cells. This phenotype of SialT2+ cells was not due to a particular genetic background of the selected SialT2+ cell clone. Different clones were analyzed for its pattern of labeling and behavior to the action of BFA. Fig. 2A shows the glycolipid pattern of a SialT2+ clone (clone B) different from the one analyzed in Fig. 1D (clone A). Both clones showed comparable levels of SialT2 activity in vitro (11.3 and 9.1 nmol of sialic acid transferred mg−1 h−1 for clone A and clone B, respectively). The glycolipid patterns of both clones in the absence and presence of BFA were undistinguishable. It was confirmed that these clones arise independently because they show different patterns of genomic insertion of the SialT2 cDNA, as determined by a restriction fragment length polymorphism experiment (Fig. 2B). The accumulation of LacCer in SialT2+ cells was not due to the generation and isolation procedures of the stable transfected clones since cell clones stably expressing c-Myc-GalNAcT and synthesizing GA2 (Fig. 1D, asterisk, co-migrating with phosphatidyl cholines), and mostly GM2, showed a pattern of labeling in the presence of BFA essentially identical to the one of WT cells (Fig. 1D) and show the expected block of the synthesis of GM2. Thus, these results leave as the most plausible explanation a modification of the topology of the glycolipid synthesis machinery by the presence of SialT2. This modification must have caused some displacement of transfer steps for LacCer, GM3, GD3, and GT3 synthesis to more distal sub-Golgi compartments, beyond the block imposed by BFA and sensitive to monensin.

Sub-Golgi Localization of GaIT1-CFP and SialT1-YFP Is Different in WT and SialT2+ Cells—To investigate the possibility that SialT2 expression altered the sub-Golgi localization of SialT1 and GaIT1, we co-transfected the N-terminal domain of GaIT1 and...
SialT1 fused to appropriate spectral variants of the GFP to WT and SialT2+ cells and examined at the single cell level their redistribution behavior upon treatment with BFA. It is known that the N-terminal domain of glycolipid glycosyltransferases is able to convey the GFP to the sub-Golgi compartment in which the endogenous transferases concentrate (7).

GalT1-CFP and SialT1-YFP co-localize in the Golgi apparatus of both WT and SialT2+ cells (Fig. 3, upper panels). However, upon BFA treatment, it was observed that GalT1-CFP and SialT1-YFP redistribution into the ER was more effective in WT than in SialT2+ cells, which consistently retained a fraction of fluorescence condensed around the nuclear area, co-localizing with the retained fraction of SialT2 (Fig. 3, SialT2+ cells, lower panels). Quantitative estimation of that fraction in SialT2+ cells as a percentage of the equivalent fraction in WT cells after BFA treatment gave values of 133 ± 27 and 170 ± 37% for SialT1-YFP and GalT1-CFP, respectively (n = 70 cells). Reciprocally, the fraction of SialT2 in the post-BFA compartment of SialT2+ cells expressing GalT1 and SialT1 was 138 ± 30% (n = 50 cells) with respect to SialT2+ cells not expressing GalT1 and SialT1 (Fig. 1B). It should be noticed that the apparent distalization of GalT1-CFP and SialT1-YFP was not a generalized phenotype of SialT2+ cells. Transfected GalNAcT-YFP was not influenced by the expression of SialT2 since its redistribution behavior in BFA was essentially the same in SialT2+ cells as in WT cells (91 ± 24%), and the endogenous cis Golgi marker GM130 was as massively redistributed in SialT2+ cells as in WT cells (Fig. 1B). Also, the glycolipid pattern in GalNAcT stable transfectant cells in the presence of BFA was identical to that in the WT cells, indicating that GalNAcT expression did not affect the localization of SialT1 and GalT1 (Fig. 1D).

The nature of the GalT1-CFP and SialT1-YFP containing Golgi elements in the post-BFA compartment of SialT2+ cells

![Figure 1. Characterization of SialT2+ cells.](image)
was investigated by comparing their localization with that of GalNAcT (Fig. 4A), a TGN resident glycosyltransferase (4), and with that of the GTPase Rab11a (Fig. 4B), an established marker of pericentriolar recycling endosomes (28, 29). GalT1-CFP and SialT1-YFP show partial co-localization with GalNAcT in the absence or presence of BFA. On the other hand, they only partially co-localize with Rab11a in the absence of BFA, but a substantial overlapping was found in BFA-treated cells, evidencing the observed fusion between the recycling endosomes and the TGN in the presence of BFA (20, 30).

**GalT1 and SialT1 Containing Membranes of BFA-treated SialT2**

**SialT2** and WT Cells Differ in Density Properties—To confirm the results of Fig. 3, membranes of WT and SialT2 cells expressing GalT1-CFP or SialT1-YFP and cultured in the presence or absence of BFA were isopycnically subfractionated by ultracentrifugation, and fractions of the gradient were Western blotted with anti-GFP and anti-HA antibodies (Fig. 5A). GalT1-CFP, SialT1-YFP, and the full-length SialT2 floated in the zone of the gradient in which Golgi membranes float (co-distributing with GM130 and mannosidase II) in both WT and SialT2+ cells in the absence of BFA. In the presence of BFA, about two-thirds of the full-length SialT2 redistributed in a zone of the gradient of higher density, partially co-distributing with α-mannosidase II and ER membranes marked by Bip. Most GalT1-CFP and an important fraction of SialT1-YFP co-distribute with heavier membranes in WT cells, but this trend was lower in SialT2+ cells. Quantification of Western blot bands from two independent experiments revealed that in BFA-treated SialT2+ cells, the percentages of SialT2, GalT1-CFP, and SialT1-YFP floating with membranes of lower densities (fractions 9–12 enriched in the endogenous TGN marker TGN38) were about 27, 54, and 38% of the total, respectively. A co-immunoprecipitation experiment was run with SialT2+ cells co-expressing FLAG-tagged full-length SialT1 (SialT1-FLAG) and GalT1-CFP in the presence and absence of BFA. Cell membranes were subjected to gradient separation, and pooled fractions 9–12 of the gradient were immunoprecipitated with anti-HA polyclonal antibody and immunoblotted with anti-HA, anti-FLAG, and anti-GFP monoclonal antibodies. GalT1-CFP, SialT1-FLAG, and SialT2-HA were physically associated in these membranes since pulling down SialT2 with anti-HA resulted in co-immunoprecipitation of SialT1-FLAG and of GalT1-CFP (Fig. 5B).

**SialT2, Rather Than Its Product or Product Derivatives, Modifies Glycolipid Synthesis Topology**—The results of Figs. 1–5 strongly suggest that the expression of SialT2 in CHO-K1 cells that normally do not express this enzyme affects the topological relationships between transfer steps for synthesis of LacCer and GlcCer. The expression of SialT2 changes the localization of GalT1 and SialT1, their subcellular distribution is affected by BFA, and co-immunoprecipitation experiments indicate that SialT2 modifies the interaction between GalT1 and SialT1. This suggests that SialT2 affects the topology of glycolipid synthesis by modifying the interaction between GalT1 and SialT1.
GM3 and also the sub-Golgi localization of the participating glycosyltransferases. This effect could be caused by changes in Golgi membrane properties due to changes of glycolipid composition (Fig. 1, A, C, and D) or by the presence of SialT2 itself, which, as a partner of a multiprotein complex, influences GalT1 and SialT1 localization. To analyze the first possibility, we transfected GalT1-CFP and SialT1-YFP to SialT2− cells depleted of glycolipids by previous treatment with P4, an inhibitor of GlcCer synthase causing depletion of all glycolipids (13, 31), during 4 days. GD3 expression was at the limit of detection in cells grown in the presence of P4 for 4 days (Fig. 6A, +P4). GalT1-CFP and SialT1-YFP still showed in +P4 cells the characteristic reduced redistribution by BFA of control (−P4) cells, indicating that changes in glycolipid composition of Golgi membranes were not determinant of the particular behavior of GalT1-CFP and SialT1-YFP in SialT2− cells.

To analyze whether it was the presence of the SialT2 itself that induced the observed topological changes, the sub-Golgi localization of GalT1-CFP and SialT1-YFP was examined in SialT2− cells in which the expression of SialT2 was knocked down by treatment of the cells with specific SialT2 siRNA. (results not shown). Results of single cell experiments and those from biochemical experiments shown in Figs. 1 and 5 support the conclusion that the presence of SialT2 affects the topological relationships of transfer steps for synthesis of LacCer and GM3, with GalT1 and SialT1 displaced toward more distal sub-Golgi compartments in SialT2− cells.

DISCUSSION

We have compared the synthesis of glycolipids in WT CHO-K1 cells, which do not express SialT2, with that of CHO-K1 cells that constitutively express SialT2 (SialT2+ cells). In addition to the expected changes in glycolipid composition due to SialT2 activity, SialT2+ cells showed a fraction of LacCer uncoupled from synthesis of higher order glycolipids, indicating a change in the functional relationships of transfer steps underlying LacCer formation and utilization. To examine these relationships, we exploited the effect of BFA on dissecting the Golgi apparatus in vivo, redistributing the proximal (cis, medial, and trans) Golgi into the ER and leaving a post-BFA compartment consisting of the distal (trans/TGN) Golgi condensed with the endosomal compartments (19, 20, 32, 33). We
SialT2 Expression Modifies GalT1 and SialT1 Localization

FIGURE 5. Isopycnic separation of membranes from WT and SialT2−/− cells. The effect of BFA is shown. A, the microsomal fraction from WT and SialT2−/− cells expressing either GalT1-CFP (GalT1) or SialT1-YFP (SialT1) in the absence (−BFA) or presence (+BFA) of BFA was centrifuged for 4 h at 200,000 × g in a continuous gradient of OptiPrep (Iodixanol 3.25%). Fractions were Western blotted with anti-GFP for GalT1-CFP (32 kDa) and SialT1-YFP (33 kDa), anti-HA for full-length SialT2-HA (47 kDa), and specific antibodies for α-mannosidase II (ManII), GM130 (medial and cis Golgi markers, respectively), Bip (ER marker), and TGN38 (TGN marker) proteins. B, the microsomal fraction from SialT2−/− cells co-expressing GalT1-CFP and full-length SialT1-YFP in the absence (−BFA) or presence (+BFA) of BFA was centrifuged as in A, and fractions of the gradient of low density (fractions 9–12) were pooled and immunoprecipitated with anti-HA polyclonal antibody. The immunocomplexes were Western-blotted with anti-GFP, anti-FLAG, and anti-HA monoclonal antibodies.

also studied the effect of monensin, a monovalent cation ionophore that exchanges Na+, K+, and H+ across membranes and that, by affecting acidification, impairs the distal Golgi function (21, 34). The pattern of glycolipid synthesis in the presence of BFA and monensin suggested that activities of transfer steps acting beyond GlcCer localize in sub-Golgi compartments more distally located in SialT2−/− cells than in WT cells. To visualize these differences, we performed dual color imaging experiments in single cells co-expressing GalT1-CFP and SialT1-YFP chimeras. Relative to WT cells, a higher fraction of the chimeras localized in a post-BFA compartment, partially co-localizing with the TGN-located GalNAcT, and with the pericentriolar recycling endosome marker Rab11. Such a displacement in SialT2−/− cells was specific for GalT1-CFP and SialT1-YFP since neither GalNAcT nor endogenous GM130 differ from WT cells in their redistribution behavior upon BFA treatment. Also, it was specific for SialT2 expression since in GalNAcT transfectant cells, the effect of BFA on the synthesis of glycolipids was essentially as in WT cells (Fig. 1D).

Sub-cellular fractionation of membranes from WT and SialT2−/− cells transfected with either GalT1-CFP or SialT1-YFP and cultured in the presence and absence of BFA showed that in SialT2−/− cells treated with BFA, a consistent fraction of about 27% of full-length SialT2 remained floating in the region of the gradient in which Golgi membranes float, and about 54% of GalT1-CFP and 38% of SialT1-YFP were also found floating at these low density fractions. Since GalT1-CFP and SialT1-YFP have the same size and are revealed with the same anti-GFP antibody, this experiment involved different transfections and different gradients. This adds variability to the average distribution of membranes coming from a population of cells with variable levels of the expression of GalT1-CFP and SialT1-YFP and contribute to make less clear subtle sublocalization changes observed at the single cell, dual color microscopy level. Even so, fractionation trends were compatible with single cell observations.

The observed topological changes in SialT2−/− cells affecting the functional coupling of GalT1 and SialT1 were caused by the presence of SialT2 and not by changes in the composition of glycolipids of the Golgi membranes caused by its enzyme activity (i.e. decrease of GM3, increase of GD3/GT3, accumulation of LacCer). This was concluded because blocking ganglioside synthesis with the specific GlcCer synthase inhibitor P4 did not revert the SialT2−/− phenotype, whereas knocking down SialT2 expression by specific siRNA did.

Since SialT2 forms a multienzyme complex with GalT1 and SialT1 in CHO-K1 cells (11), we speculate that a fraction of SialT2 complexed GalT1 and SialT1 and moved them to a more distal sub-Golgi compartment, localized beyond the block imposed by BFA and sensitive to monensin. By fluorescence microscopy, a discrete fraction of the full-length SialT2 (12) was consistently observed as resistant to redistribution in BFA.

The subcellular fractionation experiments of Fig. 5 also reveal a discrete fraction of SialT2 floating with membranes of low density in BFA-treated SialT2−/− cells, and a co-immunoprecipitation experiment showed GalT1-CFP and full-length SialT1-FLAG physically associated with SialT2-HA in SialT2−/− cells. It should be mentioned that most probably, this association does not imply coexistence in detergent-resistant domains (“rafts”) since SialT2-HA was mostly soluble in cold Triton X-100 extraction in conditions in which the “raft” marker glycosylphosphatidylinositol-YFP remains in the insoluble fraction (35). Thus, the present results are compatible with the possibility that such a distal fraction of SialT2 complexed GalT1 and SialT1 and retained them into a more distally located sub-Golgi compartment. Alternatively, SialT2 could
have sequestered an unknown, limiting partner whose availability is critical for keeping GalT1 and SialT1 concentrated in a more proximal compartment.

Although glycosyltransferases overlap along the Golgi compartments, they may not be necessarily active in all of them. Even if it may be possible to map their relative enrichment in a

FIGURE 6. SialT2, rather than its products, influences SialT1 and GalT1 sub-Golgi concentration. A, SialT2–/− cells cultured during 4 days in the absence (−P4) or presence (+P4) of the glycolipid synthesis inhibitor P4 were examined for GD3 immunoexpression and for the sub-Golgi localization of GalT1-CFP and SialT1-YFP in the absence (−BFA) and presence (+BFA) of BFA in the last 30 min of culture. B, SialT2–/− cells were treated with specific SialT2 siRNA, and homogenates were Western blotted for SialT2 with anti-HA and with anti-α-tubulin antibody (DM 1A) as internal standard. C, left panel, cells receiving nonspecific siRNA (green cells, arrows) show SialT2 immunostaining (red, arrowheads). Right panel, cells receiving the specific SialT2 siRNA (green stained cell, arrow) do not show SialT2 immunostaining; the arrowhead marks a cell in the field that did not receive siRNA (not green stained) and immunoexpress SialT2 (red). D, SialT2–/− cells treated (+siRNA) or not (−siRNA) with specific SialT2 siRNA and expressing SialT1-CFP or GalT1-CFP were treated (+BFA) or not (−BFA) with BFA. In SialT2–/− cells with knocked down SialT2 expression, GalT1-CFP and SialT1-YFP redistribute to the ER after treatment with BFA as in WT cells. The arrows point to cells in which, for clarity, the green color of the fluorescent control was omitted in the picture. Bar, 10 μm.
given sub-Golgi compartment by immunoelectron microscopy, it is difficult at present to ascertain in which subcompartment resides the functionally active fraction of the enzyme. Other factors, such as proper sugar nucleotide supply, presence of adequate acceptor, optimal luminal pH, competition with other glycosyltransferases at branching points, etc., may condition its functional activity. For the case of GalNAcT, for example, it was shown that conversion of GM3 into GM2 was not dependent on the total enzyme activity in the cell but on the compartment to which it was targeted, with the highest efficiency in the TGN (36).

These results agree with the possibility that changes of the relative levels of enzymes participating in higher order organizations may be accompanied by changes in their sub-Golgi localization. Changes in the expression levels of glycosyltransferases are common themes underlying the compositional modifications of cell surface glycolipids that occur during differentiation, development, oncogenesis, etc (37). During the ontogeny of the central nervous system of birds and mammals, SialT2 is abundantly expressed, and GalNAcT is almost absent at early embryonic stages, when cells are actively proliferating. When cells cease to proliferate and become differentiated, the expression of GalNAcT increases, and that of SialT2 decreases. These changes correlate with the respective up and down transcriptional regulation of their coding genes and with the shift from the expression of GD3 to the expression of more complex gangliosides (GM1, GD1a) that occur during development of the central nervous system (38, 39).

How a supramolecular organization cooperates with transcriptional events to respond to regulatory inputs is unclear. Increased availability of GalNAcT2 was shown to impact organelle homeostasis by up-regulation of COPII assembly and increasing of Golgi size (40). In this case, the effect was mediated by direct interaction of the conserved dibasic motif (R/K)(X)R/K in the cytoplasmic tail of Golgi glycosyltransferases with Sar1 (41). Also, recruitment of a discrete fraction of the Golgi UDP-Gal transporter to the ER for synthesis of galactosylceramide was found dependent on ceramide galactosyltransferase expression, with which it forms a complex (42). Thus, the possibility of changes in the topology of the membrane-bound machinery to adapt to variations in the mass contribution of partners is worthy of consideration.

Acknowledgments—We thank J. Valdez-Taubas and C. G. Giraudo for critical reading of the manuscript. The technical assistance of G. Schachner and S. Deza with cell cultures and of C. Mas with confocal microscopy is also acknowledged.

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