Ganglioside Glycosyltransferases Organize in Distinct Multienzyme Complexes in CHO-K1 Cells*

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The synthesis of gangliosides is compartmentalized in the Golgi complex. In most cells, glycosylation of LacCer, GM3, and GD3 to form higher order species (GA2, GM2, GD2, GM1, GD1b) is displaced toward the most distal aspects of the Golgi and the trans-Golgi network, where the involved transferases (GalNAcT and GaIT2) form physical and functional associations. Glycosylation of the simple species LacCer, GM3, and GD3, on the other hand, is displaced toward more proximal Golgi compartments, and we investigate here whether the involved transferases (GaIT1, SialT1, and SialT2) share their N-terminal domains in the TGN-dependent manner and that their property of forming physical associations. Using biochemical, pharmacological, and multiple color fluorescence imaging techniques in CHO-K1 cells expressing epitope-tagged versions of these enzymes, we found that GaIT1, SialT1, and SialT2 participate in forming physical associations. The synthesis of simple ganglioside species also show the property of forming physical associations. In the present work, we examine whether enzymes acting on the synthesis of simple ganglioside species also show the property of forming physical associations. Using biochemical, pharmacological, and multiple color fluorescence imaging techniques in CHO-K1 cells expressing epitope-tagged versions of these enzymes, we found that GaIT1, SialT1, and SialT2 participate in forming physical associations. The synthesis of simple ganglioside species also show the property of forming physical associations.
**EXPERIMENTAL PROCEDURES**

**Antibodies**—Polyclonal and monoclonal anti-c-Myc and anti-His antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA). Polyclonal and monoclonal anti-HA antibodies were from Berkeley Antibody, Richmond, CA. Monoclonal anti-GFP antibody was from Roche Applied Science. Monoclonal anti-FLAG was from Sigma.

**Molecular Constructs**—GalNAcT-Myc, GalT2-HA, and vectors containing the cDNA coding for the N-terminal domains of GalNAcT, GalT2, and ManII fused to the N terminus of the enhanced cyan fluorescent protein (CFP), to the enhanced yellow fluorescent protein.

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**Fig. 1. Pathway of ganglioside synthesis and schematic representation of constructs used in this work.** In A, the series 0, a, and b pathways of synthesis of gangliosides are indicated (32, 33). Enzymes studied in this work and their respective antigenic tags are encircled and shadowed. The sub-Golgi localization of the different transfer steps, inferred from biochemical, pharmacological, and immunocytochemical experiments, are indicated (9–12, 14, 15, 34). As shown in B, two classes of constructs were used: the one at left in which the full-length versions of the listed transferases, consisting of the cytoplasmic tail (CT), the transmembrane region (TMR), the stem region (SR), and the catalytic domain (CD), were epitope-tagged at the C terminus with His, FLAG, HA, or c-Myc, as indicated, and the one at right in which the CD was replaced with spectral variants of the green fluorescence protein (FP).
Infections were performed using Fugene 6 transfection reagent (Roche Products). For fluorescence resonance energy transfer (FRET) determinations, proteins were grown in DMEM on chambered coverglasses (LabTek, Naperville, IL) and observed on a Zeiss LSM 510 confocal microscope equipped with a culture chamber set at 5% CO2 in air and 37 °C, using a ×100 Plan Neofluor objective. The Golgi area was selected in a focus plane so that it was clearly visible. The volume of the organelle, which usually comprised about 1–2 μm above and below the focal plane, was determined defining the upper and lower Z-limits. The Golgi selected volume was irreversibly photobleached by successive scanning through the Z-series at 100% of the laser power of both 458 and 514 nm. Images were taken every 1 min during 120 min of recovery. Thirty min before and during the time course of the bleaching experiments, cells were incubated with 100 μg/ml cycloheximide to ensure that no fluorescence was gained due to protein synthesis.

**RESULTS**

SialT2, SialT1, and GalT1 Co-immunoprecipitate in Triple Transfected CHO-K1 Cells—Previous studies of the incorporation of radioactive sugars into endogenous glycolipid acceptors revealed the coexistence of glycosyltransferases in isolated Golgi membrane vesicles. This coexistence was functional since products of one transfer step coupled as substrates for the next step in vitro in the absence of vesicular transport (2). Recently, it was shown that for the distal Golgi-located GalNAcT and GalT2, the functional coupling implies a physical association between them, forming a multienzyme complex in which the substrates GM3 and GM2 are channeled toward the final product GM1 (16). To examine whether enzymes catalyzing the transfer steps operative in the proximal Golgi (GalT1, SialT1, and SialT2, Fig. 1A) organize in the form of complexes, CHO-K1 cells stably expressing the HA-tagged, full-length form of SialT2 (SialT2-HA) (18) were co-transfected with SialT1 or with GalT1 tagged at the C terminus with FLAG or His epitopes, respectively (Fig. 1B). Fifteen h after transfection, lysates of either SialT2-HA/SialT1-FLAG or SialT2-HA/GalT1-His transfected cells were immunoprecipitated respectively with anti-HA or anti-FLAG (Fig. 2A) or with anti-HA or anti-His (Fig. 2B) antibodies and Western-blotted with the appropriate antibodies. Immunoprecipitation (IP) of SialT2-HA, SialT1-FLAG, or GalT1-His revealed that SialT2-HA and SialT1-FLAG were able to mutually co-immunoprecipitate, indicating that these two enzymes form physical associations capable of being detected with this methodology. However, co-IP of GalT1-His and SialT2-HA, if it occurred, was under the limit of detection of the technique (Fig. 2B).

To further examine the lack of co-IP between GalT1-His and SialT2-HA and to examine a possible association between GalT1-His and SialT1-FLAG, we carried out a similar co-IP experiment but using lysates from triple transfected cells stably expressing SialT2-HA and transiently expressing SialT1-FLAG (A) or GalT1-His (B) or of triple transfected cells stably expressing SialT2-HA and transiently expressing SialT1-FLAG and GalT1-His (C) were immunoprecipitated using monoclonal antibodies anti-HA, anti-FLAG, or anti-His, and the immunocomplexes were Western-blotted with anti-HA or anti-His polyclonal antibodies or anti-FLAG monoclonal antibody as indicated.

(YFP), or to the De-red fluorescent protein (RFP) were constructed as indicated (16). ΔGalT2-CFP, GalT2<sup>WRES</sup>-AA-YFP, and GalNAcT<sup>THR3–AA</sup>, YFP were generated as described (17). SialT1-FLAG and GalT1-His were generated by PCR amplification of the corresponding mouse cDNAs (kindly provided by Dr. R. Proia) using reverse primers that mutated the stop codon and introduced the sequences codifying the appropriate epitopes. Immunoprecipitation (IP) of SialT2-HA/SialT1-FLAG or SialT2-HA/GalT1-His/SialT1-endogenous/SialT2-HA complex in the double transfectant CHO-K1 cells was performed in the presence of the Axivert 135M inverted fluorescence microscope at 37 °C and in an atmosphere of 5% CO2 in air for examination. Fluorescence microscopy and quantitative microscopic FRET were as described (16). Imaging processing for pseudocolor and fluorescence quantitation were carried out with the Metamorph 4.5 Imaging System (Universal Imaging Corporation, West Chester, PA); the autoscale function was applied during acquisition of images to optimize their visualization. Final images were compiled with Adobe Photoshop 6.0. Fluorescence recovery after photobleaching (FRAP) experiments were carried out with a Zeiss LSM 510 confocal microscope equipped with a culture chamber set at 5% CO2 in air and 37 °C, using a ×100 Plan Neofluor objective. The Golgi area was selected in a focus plane so that it was clearly visible. The volume of the organelle, which usually comprised about 1–2 μm above and below the focal plane, was determined defining the upper and lower Z-limits. The Golgi selected volume was irreversibly photobleached by successive scanning through the Z-series at 100% of the laser power of both 458 and 514 nm. Images were taken every 1 min during 120 min of recovery. Thirty min before and during the time course of the bleaching experiments, cells were incubated with 100 μg/ml cycloheximide to ensure that no fluorescence was gained due to protein synthesis.
GalNAcT and GalT2 Fail to Co-Immunoprecipitate with SialT2, SialT1, and SialT2—It was reported that GalNAcT and GalT2 mutually interact in CHO-K1 cells (16) and also that GalNAcT interacts with SialT2 in F-11 cells (19). We considered it of interest to examine whether interactions of GalNAcT or GalT2 with GalT1, SialT1, or SialT2 occur in CHO-K1 cells.

We first examined whether GalNAcT interacts with SialT2. To this end, CHO-K1 cells stably expressing the c-Myc-tagged full-length form of GalNAcT (GalNAcT-Myc) were co-transfected with HA-tagged forms of GalT2, SialT2, or TGN38 (Fig. 3A). TGN38 was included in the experiment as a control of a Golgi-resident protein not related to the glycosyltransferase family. It is clear in the experiment of Fig. 3A that although GalT2-HA and GalNAcT-Myc mutually co-immunoprecipitate (upper row), GalNAcT-Myc was unable to co-immunoprecipitate either SialT2-HA (middle row) or TGN38-HA (lower row) at detectable levels, and vice versa.

We then examined whether GalT2-HA and/or GalNAcT-Myc were able to co-immunoprecipitate SialT1-FLAG in cells stably expressing either GalNAcT-Myc or GalT2-HA and transiently expressing SialT1-FLAG. It was clear from data of Fig. 3B that co-IP of GalNAcT-Myc and SialT1-FLAG, (left panel) or of GalT2-HA and SialT1-FLAG (right panel), if it occurred, was below the limit of detection. Finally, we examined whether GalNAcT-Myc and GalT2-HA were able to co-immunoprecipitate GalT1-His. Fig. 3C shows that neither GalT2-HA nor GalNAcT-Myc, which mutually co-immunoprecipitate, were able to co-immunoprecipitate GalT1-His at detectable levels, and vice versa. Thus, results from co-IP experiments of Figs. 2 and 3 evidence at least two sets of interacting proteins, one formed by GalNAcT-Myc and GalT2-HA and another formed by GalT1-His, SialT1-FLAG, and SialT2-HA.

The N-terminal Domains of GalT1, SialT1, and SialT2 Participate in the Interactions—We next analyzed whether the
N-terminal domains of GalT1, SialT1, and SialT2 play a role in their mutual association. For this, CHO-K1 cells stably expressing SialT2-HA were co-transfected with GalT1-His and the N-terminal domain of SialT1 (comprising amino acids 1–54) fused to YFP. Results of Fig. 4 show that both full-length constructs, SialT2-HA and GalT1-His, were able to co-immunoprecipitate the truncated version of SialT1, and vice versa. In addition, the N-terminal domains of SialT2 and GalT1, comprising amino acids 1–57 and amino acids 1–52, respectively, fused to the YFP (Fig. 1B), were also able to co-immunoprecipitate with the other members of the complex (not shown). These results indicate, as for the case of GalNAcT and GalT2 complex (16), that the N-terminal domains are involved in the interactions among these three enzymes.

Fluorescence Resonance Energy Transfer (FRET) in Living Cells—To analyze whether the GalT1, SialT1, and SialT2 interactions observed in vitro also occur in living cells, we expressed paired constructs of the N-terminal domain of these enzymes fused to CFP or YFP (Fig. 1B) in all possible combinations, and microscopic FRET was determined. All the fluorescent constructs mainly localized in the Golgi complex (Fig. 5), colocalizing with the endogenous Golgi marker ManII (16). A significant FRET signal was detected between SialT1-CFP and GalT1-YFP (first row) and between SialT1-CFP and SialT2-YFP (second row). FRET between GalT1-CFP and SialT2-YFP was only detected in cells also co-expressing SialT1 (third row). FRET signals between either member of the GalNAcT/GalT2 complex and GalT1, SialT1, and SialT2 were at background levels. Images in the fourth and fifth row of Fig. 5 are representative of those showing no FRET between SialT1-CFP and GalNAcT-YFP and between GalNAcT-CFP and SialT2-YFP, respectively. Quantitative analyses of FRET (FRETN), which normalizes the FRET signal to the concentration of both donor and acceptor fluorophores, are given for each pair in Table I. Significant FRETN values were observed for the pairs GalT1/SialT1, SialT1/SialT2, and GalNAcT/GalT2 and for GalT1/SialT2 in cells co-expressing GalT1, SialT1, and SialT2. FRETN values between either GalNAcT or GalT2 and GalT1, or SialT1 or SialT2 (in cells co-expressing or not co-expressing SialT1), were at background level. To further analyze FRET between constructs, the alternative procedure of measuring FRET by acceptor (YFP) photobleaching under the laser scanning confocal microscope (20) was also applied in pairs SialT2-CFP/SialT1-YFP and SialT2-CFP/GalNAcT-YFP. A significant increase of about 40% in CFP fluorescence was observed in the pair SialT2/SialT1, whereas no significant increase was observed in the pair SialT2/GalNAcT (see Supplementary Fig. 1), confirming FRET data obtained by the three filter set method used in Fig. 5. These data are compatible with the possibility that the Golgi ganglioside glycosylating system is split into at least two multiprotein complexes, one containing GalT1, SialT1, and SialT2, engaged in the synthesis of simple glycolipids and gangliosides (LacCer, GM3, and GD3) and the other containing GalNAcT and GalT2, involved in the synthesis of complex gangliosides (GM2, GD2, GM1, GD1b, etc.).
Nocodazole-induced Golgi Vesicles Disclose Different Sub-Golgi Localization of GalT1/SialT1/SialT2 and GalNAcT/GalT2 Complexes—The possibility was considered that the distinct multiprotein complexes inferred from results of the experiments described above concentrate in different sub-Golgi compartments. To examine this possibility, we took advantage of the property of nocodazole of inducing the generalized appearance of Golgi membrane vesicles. This is a consequence of the disassembling of the microtubule network that impedes translocation of such vesicles to the Golgi complex on microtubule tracks. It was reported that reemerging microvesicles with trans-Golgi/TGN components appear earlier than those carrying components of other regions of the Golgi complex (21, 22).

Appropriate sets of chimeras consisting of the N-terminal domains of GalNAcT, GalT2, H92521,4GT, GalT1, SialT1, SialT2, and ManII fused to different mutants of the GFP (CFP or YFP) or the RFP were transfected to CHO-K1 cells. One day after transfection, cells were treated with nocodazole, and single cells were examined under the fluorescence microscope for coexistence of the different chimaeras in vesicles produced at short times of treatment. Fig. 6 shows that in Golgi-scattered vesicles produced at 1 h of nocodazole treatment, GalNAcT-YFP segregates from SialT1-RFP and SialT2-CFP (Fig. 6, first row), indicating a lack of concentration of the sialyltransferases in the TGN-derived vesicles. On the other hand, co-localization of GalNAcT-YFP and GalT2-CFP was observed in some early appearing vesicles, indicating a TGN concentration of these two enzymes; both chimeras segregated from GalT1-RFP.

**TABLE I**

| Construct | GalT2 | GalNAcT | SialT2 | SialT1 | GalT1 |
|-----------|-------|---------|--------|--------|-------|
| GalT1     | 2.3 ± 0.9 | 1.1 ± 0.7 | 3.0 ± 0.9<sup>a,b</sup> | 7.8 ± 0.6<sup>b</sup> |
| SialT2    | 0.6 ± 0.5 | 1.1 ± 0.7 | 11.8 ± 0.7<sup>b</sup> | 7.8 ± 0.6<sup>b</sup> |
| SialT1    | 0.8 ± 0.5; 11.8 ± 0.5<sup>a</sup> | 0.9 ± 0.5; 0.8 ± 0.5<sup>a</sup> | 11.8 ± 0.7<sup>b</sup> | 3.0 ± 0.9; 5.7 ± 0.6<sup>b</sup> |
| GalNAcT   | 20.0 ± 1.0<sup>a</sup> | 0.9 ± 0.5; 0.8 ± 0.5<sup>a</sup> | 1.1 ± 0.7 | 1.1 ± 0.7 |
| GalT2     | 20.0 ± 1.0<sup>a</sup> | 0.8 ± 0.5; 0.8 ± 0.5<sup>a</sup> | 0.6 ± 0.5 | 2.3 ± 0.9 |

<sup>a</sup> Data in italics refer to FRETN values in the presence of co-expressed SialT1.

<sup>b</sup> Values significantly higher (p < 0.02, Student’s t test) than the other analyzed pairs.

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**Fig. 6. Early appearing Golgi microvesicles induced by nocodazole bear GalNAcT and GalT2 but lack SialT1, SialT2, and GalT1.**

CHO-K1 cells were co-transfected with sets of three chimeric constructs consisting of the N-terminal domain of the indicated glycosyltransferases fused to YFP or CFP or RFP. Twenty-four h after transfection, cells were rinsed twice with culture medium and then incubated with 20 μM nocodazole. The appearance of Golgi-scattered vesicles was examined at 0, 1, 2, and 4 h after nocodazole treatment. Micrographs shown correspond to cells after 1 h treatment. Arrows point to scattered vesicles containing fluorescent constructs that segregated from Golgi. Arrowheads, indicate Golgi regions showing colocalization between YFP, RFP, and CFP. The fourth column is the merge of the images in each row.

**Nocodazole-induced Golgi Vesicles Disclose Different Sub-Golgi Localization of GalT1/SialT1/SialT2 and GalNAcT/GalT2 Complexes—**The possibility was considered that the distinct multiprotein complexes inferred from results of the experiments described above concentrate in different sub-Golgi compartments. To examine this possibility, we took advantage of the property of nocodazole of inducing the generalized appearance of Golgi membrane vesicles. This is a consequence of the disassembling of the microtubule network that impedes translocation of such vesicles to the Golgi complex on microtubule tracks. It was reported that reemerging microvesicles with trans-Golgi/TGN components appear earlier than those carrying components of other regions of the Golgi complex (21, 22). Appropriate sets of chimeras consisting of the N-terminal domains of GalNAcT, GalT2, β1,4GT, GalT1, SialT1, SialT2, and ManII fused to different mutants of the GFP (CFP or YFP) or the RFP were transfected to CHO-K1 cells. One day after transfection, cells were treated with nocodazole, and single cells were examined under the fluorescence microscope for coexistence of the different chimaeras in vesicles produced at short times of treatment. Fig. 6 shows that in Golgi-scattered vesicles produced at 1 h of nocodazole treatment, GalNAcT-YFP segregates from SialT1-RFP and SialT2-CFP (Fig. 6, first row), indicating a lack of concentration of the sialyltransferases in the TGN-derived vesicles. On the other hand, co-localization of GalNAcT-YFP and GalT2-CFP was observed in some early appearing vesicles, indicating a TGN concentration of these two enzymes; both chimeras segregated from GalT1-RFP.
which was absent from this kind of vesicle (Fig. 6, second row).

Also, GalNAcT-YFP and /H9252 1,4GT-RFP co-segregate apart from ManII-CFP (Fig. 6, third row). These results correlate with those of Figs. 2–5 and provide additional evidence for the existence of at least two distinct multiprotein complexes that, after nocodazole treatment, segregate one from the other.

**Fig. 7.** Glycosyltransferase complexes are assembled in the ER. As shown in A, CHO-K1 cells co-expressing GalT2RR7—AA-CFP and GalNAcTRR5—AA-YFP (upper row) or ΔGalT2-CFP and SialT2-YFP (middle row) or ΔGalT2-CFP and GalNAcT-YFP (lower row) were analyzed in vivo for subcellular localization and molecular proximity by FRET, as indicated under “Experimental Procedures.” Bar, 10 μm. As shown in B, statistical quantification of the ER/Golgi fluorescence intensity ratio (fluorescence intensity of the whole cell over that in the Golgi area) in single cells co-expressing the pairs of constructs corresponding from left to right to the first, second, and third row in A. Values are mean ± S.D. for n = 25 cells.
Distinct Multiglycosyltransferase Complexes in CHO-K1 Cells

Complexes Assemble in the ER—The existence of complexes of glycolipid glycosyltransferases in the Golgi poses the question of whether they are formed in the Golgi, after single species were transported from the ER, or are formed early in the ER and then moved to the Golgi as preformed complexes. We addressed this question for GalT2 and GalNAcT, which we know mutually associate them (16) and depend on RR residues in their cytoplasmic tails for Sar1 interaction during the process of selective concentration previous to ER exiting (17). We reasoned that if one partner of the complex is accumulated in the ER due to tail deletion or RR to AA substitution, the other non-mutated member of the pair should be able to revert the ER accumulation phenotype provided the cytoplasmic tail is not critical for complex formation; also, if partner associations occurred in the ER, they should be evidenced by FRET.

CHO-K1 cells were co-transfected with GalT2RR7—AA-CFP and GalNAcTRR5—AA-YFP, and the distribution of the constructs was analyzed by fluorescence microscopy. Fig. 7A shows that tail-mutated versions of GalT2 and GalNAcT accumulated in the ER membranes were close enough as to allow FRET between the fluorophores (Fig. 7, upper row). Tail-deleted GalT2-CFP (ΔGalT2-CFP, Fig. 1B) shows the same ER accumulation pattern as GalT2RR7—AA-CFP, clearly distinct from the Golgi localization pattern of the co-expressed SialT2-YFP (Fig. 7, middle row, middle column). No FRET signal was detected between these two constructs (middle row, right column). On the other hand, co-expressed ΔGalT2-CFP and GalNAcT-YFP (Fig. 7, third row) partially colocalized in the Golgi, and a significant FRET signal was detected in the Golgi area in this case. FRET determinations are given in Table II, showing significant values for the tail-deleted or tail-mutated GalT2/GalNAcT pairs but not for the ΔGalT2-CFP/SialT2-YFP pair. These results indicate that GalNAcT, but not SialT2, was able to complex ΔGalT2 in the ER and convey it to the Golgi. Fig. 7B is a statistical quantification of the results of Fig. 7A, showing that the amount of ΔGalT2-CFP at the Golgi complex is significantly higher when co-expressed with GalNAcT-YFP than when co-expressed with SialT2-YFP. In addition to showing that the cytoplasmic tails are not critical for the GalNAcT/GalT2 interaction, these results suggest that these interactions occur in early compartments of the secretory pathway, including ER membranes.

Experiments of FRAP allowed us to calculate the ER to Golgi transport rates of glycosyltransferases under the monomorphic and the associated forms. Cells were either single transfected with any of the fluorescent constructs or co-transfected with GalT2RR7—AA-CFP and either GalNAcT-CFP or SialT2-CFP. The Golgi volume of the expressing cells was photobleached at 100% of the laser power at both 458 and 514 nm, and the recovery of the fluorescence in the Golgi was monitored along the time. Fig. 8A shows that a fraction of GalT2RR7—AA-CFP was transported from the ER to the Golgi complex when co-expressed with GalNAcT-CFP but not when co-expressed with SialT2-CFP (Fig. 8B). These results and those of Fig. 7 strongly support the possibility that the enzyme complexes preassemble in the ER and then move to the Golgi complex. The ER to Golgi transport rates of monomorphic GalT2-CFP and GalNAcT-CFP, in terms of the fraction of the pool moving from the ER to the Golgi, gave similar values for both constructs (3.2 ± 0.3 and 3.4 ± 0.2 % min−1, respectively, (17)). The value calculated for GalNAcT-CFP when it was co-expressed with GalT2RR7—AA-YFP (Fig. 8A) was essentially the same, indicating that it was not delayed in its transport to the Golgi by interacting with the slowly transported GalT2RR7—AA-YFP.

**Fig. 8. The interaction between glycosyltransferases did not alter their ER to Golgi transport rates.** As shown in A, CHO-K1 cells co-expressing GalNAcT-CFP (red) and GalT2RR7—AA-YFP (green) were supplemented with 100 µg/ml cycloheximide 2 h before photobleaching. The Golgi volume was irreversibly photobleached at both 458 and 514 nm. The recovery of fluorescence (FRAP) at the Golgi region was analyzed by dual color imaging every 5 min for 120 min. Note that a fraction of GalT2RR7—AA-YFP fluorescence replenished the bleached Golgi area, as the GalNAcT-CFP did. Arrowsheads in the 120-min split image of the squared area indicate regions of co-localization between them. As shown in B, cells co-expressing SialT2-CFP (red) and GalT2RR7—AA-YFP (green) were processed for FRAP as in A. Note that SialT2-CFP, but not GalT2RR7—AA-YFP, replenished the photobleached Golgi. Arrows in the 120-min split image of the squared area indicate regions lacking co-localization between the constructs. Bar, 10 µm.

| Donor/acceptor pair* | Mean FRET* ± S.D. |
|----------------------|-------------------|
| GalT2RR7—AA-CFP/GalNAcTRR5—AA-YFP | 15.0 ± 1.0 |
| ΔGalT2-CFP/GalNAcT-YFP* | 8.1 ± 1.0 |
| ΔGalT2-CFP/SialT2-YFP | 0.9 ± 0.6 |

* In each pair, “donor” and “acceptor” stand for, respectively, the fusion of the N-terminal domain of the indicated transferase with CFP and YFP.

* FRET values were calculated as indicated in Table I in single cells co-expressing the indicated pair of constructs. Results are the mean ± S.D. of four independent experiments each one resulting from the observation of 50 cells.

* Donor/acceptor pairs showing FRET values significantly higher (p < 0.02, Student’s t test) than the ΔGalT2-CFP/SialT2-YFP pair.
In the present work, we have examined whether enzymes working on the synthesis of simple gangliosides participate in a complex between them and/or with GalNACT and GalT2. Results of co-IP experiments with membranes from double transfectant cells showed that epitope-tagged versions of GalT1 and SialT1 and of SialT1 and SialT2 interact physically so that one was able to co-immunoprecipitate the other. Co-IP of SialT2 and GalT1 was below the limit of detection under these conditions but could be evidenced if the co-IP experiment was run with membranes from triple transfectant cells expressing SialT1, GalT1, and SialT2. This result suggests the existence of a complex of SialT1, SialT2, and GalT1 in which GalT1 and SialT2 interact through a common partner involving SialT1. Also, co-IP experiments failed to evidence interactions between either GalT2 or GalNACT and GalT1, SialT1, or SialT2.

Results from co-IP experiments using different combinations of full-length and N-terminal versions of GalT1, SialT1, and SialT2 were indistinguishable from those obtained with the full-length and N-terminal versions of GalT2 and GalNACT and GalT1, SialT1, or SialT2. A complex of SialT1, SialT2, and GalT1 in which GalT1 and SialT1 and of SialT1 and SialT2 interact physically so that one was able to co-immunoprecipitate the other. Co-IP of SialT1, SialT2, and GalT1 in which GalT1 and SialT2 interact through a common partner involving SialT1. Also, co-IP experiments failed to evidence interactions between either GalT2 or GalNACT and GalT1, SialT1, or SialT2.

Results of co-IP and FRET experiments suggest that the ganglioside-synthesizing machinery is organized in distinct units. One of these units may be the GalT1, SialT1, and SialT2 complex catalyzing the conversion of glucosylceramide to lactosylceramide and to the simple gangliosides GM3 and GD3. Another unit may be the GalNACT and GalT2 complex involved in the conversion of simple gangliosides into more complex gangliosides. Results of experiments of inhibiting translocation of Golgi membrane vesicles reemerging from the ER by disorganization of microtubules in cells treated with nocodazole showed GalT2 and GalNACT in early appearing scattered microvesicles. This is compatible with a distal Golgi concentration of these two enzymes since early appearing vesicles are particularly enriched in the most distal Golgi and the TGN-located enzymes. GalT1, SialT1, and SialT2 were not detected colocalizing with GalT2 and GalNACT in these microvesicles; instead, they were found colocalizing in the Golgi remnants after 1 h of nocodazole treatment, indicating a more proximal Golgi concentration.

The topology inferred from the experiments with nocodazole is fully compatible with the one inferred from biochemical and pharmacological experiments as indicated in Fig. 1A. Although these studies localize the glycolipid glycosyltransferases to late Golgi (4, 23, 24), they also disclose differences in the fine sub-Golgi localization of the different transfer steps: those for the synthesis of complex gangliosides, catalyzed by GalNACT and GalT2, displaced toward the most distal aspects of the Golgi complex and TGN (4, 5), and those for the synthesis of simple gangliosides displaced toward more proximal Golgi compartments (10, 14, 15, 25).

Recently, a physical interaction between SialT2 and GalNACT was reported based on IP and FRET experiments of full-length forms of these enzymes expressed in a substrain of F-11 murine neuroblastoma cells (19). The present results concerning these two enzymes are at variance with the results in F-11 cells. Our quantitative FRET experiments gave FRET values near background levels for SialT2 and GalNACT, and the co-IP experiments failed to detect associations between these transferases under the same conditions in which other interactions were detected. This discrepancy might indicate that complex formation is influenced by the physiological state of the cell under examination or by the presence of accessory proteins only present in particular cell types. A question arising from the present observations is the one related to the subcellular site of formation of these complexes. Experiments in Fig. 7 indicate that tail-mutated or tail-deleted forms of GalT2 and GalNACT were unable to leave the ER. However, the ER concentrated forms are close enough as to undergo FRET between the fluorophores, giving FRET values comparable with those measured in the Golgi complex for non-mutated pairs (Tales 1 and 2). Additionally, the experiments of FRAP in Fig. 8 indicate that non-mutated GalNACT was able to rescue tail-mutated GalT2 from the ER and to convey it to the Golgi; this must have occurred as preassembled complexes since non-mutated SialT2, which does not interact with GalT2, was unable to do so. Collectively, these results suggest the ER as a site of complex formation. An additional piece of information emerging from these experiments is that the cytoplasmic tail is not critical for complex formation since both tail-deleted or tail-mutated forms can be rescued from the ER by interaction with an appropriate partner.

Other multienzyme complexes have been described that assemble in the ER and exert their function in the Golgi apparatus. Examples are the uronosyl 5-epimerase/2-O-sulfotransferase complex (26) and the EXT1/EXT2 complex (27), which are involved in the synthesis and in the co-polymerization of the glucuronic acid and N-acetylgalactosamine during heparan sulfate biosynthesis, respectively. In yeast, two different types of Golgi mannosyltransferase complexes have been described (28, 29), which actively recycle through the ER (30), a behavior shared by ganglioside glycosyltransferase complexes (17). Moreover, it has been proposed that these mannosyltransferase complexes regulate the expressed glycan diversity by altering their composition in mannan backbone (31).

The organization of the assembly line for synthesis of gangliosides in multienzyme complexes containing different sets of transferases and functionally coupled may constitute a supramolecular organization capable of finely tuning ganglioside expression in response to regulatory clues (7, 18, 19). This control level may superimpose to that exerted through the transcriptional control of the expression of individual glycosyltransferases.

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REFERENCES
1. Keenan, T. W., Morre, D. J., and Bana, S. (1974) J. Biol. Chem. 249, 310–315
2. Maxuzd, M. K., and Maccioni, H. J. (1998) J. Biol. Chem. 270, 20207–20214
3. Maxzu, M. K., and Maccioni, H. J. (2000) Neurochem. Res. 25, 145–152
4. Lanzetti, L., Gorgas, K., Meissner, I., Wieland, F. T., and Jeckel, D. (1998) J. Biol. Chem. 273, 2939–2946
5. Giraudo, C. G., Rosales Fritz, V. M., and Maccioni, H. J. (1999) Biochim. Biophys. Acta 1437, 101–118
6. Maccioni, H. J., Giraudo, C. G., and Danioptist, J. J. (2002) Neurochem. Res. 27, 629–636
7. Mollenhauer, H. H., Morre, D. J., and Rowe, L. D. (1990) Biochim. Biophys. Acta 1031, 225–246
8. Miller-Prodrroza, H., and Fishman, P. H. (1984) Biochim. Biophys. Acta 804, 44–51
9. van Echten, G., Iber, H., Stotz, H., Takatsuki, A., and Sandhoff, K. (1990) Eur. J. Cell Biol. 51, 135–139
10. Noreas, G. A., and Caputto, R. (1986) Neurochem. Int. 8, 501–506
11. Rosales Fritz, V. M., Maxud, M. K., and Maccioni, H. J. (1998) J. Neurochem.
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67, 1393–1400
13. Donaldson, J. G., and Klausner, R. D. (1994) *Curr. Opin. Cell Biol.* 6, 527–532
14. Young, W. W., Jr., Lutz, M. S., Mills, S. E., and Lechler-Osborn, S. (1990) *Proc. Natl. Acad. Sci. U. S. A.* 87, 6838–6842
15. Rosales Fritz, V. M., and Maccioni, H. J. F. (1995) *J. Neurochem.* 65, 1859–1864
16. Giraudo, C. G., Daniotti, J. L., and Maccioni, H. J. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 1625–1630
17. Giraudo, C. G., and Maccioni, H. J. F. (2000) *J. Neurochem.* 74, 1711–1720
18. Cole, N. B., Sciaky, N., Mareta, A., Song, J., and Lippincott-Schwartz, J. (1996) *J. Biol. Chem.* 271, 631–650
20. Xia, Z., and Liu, Y. (2001) *Biophys. J.* 81, 2395–2402
21. Todorow, Z., Spang, A., Carmack, E., Yates, J., and Schekman, R. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 13643–13648
22. Yang, W., and Storrie, B. (1998) *Mol. Biol. Cell* 9, 191–207
23. Trinchera, M., Pirvano, B., and Ghidoni, R. (1999) *J. Biol. Chem.* 265, 18242–18247
24. Allende, M. L., Li, J., Darling, D. S., Worth, C. A., and Young, W. W., Jr. (2000) *Glycobiology* 10, 1025–1032
25. Donaldson, J. G., Lippincott-Schwartz, J., Bloom, G. S., Kreis, T. E., and Klausner, R. D. (1990) *J. Cell Biol.* 111, 2295–2306
26. Pinhal, M. A., Smith, B., Olson, S., Aikawa, J., Kimata, K., and Esko, J. D. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 13984–13989
27. McCormick, C., Duncan, G., Goutsoos, K. T., and Tufaro, F. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 668–673
28. Jungmann, J., and Munro, S. (1998) *EMBO J.* 17, 423–434
29. Jungmann, J., Rayner, J. C., and Munro, S. (1999) *J. Biol. Chem.* 274, 6579–6585
30. van Echten, G., and Sandhoff, K. (1989) *J. Biol. Chem.* 265, 207–214
31. van Echten, G., and Sandhoff, K. (1993) *J. Biol. Chem.* 268, 5341–5344
32. Yu, R. K., and Ando, S. (1980) *Adv. Exp. Med. Biol.* 125, 33–45
33. van Echten, G., and Sandhoff, K. (1999) *J. Neurochem.* 75, 207–214
34. Gordon, G. W., Berry, G., Liang, X. H., Levine, B., and Herman, B. (1998) *Biophys. J.* 74, 2702–2713

35. Alberts, B., Johnson, A., Lewis, J., Minkle, D., and Roberts, K. (1994) *Glycobiology* 10, 1025–1032
36. Donaldson, J. G., Lippincott-Schwartz, J., Bloom, G. S., Kreis, T. E., and Klausner, R. D. (1990) *J. Cell Biol.* 111, 2295–2306