Gangliosides, complex glycosphingolipids containing sialic acids, have been found to reside in glycosphingolipid-enriched microdomains (GEM) at the plasma membrane. They are synthesized in the lumen of the Golgi complex and appear unable to translocate from the luminal toward the cytosolic surface of Golgi membrane to access the monomeric lipid transport. As a consequence, they can only leave the Golgi complex via the luminal surface of transport vesicles. In this work we analyzed the exocytic transport of the disialo ganglioside GD3 from trans-Golgi network (TGN) to plasma membrane in CHO-K1 cells by immunodetection of endogenously synthesized GD3. We found that ganglioside GD3, unlike another luminal membrane-bound lipid (glycosphosphatidylinositol-anchored protein), did not partition into GEM domains in the Golgi complex and trafficked from TGN to plasma membrane by a brefeldin A-insensitive exocytic pathway. Moreover, a dominant negative form of Rab11, which prevents exit of vesicular stomatitis virus glycoprotein from the Golgi complex, did not influence the capacity of GD3 to reach the cell surface. Our results strongly support the notion that most ganglioside GD3 traffics from the TGN to the plasma membrane by a non-conventional vesicular pathway where lateral membrane segregation of vesicular stomatitis virus glycoprotein (non-GEM resident) and glycosyolphosphatidylinositol-anchored proteins (GEM resident) from GD3 is required before exiting TGN.

Lipids are continuously transported between membranes by a non-random process since each membrane-bounded compartment along the exocytic and endocytic pathways has a unique molecular composition of lipids. Lipids can either exchange between membranes as single molecules (monomeric lipid transport) or they can be incorporated into transport vesicles destined to the plasma membrane or different organelles (vesicular transport) (1–3). Gangliosides are complex glycosphingolipids containing sialic acids, which are synthesized in the lumen of the Golgi complex. They have been found to reside in glycosphingolipid-enriched microdomains (GEM) (also called DRMs (detergent-resistant membranes) or rafts), dynamic assemblies of cholesterol, saturated phospholipids, and sphingolipids that are characterized by a light buoyant density and resistance to solubilization by Triton X-100 at 4 °C (4–6). Gangliosides appear unable to translocate from the luminal toward the cytosolic surface of Golgi membrane to access the monomeric lipid transport. As a consequence they can only leave the Golgi complex via the luminal surface of transport vesicles (7).

During vesicular transport, vesicles bud from a donor compartment by a process that allows selective incorporation of cargo into the forming vesicles while retaining resident proteins in the donor compartment. The vesicles are subsequently targeted at specific acceptor compartments into which they unload their cargo upon fusion of their limiting membranes (8).

In eukaryotic cells membrane transport between compartments requires proteins that allow the budding and cleavage of nascent cargo vesicles from the donor membrane and their latter targeting and fusion with an acceptor compartment (9). The regulatory machinery includes several cytosolic proteins such as the AP adaptor complexes and the monomeric Golgi-localized, γ-adaptin ear-containing, ARF-binding (GGAs) proteins (10) and membrane-bound GTP-binding proteins (or GTPases), such as Rabs and ARF-ribosylation factors (ARF), which are proteins that function as molecular switches cycling between their GTP- and GDP-bound states.

Members of the Rab GTPase family have been implicated in the control of various steps along the vesicular transport, including cargo selection and budding, movement, docking, and fusion (11). Among Rabs, Rab11 has been shown to be operat-
ing distally in subapical compartments in HepG2 cells (1) and to regulate the delivery of proteins from the trans-Golgi network (TGN) to the plasma membrane in both polarized cells and fibroblasts (11–15). On the other hand the endocytic transport of glycosphingolipids to the Golgi complex is dependent upon Rab7 and Rab9, as demonstrated by inhibition of Golgi targeting in normal cells by dominant-negative Rab7 and Rab9 constructs (16).

In addition to the Rab GTPases, ARF GTPases are required for intracellular transport and maintaining the integrity of organelle structure. Mammalian ARF proteins are grouped into three classes; class I includes ARF1, -2, and -3, class II includes ARF4 and -5, and class III includes ARF6. ARF6 has been the most extensively characterized and is established to be involved in the budding of vesicles from the Golgi complex. On the other hand ARF6 has been implicated in the formation of vesicles from plasma membranes (17). ARF activation and inactivation are catalyzed by guanine nucleotide exchange factors (GEFs) that facilitate GTP binding and GTPase-activating proteins that catalyze GTP hydrolysis. In general ARF6 GEFs are not inhibited by brefeldin A (BFA), in contrast with other ARF GEFs (18). BFA is a metabolite of the fungus *Eupenicillium brefeldiannum* that acts by a rare uncompetitive mechanism with formation of an abortive ARF-GDP-BFA-GEF complex, which cannot proceed to nucleotide dissociation (18).

Glycolipid transport, particularly ganglioside transport, is very poorly studied. Short chain and analogous fluorescent lipids have been used as useful tools for delineating potential pathways and mechanisms of intracellular transport and sorting (19). However, the quantitative and qualitative behavior of the analog lipids is quite different from long chain cellular lipids, since the ability of short fluorescent lipids to diffuse spontaneously between different membranes is not generally shared by their endogenous lipid counterparts (20).

In this study we analyzed the transport of the disialo ganglioside GD3 from TGN to plasma membrane in CHO-K1 cells by immunodetection of endogenously synthesized GD3. Our main approach was to accumulate GD3 at the TGN with temperature block and allow the synchronous release upon warming. GD3 arrival at cell surface was easily monitored by immunodetection since gangliosides from plasma membranes were previously depleted by incubating the cells with a potent inhibitor of complex glycolipid synthesis. We found that GD3 ganglioside, unlike another luminal membrane-bound lipid (glycosphosphatidylinositol-anchored protein) (21–24), did not partition into GEM domains in the Golgi complex and trafficked from TGN to plasma membrane via a BFA-insensitive exocytic pathway. Moreover, the dominant negative form of Rab11, which prevents the exit of vesicular stomatitis virus glycoprotein (VSVG) from the Golgi complex, did not influence the capacity of GD3 to reach the cell surface.

Taken together our results strongly support the notion that most GD3 ganglioside traffic from TGN to plasma membrane by a non-conventional vesicular pathway where lateral membrane segregation of VSVG (non-GEM resident) and GPI-anchored proteins (GEM resident) from GD3 is required before exiting TGN.

**EXPERIMENTAL PROCEDURES**

**Expression Plasmids**—Plasmids pEGFP-Rab11a wild type (wt), pEGFP-Rab11a Q70L (a GTPase-deficient mutant), and pEGFP-Rab11a S25N (a GTP binding-deficient mutant) were kindly provided by Dr. Marisa Colombo (Universidad Nacional de Cuyo, Mendoza, Argentina). Plasmids pEGFP-Rab11, Rab11S25N, and Rab11Q70L were made by subcloning the cDNA fragments in-frame to pEGFP-C3 vector (Clontech Laboratories, Palo Alto, CA). cDNAs coding for the total sequence of the yellow fluorescence protein (YFP) fused to a GPI attachment signal (GPI-YFP) and the temperature-sensitive variant of vesicular stomatitis virus glycoprotein (VSVG to O45) fused to the YFP (VSVG-YFP) were kindly supplied by P. Keller (Max-Plank Institute, Dresden, Germany) (25).

**Cell Culture and DNA Transfections**—CHO-K1 cells (clone 2), a stable CMP-NeuAcGM3 sialyltransferase (Sial-T2, tagged at the C terminus with the nanopeptide epitope of the viral hemagglutinin (HA)) transfectant expressing the ganglioside GD3 and GT3 (26, 27) were used. Cells were grown and maintained at 37°C in 5% CO2 in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum and antibiotics. Where indicated cells were transfected with 1 μg/35-mm dish of the plasmids named above using cationic liposomes (LipofectAMINE, Invitrogen) according to the manufacturer’s instructions and incubated for 14, 24, or 48 h at 37°C with the transfection reagent and DNA mixture. When VSVG-YFP plasmid was used for transfections, CHO-K1 cells grown in the presence of the inhibitor of glycolipid synthesis were transfected at 41°C (restrictive temperature) for 14 h. Under these conditions the VSVG protein accumulated in the endoplasmic reticulum (ER). Cells transfected with VSVG-YFP or GPI-YFP were then incubated at 20°C for 2 h and either processed for immunofluorescence immediately or transferred to 37°C for 1 or 2 additional hours before fixation. Where indicated, cells were supplemented with BFA from a stock solution 1 mg/ml in methanol at a final concentration of 2 μg/ml. BFA was present in the culture medium for the entire period of culture at 20°C and for 2 h before cells were supplemented with the same amount of methanol without BFA. Cycloheximide (30 μg/ml) was added to the culture medium for the last 30 min of culture at 20°C and maintained for the indicated times.

**d,l-threo-1-Phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol-HCl Treatment of Cells**—Inhibition of glycolipid synthesis with d,l-threo-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol-HCl (Brefeldin A, Inc., Pleasant Gap, PA) was carried out as follows: Cells from clone 2 in culture were treated for 5 days with 2.4 μM P4 added to the culture medium from a recently prepared 600 μM stock solution. After 3 days of treatment with P4, cells were further incubated in Dulbecco’s modified Eagle medium without serum growth factors for 34 h to synchronize them. Then, to stimulate cell growth, cells were incubated for 14 h with fresh medium containing 10% fetal bovine serum. Then, 5 days of treatment with P4 was removed, and cells were washed extensively with PBS to remove P4. Then, cells were incubated with 10% fetal bovine serum-rich medium for 2 h at 20°C to accumulate GD3 in TGN and immediately incubated at 37°C to allow GD3 transport.

**Confocal Immunofluorescence Microscopy**—Cells grown in coverslips were washed twice in PBS, fixed in acetone at −20°C for 7 min, washed in PBS, and incubated in 3% bovine serum albumin, PBS buffer for 1 h at 37°C to block nonspecific binding sites. Coverslips were then incubated overnight at 4°C with primary antibodies, washed 5 times with 1% bovine serum albumin, PBS buffer, and exposed to secondary antibodies for 90 min at 37°C. The primary antibodies were: mouse monoclonal antibody anti-GD3 (IgG3), clone 24D2 (a gift of Dr. K. Lloyd, Medical Research Council, Research Center, New York, NY); mouse monoclonal antibody anti-HA dilution 1:200; mouse monoclonal antibody anti-HA dilution 1:200 and rabbit polyclonal anti-mannose 6-phosphate receptor (M6PR, a gift of Dr. Alfredo Cáceres, Instituto Mercedes y Martín Ferreraya, Córdoba, Argentina) diluted 1:150. Secondary antibodies were Alexa 488- or Alexa 546-conjugated goat anti-mouse antibodies (Santa Cruz Biotechnology, Inc.) diluted 1:1000 or rhodamine-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch) diluted 1:1000. After final washes with 1% bovine serum albumin, PBS, cells were mounted in mounting fluid (Light Diagnostics, Temecula, CA).

Confocal images were collected using a Carl Zeiss LSM5 Pascal laser-scanning confocal microscope equipped with an argon/helium/ neon laser and a 63× (numerical aperture = 1.4) oil immersion objective (Zeiss Plan-Apochromat). Single confocal sections of 0.7 μm were taken parallel to the coverslip (xy sections). Images were acquired and processed with the Zeiss LSM image software. Images for fluorescence quantification were taken with a Zeiss LSM5 Pascal microscope equipped with epifluorescence and processed with the MetaMorph 3.0 Image System (Universal Imaging, West Chester, PA). Final images were compiled with Adobe Photoshop 5.0.
TGN and the detergent solubility/insolubility of GD3 was analyzed at different times. Cells were washed with cold PBS and harvested by scraping. Samples were treated with 0.5 ml lysis buffer containing 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% NaCO₃, 5 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin and 25 mM Tris-HCl pH 7.5 (TNE buffer) at 4 °C for 1h, and then centrifuged for 1h at 10000 g at 4 °C. The supernatant (soluble fraction) was removed, and the pellet (insoluble fraction) was resuspended in 0.2 ml lysis buffer. Glycolipids from the supernatant (soluble fraction) and from the pellet (insoluble fraction) were subjected to Folch partition (28). The resulting aqueous phases were freed from Triton X-100 (non-ionic molecule) by passing through DEAE-Sephadex column. Under this condition, neutral lipids as glucosylceramide and LacCer co-eluted with Triton X-100 in the non-retained fraction. The acidic lipid fraction retained in the column (gangliosides) was eluted with 0.1 M sodium acetate and the salt removed by passing through Sep-Pak C₁₈ cartridge column (Waters Corporation Milford, USA). The eluted lipid fraction was supplemented with appropriate amounts of standard gangliosides and chromato-graphed on high-performance TLC plates (HPTLC, Merck, Germany) using chloroform, methanol, 0.2% CaCl₂ (60:36:8 v/v) as solvent. Standard gangliosides were visualized by exposure of the plate to iodine vapors. Routinely 700–1000 cpm were spotted on each lane. Radioactive gangliosides were visualized using a Fuji Photo Film Bio Imagen analyzer or visualized by fluorography after dipping the plate in 0.4% milder alkaline methanolysis to remove glycerophospholipids, and lipids were extracted once more by the method of Folch et al. (28). The resulting aqueous phases were freed from water-soluble contaminants by passing through a Sephadex G-25 column. Lipids from the upper phase were extracted by the method of Folch et al. (28). The resulting aqueous phase (lower phase) was subjected to spotting on a HPTLC plate and developed in chloroform, methanol, 0.2% CaCl₂ (50:20:15:10:5 v/v). Standard lipids as glucosylceramide and LacCer co-eluted with Triton X-100 in the non-retained fraction. The acidic lipid fraction retained in the column (gangliosides) was eluted with 0.1 M sodium acetate and the salt removed by passing through Sep-Pak C₁₈ cartridge column (Waters Corporation Milford, USA). The eluted lipid fraction was supplemented with appropriate amounts of standard gangliosides and chromato-graphed on high-performance TLC plates (HPTLC, Merck, Germany) using chloroform, methanol, 0.2% CaCl₂ (60:36:8 v/v) as solvent. Standard gangliosides were visualized by exposure of the plate to iodine vapors. Routinely 700–1000 cpm were spotted on each lane. Radioactive gangliosides were visualized using a Fuji Photo Film Bio Imagen analyzer or visualized by fluorography after dipping the plate in 0.4% milder alkaline methanolysis to remove glycerophospholipids, and lipids were extracted once more by the method of Folch et al. (28). The chloroform phase was dried under nitrogen, and lipids were separated by HPTLC. For HPTLC analysis of ceramide, samples were spotted on an HPTLC plate and developed in chloroform, acetic acid (9:1 v/v). Ceramide (type III, Sigma) was used as a standard. For HPTLC analysis of sphingomyelin, samples were spotted on an HPTLC plate and developed in chloroform, acetone, acetic acid, methanol, water (50:20:15:10:5 v/v). Bovine sphingomyelin was used as standard. Standards lipids were visualized by exposure of the plate to iodine vapors. Radioactive lipids were visualized by fluorography.

RESULTS

GD3 Cell Depletion, de Novo Resynthesis, and TGN Accumulation at 20 °C—To investigate GD3 transport from TGN to plasma membrane, we developed a successful model in GD3-expressing CHO-K1 cells, which is established in our laboratory (23, 26). We could analyze the transport to plasma membrane of natural GD3, synthesized de novo, in cells where more than 96% of total GD3, mainly present in plasma membrane, was depleted by using P4, a potent inhibitor of complex glycolipid synthesis (23, 29) (see the scheme in Fig. 2). Cells were grown in the presence of P4 for 5 days, allowing the cells to mainly eliminate GD3 from plasma membrane (A). Then culture medium was removed, and cells were washed extensively to remove P4 and incubated with complete medium for 2 h at 37 °C to accumulate GD3 in the TGN (B). At the end of this period cells were incubated at 37 °C for during 7 (C) and 90 (F) min to allow GD3 exit and transport from TGN to plasma membrane. GD3 expression was analyzed by confocal microscopy and immunofluorescence in acetone-fixed cells using a monoclonal antibody R24 as the primary antibody and Alexa 546-conjugated goat anti-mouse IgG as the secondary antibody. Arrows in B indicate the accumulation of GD3 in the TGN. Images shown in this figure are single xy confocal sections. F, scale bar, 20 μm.

Fig. 1. Accumulation of GD3 in TGN at 20 °C and latter transport to the cell surface of CHO-K1 cells. Cells were grown in the presence of P4 for 5 days, allowing the cells to mainly eliminate GD3 from plasma membrane (A). Then culture medium was removed, and cells were washed extensively to remove P4 and incubated with complete medium for 2 h at 37 °C to accumulate GD3 in the TGN (B). At the end of this period cells were incubated at 37 °C for during 7 (C) and 90 (F) min to allow GD3 exit and transport from TGN to plasma membrane. GD3 expression was analyzed by confocal microscopy and immunofluorescence in acetone-fixed cells using a monoclonal antibody R24 as the primary antibody and Alexa 546-conjugated goat anti-mouse IgG as the secondary antibody. Arrows in B indicate the accumulation of GD3 in the TGN. Images shown in this figure are single xy confocal sections. F, scale bar, 20 μm.
other hand the presence of the inhibitor in the culture medium of CHO-K1 cells did not significantly alter the endogenous levels of sphingomyelin (Fig. 2B) and ceramide (Fig. 2C). Thus, we have established the experimental conditions to explore the role of different molecules of the sorting machinery in GD3 traffic to plasma membrane.

The Disialo Ganglioside GD3 Traffics to Plasma Membrane by a Rab11-independent Route—Rab11 is a Ras-related small GTPase that has been shown to regulate the delivery of proteins from the TGN to the plasma membrane either through endosomes or by a direct transit (13). Before evaluating the role of Rab11 on GD3 transport from TGN to plasma membrane, we investigated in CHO-K1 cells the effect of these GTPases on the sorting to plasma membrane of the temperature-sensitive VSVG protein, a useful transport marker. GD3-expressing CHO-K1 cells were grown in the presence of P4 for 5 days, and VSVG was expressed by transient DNA transfection 14 h before removing the inhibitor at 41 °C to accumulate VSVG in the ER. Then cells were incubated at 20 °C for 2 h to allow the accumulation of VSVG in the TGN in media containing cycloheximide to prevent further synthesis of VSVG (Fig. 3A, 20 °C). VSVG transport to the cell surface was initiated by changing the temperature to a permissive condition (Fig. 3A, 37 °C). Co-expression of the dominant negative Rab11S25N protein (Rab11S25N-GFP) was found to prevent the exit of VSVG protein from the Golgi complex, whereas wild type Rab11 (Rab11wt-GFP) protein expression allowed normal delivery of the protein marker to the plasma membrane (Fig. 3A, 37 °C). The expression of the constitutively activated Rab11 protein (Rab11Q70L) had no appreciable effect on VSVG delivery to the plasma membrane (data not shown). As also shown in Fig. 3, Rab11wt and Rab11S25N proteins were expressed both in the perinuclear region of the cells and dispersed throughout the cytoplasm. The perinuclear expression of Rab11wt-GFP was observed to be mainly associated with recycling endosomes, whereas Rab11S25N-GFP was found co-localizing with Golgi complex and recycling endosome markers.2 The expression of Rab11 variants did not alter the Golgi complex morphology, as evidenced by the expression of Sial-T2, a Golgi proximal resident (Fig. 3C). The analysis of Rab11 protein expression by Western blotting revealed the expected molecular mass (55 kDa) (data not shown).

Having shown that Rab11 participates in the delivery of a protein cargo (VSVG) from Golgi complex to plasma membrane in CHO-K1 cells, we next asked whether this protein might be involved in the transport of the disialo ganglioside GD3 between these organelles. To explore this we basically applied the protocol described in Fig. 1, except that Rab proteins were expressed by transient DNA transfection 2 days before removing the inhibitor of ganglioside synthesis (P4). After 2 h at 20 °C in the absence of the P4 inhibitor, GD3 synthesized de novo was mainly accumulated at the TGN (Fig. 3B, 20 °C). Under these conditions, the expression of wild type and mutant forms of Rab11 proteins had no apparent effect on cell physiology and GD3 accumulation in Golgi complex. Transfer to 37 °C caused the GD3 to become progressively concentrated at the plasma membrane in non-transfected cells (Fig. 3B, 37 °C), as shown in Fig. 1. Interestingly, the expression of wild type and dominant negative forms of Rab11 did not influence the capacity of GD3 to reach the cell surface (Fig. 3B). Moreover, the expression of the constitutively activated Rab11 protein (Rab11Q70L) had no appreciable effect on GD3 delivery to the plasma membrane (data not shown).

GD3 Exits the Golgi Complex Using a Brefeldin A-insensitive Process—Proteins like the adaptins AP1 and AP3 as well as GGA proteins have been identified in trans elements of the Golgi complex and appear to function in sorting cargo into nascent clathrin-coated vesicles. The recruitment of these proteins to

![FIG. 2. Effect of P4 on cellular glycosphingolipids, ceramide, and sphingomyelin. CHO-K1 cells were cultured for 5 days in culture medium without (−) or with (+) 2.4 μM P4. Lipids were metabolically labeled with [3H]palmitic acid for the last 18 h of culture. Lipid extracts were prepared, purified, chromatographed, and visualized as described under “Materials and Methods.” HPTLC plates were developed using chloroform, methanol, 0.2% CaCl2 (60:36:8 v/v) for GD3 and GT3 (A) or chloroform, acetone, acetic acid, methanol, water (50:20:15:10:5 v/v) for sphingomyelin (SM) and neutral lipids such as lactosylceramide (LacCer) and glucosylceramide (GlcCer) (B) or chloroform, acetic acid (9:1 v/v) for ceramide (Cer) (C). To better show lactosylceramide and glucosylceramide radioactive labels (upper part of panel B), the film was exposed for a longer time than that showing sphingomyelin expression (lower part of panel B), avoiding over-exposure of sphingomyelin bands. The positions of co-chromatographed lipid standards are indicated. A scheme of lipid biosynthesis is shown at the top of the figure. Also indicated in the scheme is the enzymatic reaction affected by the glycolipid inhibitor P4. Lipids migrated as multiple bands on the HPTLC plate because of the heterogeneity of fatty acyl chains of the molecules.

| Time/temperature | Golgi region | Rest of the cell |
|------------------|--------------|-----------------|
|                  | %            | %               |
| 120 min/20 °C    | 95.4 ± 3.2   | 4.6 ± 3.2       |
| 7 min/37 °C      | 78.7 ± 5.2   | 21.3 ± 5.2      |
| 15 min/37 °C     | 63.1 ± 5.1   | 36.9 ± 5.1      |
| 30 min/37 °C     | 44 ± 7.5     | 56 ± 7.5        |
| 90 min/37 °C     | 23.2 ± 6.2   | 76.8 ± 6.2      |
| 120 min/37 °C    | 9.2 ± 1.7    | 90.8 ± 1.7      |

2 G. Gomez and J. L. Daniotti, unpublished results.
Golgi membrane is sensitive to BFA (33) and is most likely regulated by the TGN-localized and BFA-inhibited GEFs (34). Before testing the potential role of this machinery in the exit of GD3 from Golgi complex, we first demonstrated that the constitutive transport of VSVG in GD3-expressing CHO-K1 cells is rapidly inhibited upon treatment with BFA, as already reported for BHK-21 cells (35). To test the BFA effect on VSVG transport from TGN to the cell surface, we first accumulated VSVG protein in the TGN. Thus, GD3-expressing CHO-K1 cells were grown in the presence of P4 for 5 days, and VSVG was expressed by transient DNA transfection 14 h before removing the inhibitor P4 with plasmids carrying cDNAs coding for VSVG and wild type Rab11 (Rab11wt-GFP) proteins or for VSVG and dominant negative Rab11S25N (Rab11S25N-GFP) proteins. Transfectant cells were incubated at 41 °C for 14 h to accumulate VSVG in the ER. Then cells were incubated at 20 °C for 2 h to allow the accumulation of VSVG in the TGN (VSVG, 20 °C). VSVG transport to the cell surface was initiated by changing the temperature to a permissive condition (VSVG, 37 °C). The expression of Rab11 versions was examined by the intrinsic fluorescence of GFP (GFP) fused to Rab11 proteins, and whereas VSVG expression was examined by the intrinsic fluorescence of YFP fused to VSVG protein. B, cells were grown in the presence of P4 for 5 days, allowing the cells to mainly eliminate GD3 from plasma membrane. Rab proteins (Rab11wt-GFP and Rab11S25N-GFP) were expressed by transient DNA transfection 2 days before removing the inhibitor P4. After 2 h at 20 °C in the absence of the inhibitor, GD3-synthesized de novo was mainly accumulated at the TGN (GD3, 20 °C). Transfer to 37 °C for 2 h caused the GD3 to become progressively concentrated at the plasma membrane both in Rab 11-transfected and non-transfected cells (GD3, 37 °C). GD3 expression was analyzed by confocal microscopy immunofluorescence in acetone-fixed cells using a monoclonal antibody R24 as the primary antibody and Alexa 546-conjugated goat anti-mouse IgG as the secondary antibody. Arrows in B (GFP and GD3 rows) indicate cells doubly expressing Rab11 proteins and GD3. C, CHO-K1 cells stably expressing GD3 synthase tagged at the C terminus with the epitope HA were transiently transfected to express Rab11wt-GFP or Rab11S25N-GFP. The expression of Rab11 versions was examined by the intrinsic fluorescence of GFP (GFP) fused to Rab11 proteins. The expression of Sial-T2-HA was visualized by using a monoclonal antibody anti-HA and a goat anti-mouse, Alexa 546-conjugated secondary antibody. Arrows in C indicate cells doubly expressing Rab11 proteins and Sial-T2. Images shown in A and B are single xy confocal sections. Images shown in C were acquired with a Zeiss inverted microscopy equipped with epifluorescence. Scale bar, 20 μm.

FIG. 3. GD3 traffics to the plasma membrane by a Rab11-independent pathway. A, GD3-expressing CHO-K1 cells were grown in the presence of P4 for 5 days and co-transfected before removing the inhibitor P4 with plasmids carrying cDNAs coding for VSVG and wild type Rab11 (Rab11wt-GFP) proteins or for VSVG and dominant negative Rab11S25N (Rab11S25N-GFP) proteins. Transfectant cells were incubated at 41 °C for 14 h to accumulate VSVG in the ER. Then cells were incubated at 20 °C for 2 h to allow the accumulation of VSVG in the TGN (VSVG, 20 °C). VSVG transport to the cell surface was initiated by changing the temperature to a permissive condition (VSVG, 37 °C). The expression of Rab11 versions was examined by the intrinsic fluorescence of GFP (GFP) fused to Rab11 proteins, and whereas VSVG expression was examined by the intrinsic fluorescence of YFP fused to VSVG protein. B, cells were grown in the presence of P4 for 5 days, allowing the cells to mainly eliminate GD3 from plasma membrane. Rab proteins (Rab11wt-GFP and Rab11S25N-GFP) were expressed by transient DNA transfection 2 days before removing the inhibitor P4. After 2 h at 20 °C in the absence of the inhibitor, GD3-synthesized de novo was mainly accumulated at the TGN (GD3, 20 °C). Transfer to 37 °C for 2 h caused the GD3 to become progressively concentrated at the plasma membrane both in Rab 11-transfected and non-transfected cells (GD3, 37 °C). GD3 expression was analyzed by confocal microscopy immunofluorescence in acetone-fixed cells using a monoclonal antibody R24 as the primary antibody and Alexa 546-conjugated goat anti-mouse IgG as the secondary antibody. As in A, the expression of Rab11 versions was examined by the intrinsic fluorescence of GFP (GFP) fused to Rab11 proteins. Arrows in B (GFP and GD3 rows) indicate cells doubly expressing Rab11 proteins and GD3. C, CHO-K1 cells stably expressing GD3 synthase tagged at the C terminus with the epitope HA (Sial-T2) were transiently transfected to express Rab11wt-GFP or Rab11S25N-GFP. The expression of Rab11 versions was examined by the intrinsic fluorescence of GFP (GFP) fused to Rab11 proteins. The expression of Sial-T2-HA was visualized by using a monoclonal antibody anti-HA and a goat anti-mouse, Alexa 546-conjugated secondary antibody. Arrows in C indicate cells doubly expressing Rab11 proteins and Sial-T2. Images shown in A and B are single xy confocal sections. Images shown in C were acquired with a Zeiss inverted microscopy equipped with epifluorescence. Scale bar, 20 μm.
to plasma membrane was markedly inhibited by BFA (~60%). To examine the effect of BFA on GD3 exit from TGN, we first accumulated GD3 in the TGN (Fig. 4, E and F) following the protocol described in Fig. 1. As already observed, transfer of the cells to 37 °C in the absence of BFA caused the GD3 to become progressively concentrated at the plasma membrane (Fig. 4G, -BFA). Interestingly, BFA had no significant effect both on GD3 exit from TGN and transport to the plasma membrane (Fig. 4H, +BFA). As a control of BFA action, we analyzed in all conditions the effect of BFA on the redistribution of Sial-T2 (proximal Golgi resident) into the ER (26) (data not shown). Our results indicate that in CHO-K1 cells operates a constitutive transport from the TGN to the cell surface, which is drastically inhibited by BFA. Regarding the lacking effect of BFA on GD3 exiting the TGN and exocytic transport, a preliminary interpretation supports the notion that in these processes BFA-sensitive GEF proteins might not participate, raising the existence of an alternative route(s) for glycolipid transport from Golgi to plasma membrane.

**BFA Blocks the Exit from Golgi Complex of GPI-YFP**

To evaluate whether BFA-sensitive GEFs proteins could operate in the exit and/or transport of another luminal membrane-bounded lipid, the effect of BFA in the delivery from TGN to plasma membrane of a chimeric protein containing a GPI-anchored signal fused to YFP (GPI-YFP) was investigated. GD3-expressing CHO-K1 cells were grown in the presence of P4 for 5 days. GPI-YFP was expressed by transient DNA transfection before removing the inhibitor P4. GPI-YFP-transfected CHO-K1 cells were incubated at 37 °C for 24 h. Then cells were incubated at 20 °C for 2 h to allow the accumulation of GPI-YFP from Golgi complex, cells that had been blocked at 20 °C were shifted to 37 °C in the absence (37 °C, -BFA) or presence (+BFA) of 2 μg/ml BFA. BFA was present in the culture medium during the last 15 min of culture at 20 °C.

**Scale bars:** 20 μm.
containing cycloheximide to prevent further synthesis of GPI-YFP (Fig. 5A, 20°C). Under this condition GPI-YFP was found co-localizing to a significant extent with UDP-GalNAc:LeCer/GM3/GD3 N-acetylgalactosaminyltransferase, a well-characterized TGN marker in CHO-K1 cells (26, 32) (data not shown). To test the effect of BFA in the exit of GPI-YFP from Golgi complex, cells that had been blocked at 20°C were shifted to 37°C in the absence (−BFA) or presence (+BFA) of BFA for 1 h (Fig. 5A, 37°C). As a control of BFA action, we analyzed the effect of BFA on the redistribution of Sial-T2 (proximal Golgi resident) into the ER (Fig. 5B). Results clearly demonstrated that, unlike GD3 trafficking to plasma membrane, the constitutive transport of GPI-YFP in GD3-expressing CHO-K1 cells is rapidly inhibited upon treatment with BFA, strongly suggesting in this process an active role of BFA-sensitive GEF proteins.

Triton X-100 Solubility of GD3 during Biosynthetic Pathway—One mechanism of organizing cargo into domains is that of clustering. Accumulating evidence suggests that signals such as N- and O-linked glycans and GPI anchors help sequester proteins into OGM in the TGN, which is recognized by the sorting machinery as a signal for Golgi complex exiting and apical delivery (36). We recently described that an important fraction of plasma membrane GD3 resides in OGM. However, we were unable to find evidence for OGM localization of neither newly synthesized GD3 nor GD3 synthase in the proximal Golgi (24). These results imply that nascent GD3 segregates from its synthesizing transferases and then enters OGM. This event could have taken place along the secretory pathway and/or at the cell surface or shortly after synthesis in the Golgi cisternae (e.g. TGN). To determine the time and place at which GD3 becomes Triton X-100-insoluble on its way to the plasma membrane, ceramide (precursor for GD3 synthesis) was metabolically labeled for 12 h with [3H]palmitate in the presence of the inhibitor P4 to block synthesis of GD3. Then culture medium was removed; cells were washed extensively to remove P4 and incubated with complete medium for 2 h at 20°C to synthesize and accumulate GD3 in TGN. After a 20°C treatment, cells were incubated at 37°C to allow GD3 exit from TGN. Membranes were extracted with 1% Triton X-100 at 4°C at different times (0, 7, 15, 30, 45, and 90 min), and lysates were centrifuged for 1 h to 100,000 × g at 4°C. The supernatants (S, soluble fraction) were removed from the pellets (I, insoluble fraction), and gangliosides were purified and analyzed by HPTLC as indicated in “Materials and Methods.” St, radioactive glycolipids obtained by metabolic labeling of GD3-expressing clone. The positions of glycolipids are indicated. The radioactive band labeled with an asterisk corresponds to a non-characterized lipid.

DISCUSSION
The topological orientation of ganglioside synthesis on the luminal surface of the Golgi complex implies a priori a vesicular transport process of these glycosphingolipids from Golgi complex to cell surface. Supporting this notion, it was concluded from experiments with a cell-free assay system that transport of glycosphingolipids and glycoproteins through the Golgi complex stacks occurs by a vesicular process that is biochemically and kinetically similar for both types of molecules (37). Furthermore, it has been indicated that transport vesicles containing ganglioside GM3 are transported to the plasma membrane of CHO-K1 cells.

Rab11 is a Ras-related small GTPase that has been shown to regulate the delivery of proteins from the TGN to the plasma membrane either through endosomes or by a direct transit (13). Although the dominant negative form of Rab11 efficiently affected the Golgi exiting and delivery of VSVG to the plasma membrane of CHO-K1 cells, it did not affect the efficiency of GD3 transport to the cell surface. Although we cannot absolutely discard with these experiments the existence of a GD3 transport dependent on Rab11 proteins, the Rab protein-independent route(s) is able to move almost all GD3 molecules from the TGN to plasma membrane in CHO-K1 cells.

Adaptons and GGAs proteins have been identified in trans elements of the Golgi complex and appear to function in sorting cargo into nascent clathrin-coated vesicles. The recruitment of these proteins to Golgi membrane is sensitive to BFA (33) and is most likely regulated by the TGN-localized and BFA-inhibited GEFs (34). At concentrations at which it blocks VSVG transport from the Golgi complex to the plasma membrane in CHO-K1 cells, BFA had no appreciable effect on GD3 transport from TGN to the cell surface, suggesting that in these processes BFA-sensitive GEF proteins might not participate.

It was described in chick embryo retina cells that the proximal-synthesized GD3 uses a transport mechanism dependent on ADP-ribosylation factor (ARF, BFA-affected transport) (39). The reasons for the apparent discrepancy between this result and those described in this work may be due to the conditions of metabolic GD3 labeling and BFA treatment, which is essential in the interpretation of these results since both studies could be discriminating against different transport stages or, perhaps, to the different cell types used. Accumulating evidence suggests that the site of synthesis of GD3 in CHO-K1 cells is the proximal Golgi. Thus, most of the N-linked oligosaccharides from Sial-T2 (GD3 synthase) were found to be both endoglucosidase H-sensitive and neuraminidase-insensitive, and in cells treated with BFA, a major fraction of the enzyme redistributed to the ER, indicative of a proximal Golgi location (26). Under our experimental conditions and given the above-mentioned information, it is assumed that GD3 is mostly synthesized in the proximal Golgi and then conveyed and accumulated in the TGN at 20°C. At the end of this process the effect of BFA on TGN exit and transport of GD3 to the cell surface of...
CHO-K1 cells was analyzed. On the contrary, in experiments done in chick embryo retina cells (39), BFA was present during the metabolic labeling of the proximal-synthesized GD3, raising the possibility that GD3 transport from the proximal Golgi to TGN is an ARF-mediated process in neural cells, whereas the exit of GD3 from the TGN remains to be evaluated in these cells. The same assumption could explain the results obtained by Warnock et al. (40). The authors described GM3 transport in CHO cells as inhibited by BFA. However, like experiments carried in neural cells (39), BFA was present during the metabolic labeling of the proximal-synthesized GM3.

It was hypothesized early that genesis of GEM occurs by a dynamic process in the TGN (6, 41). Relying on the localization of GEM at the TGN and the affinity of glycosphosphatidylinositol-anchored proteins for GEM (23–25), it was demonstrated that the GPI-linked proteins segregate into GEM-enriched carriers in the TGN and then are directed to the plasma membrane (25, 42, 43). More recently, an alternative model proposes that glycosphosphatidylinositol-anchored protein exits the TGN in membrane-bounded carriers that also contain non-resident GEM, although the two cargoes were laterally segregated (36). In this work we demonstrated that the constitutive transport of GPI-YFP in GD3-expressing CHO-K1 cells is rapidly inhibited upon treatment with BFA, strongly suggesting in this process an active role of BFA-sensitive GEF proteins.

It was described that high concentrations (>100 μM) of D,L-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol, a similar compound to P4, antagonizes the effect of BFA on retrograde membrane transport from Golgi to ER (44–46) and inhibits anterograde membrane transport from the Golgi complex to the plasma membrane (47, 48). It was suggested that the effect of D,L-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol on these processes is probably attributed to an elevation of the intracellular ceramide and a reduction of sphingomyelin levels (47, 48), changes in calcium homeostasis (44), or inhibition of BFA-induced ADP-ribosylation of specific proteins (45). In this work P4 depletes cellular gangliosides at a very low concentration (2.4 μM) without causing both an increase in ceramide and a decrease in sphingomyelin levels. Moreover, P4 was removed before analyzing the effect of BFA, which effectively inhibited the constitutive transport of GPI-YFP from the Golgi to the plasma membrane and led to a dispersion of most proximal Golgi resident Sial-T2 throughout the ER. Taking these results into consideration, it is highly improbable that P4 antagonizes the effect of BFA on the GD3 exit from TGN and transport to the plasma membrane of CHO-K1 cells.

Like glycosphosphatidylinositol-anchored proteins, GD3 was found to be a constituent of GEM or DRMs in the plasma membrane of CHO-K1 cell, but GD3 was almost absent from GEM when it was newly synthesized in the proximal Golgi (24). We took into consideration the possibility that GD3 could partition into GEM shortly after the synthesis in the Golgi cisternae (e.g. TGN), which could represent a sorting signal. Results shown in this work indicate that GD3 exits the TGN mainly divided from DRMs and strongly suggests that partitioning of GD3 into DRMs at the TGN is not absolutely necessary for its exit from the organelle. Moreover, it seems that alternative sorting signals could operate in the exiting of the glycolipid from the Golgi complex. Although we produced in CHO-K1 cells a block in glycosphingolipid synthesis, it does not seem to influence the distribution of membrane components on the cell surface of CHO-K1 cells, as already reported (23, 49). Indeed, it was described that glycosphingolipids are not essential for the formation of DRMs and for efficient packaging and cell surface transport of placental alkaline phosphatase, a GPI-anchored protein resident in detergent-insoluble domains (50).

Concerning possible mechanisms of GD3 transport from Golgi to plasma membrane, we cannot entirely exclude the possibility that GD3 is transported to the plasma membrane by conventional vesicle coats. However, a plausible alternative is that the GD3 transport occurs by mechanisms other than coat-mediated budding (8). In this sense the existence of a mechanism of constitutive export from the Golgi complex by formation of Golgi-to-plasma membrane carriers (GPCs) was recently suggested (51). GPCs emerge from the Golgi through a bulk flow process involving the formation of tubular TGN cargo domains that later detach en bloc from the TGN. The formation of GPCs does not involve cargo concentration and is not associated with the presence of known adapters (AP1, AP2, AP4, and GGA) and coats (clathrin and β-COP) proteins on the GPC precursor. In light of these antecedents it is particularly attractive to investigate if GD3 conveys to plasma membrane by using GPC carriers and whether this putative mechanism of intracellular transport of GD3 is also used for other glycolipids. Finally, whatever the mechanism of GD3 exiting from Golgi complex, it is clear that lateral membrane segregation of VSVG (non-GEM resident) and GPI-anchored proteins (GEM resident) from GD3 is at least required before exiting TGN.

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Ganglioside GD3 Traffics from the trans-Golgi Network to Plasma Membrane by a Rab11-independent and Brefeldin A-insensitive Exocytic Pathway
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