Ganglioside glycosyltransferases and newly synthesized gangliosides are excluded from detergent-insoluble complexes of Golgi membranes

Pilar M. CRESPO, Adolfo R. ZURITA, Claudio G. GIRAUDO, Hugo J. F. MACCIONI and Jose L. DANIOTTI¹

Centro de Investigaciones en Química Biológica de Córdoba, CIQUIBIC (UNC-CONICET), Departamento de Química Biológica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Ciudad Universitaria, 5000 Córdoba, Argentina

GEM (glycosphingolipid-enriched microdomains) are specialized detergent-resistant domains of the plasma membrane in which some gangliosides concentrate. Although genesis of GEM is considered to occur in the Golgi complex, where the synthesis of gangliosides also occurs, the issue concerning the incorporation of ganglioside species into GEM is still poorly understood. In this work, using Chinese hamster ovary K1 cell clones with different glycolipid compositions, we compared the behaviour with cold Triton X-100 solubilization of plasma membrane ganglioside species with the same species newly synthesized in Golgi membranes. We also investigated whether three ganglioside glycosyltransferases (a sialyl-, a N-acetylgalactosaminyl- and a galactosyl-transferase) are included or excluded from GEM in Golgi membranes. Our data show that an important fraction of plasma membrane G_{M3} , and most G_{D3} and G_{T3} , reside in GEM. Immunocytochemical examination of G_{D3} -expressing cells

showed G_{D3} to be distributed as cold-detergent-resistant patches in the plasma membrane. These patches did not co-localize with a glycosylphosphatidylinositol-anchored protein used as GEM marker, indicating a heterogeneous composition of plasma membrane GEM. In Golgi membranes we were unable to find evidence for GEM localization of either ganglioside glycosyltransferases or newly synthesized gangliosides. Since the same ganglioside species appear in plasma membrane GEM, it was concluded that *in vivo* nascent G_{D3} , G_{T3} and G_{M3} segregate from their synthesizing transferases and then enter GEM. This latter event could have taken place shortly after synthesis in the Golgi cisternae, along the secretory pathway and/or at the cell surface.

Key words: detergent-resistant membrane, endomembrane, ganglioside, Golgi complex, glycolipid, glycolipid glycosyltransferase.

INTRODUCTION

Glycolipids, particularly gangliosides (named according to [1]), are important constituents of biological membranes of vertebrate cells. It has been speculated that they participate in cell-surface events such as signal transduction and cell adhesion [2–6]. Glycosphingolipids reside preferentially in GEM (glycosphingolipid-enriched microdomains) at the cell surface [7–10], and it was hypothesized that glycosphingolipids segregated in specialized microdomains could regulate the distribution and function of membrane proteins [4,11–14].

GEM are dynamic assemblies of cholesterol, saturated phospholipids and sphingolipids, which are characterized by their insolubility in Triton X-100 under cold conditions and by a light buoyant density [9,11]. The presence of specific ganglioside species in GEM has been demonstrated in different systems with diverse experimental approaches. Segregation of ganglioside G_{M1} from G_{D3} on plasma membrane and isolated GEM from neurons has been reported [12]. G_{M1} was also found to be associated with GEM in the plasma membrane from CaCo-2 human intestinal epithelial cells [15] and COS-7 cells [16], and a distinctive distribution of individual ganglioside species was found in CHO (Chinese hamster ovary) K1 cells, some of them in physical association with a GPI-AP [GPI (glycosylphosphatidylinositol)-anchored protein], a typical GEM constituent [14]. Also, partition of gangliosides into lipid clusters reconstituted in a supported model membrane monolayer has been reported [12,13].

It was early hypothesized that genesis of GEM occurs by a dynamic process in the *trans*-Golgi network where polarized sorting of apical and basolateral cargo occurs [7,17]. However, few works refer to the existence of detergent-resistant domains in membranes of the secretory pathway. In this regard, the behaviour to detergent extraction of molecules such as GPI precursors, GPI-AP and sphingomyelin has been analysed in the ER (endoplasmic reticulum) and Golgi complex [18–22].

So far, the membrane distribution of glycolipids, particularly gangliosides, at the Golgi complex has not been investigated. Most of the GEM constituents are synthesized in the ER and in the Golgi complex by enzymes resident in these organelles. Particularly, the synthesis of ganglioside molecules starts in the ER, where the ceramide moiety is synthesized, and continues in the lumen of the Golgi complex by the action of specific glycosyltransferases, which build up the oligosaccharide moiety (Figure 1). A major limitation in the search for GEM along the endomembrane system resides in the difficulty inherent in the collection of these membranes freed from contaminating plasma membrane, the main site of deposition of gangliosides, and in quantities sufficient to undertake this type of analysis. In this work, applying to CHO-K1 cells an experimental approach that overcomes the necessity of separation of Golgi membranes from plasma membranes, we have compared the behaviour of their respective constituent gangliosides to cold Triton X-100 solubilization. Gangliosides of Golgi membranes were radioactively labelled *in vitro* from appropriate radioactive sugar nucleotides and plasma

Abbreviations used: CHO, Chinese hamster ovary; CFP, cyan fluorescent protein; ER, endoplasmic reticulum; GEM, glycosphingolipid-enriched microdomains; GFP, green fluorescent protein; Gal-T2, UDP-Gal:G_{A2}/G_{M2}/G_{D2} galactosyltransferase; GalNAc-T, UDP-GalNAc:LacCer/G_{M3}/G_{D3} N-acetyl-galactosaminyltransferase; GPI, glycosylphosphatidylinositol; GPI-AP, GPI-anchored protein; HA, YPYDVPDYA nanopptide of influenza virus haemagglutinin; HPTLC, high-performance TLC; mAb, monoclonal antibody; M6PR, mannose-6-phosphate receptor; NANase, neuraminidase; Sial-T2: CMP-NeuAc:G_{M3} sialyltransferase; VSVG, vesicular stomatitis virus glycoprotein; YFP, yellow fluorescent protein.

¹ To whom correspondence should be addressed (e-mail daniotti@dqf.fcq.unc.edu.ar).

Figure 1 Glycolipid labelling of wild-type cells and of stably transfected CHO-K1 cell clones

Wild-type cells (WT) and clones 2, 3 and 4 were metabolically labelled from [14 C]Gal during 24 h. Lipid extracts were prepared, purified, chromatographed and visualized as indicated in the Materials and methods section. The positions of co-chromatographed glycolipid standards are indicated. A scheme of glycolipid biosynthesis is shown at the top of the Figure indicating the step of the pathways of ganglioside synthesis opened by transfection of Sial-T2 (clone 2), GalNAc-T (clone 3) or GalNAc-T and Gal-T2 (clone 4) to the wild-type cells expressing only G_{M3} (WT).

membrane gangliosides were metabolically labelled by long-term feeding of cells with radioactive galactose. Different CHO-K1 cell clones were used, each stably transfected with particular glycosyltransferases and hence expressing different sets of ganglioside species, which allowed the examination of the detergent-solubility behaviour of individual ganglioside species, and of the acting glycosyltransferases. Our data show that an important fraction of plasma membrane G_{M3} , and most G_{D3} and G_{T3} , reside in GEM. Immunocytochemical examination of G_{D3} -expressing cells showed G_{D3} distributed as detergent-resistant patches in the plasma membrane. These patches did not co-localize with the GEM marker GPI-AP [23], indicating a heterogeneous composition of the plasma membrane GEM. In Golgi membranes we were unable to find evidence for GEM localization of either ganglioside glycosyltransferases or newly synthesized gangliosides.

MATERIALS AND METHODS

Cell lines, cell culture and DNA transfections

The following CHO-K1 cell clones expressing different ganglioside glycosyltransferases were used: wild-type CHO-K1 cells

(ATCC, Manassas, VA, U.S.A.); clone 2, a stable Sial-T2 [CMP-NeuAc: G_{M3} sialyltransferase; tagged at the C-terminus with the YPYDVPDYA nanopeptide epitope of the viral HA (haemagglutinin)] transfectant expressing the ganglioside G_{D3} and G_{T3} [5,24]; clone 3, a stable GalNAc-T (UDP-GalNAc:LacCer/ G_{M3} / G_{D3} N-acetylgalactosaminyltransferase; tagged at the C-terminus with ten amino acids of human c-Myc) transfectant mostly expressing gangliosides G_{M3} , G_{M2} and to a lesser extent G_{M1} and G_{D1a} [25], and clone 4, a stable double transfectant expressing GalNAc-T and Gal-T2 (UDP-Gal: G_{A2} / G_{M2} / G_{D2} galactosyltransferase; tagged at the C-terminus with the HA epitope) [14] having increased expression of G_{M1} and G_{D1a} .

Cells were maintained at 37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics. Cells were transfected with 1 μ g/dish of plasmids carrying cDNAs coding for GPI-YFP [the total sequence of the YFP (yellow fluorescent protein) fused to a GPI-attachment signal] or VSVG-CFP [the VSVG (vesicular stomatitis virus glycoprotein) fused to CFP (cyan fluorescent protein)] using Lipofectamine (Gibco-BRL, Gaithersburg, MD, U.S.A.) or FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN, U.S.A.) as DNA carriers, essentially according to the manufacturers' recommendations. GPI-YFP and VSVG-CFP fusion constructs were kindly supplied by Dr P. Keller, Max-Planck Institute, Dresden, Germany. After transfection (15 h) the cells were washed with cold PBS, harvested by scraping and lysed as indicated below or washed with PBS and fixed for immunofluorescence microscopy.

Triton X-100 membrane extraction

Cells were washed with cold PBS and harvested by scraping. Samples were treated with 0.5 ml of lysis buffer containing 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1 M Na_2CO_3 , 5 μ g/ml aprotinin, 0.5 μ g/ml leupeptin, 0.7 μ g/ml pepstatin and 25 mM Tris/HCl, pH 7.5 (TNE buffer), at 4 °C for 1 h, and then centrifuged for 1 h at 100 000 g at 4 °C. The supernatant (soluble fraction) was removed, and the pellet (insoluble fraction) was resuspended in 0.2 ml of lysis buffer. Proteins from soluble and insoluble fractions were precipitated with chloroform/methanol (1:4, v/v). The pellets were resuspended in Laemmli buffer [26] and subjected to SDS/PAGE and Western blotting.

Sucrose-density-gradient separation

Cells were lysed in 0.5 ml of TNE lysis buffer at 4 °C for 1 h. Lysates were centrifuged for 10 h at 150 000 g at 4 °C on continuous sucrose gradients (5–35%) in TNE buffer without Triton X-100. Twelve fractions were collected from the bottom of the tube with a fraction collector. Proteins in each fraction were precipitated with 10% trichloroacetic acid, resuspended in Laemmli buffer and subjected to SDS/PAGE and Western blotting. Sucrose concentration in each fraction was determined by refractometry.

Electrophoresis and Western blotting

Proteins were resolved by electrophoresis through SDS/PAGE gels (4–20%) under reducing conditions [26] and then electrophoretically transferred to nitrocellulose membranes [27] for 1 h at 300 mA. Protein bands in nitrocellulose membranes were visualized by Ponceau S staining. For immunoblotting, non-specific binding sites on the nitrocellulose membrane were blocked with 5% non-fat dry milk or with 2.5% BSA/2.5%

polyvinylpyrrolidone 40 in Tris-buffered saline (400 mM NaCl/100 mM Tris/HCl, pH 7.5), depending on the antibody. Anti-GFP (green fluorescent protein) polyclonal antibody (Roche Molecular Biochemicals), anti-HA and anti-c-Myc [mouse mAbs (monoclonal antibodies)] were used at a dilution of 1:800, 1:500 and 1:300, respectively. Bands were detected by Protein A coupled to horseradish peroxidase combined with the chemiluminescence detection kit (Western lightning; PerkinElmer Life Sciences, Indianapolis, IN, U.S.A.) and Kodak Biomax MS films. The molecular masses were calculated based on calibrated standards (Gibco-BRL) run in every gel. The relative contribution of individual bands was calculated using the computer software Scion Image on scanned films of low-exposure images.

Radioactive labelling of gangliosides

For *in vitro* labelling of endogenous gangliosides in Golgi membranes, CHO-K1 cells were suspended in 10 mM Tris/HCl buffer (pH 7.2) containing 0.25 M sucrose, washed twice in the same solution and homogenized. A microsomal membrane fraction (containing Golgi membranes) was collected by centrifugation for 1 h at 100 000 *g*. One-step labelling of endogenous ganglioside acceptors [28] was carried out as follows: membranes (80 µg of protein) were incubated for 2 h in an incubation system that contained, in a final volume of 20 µl, 45 mM MnCl₂, 20 µM CMP-[³H]NeuAc or 50 µM UDP-[³H]Gal or 50 µM UDP-[³H]GalNAc, 25 mM Hepes/KOH (pH 7.0), 25 mM KCl and 2.5 mM magnesium acetate. For each condition, the labelled nucleotide was added to give a specific radioactivity of 12 500 c.p.m./pmol for CMP-[³H]NeuAc or 5000 c.p.m./pmol for UDP-[³H]Gal and UDP-[³H]GalNAc. For metabolic labelling *ex vivo*, cells in culture (3 × 10⁵ cells/35 mm dish) were labelled with 2 µCi/ml of D-[U-¹⁴C]galactose ([¹⁴C]Gal; DuPont NEN; 329.5 mCi/mmol) during 24 h.

Lipid extraction and chromatography

For glycolipid analysis, cells after metabolic labelling were washed with cold PBS, scraped from the plate and pelleted by centrifugation. Then lipids were extracted from the pellet with chloroform/methanol (2:1, v/v) and freed from water-soluble contaminants by passing through a Sephadex G-25 column. The lipid extract was supplemented with appropriate amounts of standard gangliosides and chromatographed as indicated below.

For GEM analysis, membranes after labelling endogenous gangliosides *in vitro* or cells after metabolic labelling were extracted with 1 % Triton X-100 at 4 °C for 1 h, and then centrifuged for 1 h at 100 000 *g* at 4 °C. Glycolipids from the supernatant (soluble fraction) and from the pellet (insoluble fraction) were subjected to Folch-Pi partition [29]. The resulting aqueous phases were freed from Triton X-100 and sucrose (non-ionic molecules) by passing through DEAE-Sephadex column. Under this condition, neutral lipids such as glucosylceramide and lactosylceramide 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, MA, U.S.A.). The eluted lipid fraction was supplemented with appropriate amounts of standard gangliosides and chromatographed on HPTLC plates (high-performance TLC plates; Merck, Darmstadt, Germany) using chloroform/methanol/0.2 % CaCl₂ (60:36:8, by vol.) as solvent. Standard gangliosides were visualized by exposure of the plate to iodine vapours. Routinely 2000–3000 c.p.m. were spotted on

each lane. Radioactive gangliosides were visualized using a Fuji Photo Film Bio Imagen analyser or visualized by fluorography after dipping the plate in 0.4 % melted 2,5-diphenyloxazole in 2-methylnaphthalene and exposure to a radiographic film at –70 °C, usually for 4–6 days [5].

Immunofluorescence microscopy

Cells grown on coverslips were washed twice in PBS, fixed in acetone at –20 °C for 7 min, washed in PBS and incubated in 3 % BSA/PBS buffer for 1 h at 37 °C to block non-specific binding sites. Coverslips were then incubated overnight at 4 °C with primary antibodies, washed five times with 1 % BSA/PBS buffer, and exposed to secondary antibodies for 1.5 h at 37 °C. The primary antibodies were: mouse mAb anti-G_{D3} (IgG), clone R24 (a gift from Dr K. Lloyd, Memorial Sloan Kettering Cancer Research Center, New York, NY, U.S.A.), diluted 1:200; rabbit polyclonal anti-calnexin (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) diluted 1:200; rabbit polyclonal anti-mannose-6-phosphate receptor (M6PR; a gift from Dr A. Cáceres, Instituto Mercedes y Martín Ferreyra, Córdoba, Argentina) diluted 1:150; and rabbit polyclonal anti-mannosidase II (from Dr K. Moremen, University of Georgia, Athens, GA, U.S.A.) diluted 1:300. Secondary antibodies were Alexa 488- or Alexa 546-conjugated goat anti-mouse antibodies (Santa Cruz Biotechnology), diluted 1:1000, or rhodamine-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA, U.S.A.), diluted 1:700. After final washes with 1 % BSA/PBS, cells were mounted in mounting fluid (Light Diagnostics, Temecula, CA, U.S.A.).

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).

Neuraminidase treatment

Cells from clone 4 metabolically labelled as indicated above were incubated for 2 h at 37 °C in Dulbecco's modified Eagle's medium containing 2 units/ml neuraminidase (NANase) type V from *Clostridium perfringens* (Sigma-Aldrich, St. Louis, MO, U.S.A.), washed with PBS and lipids were extracted and chromatographed as indicated above.

RESULTS

CHO-K1 cell clones

The pattern of radioactive gangliosides of cell clones expressing different ganglioside glycosyltransferases is shown in Figure 1. We have previously shown that after 24 h of metabolic labelling most radioactive gangliosides localize at the plasma membrane [14]. Wild-type CHO-K1 cells predominantly express the ganglioside G_{M3} (Figure 1, WT), while those stably expressing the Sial-T2 cDNA (clone 2) [5,24] synthesize mostly G_{D3} and G_{T3}, accumulate lactosylceramide and accumulate practically no G_{M3} (Figure 1, clone 2). CHO-K1 cells stably expressing the human full-length GalNAc-T cDNA (clone 3) [25] synthesize the a-series ganglioside G_{M2} and, to a lesser extent, G_{M1} and G_{D1a} because of the constitutive expression in these cells of the enzymes involved in the synthesis of G_{M1} and G_{D1a} [30] (Figure 1, clone 3). Clone 4, stably expressing both GalNAc-T and Gal-T2, shows increased expression of G_{M1} and G_{D1a} (Figure 1, clone 4).

Figure 2 Metabolic labelling and Triton X-100 extraction of total gangliosides expressed in wild-type and genetically modified CHO-K1 cells

Lipids from wild-type CHO-K1 cells (WT) and cells from clones 2, 3 and 4 were metabolically labelled from [14 C]Gal for 24 h. Aliquots of homogenates from these cells, or from cells of clone 4 previously incubated for 2 h with NANase (Clone 4 + NANase), were used for total lipid analysis (T) and for cold-detergent solubility analysis (S, soluble fraction; I, insoluble fraction). The positions of co-chromatographed glycolipid standards are indicated.

Triton X-100 extraction of different plasma membrane gangliosides

Clones metabolically labelled as indicated in Figure 1 were extracted with 1% Triton X-100 at 4 °C and lysates were separated in a soluble and an insoluble fraction by centrifugation for 1 h at 100 000 *g* at 4 °C. Gangliosides were purified from the soluble and insoluble fractions and analysed by HPTLC as indicated in the Materials and methods section. G_{M3} from wild-type cells partitioned in equal percentage between the soluble and the insoluble fractions (Figure 2) and G_{D3} and G_{T3} from clone 2 were about 80% insoluble to Triton X-100 extraction. On the other hand, more complex gangliosides such as G_{M2} in clone 3 or G_{D1a} and G_{M1} in clone 4 were mainly found in the soluble fraction. To better analyse the behaviour of G_{M1} to detergent extraction, the amount of G_{M1} was increased in clone 4 by incubating the cells with *C. perfringens* NANase to convert G_{D1a} to G_{M1} (Figure 2, Clone 4 + NANase). Even under these conditions, G_{M1} was found mainly in the soluble fraction after detergent extraction. Results from this experiment disclose a different behaviour of individual ganglioside species to detergent extraction, which probably reflect the heterogeneous composition of GEM present in membranes from different CHO-K1 cell clones [12,14].

The disialo ganglioside G_{D3} is enriched in patches in the plasma membrane of CHO-K1 cells expressing Sial-T2

Biochemical results from Figure 2 disclosed that G_{D3} from clone 2 was about 80% insoluble to Triton X-100 extraction. We next characterized in this clone the subcellular location of G_{D3} and analysed its behaviour to Triton X-100 extraction of cells before fixation. As shown in Figures 3(A) and 3(B) G_{D3} immunostaining with mAb R24 was typical of a plasma membrane constituent with a patchy distribution, although a minor fraction was also observed in internal membranes. The expression of G_{D3} was under the limit of detection in wild-type CHO-K1 cells, which only express G_{M3} , thus confirming the specificity of the antibody (result not shown). Membrane patches of G_{D3} remained as such after extraction of cells with 1% Triton X-100 at 4 °C, before fixation (Figure 3C). Under the same conditions VSVG-CFP, a non-GEM marker, was efficiently removed while GPI-YFP, a GEM marker,

remained associated to the cells (result not shown). The fraction of G_{D3} observed in internal membranes was further investigated by examining co-localization with appropriated markers of organelles. Co-localization of G_{D3} was observed neither with calnexin, an ER marker (Figures 3D–3F), nor with mannosidase II, a medial Golgi marker (Figures 3G–3I). However, G_{D3} was found partially co-localizing with M6PR, a marker of the *trans*-Golgi network and late endosomes [24] (Figures 3J–3L, see yellow spots in merge panel 3L). The co-localization of G_{D3} with M6PR observed on vesicular structures close to the plasma membrane probably represent late endosomes because no co-localization was observed of G_{D3} and GalNAc-T, a *trans*-Golgi network resident protein in CHO-K1 cells [25] (result not shown).

To determine if other GEM-associated molecules co-localize with G_{D3} in membrane patches, cells expressing G_{D3} (clone 2) were transiently transfected with a cDNA encoding GPI-YFP, a chimaeric protein containing a GPI-anchored signal fused to the YFP. Interestingly, G_{D3} was essentially found not to co-localize with GPI-YFP (Figures 3M–3O). Altogether, results of Figure 3 show that G_{D3} is mainly expressed as detergent-resistant patches on the plasma membrane of cells (in line with biochemical experiments shown in Figure 2), although it did not co-localize mainly with GPI-YFP, a previously well-characterized GEM marker [23].

Newly synthesized gangliosides are excluded from GEM in Golgi membrane

To investigate if GEM localization of plasma membrane gangliosides is defined at the stage of their synthesis in the Golgi complex, microsomal membranes (containing Golgi membranes) were collected from wild-type CHO-K1 cells or from CHO-K1 clones 2, 3 and 4 and incubated for 2 h with different labelled nucleotides as follows (see Figure 1): membranes from wild-type CHO-K1 cells and clone 2 were incubated with CMP-[3 H]NeuAc; those from clone 3 were incubated with UDP-[3 H]GalNAc and those from clone 4 were incubated with UDP-[3 H]Gal or CMP-[3 H]NeuAc. After incubation, the pattern of radioactive glycolipids was analysed in the cold Triton X-100-soluble and -insoluble fractions by HPTLC. Figure 4 shows the behaviour to

Figure 3 For legend see next page

Figure 4 Radioactive labelling and Triton X-100 extraction of endogenous glycolipid acceptors from wild-type and genetically modified CHO-K1 cells

Microsomal membranes (containing Golgi membranes) were collected from wild-type CHO-K1 cells (WT) or cells from CHO-K1 clones (clones 2, 3 and 4) and incubated for 2 h with different labelled nucleotides as follows: membranes from wild-type CHO-K1 cells, clone 2 and clone 4 were incubated with CMP-[3 H]NeuAc; membranes from clone 3 were incubated with UDP-[3 H]GalNAc and membranes from clone 4 were incubated with UDP-[3 H]Gal. After incubation, membranes were extracted with 1% Triton X-100 at 4 °C 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 analysed by HPTLC as indicated in the Materials and methods section. St, radioactive glycolipids obtained by metabolic labelling of each clone as indicated in lanes T of Figure 2. Radioactive bands, which are labelled with asterisks, correspond to unincorporated sugar nucleotides. The positions of glycolipids are indicated.

Triton X-100 extraction of different newly synthesized ganglioside species. Interestingly, all ganglioside species were found to partition into the soluble fraction of Triton X-100 extraction. A minor percentage (<5%) of G_{D3} and G_{T3} from clone 2 was also found in the insoluble fraction. These results indicate that newly synthesized gangliosides are not typical components of GEM in membranes from Golgi complex.

Ganglioside glycosyltransferases are excluded from GEM in Golgi membranes

We next explored the behaviour of three different ganglioside glycosyltransferases to the extraction with Triton X-100 at 4 °C. The Golgi expression of these full-length, tagged forms of sialyl-, N-acetylgalactosaminyl- and galactosyl-transferases has been described already [24,25,31,32]. The solubility or insolubility of the enzymes in the non-ionic detergent was analysed by both velocity sedimentation and isopycnic separation in a continuous sucrose gradient. Cell homogenates from CHO-K1 clones constitutively expressing Sial-T2 (clone 2) and stable double-transfectant cells expressing GalNAc-T and Gal-T2 (clone 4) were extracted with Triton X-100 at 4 °C and then ultracentrifuged for 1 h at 100 000 *g* at 4 °C. The supernatants (soluble fraction) and the pellets (insoluble fraction) were resuspended in lysis buffer, resolved in SDS/PAGE and Western blotted with the appropriate antibodies. As shown in Figure 5, Sial-T2, GalNAc-T

Figure 5 Detergent solubility of ganglioside glycosyltransferases, GEM and non-GEM markers

Cell homogenates from CHO-K1 clones constitutively expressing Sial-T2-HA (clone 2), stable double transfectant expressing GalNAc-T-*myc* (where *myc* is 10 amino acids of human c-Myc) and Gal-T2-HA (clone 4) and CHO-K1 cells transiently expressing GPI-YFP (GEM marker) or VSVG-CFP (non-GEM marker) were extracted with Triton X-100 at 4 °C and then ultracentrifuged for 1 h at 100 000 *g* at 4 °C. The supernatants (S, soluble fraction) were removed, and the pellets (I, insoluble fraction) were resuspended in lysis buffer. Proteins from soluble and insoluble fractions were resolved in SDS/PAGE and Western blotted with the appropriate antibody (anti-GFP to reveal GPI-YFP and VSVG-CFP, anti-HA to reveal Sial-T2 and Gal-T2 or anti-c-*myc* to reveal GalNAc-T).

and Gal-T2 were 97, 98 and 90% soluble, respectively. As a control, we analysed the behaviour to Triton X-100 extraction of GPI-YFP, a GEM marker, and of VSVG-CFP, a non-GEM marker. As expected [14], the GEM marker (GPI-YFP) was 91% insoluble, whereas the non-GEM marker (VSVG-CFP) was less than 30% insoluble (Figure 5). Additionally, we also examined the distribution of the glycosyltransferases in sucrose density gradients (Figure 6). For this, homogenates from

Figure 3 Steady-state location of G_{D3} in CHO-K1 cells

Representative images of subcellular G_{D3} location in CHO-K1 cells stably expressing Sial-T2 (clone 2) and transiently expressing GPI-YFP. Cells were immunostained for G_{D3} with mAb R24 (A–D, G, J and M, green) and with appropriate antibodies for an ER marker (calnexin, E, red), a Golgi marker (mannosidase II, H, red), a *trans*-Golgi and late endosomal marker (M6PR, K, red). The GEM marker (GPI-YFP, N) was also examined by the intrinsic fluorescence of YFP (pseudo-coloured red). F, I, L and O are merged images from D and E, G and H, J and K, and M and N, respectively. Panel C shows cells extracted with cold 1% Triton X-100 before fixation, and then immunostained for G_{D3} expression. The insets in A and C show details of the boxed areas at higher magnification with arrows pointing to patches of G_{D3}. Single confocal sections of 0.2 μ m were taken parallel to the coverslip except for B in which a series of z sections were collected and displayed using the ortho mode in LSM5 Pascal software provided with the microscope. A single xy section of the stack is shown in B. An xz section (a') is shown at the bottom of B and an yz section (b') at the right of B. Scale bars: A, 20 μ m (for panels A–C and G–O); D, 15 μ m (for panels D–F).

Figure 6 Sucrose density gradient analysis of ganglioside glycosyltransferases in CHO-K1 transfectants

Cells from CHO-K1 clones constitutively expressing Sial-T2-HA (clone 2), stable transfectant expressing both GalNAc-T-myc (where *myc* is 10 amino acids of human c-Myc) and Gal-T2-HA (clone 4) and CHO-K1 cells transiently expressing GPI-YFP or VSVG-CFP were lysed in lysis buffer at 4 °C for 1 h and centrifuged for 10 h at 150 000 *g* at 4 °C on continuous sucrose gradients (5–35 %). Twelve fractions were collected from the bottom of the sucrose density gradient with a fraction collector. Proteins were precipitated with 10 % trichloroacetic acid and resolved by electrophoresis through SDS/PAGE (4–20 % gels) and later analysed by Western blot. Immunoblotting were carried out with anti-GFP to reveal GPI-YFP and VSVG-CFP, anti-HA to reveal Sial-T2 and Gal-T2 or anti-c-myc to reveal GalNAc-T. The positions (molecular masses) of recombinant proteins are indicated. The sucrose profile of the gradient is shown at the top of the Figure.

transfected CHO-K1 cells were extracted with Triton X-100 at 4 °C and lysates were subjected to continuous sucrose gradient ultracentrifugation, fractionation and detection of the fusion proteins and protein markers by Western blotting. Under these conditions, proteins and lipids resistant to 1 % Triton X-100 extraction float at low-density fractions as is the case for the GEM marker GPI-YFP [14]. In contrast, VSVG-CFP (a non-GEM marker) distributed in higher-density fractions, behaving essentially as Triton X-100-soluble proteins [18] (Figure 6). Sial-T2, GalNAc-T and Gal-T2 showed a clear co-distribution with VSVG-CFP, reinforcing the solubility behaviour with cold

Triton X-100 observed in Figure 5. These results, together with those showing the solubility behaviour of newly synthesized gangliosides to cold detergent, indicate that Sial-T2, GalNAc-T, Gal-T2 and the precursor pool of gangliosides are mainly excluded from GEM in Golgi complex.

DISCUSSION

We have compared the behaviour to cold detergent extraction of individual ganglioside species of plasma membrane with that of the same species newly synthesized in Golgi membranes from CHO-K1 cells. We also investigated if ganglioside glycosyltransferases are included in Golgi membrane GEM. The analysis of plasma membrane gangliosides revealed that G_{D3} and G_{T3} were almost completely insoluble to detergent extraction. G_{M3} was found to partition equally between soluble and insoluble fractions while more complex gangliosides such as G_{M2} , G_{M1} and G_{D1a} were mainly found in the soluble fraction.

Immunocytochemical examination of G_{D3} -expressing cells showed this ganglioside expressed at the cell surface as patches resistant to cold-detergent extraction of the cells before fixation. Interestingly, these patches did not co-localize, in general, with the well-characterized GEM marker GPI-YFP [23]. This is consistent with the already observed coexistence of compositionally distinct lipid microdomains within a given membrane [12,14,33,34]. Other protein components like Lyn and caveolin have been reported to associate to G_{D3} in GEM from neural cell lines and CHO cells, lending support to the idea that GEM containing G_{D3} could represent a vehicle for transporting signalling molecules to sites of signal coupling [35].

In contrast to what was observed for G_{M3} , G_{D3} and G_{T3} in the plasma membrane, most of these newly synthesized species in Golgi membranes partitioned into the soluble fraction of cold-detergent extraction. Less than 5 % of G_{D3} and G_{T3} from clone 2 were found in the insoluble fraction. Similar behaviour was observed for the Golgi-resident ganglioside glycosyltransferases Sial-T2, GalNAc-T and Gal-T2, which were almost completely soluble to detergent extraction. Glycolipid glycosyltransferases form multienzyme complexes in the Golgi membranes [36–38] on which the substrates are channelled from the position of product to the position of acceptor for the next transferase [36]. It is possible that by virtue of the size of the complexes, or as it has been hypothesized, because on average the transmembrane domain of transferases is too short to span the transport vesicle membrane [39], they do not enter GEM in the Golgi. As a consequence, it is reasonable that gangliosides in the pathway of synthesis labelled *in vitro* by incubation of the Golgi membranes with radioactive sugar nucleotides behave also as if excluded from GEM. Since the same ganglioside species appear in plasma membrane GEM, this implies that nascent gangliosides such G_{D3} , G_{T3} and G_{M3} segregate from their synthesizing transferases and then enter GEM. This event could have taken place along the secretory pathway and/or at the cell surface or shortly after synthesis in the Golgi cisternae. In the conditions of the *in vitro* labelling experiment the genesis of transport vesicles is limited by the absence of cytosol and ATP. However, we cannot reject the idea that the minor fraction of G_{D3} and G_{T3} insoluble in cold detergent represent molecules segregated to transport vesicle membranes or to *trans*-Golgi membranes just before budding. In these structures the lipid gradient of ER-synthesized cholesterol [21] could favour the conditions for GEM formation [34,40,41].

The use of CHO-K1 cell clones expressing particular sets of gangliosides, and the possibility of comparison of the detergent solubility properties of newly synthesized molecular species with

that of the same species delivered to the plasma membrane, provides an excellent system to explore the cell biology of GEM assembly, a still poorly understood issue.

This work was supported in part by grants from SECyT-Universidad Nacional de Córdoba (to J. L. D. and H. J. F. M.), "Ramon Carrillo-Arturo Oñativia" from Ministerio de Salud de la Nación Argentina (to J. L. D. and H. J. F. M.), 75197-554001 from Howard Hughes Medical Institute (U.S.A.) (to H. J. F. M.), 10087 from Mitzutami Foundation (to H. J. F. M.), 01-5185 from Agencia Nacional de Promoción Científica y Tecnológica de Argentina (to H. J. F. M.), 14116-112 from Fundación Antorchas (to J. L. D.) and The International Society for Neurochemistry (Special ISN grant to J. L. D.). We thank G. Gomez (Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Córdoba, Argentina) for helpful discussions and comments. We also thank G. Schachner and S. Deza for technical assistance with cell culture and Dr Carlos Mas for the excellent assistance with the confocal microscopy and image analysis. J. L. D. and H. J. F. M. are Career Investigators, and P. M. C., A. R. Z. and C. G. G. are Fellows of CONICET, Argentina.

REFERENCES

- Svennerholm, L. (1963) Chromatographic separation of human brain gangliosides. *J. Neurochem.* **10**, 613–623
- Hakomori, S. (1990) Bifunctional role of glycosphingolipids. Modulators for transmembrane signaling and mediators for cellular interactions. *J. Biol. Chem.* **265**, 18713–18716
- Hakomori, S.-I., Yamamura, S. and Handa, K. (1998) Signal transduction through glycosphingolipids. *Ann. N.Y. Acad. Sci.* **845**, 1–10
- Kasahara, K., Watanabe, K., Takeuchi, K., Kaneko, H., Oohira, A., Yamamoto, T. and Sanai, Y. (2000) Involvement of gangliosides in glycosylphosphatidylinositol-anchored neuronal cell adhesion molecule TAG-1 signaling in lipid rafts. *J. Biol. Chem.* **275**, 34701–34709
- Zurita, A. R., Maccioni, H. J. and Daniotti, J. L. (2001) Modulation of epidermal growth factor receptor phosphorylation by endogenously expressed gangliosides. *Biochem. J.* **355**, 465–472
- Yamashita, T., Hashimoto, A., Haluzik, M., Mizukami, H., Beck, S., Norton, A., Kono, M., Tsuji, S., Daniotti, J. L., Werth, N. et al. (2003) Enhanced insulin sensitivity in mice lacking ganglioside GM3. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 3445–3449
- Simons, K. and Ikonen, E. (1997) Functional rafts in cell membranes. *Nature (London)* **387**, 569–572
- Prinetti, A., Iwabuchi, K. and Hakomori, S. (1999) Glycosphingolipid-enriched signaling domain in mouse neuroblastoma Neuro2a cells. Mechanism of ganglioside-dependent neuritogenesis. *J. Biol. Chem.* **274**, 20916–20924
- Simons, K. and Toomre, D. (2000) Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell. Biol.* **1**, 31–39
- Galbati, F., Razani, B. and Lisanti, M. P. (2001) Emerging themes in lipid rafts and caveolae. *Cell* **106**, 403–411
- Iwabuchi, K., Yamamura, S., Prinetti, A., Handa, K. and Hakomori, S. (1998) GM3-enriched microdomain involved in cell adhesion and signal transduction through carbohydrate-carbohydrate interaction in mouse melanoma B16 cells. *J. Biol. Chem.* **273**, 9130–9138
- Vyas, K. A., Patel, H. V., Vyas, A. A. and Schnaar, R. L. (2001) Segregation of gangliosides GM1 and GD3 on cell membranes, isolated membrane rafts, and defined supported lipid monolayers. *Biol. Chem.* **382**, 241–250
- Dietrich, C., Volovyk, Z. N., Levi, M., Thompson, N. L. and Jacobson, K. (2001) Partitioning of Thy-1, GM1, and cross-linked phospholipid analogs into lipid rafts reconstituted in supported model membrane monolayers. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 10642–10647
- Crespo, P. M., Zurita, A. R. and Daniotti, J. L. (2002) Effect of gangliosides on the distribution of a glycosylphosphatidylinositol-anchored protein in plasma membrane from Chinese hamster ovary-K1 cells. *J. Biol. Chem.* **277**, 44731–44739
- Orlandi, P. A. and Fishman, P. H. (1998) Filipin-dependent inhibition of cholera toxin: evidence for toxin internalization and activation through caveolae-like domains. *J. Cell Biol.* **141**, 905–915
- Nichols, B. J., Kenworthy, A. K., Polishchuk, R. S., Lodge, R., Roberts, T. H., Hirschberg, K., Phair, R. D. and Lippincott-Schwartz, J. (2001) Rapid cycling of lipid raft markers between the cell surface and Golgi complex. *J. Cell Biol.* **153**, 529–541
- Simons, K. and van Meer, G. (1988) Lipid sorting in epithelial cells. *Biochemistry* **27**, 6197–6202
- Brown, D. A. and Rose, J. K. (1992) Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell* **68**, 533–544
- Sevlever, D., Pickett, S., Mann, K. J., Sambamurti, K., Medof, M. E. and Rosenberry, T. L. (1999) Glycosylphosphatidylinositol-anchor intermediates associate with triton-insoluble membranes in subcellular compartments that include the endoplasmic reticulum. *Biochem. J.* **343**, 627–635
- Bagnat, M., Keranen, S., Shevchenko, A., Shevchenko, A. and Simons, K. (2000) Lipid rafts function in biosynthetic delivery of proteins to the cell surface in yeast. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 3254–3259
- Heino, S., Lusa, S., Somerharju, P., Ehnholm, C., Olkkonen, V. M. and Ikonen, E. (2000) Dissecting the role of the golgi complex and lipid rafts in biosynthetic transport of cholesterol to the cell surface. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 8375–8380
- Gkantiragas, I., Brugger, B., Stuvén, E., Kaloyanova, D., Li, X. Y., Lohr, K., Lottspeich, F., Wieland, F. T. and Helms, J. B. (2001) Sphingomyelin-enriched microdomains at the Golgi complex. *Mol. Biol. Cell* **12**, 1819–1833
- Keller, P., Toomre, D., Diaz, E., White, J. and Simons, K. (2001) Multicolour imaging of post-Golgi sorting and trafficking in live cells. *Nat. Cell Biol.* **3**, 140–149
- Daniotti, J. L., Martina, J. A., Giraudo, C. G., Zurita, A. R. and Maccioni, H. J. (2000) GM3 alpha2,8-sialyltransferase (GD3 synthase): protein characterization and sub-golgi location in CHO-K1 cells. *J. Neurochem.* **74**, 1711–1720
- Giraudo, C. G., Rosales Fritz, V. M. and Maccioni, H. J. (1999) GA2/GM2/GD2 synthase localizes to the trans-Golgi network of CHO-K1 cells. *Biochem. J.* **342**, 633–640
- Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685
- Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- Maxzúd, M. K., Daniotti, J. L. and Maccioni, H. J. F. (1995) Functional coupling of glycosyltransfer steps for synthesis of gangliosides in Golgi membranes from neural retina cells. *J. Biol. Chem.* **270**, 20207–20214
- Folch-Pi, J., Arsove, S. and Meath, J. A. (1951) Isolation of brain strandin, a new type of large molecule tissue component. *J. Biol. Chem.* **191**, 819–831
- Rosales Fritz, V. M., Daniotti, J. L. and Maccioni, H. J. F. (1997) Chinese hamster ovary cells lacking GM1 and GD1a synthesize gangliosides upon transfection with human GM2 synthase. *Biochim. Biophys. Acta* **1354**, 153–158
- Daniotti, J. L., Martina, J. A., Zurita, A. R. and Maccioni, H. J. F. (1999) Mouse beta1,3-galactosyltransferase (GA1/GM1/GD1b Synthase): protein characterization, tissue expression, and developmental regulation in neural retina. *J. Neurosci. Res.* **56**, 318–327
- Giraudo, C. G. and Maccioni, H. J. F. (2003) ER export of glycosyltransferases depends on interaction of a cytoplasmic di-basic motif with Sar1. *Mol. Biol. Cell* **14**, 3753–3766
- Madore, N., Smith, K. L., Graham, C. H., Jen, A., Brady, K., Hall, S. and Morris, R. (1999) Functionally different GPI proteins are organized in different domains on the neuronal surface. *EMBO J.* **18**, 6917–6926
- Ikonen, E. (2001) Roles of lipid rafts in membrane transport. *Curr. Opin. Cell Biol.* **13**, 470–477
- Kasahara, K., Watanabe, Y., Yamamoto, T. and Sanai, Y. (1997) Association of Src family tyrosine kinase Lyn with ganglioside GD3 in rat brain. Possible regulation of Lyn by glycosphingolipid in caveolae-like domains. *J. Biol. Chem.* **272**, 29947–29953
- Giraudo, C. G., Daniotti, J. L. and Maccioni, H. J. (2001) Physical and functional association of glycolipid N-acetyl-galactosaminyl and galactosyl transferases in the Golgi apparatus. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 1625–1630
- Bieberich, E., MacKinnon, S., Silva, J., Li, D. D., Tencnmao, T., Irwin, L., Kapitonov, D. and Yu, R. K. (2002) Regulation of ganglioside biosynthesis by enzyme complex formation of glycosyltransferases. *Biochemistry* **41**, 11479–11487
- Giraudo, C. G. and Maccioni, H. J. (2003) Ganglioside glycosyltransferases organize in distinct multienzyme complexes in CHO-K1 cells. *J. Biol. Chem.* **278**, 40262–40271
- Bretscher, M. S. and Munro, S. (1993) Cholesterol and the Golgi apparatus. *Science* **261**, 1280–1281
- Brugger, B., Nickel, W., Weber, T., Parlati, F., McNew, J. A., Rothman, J. E. and Sollner, T. (2000) Putative fusogenic activity of NSF is restricted to a lipid mixture whose coalescence is also triggered by other factors. *EMBO J.* **19**, 1272–1278
- Pelham, H. R. and Rothman, J. E. (2000) The debate about transport in the Golgi – two sides of the same coin? *Cell* **102**, 713–719