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Critical Review

Metabolic Pathways and Intracellular Trafficking of Gangliosides

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Summary

Gangliosides constitute a large and heterogeneous family of acidic glycosphingolipids that contain one or more sialic acid residues and are expressed in nearly all vertebrate cells. Their *de novo* synthesis starts at the endoplasmic reticulum and is continued by a combination of glycosyltransferase activities at the Golgi complex, followed by vesicular delivery to the plasma membrane. At the cell surface, gangliosides participate in a variety of physiological as well as pathological processes. The cloning of genes for most of the glycosyltransferases responsible for ganglioside biosynthesis has produced a better understanding of the cellular and molecular basis of the ganglioside metabolism. In addition, the ability to delete groups of glycosphingolipid structures in mice has been enormously important in determining their physiological roles. Recently, a number of enzymes for ganglioside anabolism and catabolism have been shown to be associated with the plasma membrane, which might contribute to modulate local glycolipid composition, and consequently, the cell function.
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Keywords sphingolipids; gangliosides; glycolipids; biosynthesis; intracellular trafficking; glycosyltransferases; cholera toxin.

INTRODUCTION

Glycosphingolipids (GSLs) are amphipathic molecules consisting of a ceramide lipid moiety linked to a glycan chain of variable length and structure (Fig. 1). Among these are found gangliosides, which are mono- or multisialosylated GSLs mainly located in the outer layer of the plasma membrane of vertebrate cells. In addition, they are expressed in cell-type- and developmental-specific patterns and are major components of nerve cells, where they can represent more than 10% of the total lipid content and, on the neuronal surface, contribute more than 30% of the *N*-acetylneuraminic acid (NeuAc or sialic acid) (*1, 2*). Gangliosides have been implicated in many physiological processes, including growth, differentiation, migration, and apoptosis through modulating both cell signaling processes and cell-to-cell and cell-to-matrix interactions (*3–11*). Moreover, gangliosides have been associated with a wide range of pathological processes, being receptors for viruses (i.e., simian virus 40, SV40), toxins [i.e., cholera (CTx); tetanus and botulinus toxins], lectins and antibodies (*12–15*). In particular, antibodies to ganglioside GM1 (ganglioside named according to Svennerholm (*16*), see Table 1) have been associated with neuropathologic syndromes, such as Guillain-Barré syndrome (*17–19*). Also, inherited defects in the degradation of gangliosides cause a group of severe diseases known as GSL storage disorders (*20, 21*). However, to date, only two human diseases have been associated with a direct defect in ganglioside biosynthesis (*22*) or in biosynthetic precursors (*23*).

The development of genetically engineered mice with defects in distinct biosynthetic steps of ganglioside biosynthesis has revealed the critical role played by glycolipids in a number of important processes, especially in the nervous system. Mutant mice lacking the GM3 synthase (CMP-NeuAc: lactosylceramide sialyltransferase, Sial-T1), although appearing to be normal, exhibited an enhanced insulin sensitivity (*24*). Furthermore, knockout mice for UDP-GalNAc: lactosylceramide/GM3/GD3/GT3 *N*-acetylgalactosaminyltransferase (GalNAc-T) had defects in the maintenance and repair of nervous tissues and in the differentiation of spermatocytes (*25, 26*), whereas the double mutant mice lacking GalNAc-T and CMP-NeuAc: GM3 sialyltrans-

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This work is dedicated to Professor Emeritus Dr. Hugo J. F. Maccioni (Universidad Nacional de Córdoba, Córdoba, Argentina) on the occasion of his 70th birthday and for his many outstanding contributions to the field of Neurochemistry, particularly in the molecular and cell biology of sphingolipids.

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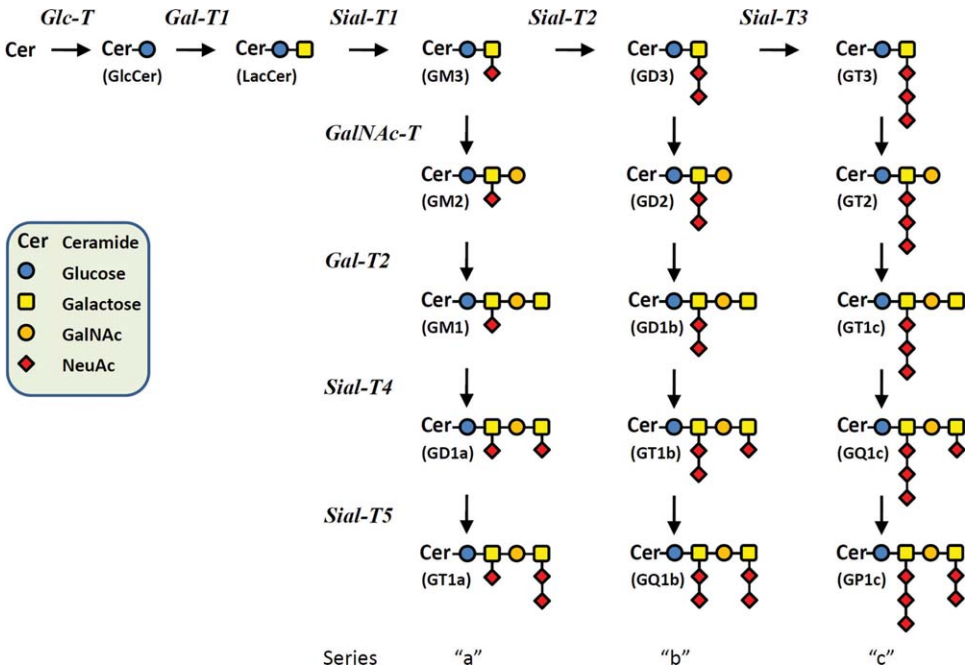

Figure 1. Scheme of the biosynthesis of oligosaccharide moieties of gangliosides. Transfer steps for synthesis of a-, b-, and c-series gangliosides are shown. Glc-T, UDP-Glc: ceramide glucosyltransferase; Gal-T1, UDP-Gal: glucosylceramide galactosyltransferase; Sial-T1, CMP-NeuAc: lactosylceramide sialyltransferase; Sial-T2, CMP-NeuAc: GM3 sialyltransferase; Sial-T3, CMP-NeuAc: GD3 sialyltransferase; GalNAc-T, UDP-GalNAc: lactosylceramide/GM3/GD3/GT3 *N*-acetylgalactosaminyltransferase; Gal-T2, UDP-Gal: GA2/GM2/GD2/GT2 galactosyltransferase; Sial-T4, CMP-NeuAc: GA1/GM1/GD1b/GT1c sialyltransferase; Sial-T5, CMP-NeuAc: GM1b/GD1a/GT1b/GQ1c sialyltransferase.

ferase (Sial-T2) developed skin injuries more rapidly and were susceptible to the induction of lethal seizures by sound stimulus (*27, 28*).

In this review, we summarize the cellular and molecular basis involved in the metabolic pathways and intracellular trafficking of gangliosides, with particular attention paid to reviewing

Table 1
Chemical structure and abbreviations for representative gangliosides belonging to the a-, b-, and c-series

Abbreviation	Structure
GM3	NeuAcα2,3Galβ1,4Glc-Cer
GM2	GalNAcβ1,4(NeuAcα2,3)Galβ1,4Glc-Cer
GM1	Galβ1,3GalNAcβ1,4(NeuAcα2,3)Galβ1,4Glc-Cer
GD1a	NeuAcα2,3Galβ1,3GalNAcβ1,4(NeuAcα2,3)Galβ1,4Glc-Cer
GT1a	NeuAcα2,8NeuAcα2,3Galβ1,3GalNAcβ1,4(NeuAcα2,3)Galβ1,4Glc-Cer
GD3	NeuAcα2,8NeuAcα2,3Galβ1,4Glc-Cer
GD2	GalNAcβ1,4(NeuAcα2,8NeuAcα2,3)Galβ1,4Glc-Cer
GD1b	Galβ1,3GalNAcβ1,4(NeuAcα2,8NeuAcα2,3)Galβ1,4Glc-Cer
GT1b	NeuAcα2,3Galβ1,3GalNAcβ1,4(NeuAcα2,8NeuAcα2,3)Galβ1,4Glc-Cer
GQ1b	NeuAcα2,8NeuAcα2,3Galβ1,3GalNAcβ1,4(NeuAcα2,8NeuAcα2,3)Galβ1,4Glc-Cer
GT3	NeuAcα2,8NeuAcα2,8NeuAcα2,3Galβ1,4Glc-Cer
GT2	GalNAcβ1,4(NeuAcα2,8NeuAcα2,8NeuAcα2,3)Galβ1,4Glc-Cer
GT1c	Galβ1,3GalNAcβ1,4(NeuAcα2,8NeuAcα2,8NeuAcα2,3)Galβ1,4Glc-Cer
GQ1c	NeuAcα2,3Galβ1,3GalNAcβ1,4(NeuAcα2,8NeuAcα2,8NeuAcα2,3)Galβ1,4Glc-Cer
GP1c	NeuAcα2,8NeuAcα2,3Galβ1,3GalNAcβ1,4(NeuAcα2,8NeuAcα2,8NeuAcα2,3)Galβ1,4Glc-Cer

Cer, ceramide; Glc, glucose; Gal, galactose; GalNAc, *N*-Acetylgalactosamine; NeuAc, *N*-acetylneuraminic acid.

recent findings concerning ganglioside metabolic remodeling at the plasma membrane and cellular binding and internalization of the ganglioside ligands.

THE SYNTHESIS OF GANGLIOSIDES

The biosynthesis of gangliosides results from the combinatorial action of membrane-bound enzymes resident in the endoplasmic reticulum and the Golgi complex (Fig. 1) (*1, 2*). Condensation of palmitoyl- or stearoyl-coenzyme A with L-serine gives rise to 3-ketosphinganine, which is reduced to D-erythro-sphinganine by 3-ketosphinganine reductase in a NADPH-dependent reaction. This compound is acylated to dihydroceramide, which is then unsaturated at the 4,5 position with the formation of ceramide. All these above reactions have been mapped in membranes from the endoplasmic reticulum and topologically oriented toward the cytosol [for review see (*2, 21*)]. The newly synthesized ceramide is then transported from the endoplasmic reticulum to the Golgi complex, where it is catalytically converted to glucosylceramide (GlcCer) through UDP-Glc: ceramide glucosyltransferase (Glc-T) and to sphingomyelin (SM) through SM synthase 1, which transfers the choline headgroup from phosphatidylcholine to ceramide.

It was reported that the synthesis of SM but not GlcCer depends on nonvesicular ceramide transport to the *trans* Golgi network (TGN) by ceramide-transfer protein (CERT) (*29*), a lipid-transfer protein containing a N-terminal pleckstrin-homology domain that binds phosphatidylinositol-4-phosphate (PI4P) at the TGN and an FFAT (two phenylalanines in an acidic tract) motif, which mediates the binding with the endoplasmic reticulum membrane protein VAP (vesicle-associated membrane-protein-associated protein) (*30*). However, more recently published results have suggested that CERT could also transport ceramide to the *trans* Golgi to be used for GlcCer synthesis on the cytosolic surface of the organelle (*31*). Then, most GlcCer could be transported back to the endoplasmic reticulum via a four-phosphate adaptor protein (FAPP2, a glycolipid-transport protein carrying a PI4P binding domain), thereby entering the secretory pathway for further conversion to lactosylceramide, GM3 or to more complex gangliosides in the luminal face of the *trans* Golgi and TGN. Other evidence also indicate that ceramide can be glycosylated to GlcCer on the cytosolic leaflet of the *cis* Golgi membranes by Glc-T and that FAPP2 is then required for the nonvesicular transport of GlcCer to distal Golgi compartments, where it translocates for further glycosylation steps leading to more complex GSLs synthesis, which eventually includes gangliosides (Fig. 2) (*32*).

Glc-T is a type III integral membrane protein, with a N-terminal uncleaved signal anchor sequence and a long cytoplasmic tail bearing the catalytic domain. On the other hand, the glycosyltransferases involved in the building up of the oligosaccharide chain of higher order gangliosides starting from GlcCer are type II integral membrane proteins with a N-terminal domain consisting of a relatively short cytoplasmic tail facing the cytoplasm, a transmembrane uncleaved signal-anchor region and a lumenally oriented C-terminal domain that bears the catalytic and the sugar nucleotide-binding sites (*1*). The synthesis of lactosylceramide occurs by the action of UDP-Gal: glucosylceramide galactosyltransferase (Gal-T1), which transfers a galactose residue from UDP-Gal to GlcCer. Then, monosaccharide units, including sialic acid, are transferred from the cognate sugar nucleotide donor to glycolipids acceptors produced by the transferases acting in the preceding steps in the pathway of synthesis. Sialylated derivatives from lactosylceramide are produced by the action of Sial-T1, Sial-T2, and Sial-T3 (CMP-NeuAc: GD3 sialyltransferase), which specifically catalyze the formation of the gangliosides GM3, GD3, and GT3, respectively. Lactosylceramide, GM3, GD3, and GT3 are potentially converted to more complex gangliosides of the 0-, a-, b-, or c-series by sequential glycosylations catalyzed by GalNAc-T, Gal-T2, Sial-T4, and Sial-T5 (Fig. 1). These transferases are nonredundant and specific, and catalyze a sugar transfer to a glycosyl acceptor that differs only in the number of sialic acids bound to the inner galactose [none (0-series), one (a-series), two (b-series), or three (c-series)]. Exceptionally, the ganglioside GM4 (NeuAcα2,3Gal-Ceramide), a major component of the myelin, does not derive from lactosylceramide.

Some lessons from biochemical (*33, 34*) and immunocytochemical (*35, 36*) studies have concluded that all the enzymes catalyzing the conversion of lactosylceramide to complex gangliosides are distally located in the Golgi complex (*trans* Golgi and TGN). However, from results of studies with pharmacological agents that block intra-Golgi transport or impair the distal Golgi function, it was hypothesized that although Gal-T1, Sial-T1, Sial-T2, and Sial-T3 and their corresponding acceptors are present in the proximal Golgi compartment, their presence extends to the TGN (*34, 37*). Saul Roseman (*38*) predicted that enzymes of ganglioside biosynthesis might form functional complexes. This was later confirmed experimentally by finding from Hugo Maccioni's laboratory (*39, 40*), who concluded that ganglioside synthesis is organized in different complexes of particular glycosyltransferses [GalNAc-T/UDP-Gal:GA2/GM2/GD2/GT2 galactosyltransferase (Gal-T2) and Gal-T1/Sial-T1/Sial-T2 complexes] in which the transmembrane domains are relevant to the associations. Interestingly, it was also reported that the sub-Golgi localization of ganglioside glycosyltransferase complexes may change according to the relative levels of the expression of participating enzymes, thus revealing the capacity of the organelle to adapt the topology of the glycolipid synthesis machinery to functional states of the cell (*41*). However, this probably does not involve the segregation of glycosyltransferase complexes into specialized membrane domains of the Golgi complex, such as the GSL-enriched microdomains (GEM). Related to this, it was earlier reported by Crespo et al. (*42*) that GM3, GD3, and GT3 reside preferentially in the GEM at the cell surface from Chinese hamster ovary (CHO)-K1 cells. However, the authors were unable to find any evidence of GEM localization of either the ganglioside glycosyltransferases or the

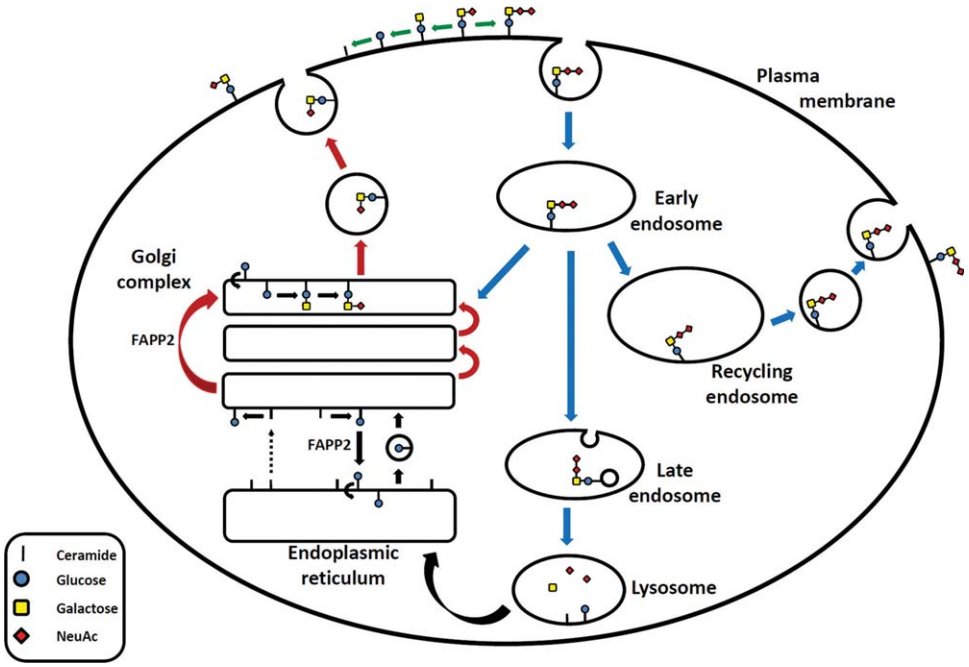

Figure 2. Simplified scheme of metabolic pathways and intracellular trafficking of gangliosides. Red arrows indicate the exocytic/biosynthetic pathway. Blue arrows indicate the endocytic, recycling and catabolic pathways. Dotted arrow indicates the vesicular or protein mediated transport of ceramide. Green arrows indicate remodeling of glycosphingolipids by plasma membrane-associated glycohydrolases and glycosyltransferases. The hypothetical neobiosynthesis of GM3 at the Golgi complex and later vesicular transport to plasma membrane is indicated. At the cell surface, GM3 could be catabolized by glycohydrolases or used for GD3 biosynthesis by plasma membrane-associated Sial-T2. GD3 can undergo endocytosis through clathrin-independent vesicles (caveolae), and once internalized, it can be recycled back to the plasma membrane directly from recycling endosomes or sorted from endosomes to the Golgi complex, where it could be reglycosylated, or transported to lysosomes for total or partial degradation. See text for more details. The schematic representation and colors of ganglioside structures are the same as indicated in Figure 1.

newly synthesized gangliosides. Thus, it was concluded that nascent gangliosides segregated from their synthesizing transferases before entering the 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 (*42*).

EXOCYTIC TRAFFICKING OF GANGLIOSIDES

After synthesis, gangliosides leave the Golgi complex via the lumenal surface of the transport vesicles (Fig. 2). This was demonstrated in nonpolarized CHO-K1 cells that gangliosides trafficked from the TGN to the plasma membrane by a Rab11-independent and brefeldin A (BFA)-insensitive exocytic pathway (*43*). In polarized epithelial cells, apical and basolateral domains possess distinct protein and lipid compositions, with the polarized distribution of sphingolipids having been reported in different cell types. In migrating lymphocytes, GM1 localizes to the uropods, whereas another ganglioside, GM3, segregates to the leading edge (*44*). In fully polarized human hepatoma HepG2 cells, C6-NBD-GlcCer, a fluorescent sphingolipid analogue of glucosylceramide, recycles between the subapical compartment (SAC) and the apical bile canalicular membrane. By contrast, C6-NBD-SM, a fluorescent sphingolipid analogue of SM, initially accumulates in the SAC compartment but is ultimately transported to the basolateral membrane (*45*). Apical membranes from renal epithelial Madin-Darby canine kidney (MDCK) cells have generally been found to be enriched in neutral GSLs and SM whereas phosphatidylcholine is concentrated in the basolateral domain (*46, 47*). More recently, it was elegantly demonstrated that complex gangliosides (GD3 and GM1), after being synthesized at the Golgi complex, were transported and accumulated at the apical domain of polarized MDCK cells (*48*).

After arrival to the plasma membrane, the gangliosides can undergo endocytosis, and once internalized, they can be recycled back to the plasma membrane directly from early or recycling endosomes or sorted from endosomes at the Golgi complex, where they can then be reglycosylated or degraded at the lysosomal level (*2, 13, 49*) (see later).

PLASMA MEMBRANE: DOES IT SUPPORT ANABOLISM AND CATABOLISM OF GANGLIOSIDE?

The level of expression and diversity of GSLs, including the gangliosides, can be controlled by regulating the sugar nucleotide and acceptor availability and the enzymatic degradation as well as by the presence and activity of the glycosyltransferases

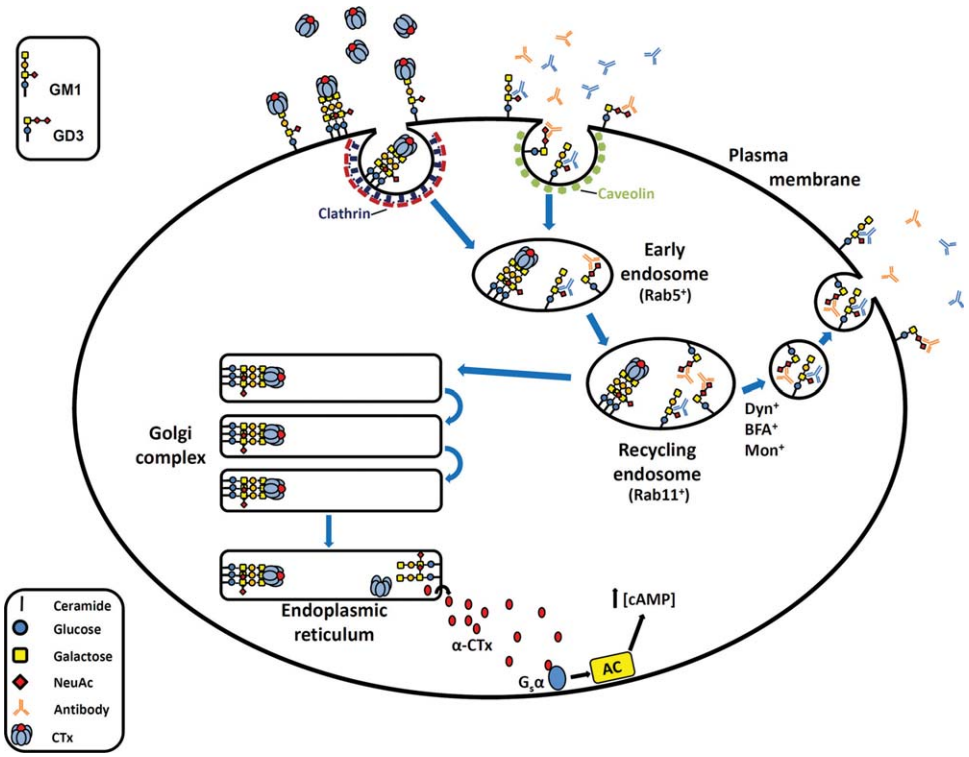

Figure 3. Endocytic trafficking of ganglioside ligands. The scheme shows the main intracellular routes followed by cholera toxin (CTx) and antibodies to gangliosides GM1 and GD3. CTx can bind up to five molecules of GM1 at the plasma membrane. This clustering is thought to promote CTx internalization to early endosome ($Rab5^+$), recycling endosomes ($Rab11^+$) and then sorting to the Golgi complex and endoplasmic reticulum (ER). After reaching the ER, the α-subunit of CTx is translocated into the cytoplasm by the Sec61 retrotranslocon, where it activates the G protein $G_s\alpha$ through an ADP-ribosylation reaction that locks the G protein in its GTP-bound form, thereby continually stimulating adenylate cyclase (AC) to produce cyclic adenosine monophosphate (cAMP). To simplify, it is shown that CTx is internalized by a clathrin-dependent pathway. However, CTx and other GSL-binging toxins can use different endocytic mechanisms to reach endosomes. In the case of antibodies to gangliosides, most of the complex is internalized by a clathrin-independent mechanism, mainly through the caveolae. Interestingly, in cells lacking caveolin-1, the internalization of the antibody-ganglioside complex is impaired. This can affect the time of residency of the complex in the plasma membrane and, consequently, affect the extent of the immune response to the cells. After internalization, the antibody accumulates transiently in Rab11 positive recycling endosomes, from which is trafficked back to the plasma membrane. For the antibody to GD3 (R24), this recycling has been shown to be a dynamin-2 (Dyn^+)-dependent pathway sensitive to brefeldin A (BFA^+) and monensin (Mon^+) (*55*). The schematic representation and colors of ganglioside structures are the same as indicated in Figure 1.

that participate in their biosynthesis. Related to this, different levels of regulation of glycosyltransferases have been reported, i.e., transcriptional, translational, post-translational and organellar topology (*1, 41, 50*). However, an additional level of regulation of GSL expression has been suggested to occur at the plasma membrane level, with the existence also having been reported of a plasma membrane-associated sialidase, termed Neu3, which is able to trigger selective ganglioside desialylation in different cell types (*51*). Moreover, β-hexosaminidase, β-glucosidase, and β-galactosidase activities have also been demonstrated at the cell surface, suggesting a role of these enzymes in the remodeling of plasma membrane GSLs, thus contributing to modulate lipid composition and membrane organization, and consequently, to the different signaling processes (*51*). On the other hand, the existence of ganglioside sialylation was previously reported in the synaptosomal membrane of calf and rat brains, during the development of neuronal cell cultures and in the thymuses of dexamethasone-administered mice (*52*). Recently, the biosynthesis of ganglioside at the plasma membrane was undoubtedly demonstrated by Crespo et al. (*53*). In particular, these authors provided the first direct evidence indicating that both ectopically and endogenously expressed Sial-T2 is able to sialylate GM3 at the plasma membrane by using both the exogenous and endogenous donor (cytidine monophospho-NeuAc, CMP-NeuAc) and acceptor (GM3) substrates (Fig. 2). Additionally, the expression of GalNAc-T was also detected at the cell surface of epithelial cells, whose catalytic activity was only observed after feeding the cells with exogenous GM3 substrate.

These findings provide strong evidence that ecto-glycosyltransferases catalyze ganglioside synthesis outside the Golgi compartment, particularly at the cell surface, which might contribute, together with the glycolipid catabolizing enzymes, to the local regulation of GSL composition at the plasma membrane.

ENDOCYTIC TRAFFICKING OF GANGLIOSIDES AND GANGLIOSIDE LIGANDS

The endocytic trafficking of gangliosides has been of interest since gangliosides at the plasma membrane can be receptors for different toxins and viruses that are dependent on them for their internalization and toxicity/infection. Thus, the *Vibrio cholerae* CTx binds with high affinity the GM1 ganglioside through its β-subunit and *Escherichia coli* heat label toxins I and IIb bind GM1 and GD1a, respectively. Gangliosides are the receptors for the clostridial neurotoxin tetanus (GM1 and GD3) and botulinum (GT1b and GD1a), and in addition, the nonenveloped DNA viruses SV40 and the murine polyoma use GM1 and GD1a/GT1b, respectively, to bind and infect cells (*14*).

The internalization and intracellular pathways followed by gangliosides have been mainly studied through the use of toxins and exogenously added fluorescent or radioactive lipids. However, the use of these tools presents some disadvantages. For instance, the membrane behavior and metabolism of short-chain fluorescent lipid analogs may be different from those of endogenous long-chain lipids (*54*). Also, it is known that toxins can change the membrane behavior of the glycolipid receptor (see below), and that, the exogenous addition of lipids, which produces a rapid change in the cellular membrane composition, can result in aberrant lipid sorting and transport. More recently, another technique involving the use of antibodies has been deployed to study the endocytic trafficking of the endogenously synthesized gangliosides (*13, 55*). Antibodies tend not to have a significant effect on the endocytic behavior of proteins (*56*), and evidence indicates it is plausible that the itinerary of the antibodies to gangliosides reflect the intracellular transit of the glycolipids (*55*) (Fig. 3). Antibodies to gangliosides, in particular to GM1, GD1a, and GQ1b, have been associated with a wide range of clinically identifiable acute and chronic neuropathy syndromes, including the Guillain-Barré and Miller-Fisher syndromes (*17–19*), and antibodies to tumor-associated gangliosides are considered to be potential therapeutic agents (*57, 58*).

The internalization of gangliosides starts with their recruitment into endocytic vesicles. Different studies have indicated that gangliosides are internalized through clathrin independent vesicles (*48*), mainly through the caveolae (*59, 60*) where they are also enriched (*61*). In agreement with this, it was found that GM1 was almost never internalized in cells that expressed reduced or undetectable levels of caveolin-1, but that this internalization defect could be reverted by the ectopic expression of this protein (*13*), indicating that caveolin may increase the basal level of ganglioside endocytosis. Remarkably, these differences were only detected when tracking the internalization with an antibody against GM1, and not when internalization was followed with CTx (*13*) (Fig. 3). In fact, CTx and other GSL-binging toxins can use different internalization pathways (both clathrin dependent and independent pathways). In this way, CTx can induce and increase GM1 endocytosis by switching the internalization pathways (*60*). Moreover, biophysical studies of model membranes indicate that CTx can perturb the membrane structure, leading to an initiation of its own endocytosis (*62*). It is also worth noting that sphingolipids, in general, are required for the proper function of clathrin independent endocytosis, with GSL depletion inhibiting endocytosis and caveolae formation (*63*). This indicates that gangliosides may be internalized through caveolae but also may contribute to the proper function of this pathway.

Once gangliosides reach the endosomal compartment, they can follow different intracellular routes (Fig. 2). By using GD3 and GM1 antibodies, it was shown that most of the internalized gangliosides accumulate transiently in Rab11 and transferrin positive recycling endosomes, from which they are trafficked back to the plasma membrane (*13, 55*) (Fig. 3). For the GD3 ganglioside, this endosomal recycling pathway was demonstrated to be partially dependent on clathrin-coated vesicles (*55*). The gangliosides can also be transported from endosomes to the Golgi complex, where they undergo direct glycosylation, leading to the formation of more complex glycolipids (*2*). Finally, gangliosides can be sorted to lysosomes, where the glycosidases sequentially cleave off the sugar residues from the nonreducing end of the glycolipid substrate (Fig. 2). In some cases, activator proteins assist in the ganglioside degradation by presenting the substrate to the hydrolytic enzymes. The resulting catabolic products can escape from the lysosomes and then be used for biosynthetic purpose (salvage pathway) or are further degraded (*2, 20, 21*).

CONCLUDING REMARKS

Since the discovery of gangliosides by Ernst Klenk in 1935, we have learnt a lot about chemical structures, metabolism and the physicochemical properties of these membrane lipids. In recent years, molecular cloning of the enzymes involved in the GSL metabolism has enabled us to modulate both *in vivo* and *in vitro* the expression profiles of gangliosides, which in turn has markedly promoted a rapid progress in the understanding of their biological function. Emerging evidence has indicated a novel level of regulation of GSL expression by plasma membrane associated glycohydrolases and glycosyltransferases. This appears to be an essential and fast mechanism to locally control the ganglioside composition, membrane organization, and consequently, the different signaling processes in response to external and internal stimuli. Future studies in this direction should be able to elucidate the molecular mechanisms and effects underlying the fine changes in ganglioside pattern at cell surface.

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