Minireview

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Combinatorial Ganglioside Biosynthesis*

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Ganglioside Structure and Function

Sphingolipids are building blocks of eukaryotic cell surfaces (1). They anchor lipid-bound carbohydrates to the plasma membrane and contribute to the epidermal water permeability barrier of the skin. Glycolipid patterns can reach a high degree of complexity that constitutes a paradigm of combinatorial biosynthesis. During combinatorial biosynthesis, a variety of biomolecules is generated by the combination of different building blocks (2). A paradigm for this process is the humoral immune response that relies on somatic recombination of gene fragments encoding proteins of the immunoglobulin family. In contrast to the formation of antibodies, the formation of the glycolipid variety is controlled at the transcriptional and post-transcriptional level. Glycolipid patterns are generated by only a few enzymes that are expressed in a cell type- and development-dependent fashion. The limited substrate specificity of some of the glycosyltransferases involved allows combination of the carbohydrate building blocks. On the other hand, the large number of theoretically possible glycolipid structures that can be formed by combination of different carbohydrate building blocks, linkage positions, and stereochemistry is realized only to a certain extent. Only a few glycolipid series are actually found in evolutionary related species. A series is defined by sugar sequences that are part of a common carbohydrate core. Gangliosides are acidic glycosphingolipids that contain one or more sialic acid residues and are particularly prevalent on neuronal cells. They belong to the so-called ganglio-, lacto-, and neolacto-series (Fig. 1). Other glycolipid series found in human tissues are the globo-, isoglobo-, and muco-series, which are all derived from lactosylceramide (Fig. 1). Sialic acids can be attached to several positions within these core structures but also to galactosylceramide, a glycolipid of the gala-series. Additional heterogeneity in the glycolipids arises from variations within the ceramide moiety, which are due to different alkyl chain lengths, the degree of hydroxylation, and desaturation. Functional consequences of heterogeneities in the lipid component have been discussed (3). Moreover, the sialic moiety can be heterogeneous with respect to modifications of the *N*-acetylneuraminic acid by *O*-acetylation, *N*-glycolylation in some species, lactonization, and others (4).

Glycolipid patterns vary between different cell types and change with the differentiation of the cell (5). Differences in glycolipid composition have even been found between different neuronal cell types (6). For example, β1,3-*N*-acetylglucosaminyltransferase expression, which leads to the formation of glycolipids of the lacto- and neolacto-series, is highly regulated during embryonic development in mice and decreases after birth to undetectable levels in most cell types (7). In adult cerebellum, it is restricted to Purkinje cells (8). The biological function of this molecular diversity is unclear, but the vital role of glycosphingolipids has been demonstrated by the fact that their complete deficiency in mice leads to early embryonic lethality (see below). Gangliosides seem to be involved in cell-to-cell interactions (9) and are also reported to regulate the activities of receptor tyrosine kinases in the plasma membrane such as the receptors of epidermal growth factor (10), nerve growth factor (11), and insulin (12) and in this way might regulate cell signaling. Gangliosides are not homogeneously distributed on the cell surface; together with glycosylphosphatidylinositol-anchored proteins, sphingomyelin, and cholesterol, they can segregate into membrane domains (rafts), which might be the physiological surroundings of many membrane proteins (13). Several reports indicate that physiological processes like embryogenesis and differentiation of neuronal cells and leukocytes might be influenced by gangliosides (for review, see Ref. 14). Just as with their functions, the mechanisms by which glycosphingolipid diversity is controlled are also beginning to be understood. We report on the principles of the ganglioside-synthesizing machinery and on recent findings from genetically engineered animals that shed light on regulation and function of these complex lipid patterns.

Ganglioside Metabolism

Glycosphingolipids including gangliosides are formed biosynthetically at intracellular membranes (1, 14) from which they are transported to the plasma membrane by exocytotic membrane flow (15). They are degraded after endocytosis within the acidic compartments of the cell by a complex machinery of degrading enzymes, activator proteins, and negatively charged lipids (1). Several human diseases are caused by defects in glycolipid degradation that lead to the accumulation of metabolites upstream of the defective reactions (1). Only one human disease is caused by a biosynthetic defect (16).

A principal difference between ganglioside anabolism and catabolism is that during glycolipid formation glycolipid substrates and enzymes are membrane-bound, whereas during degradation most glycosidases are soluble enzymes that act on membrane-bound substrates. This explains in part the requirement for activator proteins for glycolipid degradation (1), which seems not to be the case in biosynthesis. In addition, membrane-bound glycosyltransferases interact with their membrane-bound glycolipid substrates by diffusion within the two-dimensional plane of the lipid bilayer. This explains the observation that reaction rates can become independent of the reaction volume and may obey two-dimensional enzyme kinetics (17, 18).

Ceramide Formation

Ganglioside biosynthesis starts with the formation of ceramide at the membranes of the endoplasmic reticulum (ER)[1] (for review, see Refs. 1 and 19). Ceramide is the common precursor of glycosphingolipids and sphingomyelin and is transported to the Golgi apparatus by unknown mechanisms (for review, see Ref. 15). Glycosphingolipids are synthesized by the stepwise addition of monosaccharides onto ceramide. The synthesis is coupled to bulk exocytotic vesicle flow through the Golgi apparatus to the plasma membrane (15).

Galactosylceramide

Galactosylceramide (GalCer)[2] and sulfatide (GalCer-3-sulfate) are present in high concentrations in the multilamellar layers of the myelin that surrounds the axons of neuronal cells (20). The reaction of the 3′-OH group in the galactose residue of GalCer with sulfate

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[1] The abbreviations used are: ER, endoplasmic reticulum; SAT, sialyltransferase.

[2] Nomenclature of glycolipids is that of Svennerholm (34) in accordance with the IUPAC recommendations (*Pure Appl. Chem.* (1997) **69,** 2475–2487).

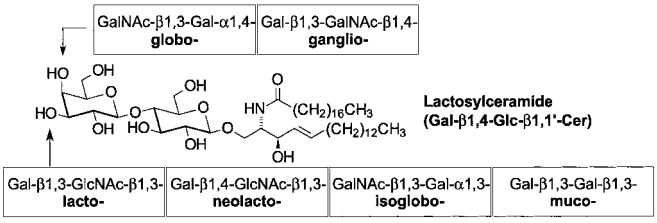

FIG. 1. **Structures and trivial names of the mammalian glycosphingolipid series derived from lactosylceramide (modified from Ref. 1).** Members of the individual series can be formally obtained by elongation of the lactosylceramide structure at the 3′- and 4′-position with the given disaccharide units.

activated as PAPS (3′-phosphoadenosine 5′-phosphosulfate) yields sulfatide (21). In contrast to most glycosyltransferases involved in ganglioside biosynthesis, which are type II transmembrane proteins, ceramide galactosyltransferase is a type I transmembrane protein. It has a C-terminal ER retention signal and the catalytic domain is on the luminal side of the ER (22, 23). In addition to their lipid substrates, the transferases require nucleotide-activated sugars that are transported through the organellar membranes by antiporters (24). Although formation of galactosylceramide occurs on the luminal face of the ER membrane, the biosynthesis of sulfatide (25) and digalactosylceramide (26) takes place in the lumen of the Golgi apparatus.

Glucosylceramide

Ceramide glucosyltransferase transfers a glucose residue from UDP-glucose to ceramide (27, 28). Although GlcCer and GalCer synthase catalyze similar reactions, there is no sequence homology between the cDNAs that encode these enzymes. This is obviously because of the different topology of their active sites. In contrast to most of the other sphingolipid glycosyltransferases that structurally resemble the protein glycosyltransferases, ceramide glucosyltransferase is a type III transmembrane protein. It forms noncovalent dimers or oligomers (29) with their C-terminal catalytic domains in the cytosol (28). Because the formation of glucosylceramide occurs on the cytosolic face of the Golgi apparatus or a pre-Golgi compartment (31, 32), ceramide, and in part dihydroceramide, has to be transported from the ER to the Golgi apparatus. Introduction of the next sugar residue that leads to the formation of lactosylceramide occurs on the luminal site of the Golgi apparatus (33). This implicates a membrane translocation of glucosylceramide, which might be mediated by an as yet uncharacterized "flippase." Translocation is energy-independent (26) and presumably incomplete, because a part of the GlcCer pool can reach the cytosolic leaflet of the plasma membrane from the cytosolic face of the Golgi apparatus (35).

The biosynthesis of higher gangliosides then occurs on the luminal face of the Golgi apparatus. Consequently, the glycan chains on the membrane-bound glycosphingolipids are orientated extracytosolically. This orientation is topologically equivalent to the situation in the plasma membrane, where the carbohydrate residues of complex glycolipids project into the extracellular space.

The Assembly Line

Ganglioside biosynthesis is catalyzed by glycosyltransferases in the lumen of the Golgi apparatus (1, 36–39, 52). With the exception of ganglioside GM4 (3′-sialyl-GalCer), a major component of myelin (40), gangliosides are structurally and biosynthetically derived from lactosylceramide. Lactosylceramide, the common precursor of the glycosphingolipid series that are found in vertebrates, is formed by the action of galactosyltransferase I, which transfers a galactose residue from UDP-galactose to glucosylceramide. The enzyme has been purified from rat brain and cloned (41). Further sugar residues, including sialic acids, are transferred in a stepwise manner to the growing glycan chains. Lactosylceramide and its sialylated derivatives, the hematosides GM3, GD3, and GT3, serve as precursors for complex gangliosides of the 0-, a-, b-, and c-series (Fig. 2). These different series are characterized by the presence of no (0-series), one (a-series), two (b-series), or three sialic acid residues (c-series) linked to the 3-position of the inner galactose moiety. In adult human tissues, gangliosides from the 0- and c-series are found only in trace amounts. The transferases that catalyze the first steps in ganglioside biosynthesis show high specificity toward their glycolipid substrates, *i.e.* the formation of LacCer, GM3, and GD3. The relative amounts of these glycolipids in the steady state seem to determine the amount of 0-series glycolipids that are derived only from LacCer, a-series gangliosides that are only derived from ganglioside GM3, and b-series gangliosides that are only derived from ganglioside GD3.

Surprisingly, the stepwise glycosylation of these precursors is performed by only a few glycosyltransferases of limited specificity, like on an assembly line. They transfer the respective sugar residue to glycosyl acceptors that differ only in the number of sialic acids bound to the inner galactose. Sialyltransferases I and II are much more specific for their glycolipid substrates than sialyltransferases IV and V or than galactosyltransferase II and GalNAc-transferase. This finding was completely unexpected, and to our knowledge there is also no close parallel in another metabolic pathway. It was assumed that different transferases catalyze the formation of the different gangliosides occurring in Fig. 2. The identity of the transferases was demonstrated by enzyme kinetics (42, 43) and resulted in the actual biosynthetic scheme. It shows the major anabolic routes and does not exclude side activities of other enzymes as well as the existence of isoenzymes that might contribute to this pathway to only a minor extent. The results have been confirmed later on by the analysis of genetically engineered animals (see below).

The complex gangliosides with sialic acid moieties α2,6-glycosidically linked to *N*-acetylgalactosamine residues have been added later to the biosynthetic scheme (44). There has been some confusion on the identity of the enzymes that catalyze the formation of gangliosides GD3 and GT3. Based on *in vitro* experiments, the cDNAs for GD3 and GT3 synthase have been claimed to be identical and, therefore, both gangliosides, GD3 and GT3, should be biosynthesized by the same enzyme (45). There is, however, evidence against this identity, *e.g.* the exclusive occurrence of gangliosides of the c-pathway in early mammalian development (for review, see Ref. 5) and in the adult brain of vertebrates like bird and fish.

Regulation

It is not clear how the relative amounts of the various glycosphingolipids are controlled. The kinetic parameters of the transferases, their topological organization within the Golgi apparatus, or spatial neighborhood to other transferases will influence the resulting ganglioside pattern. Furthermore, it cannot be excluded that the flow rates through the Golgi apparatus and the trans-Golgi network differ for the different glycolipid metabolites. The glycolipid patterns might also be modified by transcriptional regulation of transferases and translocators, and other means. Besides these factors, modification of glycolipid patterns can also take place in the plasma membrane, especially by membrane-bound sialidase of synaptosomes and myelin (for reviews, see Refs. 5 and 14).

Two enzymes accept LacCer as a substrate: sialyltransferase I that forms ganglioside GM3 and GalNAc-transferase that forms glycolipid GA2. Based on *in vitro* experiments, it has been postulated that the biosynthesis of 0-series glycolipids occurs in this way (42), but these lipids are usually not observed *in vivo*. They have only been recently demonstrated in sialyltransferase I knockout mice, as predicted by the biosynthetic scheme (see below). The selective formation of ganglioside GM3 can be explained by different possibilities. Because of the different enzyme kinetic constants, much more ganglioside GM3 is formed than GA2 (46). Representative Michaelis-Menten constants for LacCer are 72 μM for SAT I and 437 μM for GalNAc-transferase, as determined from rat liver Golgi in a micellar assay (47). An initial attempt has been made to calculate glycolipid pattern on the bases of the kinetic constants of the transferases that were estimated from the steady state concentrations of the glycolipid substrates in intact cells (48). On the other hand, it is possible that ganglioside GM3 might be synthesized in an earlier compartment than glycolipid GA2, as suggested by experiments with inhibitors of vesicular membrane flow. Although glycosyltransferase activity has been detected in many Golgi subcompartments (49), current data suggest that the precursor molecules GM3 and GD3 are formed in Golgi compartments and the complex gangliosides predominantly in the trans-Golgi network (for review, see Refs. 15 and 38). These findings have been con-

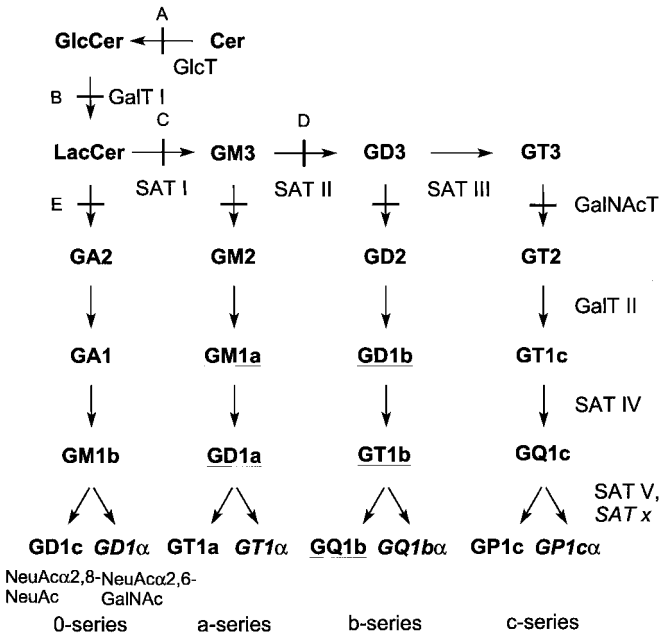

FIG. 2. **Scheme of ganglioside biosynthesis.** Major gangliosides occurring on neurons in adult mammalian brain are *underlined*. Enzyme defects in knockout mice are indicated. A, ceramide glucosyltransferase −/−; *B*, galactosyltransferase I −/−; *C*, sialyltransferase I −/−; *D*, sialyltransferase II −/−; *E*, GalNAc-transferase −/−; "GM3 only" mouse = *D* + *E*; "LacCer only" mouse = *C* + *E*. GD1α, GT1α, GQ1bα, and GP1cα with a sialic acid residue α2,6-glycosidically linked to *N*-acetylgalactosamine are formed by a yet uncharacterized sialyltransferase, whereas GD1c, GT1a, GQ1b, and GP1c are formed by SAT V. *T*, transferase (modified from Ref. 1).

firmed to some extent by biochemical and immunohistochemical studies that, for example, localized SAT I to the medial and trans-Golgi cisternae (50, 51).

An additional possibility is that galactosyltransferase I and sialyltransferase I form a functional complex and that lactosylceramide is transferred directly after its formation to the next enzyme. Roseman (52) predicted that enzymes of ganglioside biosynthesis might form such functional complexes. This has been confirmed for GalNAc-transferase and galactosyltransferase II by coimmunoprecipitation (53). This enzyme complex may accept ganglioside GM3 and finally release ganglioside GM1. This might explain why the brain contains larger amounts of gangliosides GM1 and GD1a but little ganglioside GM2. These type II glycosyltransferases interact via their N-terminal domains. Because GalNAc-transferase forms disulfide-bonded homodimers (54), the glycosyltransferases might occur as multicomponent complexes. During ontogenesis and cell transformation, a correlation between glycosphingolipid expression and the activity of glycosyltransferases that lead to their synthesis has been observed. Therefore, control of glycosyltransferases, possibly at the transcription level, appears to be a significant regulation point. Because most glycosyltransferases have been cloned in the recent past (55) it is expected that information for an understanding of the control at the transcription level will be available in the near future. In addition to regulation at a genomic level, some observations also suggest epigenetic regulation mechanisms. Feedback control of several glycosyltransferases either by their respective reaction product or by an end product of the respective glycosphingolipid series has been observed at least *in vitro* (56). The phosphorylation status of glycosyltransferases (57) and the pH value of their environment (58) can also affect their activity.

Genetically Engineered Mice

A significant advance toward understanding the function of the complex ganglioside pattern found on eukaryotic cells is the development of mice with defects in distinct biosynthetic steps (59). Blocks in the early steps of this pathway have been indicated by *A–E* on Fig. 2.

In 1994 a mouse melanoma cell line deficient in glycolipids was described. The cells were viable and showed only minor changes in cellular morphology and growth rate. From these observations it was concluded that glycolipids including gangliosides might not be essential for animal survival (60). Later, the development of animals deficient in glycolipids led to the conclusion that they are indeed required for the development of a multicellular organism; mice with targeted disruption of the ceramide glucosyltransferase gene (*A* on Fig. 2) are not viable and are beginning to die around day 7.5 of embryonic development, presumably by increased apoptosis in the ectoderm. There was no cellular differentiation beyond the primitive germ layers. Embryonic stem cells with both alleles of the ceramide glucosyltransferase gene disrupted were also severely compromised in their ability to form mature differentiated tissues in teratomas grown in mice. This indicates that formation of glucosylceramide, and subsequently higher glycolipids, might be essential events in the process of differentiation and development (61). However, the possibility that accumulation of the upstream substrate, ceramide, might account for some of the characteristics of the knockout has not been ruled out.

The phenotype of the knockout animals was not evident from cell culture experiments using ceramide glucosyltransferase-deficient embryonic stem cells. The mutant cells displayed similar growth curves as normal control cells and showed the ability to differentiate *in vitro*, which indicates that *in vitro* models may be of limited use to elucidate the function of metabolites that are directly or indirectly involved in cell-cell interactions in the complex morphogenesis of multicellular aggregates (62).

Negatively charged glycolipids of the cell surface are not necessarily derived from glucosylceramide but also from galactosylceramide. Knockout mice, in which the gene of ceramide galactosyltransferase was inactivated and which consequently cannot form any galactosylceramide, sulfatide, or ganglioside GM4, are still able to form myelin of essentially normal structure. In place of the absent GalCer they incorporate a GlcCer with hydroxylated fatty acid moieties into their myelin. Stability and function of these myelin sheets, however, were considerably impaired, and the animals suffered from generalized tremor, ataxia, conduction deficits, and further symptoms (63, 64). Spermatogenesis was impaired in these mice because of a defect in the formation of monogalactosylalkylglycerol and, subsequently, seminolipid (65). Also mice deficient in the sulfotransferase that is required for the formation of sulfatide, sulfolactosylceramide, and seminolipid have been generated (66). The animals showed similar neurological abnormalities like the animals with defective galactosylceramide formation.

Two different strains of mice have been generated that are not able to synthesize the major gangliosides especially found in the brain of the animals. In accordance with the scheme of ganglioside biosynthesis, mice with a deficiency of GalNAc-transferase (*E* on Fig. 2) are not able to form the major gangliosides GM2, GD2, GM1a, GD1b, GD1a, GT1b, GT1a, and GQ1b, and mice with deficient sialyltransferase II (*D* on Fig. 2) lack gangliosides GD3, GD2, GD1b, GT1b, and GQ1b. Surprisingly, both strains showed only subtle impairment of brain function. GalNAc-transferase-deficient homozygous mutant mice (67) developed normally without significant histological defects in the central nervous system. Their life spans were nearly normal. Further examination revealed axonal degeneration and myelination defects in the nervous system (68). Consistent with these findings the mutant mice displayed progressive defects in motor function (69). Unexpectedly, defects were found also in non-neuronal tissues. The mutant male mice were sterile and showed morphological and functional defects in the testis (70). Within their immune system, the response of spleen T cells to interleukin 2 was impaired (71). The molecular bases for these observations are currently unknown.

Disruption of the gene encoding sialyltransferase II, which is required for the formation of ganglioside GD3 and subsequently the b-series gangliosides led to homozygous animals with a normal life span and without detectable developmental defects (72). The dramatic changes in the expression of GD3 and other b-series gangliosides during neuronal development and morphogenesis have led to the belief that these gangliosides might be required for neuronal differentiation. However, it has been shown before that embryonic stem cells with a disrupted sialyltransferase II gene underwent neuronal differentiation even in the absence of b-series ganglio-

sides (73). This is also in agreement with the phenotype of the knockout animals.

To further restrict ganglioside expression, sialyltransferase II mutant mice were crossbred with mice carrying a disrupted gene encoding β1,4-GalNAc-transferase. The double mutant mice (*D* and *E* on Fig. 2) expressed only ganglioside GM3 as their major ganglioside (72). In contrast to sialyltransferase II mutant mice, the double mutants were extremely susceptible to induction of lethal seizures by sound stimulus and displayed a sudden death phenotype. This indicates a vital role of the physiological ganglioside pattern for neuronal function and enables further functional studies.

A recent mouse model generated is a sialyltransferase I-deficient strain (*C* on Fig. 2). The mice are not able to form ganglioside GM3 and, subsequently, none of the gangliosides found in the nervous systems of humans and mice, at least of the ganglio-series.[3] As could have been predicted from the scheme of ganglioside biosynthesis, they form gangliosides of the 0-series (GM1b, GD1c, and GD1α) in amounts that correspond to the total ganglioside content of normal animals. The knockout mice display altered glucose homeostasis with an accelerated insulin receptor signaling pathway. The results indicate that ganglioside GM3 is an important regulator of insulin-mediated signaling and support recent evidence that GM3 overexpression could contribute to type II diabetes (30).

The most recently developed animal model is the double knockout mouse with deficient GalNAc-transferase and sialyltransferase I (*C* and *E* on Fig. 2).[4] These mice are not able to form any glycolipid of the ganglio-series. Lactosylceramide concentrations are up to 100-fold elevated in the brains of the animals. In addition, lactosylceramide sulfate with sphingoid and fatty acid composition similar to gangliosides has been detected in these mice. The usually found ganglio-series gangliosides are completely absent, although traces of other sialic acid-containing glycolipids that are present also in normal brain were still found. The recent development of genetically engineered mice with defects in definite steps of ganglioside synthesis will improve our understanding of the function of ganglioside diversity found on animal cells.

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