Regulation of membrane-bound enzymes of glycosphingolipid biosynthesis

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Abstract The descriptive phase of glycosphingolipid bio-chemistry has blossomed over the past decade with the application of exquisitely sensitive analytical methods of mass spectrometry, NMR, and monoclonal antibody technology. Consequently, appreciation of the interrelation among the complex carbohydrate structures that are expressed on the cell surface has focused attention on the regulation of the glycosyltranferases responsible for such diversity. This review explores some aspects of several potential molecular mechanisms of regulation—compartmentation of glycosyltransferases and effect of the immediate lipid environment; modulation of enzyme activity by acceptor substrate, divalent cations, and availability of sugar nucleotides; modifications of the enzyme operon elicited by viral transformation integration sites, or to the enzyme by phosphorylation-dephosphorylation—which are presently at the cutting edge of glycosphingolipid science.—**Burczak, J. D., R. M. Soltysiak, and C. C. Sweeley.** Regulation of membrane-bound enzymes of glycosphingolipid biosynthesis. *J. Lipid Res.* 1984. **25:** 1541–1547.

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The first issue of the Journal of Lipid Research contained a report from this laboratory on the analysis of long-chain sphingolipid bases by gas–liquid chromatography (1). Since that time methods for the analysis of ceramide composition and the structural characterization of complex carbohydrate chains of glycosphingolipids (GSL), including mass spectrometry and nuclear magnetic resonance spectroscopy, have become very sophisticated. The structures of approximately 200 GSL are now known and the pathways for their biosynthesis are relatively well understood. However, GSL biochemistry is at a rudimentary state insofar as the regulation of GSL biosynthesis is concerned. Much of the literature is descriptive, and there is no definitive information about molecular mechanisms. This review is oriented toward various aspects of regulation, which is pertinent at this time when the field seems to be at the threshold of important discoveries.

Glycosphingolipids are plasma membrane molecules composed of three components: long-chain aliphatic amines, fatty acids, and carbohydrates (2). The long-chain aliphatic amine has been found to consist of sphingosine (4-sphingenine) and related substances. Fatty acids are linked to the long-chain bases via amide linkages; the fatty acids can be either saturated or unsaturated and vary in length from 14 to 26 carbon atoms. The sphingosine and fatty acid components constitute the hydrophobic portion (ceramide) of the molecule, which is anchored in the membrane. The hydrophilic carbohydrate chains, which vary in size from monosaccharides to complex oligosaccharides of 30 or more glycose residues, are attached to the C-1 hydroxyl group of ceramide by a glycosidic linkage (2). Glycosphingolipids containing sialic acid in their carbohydrate portion are referred to as gangliosides (2).

The biosynthesis of sphingosine is initiated by the reaction of palmitoyl CoA with L-serine, followed by reduction and oxidation reactions to form sphingosine (3, 4). The biosynthesis of GSL from sphingosine occurs by two alternative pathways. The major pathway probably involves acylation of the sphingosine, followed by the transfer of a monosaccharide from a sugar nucleotide to the ceramide moiety (5, 6). A minor pathway may involve the tranfer of galactose or glucose from a sugar nucleotide to sphingosine, followed by acylation (7, 8). Oligosaccharide chains of GSL are belived to be synthesized by the stepwise transfer of monosaccharides from sugar nucleotides to the non-reducing end (9). Ceramides may also be synthesized from long-chain bases and free fatty acids, by a reversal of the ceramidase reaction, as characterized by Gatt and coworkers (10, 11). Recent studies with α-fluoropalmitic acid have revealed a capacity of some tumor cells to form GSL by such a CoA thiol ester-independent mechanism (12).

Abbreviations: GSL, glycosphingolipid; PMA, phorbol-12-myristate-13-acetate.

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Based on precursor-product studies in cell-free extracts, the major pathways for neutral GSL and ganglioside biosynthesis have been formulated, largely due to the work of Basu and coworkers (13). These GSL may be classified into distinct families of oligosaccharide core structures related by their biosynthetic pathways (14).

The subcellular location of the glycosyltransferases involved in GSL biosynthesis has been the subject of much controversy. Subcellular fractionation (15) and radioautography (16) indicate that glycosylation generally occurs in the Golgi apparatus. Several other studies have indicated the existence of cell surface glycosyltransferases which perhaps function to a lesser extent in the biosynthesis of GSL (17). Glycosyltransferase activities are generally found to be highest at pH 6.0–6.5. This pH range may indicate that the lumen of the Golgi apparatus is acidic. Studies involving subcellular fractionation and isolation of Golgi membranes indicate that the Golgi apparatus contains a proton pump capable of establishing a lumenal acidic pH (18).

Nothing is known about the compartmentalization of GSL glycosyltransferases within the Golgi apparatus. Glycoprotein glycosyltransferases have been localized in different Golgi membrane cisternae (19). Their distribution across the Golgi apparatus seems to reflect the order by which monosaccharides are sequentially added to the carbohydrate chains of glycoproteins as the glycoproteins move from the *cis* (nucleus/endoplasmic reticulum) face to the *trans* (plasma membrane) face of the Golgi apparatus (20). Drugs such as monensin inhibit glycoprotein transport between Golgi compartments, resulting in the accumulation of incomplete glycoprotein carbohydrate structures (21). It is reasonable to assume that GSL glycosyltransferases are also compartmentalized within the Golgi apparatus, and that this may in part regulate the sequential addition of monosaccharides to GSL. This is not inconsistent with the concept of a multiglycosyltransferase complex suggested by Roseman (9).

There is evidence which suggests that both the chain-length and type of fatty acid group of the GSL ceramide moiety can influence the orientation of GSL in the membrane to modulate antigenicity (22), and the extent of reactivity with other ligands and glycosyltransferases (23). Indeed, the composition of other lipids in membranes of the Golgi apparatus is known to vary from the *cis* to the *trans* face of the Golgi stack (20). Monensin treatment of cultured human fibroblasts resulted in a rapid accumulation of glucosylceramide (GlcCer) and lactosylceramide (LacCer) (24). Moreover, increased incorporation of [^{3}H]galactose into GlcCer, LacCer, and ganglioside G_{M3} was noted, while a decreased incorporation of labeled galactose into more highly glycosylated neutral glycosphingolipids and gangliosides was seen. These results suggest a difference between initial and later intracellular glycosylation sites of GSL biosynthesis, as previously observed with glycoproteins (21).

Assays to determine glycosyltransferase activity generally involve incubation of a GSL substrate and the appropriate sugar nucleotide containing a radiolabel in the sugar moiety (25). After a chromatographic step to separate substrates and products, incorporation of radiolabel into the GSL product is determined. Such assays include a metal ion activator, such as manganese, and a detergent to solubilize membranes and GSL substrates. Assays in vitro for different GSL glycosyltransferases have required different types and amounts of detergents (26, 27), which may reflect differing lipid environments in Golgi membranes (28). The explanation generally offered for the Mn^{2+} requirement is that it reflects localized concentrations of cellular divalent metal cations. However, it is unlikely that such Mn^{2+} concentrations exist within the Golgi apparatus. Mn^{2+} may therefore be a less specific substitute for another divalent cation. Mn^{2+} substitutes for Mg^{2+} as an activator of the restriction enzyme Eco R1 and for DNA polymerases (29, 30), and the same may be the case with GSL glycosyltransferases. The Mn^{2+}-activated enzymes are, however, less restrictive in cutting DNA sequences and in incorporating nucleotides into DNA. Exogenous GSL added with detergent to glycosyltransferase assays probably does not accurately approximate the conditions of GSL in membranes, and Mn^{2+} may allow the glycosyltransferase to be less specific for the arrangement of substrate molecules.

Phospholipid addition to glycosyltransferase assays in crude homogenates has been found to modulate transferase activities (31). Phosphatidylcholine gave the greatest activation of CMP-sialic acid:lactosylceramide sialyltransferase, while phosphatidylethanolamine slightly enhanced activity and phosphatidic acid had no effect (**Fig. 1A**). Phosphatidylserine, phosphatidylinositol, lysophosphatidylcholine, and lysophosphatidylethanolamine inactivated the enzyme (Fig. 1B). Enhanced activity of the sialyltransferase in the presence of added diacylphospholipids may be due to partial replacement of boundary lipids of the enzyme or titration of detergent used in assays. Inactivation of CMP-sialic acid:lactosylceramide sialyltransferase by detergent-like lysophospholipids is consistent with inactivation of the sialyltransferase by ionic detergents (27). In contrast to this sialyltransferase activity, other glycosyltransferases have shown increased activity in the presence of other phospholipids (31). This is consistent with the possibility that various types of glycosyltranferases may exist in characteristic membrane lipid environments, and that the varied effects of deter-
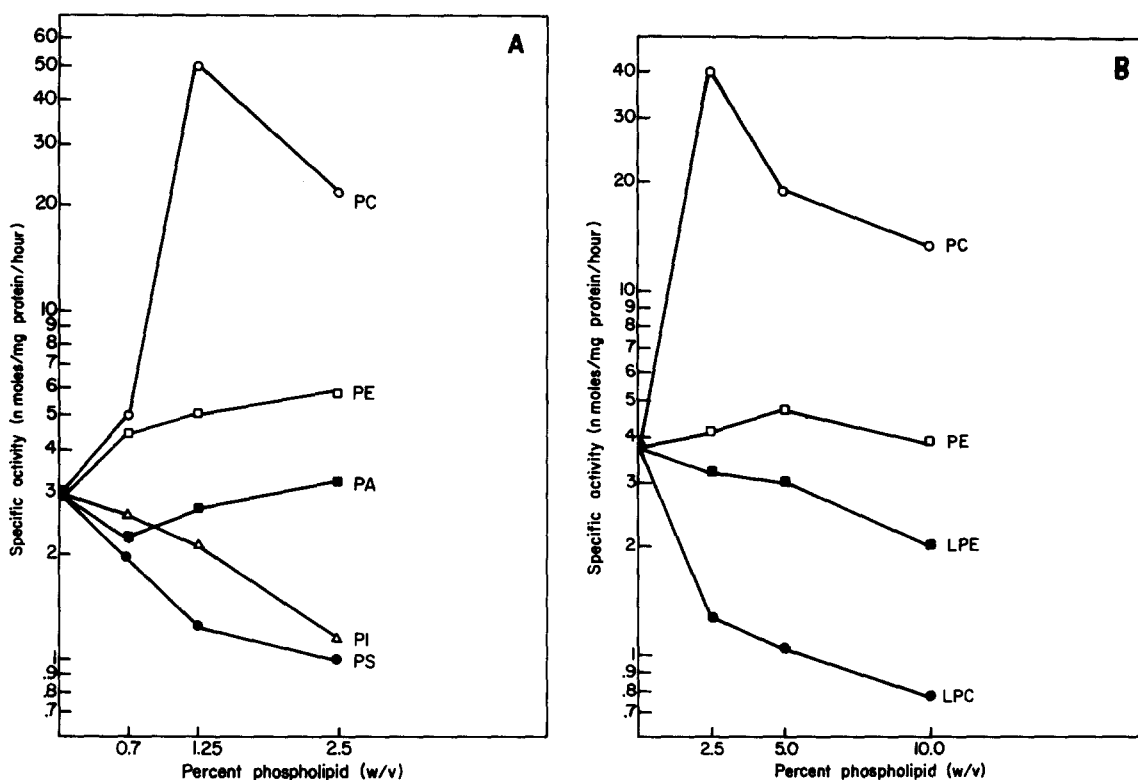
Fig. 1. A: The effect of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylinositol (PI), and phosphatidylserine (PS) on sialyltransferase activity; B: the effect of phosphatidylcholine (PC), phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE) and lysophosphatidylcholine (LPC) on sialyltransferase activity.

gents on Golgi membrane glycosyltransferase activities reflect the interactions with these lipid environments (28).

The changes in GSL composition and metabolism associated with oncogenic transformation suggest a specific role for GSL in the regulation of cell growth and differentiation, and may further an understanding of the regulation of GSL biosynthesis. Studies examining such changes are mainly the work of Hakomori and colleagues and have been reviewed (14). Two general types of GSL changes which have been seen during transformation are *a*) deletion of complex GSL due to a block or impairment in specific GSL pathways, and *b*) synthesis of new GSL due to activation of a normally unexpressed glycosyltransferase(s). In some cases, alteration of GSL biosynthesis has led to formation of novel tumor-associated antigens or markers (14).

Changes in the levels of certain GSL caused by chemical compounds have also been correlated with modulation of cell growth. Sodium butyrate, a potent inducer of differentiation for several transformed cell types (32), reduces cell saturation densities and restores contact-inhibition of cultured cells (33). Associated with these changes in cell growth behavior is a greatly increased level of G_{M3}, which results from increased CMP-

sialic acid:lactosylceramide sialyltransferase activity (34, 35). The tumor promoter, phorbol-12-myristate-13-acetate (PMA), induces differentiation of human melanoma cells. It also induces the synthesis of ganglioside G_{M3} (36, 37). Finally, various retinoid compounds, when added to culture media, cause transformed cells to regain their normal growth behavior and show density-dependent growth inhibition (38). An increased synthesis of G_{M3} in NIL cells has been demonstrated after the administration of retinol (39).

To examine this phenomenon in more detail, we have studied the effects of retinoic acid and PMA on three glycosyltransferase activities of NIL-8 and hamster sarcoma virus-transformed NIL-8 (NIL-8HSV) cell lines in tissue culture (Burczak, J. D., J. R. Moskal, M. W. Lockney, J. E. Trosko, and C. C. Sweeley, unpublished results). CMP-sialic acid:lactosylceramide sialyltransferase activity was elevated 3-fold in NIL-8 and 15-fold in NIL-8HSV cells by retinoic acid. Final activities were similar since control NIL-8HSV cells had about one-fifth the activity of control NIL-8 cells. PMA treatment resulted in a 6-fold increase in activity in NIL-8HSV cells, but a 50% decrease in that of NIL-8 cells. Combined PMA and retinoic acid treatment gave only a small increase in activity above that of retinoic acid

alone for NIL-8HSV cells, but a significantly greater than additive effect of NIL-8 cells to more than 6-fold the control activity. The controls, UDP-GalNAc:globotriaosylceramide N-acetylgalactosaminyltransferase and UDP-Gal:glycoprotein galactosyltransferase activities, were not greatly affected by treatment of these cells with either retinoic acid or PMA. Taken together, the results suggest that CMP-sialic acid:lactosylceramide sialyltransferase activity is specifically stimulated by both PMA and retinoic acid. The molecular mechanism(s) of these effects has not been elucidated.

Dawson, McLawhon, and Miller (40) have proposed that phosphorylation-dephosphorylation may be involved in the regulation of a glycosyltransferase in the ganglioside biosynthetic pathway (41), catalyzing the transfer of N-acetylgalactosamine (GalNAc) to G_{M3} ganglioside. G_{M3} is a key intermediate in that it may be the substrate either for the GalNAc-transferase or for a second sialyltransferase; these enzymes, therefore, initiate separate ganglioside biosynthetic pathways. The GalNAc-transferase activity was increased in cells treated with prostaglandin E1 (PGE$_1$), cholera toxin, 8-bromo-cAMP, dibutyryl-cAMP, or phosphodiesterase inhibitors (40, 41), all of which are known to stimulate the activity of cAMP-dependent protein kinase in vivo. When enkephalins were added to these cells to block PGE$_1$ or cholera toxin stimulation of adenyl cyclase, the induction of GalNAc-transferase activity was inhibited (40, 41). In other experiments involving the addition of ATP and the catalytic subunit of cAMP-dependent protein kinase to microsomes derived from neonatal rat brain, phosphorylation of microsomal protein and increased GalNAc transferase activity were observed (G. Dawson, personal communication). However, the results were difficult to reproduce and seen only in some microsomal preparations. This apparent paradox seems to be resolved by the demonstration of a cAMP-dependent protein kinase activity endogenous to the microsomes. This activity could be specifically activated in a dose-dependent manner by the addition of cAMP (but not cGMP) which, with endogenous kinase, phosphorylates proteins. Subsequent transferase assays revealed a doubling of GalNAc transferase activity (G. Dawson, personal communication).

CMP-sialic acid:lactosylceramide sialyltransferase is also a good candidate to be a regulated enzyme in the ganglioside pathway since its substrate (lactosylceramide) and product (G_{M3} ganglioside) are key intermediates initiating most of ganglioside biosynthesis, and several other GSL biosynthetic pathways as well (13). In support of this hypothesis, changes of CMP-sialic acid:lactosylceramide sialyltransferase activity or in the levels of LacCer and G_{M3} have been demonstrated in several

cultured cell lines in parallel with protein phosphorylation. Tumor-promoting phorbol esters are thought to bind to and activate protein kinase C, which is capable of phosphorylating other proteins (42). As described earlier, CMP-sialic acid:lactosylceramide sialyltransferase activity in cultured cells is modulated by phorbol ester treatment (36, 37). Insulin treatment of 3T3-C2 cells results in an increased cellular content of G_{M3} and G_{D1a} gangliosides (43). This particular clone of 3T3 cells does not differentiate into adipocytes; thus, the increase in ganglioside content can be attributed to the insulin treatment and not differentiation (43). The addition of insulin to cultured cells results in increased phosphorylation of several proteins (44). Purified insulin receptor has been found to phosphorylate both itself (45) and other proteins (44) upon the addition of insulin. Cyclic nucleotides are well known activators of cyclic nucleotide-dependent protein kinases, which may then phosphorylate other proteins (46), and cAMP increases G_{M3} ganglioside content of cells (47). Thus, there is some correlative evidence in support of the hypothesis that CMP-sialic acid:lactosylceramide sialyltransferase activity may be regulated by phosphorylation-dephosphorylation.

We have observed that solubilized CMP-sialic acid:lactosylceramide sialyltransferase activity from chicken liver Golgi membrane is decreased by bovine intestinal alkaline phosphatase. The amount of recovered enzyme activity was dependent upon the amount of phosphatase used and length of treatment. Wheat germ acid phosphatase and bacterial alkaline phosphatase were without effect on sialyltransferase activity. Phosphate addition to preincubations of solubilized sialyltransferase preparations ordinarily high in endogenous phosphatase activity increased the activity of this sialyltransferase. As the time of phosphate preincubation was decreased, the sialyltransferase activity decreased as well. Preincubation of the partially purified chicken liver CMP-sialic acid:lactosylceramide sialyltransferase with ATP and protein kinase increased enzyme activity (**Table 1**); however, this phenomenon was not always repeatable. ATP alone seemed sufficient to activate the enzyme to an extent that sometimes approached the activity observed with added kinase.

Although our work and the results reported by Dawson and coworkers suggest that phosphorylation-dephosphorylation is involved in the regulation of some glycosyltransferases, the experiments have been difficult to perform and reproduce. The poor reproducibility may be due to a) high levels of endogenous phosphatase and/or kinase activities in the enzyme preparation, or b) alteration of potential phosphorylation sites prior to or during the assay by detergents added to the preparation.

TABLE 1. The effect of ATP and protein kinase on CMP-sialic acid:lactosylceramide sialyltransferase activity

Preparation	Percent of Control
Control	100
Complete[a]	155
minus ATP	107
minus ATP; plus hexokinase and glucose	87
minus ATP, phosphocreatine and creatine phosphokinase	89
minus cAMP-dependent protein kinase	129
minus cAMP	133
minus cAMP and cAMP-dependent protein kinase plus protein	126
plus protein kinase inhibitor	75

[a] Complete incubations included ATP, phosphocreatine, creatine phosphokinase, cAMP, and cAMP-dependent protein kinase.

Finally, the biosynthesis of GSL may be regulated by the availability of sugar nucleotides. Rats injected with radiolabeled galactose after galactosamine administration incorporated less label than controls into the hepatic gangliosides G_{M1} and G_{D1a} (48). This amino sugar is known to lower the content of hepatic UDP-Glc and UDP-Gal, while increasing the UDP-hexosamine and UDP-N-acetylhexosamine levels. The synthesis of G_{M1} involves the transfer of galactose from UDP-Gal to G_{M2} ganglioside (31). The biosynthesis of G_{M1} and G_{D1a} may therefore be impaired by decreased levels of UDP-Gal, or perhaps by the inhibition of a specific galactosyltransferase by increased UDP-hexosamine or UDP-N-acetylhexosamine. Other studies have shown that sugar nucleotides are transported across the Golgi membrane from the cytosol by an antiport mechanism, with cotransport of a nucleoside monophosphate from the lumen to the cytosol (49). CMP-sialic acid can penetrate microsomes in a manner suggesting carrier-mediated transport (50). Upon subfractionation of rat liver, both the highest CMP-sialic acid specific transport activity and total transport activity were localized in the Golgi apparatus. Any transport activity in the smooth and rough endoplasmic reticulum could be accounted for by contamination with Golgi membranes, as determined by Golgi membrane marker enzyme activities. Transport of CMP-sialic acid was temperature-dependent, inhibited by pronase as well as substrate analogues, and saturable (51). Such a transport mechanism could regulate the availability of sugar nucleotides for GSL biosynthesis in the lumen of the Golgi. As an example, albeit contrived, tunicamycin has been found to inhibit ganglioside biosynthesis in neuronal cells (52), presumably by blocking sugar nucleotide transport across the Golgi membrane (53, 54).

If current interest in glycosyltransferase regulation continues unabated, the next few years should bring increasingly sophisticated techniques of subcellular molecular dissection to bear on the question of control of the cascade of glycosylation reactions occurring within the Golgi. Cellular control of glycosphingolipid expression at the genomic level may be deciphered by use of temperature-sensitive viral transformants to relate the association of viral integration sites with characteristic glycosyltransferase operons. Analytical methods to determine the extent and consequence of covalent modification of protein for enzyme activity will continue to be refined, and boundaries of its involvement expanded. The next phase of glycosphingolipid research may well begin by the close of this decade with a reassessment of the descriptive phase with biomedical application in the light of a control theory integrating the current concepts of glycosyltransferase regulation.

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