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GLYCOPINION

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This mini-review by Ana Olivera and Sarah Spiegel focuses on the role of ganglioside G_{M1} and its backbone sphingosine in cell growth regulation, and the ways in which these glycoconjugates may act as mediators of cellular activities.

Glycosphingolipids, as their name implies, are sugar-containing lipids. They consist of three characteristic building block components: one molecule of sphingosine (or one of its derivatives) and one molecule of a fatty acid with carbohydrate residues of varying complexity as their polar head groups. Sphingosine itself is a nonglycosylated long-chain amino alcohol (Fig. 1). The amino group of the sphingoid base is attached to a long chain fatty acyl group (that can be saturated or monounsaturated depending on different factors, such as, cell type, diet or age) to form ceramide. While the ceramide moiety is located in the membrane lipid bilayer, the polar head group residues (phosphorylcholine or the oligosaccharide chain) extend out from the plasma membrane surface to the extracellular space. The most complex sphingolipids are the glycosphingolipids, which include neutral lipids containing from one (cerebrosides) to twenty or more glycose units and acidic glycosphingolipids containing one or more sialic acid residues (gangliosides) or sulfate esters (sulfatides).

Glycosphingolipids are classified according to their carbohydrate components. These complex lipids can be divided into two major groups: neutral and acidic. Cerebrosides are the simplest glycosphingolipids and consist of sphingosine, a fatty acid residue, and a single monosaccharide polar head group (Fig. 2). More complex neutral glycosphingolipids have unbranched or branched oligosaccharide head groups containing up to twenty sugar residues. Acidic glycosphingolipids are sulfatides or gangliosides. Gangliosides have a similar basic structure to the neutral glycosphingolipids but are differentiated from each other by the fatty acid residue they contain and by the sialylated oligosaccharides which comprise the polar head group.

Over 100 gangliosides have been identified. Gangliosides are found in all vertebrate cells but are highly enriched in nervous tissues. They have considerable physiological and medical significance. The expression of glycosphingolipids on the cell surface changes as the cells divide and differentiate. There is considerable evidence that glycosphingolipids are specific determinants of cell–cell recognition, so that they probably have an important role in growth and differentiation of tissues. Specific glycosphingolipids of host cells interact with the proteins of viral and bacterial parasites. Ganglioside G_{M1} acts as the cell surface receptor for the bacterial toxin that causes the debilitating diarrhea of cholera.

The implications of this work are exciting. The initial recognition of the oligosaccharide portion of the glycosphingolipid is obviously critical, and the binding site of the enterotoxin from *E. coli*, which is closely related to the cholera toxin (CT) has recently been established (*Nature* **335**:561–4 (1992)). It may be that gangliosides are yet another class of receptors which can be recognized by viruses and bacteria, allowing them to interrupt the normal processes of the cell.

This review highlights the role of the ganglioside G_{M1} and its breakdown product, sphingosine, in transmembrane signalling and the regulation of cell proliferation. Advances have been made by using the B subunit of CT, which binds specifically and with high affinity to the oligosaccharide of ganglioside G_{M1}, affecting the stimulation of the cell by growth factors and phorbol esters. The possibility that G_{M1} acts via a growth signalling pathway is also discussed.

The authors propose that a turnover of sphingolipids with the messenger transducing products may play a role in initiating cellular responses. Although the product sphingosine has been proposed to be a potent and specific inhibitor of protein kinase C, evidence is reviewed here which suggests that it (or a phosphate derivative) may act as a positive regulator of cell growth, acting through a protein kinase-C independent pathway.

Some questions suggested by this article are:

- The B subunit of CT binds to the carbohydrate in gangliosides. Is the inhibition of this process by oligosaccharides a possible route to therapy?
- How critical is the structure of the oligosaccharides on the ganglioside G_{M1} to the binding of the B subunit of CT (there is substantial evidence documenting that the oligosaccharide is crucial for the binding of the B subunit of CT: see Fishman PH (1990) In *ADP-ribosylating Toxins and G Proteins: Insights into Signal Transduction* (Moss J and Vaughan M, eds), pp. 127–140. Washington, DC: American Society of Microbiology).
- Is the recognition of the carbohydrate receptors of the B subunit of CT able to initiate an intracellular secondary response system?
- What factors control the concentration of gangliosides on the cell surface, and to what extent is multiple presentation necessary?
- What part does the developmental stage of the cell play in the transduction of the signal generated by the interaction of the B subunit with G_{M1}.

Ganglioside G_{M1} and sphingolipid breakdown products in cellular proliferation and signal transduction pathways

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This review focuses on our current work on the role of ganglioside G_{M1} and its backbone building unit, sphingosine, in transmembrane signaling mechanisms, resulting in regulation of cell proliferation.

Abbreviations: EGF, Epidermal growth factor; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; SPC, sphingosinephosphorylcholine; DAG, diacyl glycerol; IP_3, inositol trisphosphate. The ganglioside nomenclature is used according to Svennerholm's system [5].

Sphingolipids comprise a complex family of lipid components of membranes whose role and importance in eukaryotic cells have been studied and reviewed in detail recently [1, 2]. One of the unique characteristics of this class of lipids is their diversity which is a consequence of their structural complexity. All sphingolipids (sphingomyelin, cerebrosides, sulfatides, etc.) contain, as their backbone, sphingosine or a related long chain sphingoid base, an amide-linked fatty acid, and a polar head group (hydroxyl for ceramide, phosphorylcholine for sphingomyelin, or carbohydrate residues of varying complexity for glycosphingolipids). The most predominant sphingoid base in mammalian cells is sphingosine (4-*trans*-sphingenine). Dihydrosphingosine (sphinganine) and phytosphingosines (4-hydroxysphinganines) are minor constituents. The amino group of the sphingoid base is attached to a long chain fatty acyl group (that can be saturated or monounsaturated depending on factors such as, cell type, diet, or age) to form ceramide. The most complex sphingolipids are the glycosphingolipids, which include neutral lipids containing from one (cerebrosides) to more than twenty sugar residues, and acidic glycosphingolipids containing one or more sialic acid residues (gangliosides) or sulfate esters (sulfatides). A large degree of heterogeneity exists in the carbohydrate moiety with respect to the composition, sequence, number, and linkage of the sugar residues [3, 4].

Sphingolipids have long been implicated in diverse cellular functions, including cellular communication, transformation, proliferation, differentiation, and modulation of receptor function [reviewed in 1, 2, 5, 7]. Glycosphingolipids are receptors for viral and bacterial toxins and can serve as mediators for cell–cell or cell–substratum recognition [reviewed in 6].

Gangliosides regulate cell growth

The possible importance of gangliosides in cell growth began to be recognized more than two decades ago following the demonstration of profound changes in ganglioside composition and biosynthesis during differentiation, transformation, and the cell cycle [reviewed in 8–10]. In recent years, several different approaches have been used to study the role of gangliosides in cell growth regulation. Some laboratories have convincingly demonstrated that exogenous gangliosides can directly affect growth and differentiation, rather than merely change as consequences of them. For example, addition of ganglioside G_{M3} to A431 carcinoma and Swiss 3T3 fibroblasts reduced EGF-dependent proliferation and the associated tyrosine kinase activity of the EGF receptor [11, 12]. Slight modification in G_{M3} structure led to opposite effects on cell growth and tyrosine kinase activity [13]. Recently, it has been shown that ganglioside G_{M3} undergoes growth-regulated turnover [14] and this has led to the suggestion that an extracellular or membrane bound-sialidase activity may play a role in the modulation of cell growth by regulating the levels of G_{M3} [14, 15]. Another useful approach to examine potential physiological functions of sphingolipids is the inhibition of ganglioside biosynthesis. There are several excellent examples of modifying gangliosides biosynthesis by competitive substrate inhibition, resulting in dramatic changes of cell growth and morphology [14–17]. A different approach to determine whether endogenous gangliosides are physiologically relevant biomodulating molecules has emerged through the use of ligands which interact specifically with individual gangliosides, such as anti-ganglioside antibodies [18–21] or ganglioside-binding toxins [22–30]. Our laboratory has developed the B subunit

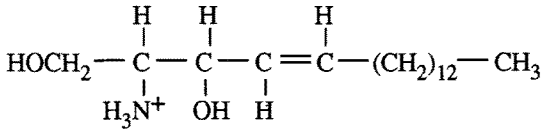

Figure 1. Sphingosine.

of cholera toxin (CT), which binds specifically and with high affinity to ganglioside G_{M1}, as a tool to study the function of endogenous ganglioside G_{M1}. This approach has been extensively employed to accumulate important clues to the role of ganglioside G_{M1} in cellular proliferation and signal transduction in a variety of cellular systems [22–30]. In our early studies, we found that the B subunit stimulated the growth of resting thymocytes [22–24] and quiescent cultures of murine 3T3 fibroblasts [25, 26]. Extension of these studies

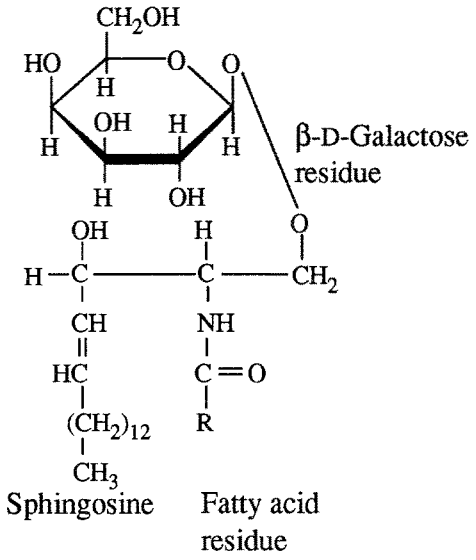

Figure 2. A galactocerebroside.

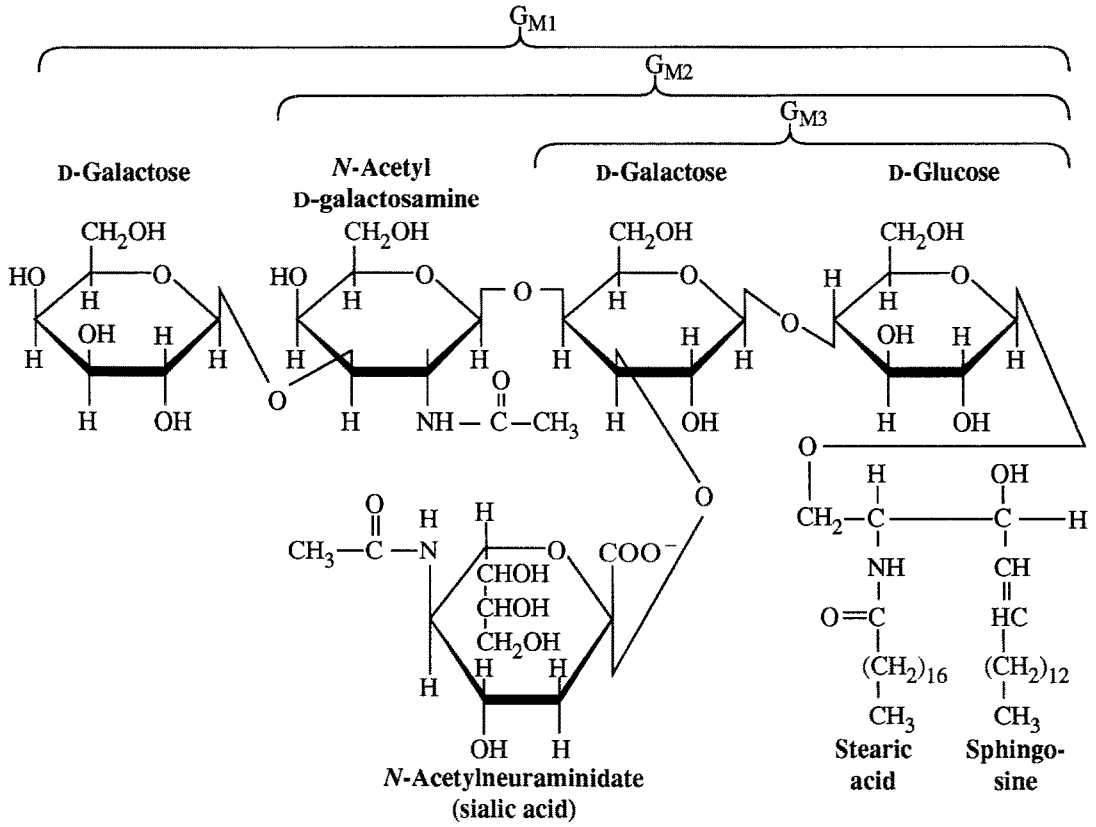

Figure 3. Gangliosides G_{M1}, G_{M2} and G_{M3}.

to transformed cells revealed that, in contrast to its effect on resting cells, the B subunit inhibited the growth of *ras*-transformed 3T3 fibroblasts [23, 25] and rat glioma C6 cells with elevated levels of G_{M1} [27, 31]. Furthermore, the B subunit not only inhibited the growth of astroglial cells [32, 33] and mouse neuroblastoma N18 cells [34], but it also induced marked differentiation of these cells. Recently, the effect of the B subunit on various other cell lines has been studied [35–39].

The biphasic response to the B subunit of the same 3T3 fibroblasts depending on their state of growth led us to suggest that endogenous ganglioside G_{M1} may play a fundamental role as a bimodal regulator of cell growth [23, 25]. Although at first glance it appeared that the ability of ganglioside G_{M1} to modulate negative or positive growth signals correlated with its density on the cell surface (levels of cell surface G_{M1} are reduced in growing 3T3 cells and increase as the cells become contact-inhibited), further studies revealed that this was a premature conclusion. Our recent results indicate that the bimodal response to the B subunit is not solely a function of the concentration of cell surface G_{M1}, but rather that the growth stage may determine the fate of the signal transduced by the interaction of the B subunit and ganglioside G_{M1} [40]. In agreement with this hypothesis, we observed that the B subunit has a dual action in quiescent Swiss 3T3 fibroblasts, depending on the context of other growth factors [28]. While the B subunit potentiated the effect of EGF, insulin, bombesin, and platelet-derived growth factor, it diminished the stimulation of DNA synthesis induced by phorbol esters via protein kinase C [28]. The dual effects of the B subunit were also related to different phases of the cell cycle [28]. Similar dual effects of the B subunit were also found in quiescent rat thyroid FRTL-5 cells [41].

Ganglioside G_{M1} modulates transmembrane signalling mechanisms

The use of the B subunit has provided a unique tool to investigate the molecular mechanisms underlying the action of ganglioside G_{M1}. The binding of the B subunit to endogenous ganglioside G_{M1} does not elicit the classical intracellular second messenger systems, such as cAMP, DAG (an endogenous activator of protein kinase C), or IP_3 (which mobilizes calcium from internal stores), since the B subunit did not activate adenylate cyclase, Na^+/H^+ exchange, phospholipase C, or protein kinase C [23–26]. However, the B subunit mediated a large increase of intracellular free calcium resulting from a net influx from extracellular sources [23, 24, 26]. Further studies demonstrated that the rise in $[Ca^{2+}]_i$ by itself was not sufficient to explain the effects of the B subunit, since Ca^{2+} ionophores, in contrast to the B subunit, did not stimulate DNA synthesis in quiescent 3T3 fibroblasts and did not increase the synthesis of numatrin, a nuclear protein whose synthesis is closely correlated to cellular commitment for mitogenesis [26, 29]. Recently, we have shown the involvement of a pertussis toxin-sensitive GTP-binding protein in a late event of DNA synthesis mediated through the interaction of endogenous gangliosides with the B subunit [30]. The underlying mechanism of the involvement of this Gi protein in DNA synthesis is not yet clear, but it is evidently not related to any effects of coupling to phospholipase C or adenylate cyclase, or on calcium influx [30]. Taken together, the data described above [23–27, 28–30] imply that ganglioside G_{M1} may modulate cellular proliferation through a growth signaling pathway that is still undefined.

Recent results from various laboratories suggest two other possible mechanisms that could explain the actions of gangliosides in cell growth regulation. Gangliosides could act by modifying growth factor receptors directly [2, 12, 13, 42] or by modifying the activity of novel protein kinases within the cell [43–45]. Another exciting possibility is that the products of sphingolipid turnover could mediate cellular responses.

Sphingosine: a member of a new class of intracellular second messengers?

The turnover of phosphoglycerolipids, often related to the phosphoinositide cycle and more recently to phosphatidylcholine hydrolysis, plays an important role in the initiation of a variety of cellular responses, including cell growth [46, 47]. A cycle of sphingolipids, comparable to the phosphoglycerolipid cycles, with its own messenger transducing products may play a similar role [5, 48, 49]. Furthermore, since sphingolipids are mainly located in the outer leaflet of the plasma membrane, whereas most effector targets are intracellular, it is logical to search for breakdown products of complex sphingolipids as possible transducers of information into cells. Unfortunately, previously it was not possible to determine accurately the intracellular levels of these metabolites because of their low concentration in cells. Some of these metabolites were detected only in sphingolipidoses—pathological conditions in which sphingolipid metabolism is altered [48, 50]. The pathophysiology of such diseases may result from abnormal accumulation of lysosphingolipids [48].

A provocative link between sphingolipids and signal transduction emerged recently when sphingosine, a metabolite of membrane sphingolipids, was found to inhibit potently and specifically protein kinase C activity *in vitro* [5, 48, 49, 51]. Sphingosine and other long-chain aliphatic amines compete with diacylglycerol and phorbol dibutyrate for specific binding sites on protein kinase C. Excitement in this field grew with the demonstration that exogenous sphingosine could inhibit a variety of protein kinase C-dependent processes *in vivo*. Sphingosine inhibits platelet [52] and neutrophil responses [53], inhibits differentiation

of HL-60 cells induced by phorbol ester [54], and also has antitumor promoter activity [48].

If indeed sphingosine serves as another class of lipid second messengers, it must be present in cells in sufficient amounts to produce the observed effect. Only recently quantitation of free sphingosine in cells has been possible due to the development of an HPLC method by Merril *et al.* which enables accurate detection of minute amounts of different sphingoid bases [55, 56]. By this method, it was shown that neutrophils not only contain appreciable quantities of free sphingosine, but they also have metabolic mechanisms for regulating its level, depending on the responses to particular stimuli [56]. In other studies, differentiation of 3T3-L1 preadipocyte cells in response to dexamethasone was accompanied by an elevation of sphingosine levels resulting from activation of membrane neutral sphingomyelinase [57]. Furthermore, exogenous sphingosine mimics the effects of dexamethasone on 2-deoxyglucose uptake [58]. These results indicate that sphingolipid turnover may be involved in the mechanism of action of corticosteroid hormones [57, 58].

In sum, taking into consideration the findings discussed above, sphingosine has appropriate properties that make it a suitable candidate to function as an intracellular second messenger [reviewed in 5]: (1) sphingosine elicits different responses in many cell types; (2) the structural properties of this molecule allow for its rapid mobility and incorporation in membranes and make it accessible to different effector systems; (3) the level of free sphingosine in cells is very low and can be regulated by physiological stimuli [56]; and (4) the turnover of sphingosine by phosphorylation to form sphingosine 1-phosphate followed by cleavage to a long chain aldehyde and phosphorylethanolamine is extremely rapid. Recognizing the importance of this, Hannun, Bell and Merrill and their colleagues proposed that sphingosine may function as an endogenous inhibitor of protein kinase C, opposing the action of diacylglycerol [5, 48, 49] either tonically in resting conditions, or acutely in response to agonists, and represents the missing functional link to the pathology of gangliosidoses [59].

Although a wealth of information exists that sphingosine has diverse biological functions which are dependent on protein kinase C, other studies indicate a more versatile role of sphingosine, which does not appear to be restricted to its action via protein kinase C [60–67]. Sphingosine inhibits thyrotropin-releasing hormone binding to pituitary cells [63] and insulin receptor tyrosine kinase activity [66], increases affinity and number of cell EGF-receptor in A431 human epidermoid carcinoma cells [60, 61], and produces retraction/degeneration of neurites [68]. Furthermore, sphingosine has been shown to act on a number of other targets, causing inhibition of CTP-phosphocholine cytidyl transferase [69], a key enzyme in the synthesis of phosphatidylcholine, Na^+/K^+ ATPase [70], calmodulin-dependent enzymes [71], casein kinase II [72], phosphatidyl-phosphohydrolase, DAG kinase, and activation of phospholipase D (see below). Even though these data may be valuable from a pharmacological viewpoint, caution should be observed when considering these targets as physiological effectors of sphingosine action, since most studies have been performed with high concentrations of sphingosine *in vitro*.

Sphingosine regulates cellular proliferation

Since protein kinase C appears to play a pivotal role in cellular proliferation, we investigated the effect of sphingosine on the growth of Swiss 3T3 fibroblasts, a convenient model system for the study of cell activation and growth. Surprisingly, in contrast to previous reports that sphingosine is highly cytotoxic for a variety of cell types, we have found that sphingosine at low concentrations stimulates DNA synthesis and cell proliferation of quiescent cultures of Swiss 3T3 fibroblasts [67]. In contrast to its effect on protein kinase C [48, 49, 51], the mitogenic effect of sphingosine is very specific, since structurally related analogs of sphingosine did not mimic the mitogenic effects of sphingosine or potentiate the effect of any combination of other growth factors. Furthermore, only the naturally occurring isomer of sphingosine, D-*erythro*-sphingosine, has this biological effect, while L-*threo*-sphingosine is completely inactive.

The effect of sphingosine on cellular proliferation was clearly independent of protein kinase C [67]. Several lines of evidence support this conclusion. First, sphingosine potentiates rather than inhibits the mitogenic effect of the tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate (TPA). The synergistic effect of optimal concentrations of sphingosine and TPA suggests that they do not share a common pathway of mitogenic action. Second, in contrast to sphingosine, H-7, an inhibitor of protein kinase C, inhibits the mitogenic response to TPA. It is important to note that sphingosine, unlike H-7, did not inhibit the stimulation of TPA-induced phosphorylation of an acidic cellular 80 kDa protein, which is a specific marker for the activation of protein kinase C in Swiss 3T3 fibroblasts [67]. Interestingly, stearylamine, which has been reported to be as potent an inhibitor of protein kinase C *in vitro* and *in vivo* as sphingosine [48, 49], did not mimic the mitogenic effect of sphingosine nor potentiate the effect of other growth factors. Rather, similar to the effect of H-7, it inhibited TPA-induced mitogenesis. Third, mitogenic concentrations of sphingosine do not compete with phorbol dibutyrate for specific receptors on intact cells (protein kinase C). Finally, down regulation of protein kinase C has no effect on sphingosine-mediated mitogenesis.

Our results suggest that sphingosine may play an important role as a positive regulator of cell growth acting via a protein kinase C-independent pathway. Only high,

cytotoxic concentrations of sphingosine have any effect on protein kinase C in our system, indicating that sphingosine may not be a physiological modulator of protein kinase C at all but may modulate some other novel signal transduction pathway, and that the target of sphingosine action still remains to be uncovered.

What are the cellular targets of sphingosine?

There is abundant evidence demonstrating that many growth factors mediate their action through increased phosphoinositide metabolism [73, 74]. In many cases there is an increased breakdown of other phospholipids as a result of activation of phospholipases C, D and A_2 [75]. Thus, it has been suggested that in addition to the two lipid intermediates, diacylglycerol and inositol trisphosphate (IP_3), other metabolites of membrane lipids such as phosphatidic acid [75–77] and arachidonic acid also play crucial roles in signal transduction and cellular proliferation. Recently, we observed that the mitogenic effect of sphingosine was accompanied by an increase in the levels of phosphatidic acid [78], which is a potent mitogen for Swiss 3T3 fibroblasts [76–78]. Thus, sphingosine may play an important role as a positive regulator of cell growth acting through phosphatidic acid.

Phosphatidic acid is not only a potent mitogen for a variety of cell types, but it also induces expression of *c-fos* and *c-myc* protooncogenes [76, 77]. Recently, it has been shown that some growth factors stimulate the production of phosphatidic acid by activation of phospholipase D, and it has been suggested that phosphatidic acid is essential in mitogenic signal transduction cascades, and may function as an intracellular second messenger [75, 79, 80]. Furthermore, phosphatidic acid might be involved in positively modulating *ras* activity during mitogenic stimulation through its inhibiting effect on the GTPase activating protein [81]. Thus, in view of the prominent role of phosphatidic acid in signal transduction and cellular proliferation, our observations that sphingosine, at mitogenic concentrations, increases the level of phosphatidic acid and also mimics the effects of phosphatidic acid on signal transduction, have important implications for the biological actions of sphingosine.

How does sphingosine stimulate phosphatidic acid levels?

An increase in cellular phosphatidic acid levels can occur through several known pathways, such as stimulation of phospholipase D, inhibition of phosphatidic acid phosphohydrolase, enhancement of acylation of glycerol 3-phosphate, or enhancement in phosphorylation of diacylglycerol catalyzed by DAG kinase. Recently, sphingosine has been shown to stimulate phospholipase D activity in several cell types [78, 82]. Sphingosine decreases phosphatidylcholine levels in NG108-15 neuroblastoma-glioma hybrid cells [83] and in Swiss 3T3 fibroblasts [78], and stimulates the hydrolysis of phosphatidylethanolamine in NTH 3T3 fibroblasts [83]. Sphingosine also inhibits phosphatidic acid phosphohydrolase activity *in vitro* [84, 85]. Furthermore, *in vitro* studies demonstrate that sphingosine activates the 80 kDa isoenzyme of DAG kinase while inhibiting the 150 kDa isoenzyme [86]. Thus, the effect of sphingosine on DAG kinase could be different depending on the type of DAG kinase isozyme present in a particular cell type. In this regard, it has been observed that sphingosine causes accumulation of phosphatidic acid in the human leukemic Jurkat cell line which has been reported to be enriched in the 80 kDa isozyme form of DAG kinase [86]. It appears that sphingosine could have multiple actions on the regulation of phosphatidic acid levels in different cell types. Recently, it has been suggested that the DAG cycle could influence the sphingolipid cycle [87, 88]. An important addition to these pathways is the regulation of phosphatidic acid levels by sphingosine, which indicates that, conversely, sphingolipid turnover could regulate the DAG cycle. The crosstalk between these lipid metabolites which act as second messengers and the function of these interlocking pathways remains unclear.

Sphingosine metabolites may mediate the actions of sphingosine

To add even more complexity, metabolites of sphingosine have recently been shown to be produced in cells and to have potent effects on cell growth [2, 89]. Hakamori and coworkers found that a methylated derivative of sphingosine, *N,N*-dimethylsphingosine, was a much more potent inhibitor of protein kinase C and enhanced the tyrosine kinase activity of src-kinase and EGF receptor [65, 90]. They suggested that some, if not all, of the effects of sphingosine may operate through *N,N*-dimethylsphingosine. In addition, experiments performed in our laboratory have shown that two more sphingosine-derived metabolites, sphingosine 1-phosphate (see below) and sphingosine-phosphorylcholine (SPC) or lysosphingomyelin are also very potent mitogens in Swiss 3T3 fibroblasts [89, 91]. The possibility that these novel sphingosine derivatives may regulate cellular processes points to a potentially new frontier of research.

Recently, we studied the formation and function of a unique phospholipid, a metabolite of sphingosine, which was unequivocally identified as sphingosine 1-phosphate [89]. We found that sphingosine induced a rapid rise in the levels of sphingosine 1-phosphate with a dose–response that correlated closely with its mitogenic effect [89]. Similar to sphingosine, sphingosine 1-phosphate stimulated DNA synthesis and cell division in quiescent cultures of Swiss 3T3 fibroblasts via a protein kinase C-independent pathway. Although both sphingosine and sphingosine 1-phosphate

acted synergistically with a wide variety of growth factors, there was no additive or synergistic effect in response to a combination of sphingosine and sphingosine 1-phosphate, indicating that they modulate cellular proliferation through a common pathway. It should be noted that sphingosine 1-phosphate stimulated DNA synthesis to a higher extent than sphingosine and required much lower concentrations for the maximum response, suggesting that it may mediate the mitogenic activity of sphingosine.

Recently, Ghosh *et al.* reported that sphingosine induced a rapid and profound release of calcium from IP_3-sensitive and insensitive intercellular pools in permeabilized smooth muscle cells [92], and they proposed that this effect was mediated via the conversion of sphingosine to sphingosine 1-phosphate [92]. However, direct experimental evidence that sphingosine 1-phosphate itself can induce release of calcium from internal sources was not presented. Using a digital imaging system for measurement of cytosolic free calcium, we observed that sphingosine 1-phosphate is a very potent calcium-mobilizing agonist in viable 3T3 fibroblasts [89]. The rapid rise in cytosolic free calcium was independent of the presence of calcium in the external medium, indicating that the response was due to mobilization of calcium from internal stores. In contrast to sphingosine, sphingosine 1-phosphate stimulated rapid release of calcium from internal sources at room temperature [89]. Furthermore, the concentration of sphingosine required to release calcium at 37 °C is much higher than the effective concentration of sphingosine 1-phosphate. The temperature dependency of the effect of sphingosine on intracellular calcium, together with the increased lag in its action, support the suggestion that sphingosine must require an enzymatic conversion to sphingosine 1-phosphate for its function [92]. Sphingosine 1-phosphate is produced from sphingosine by the action of a specific kinase in the major sphingolipid degradation pathway [93, 94]. Sphingosine 1-phosphate is rapidly degraded to *trans*-2-hexadecanal and phosphorylethanolamine by the action of a microsomal lyase [93], recently reported to be located in the endoplasmic reticulum [95]. Thus, sphingosine 1-phosphate could be a suitable candidate as an intracellular second messenger. Our recent results are consistent with such an hypothesis. Sphingosine 1-phosphate is rapidly produced in response to mitogenic concentrations of sphingosine, is mitogenic by itself for 3T3 fibroblasts, induces a rapid increase in phosphatidic acid levels, and mediates calcium release in viable cells. Thus, sphingosine 1-phosphate may be an important component of the intracellular second messenger system that is involved in calcium release and the regulation of cell growth induced by sphingosine. However, there are some obvious caveats to this hypothesis. Sphingosine 1-phosphate was added exogenously to Swiss 3T3 cells and it is difficult to compare its effects to those of the endogenously generated compound. In this regard, we would like to point out that the amount of sphingosine 1-phosphate formed intracellularly in response to a mitogenic concentration of sphingosine was approximately the same order of magnitude as that taken up by the cells after treatment with sphingosine 1-phosphate. Unfortunately, the subcellular localizations of sphingosine and sphingosine 1-phosphate are still unknown and this makes the comparison of extracellular to intracellular concentrations ambiguous.

Some future consideration should also be given to SPC, a remarkably potent mitogen for a variety of cell types [91]. SPC is also extremely potent in releasing calcium in viable cells. However, its biosynthetic pathways and mechanisms of turnover remain unexplored and may be a rewarding new area of research.

Our results raise the important question of whether other physiological growth regulating agents will alter degradation of glycosphingolipids [96] or sphingomyelin [97] to regulate endogenous sphingosine levels which, in turn, will modulate sphingosine 1-phosphate levels. If so, sphingosine 1-phosphate could function not only as a novel intracellular second messenger, inducing the release of calcium from internal stores, but also, in a positive feedback loop, as an amplifier of the cascade of events following mitogenic stimulation via its effect on phosphatidic acid levels. Whether this is a plausible speculation for one of the cellular functions of sphingosine and its metabolites awaits further study.

Although a cycle of sphingolipids, comparable to the phosphoglycerolipid cycles, with its own messenger transducing products (sphingosine or ceramide) is a very fascinating hypothesis [5, 48, 49], many questions still remain open. Is sphingosine the actual bioeffector molecule in this cycle, or are other intermediates involved? Does the cycle have universal biological meaning? Does it work similarly in all tissues and cells? Are there different routes being activated and, if so, are there different messengers generated depending on cell types or stimuli? Recent discoveries from different laboratories provide new insights into the complexity of the proposed cycle. In addition to the sphingosine metabolites discussed above, it has been suggested that ceramide might have an important role in cellular differentiation [98]. A new metabolite of ceramide, ceramide 1-phosphate, has been described but its function has not yet been studied [99, 100]. Since sphingosine and ceramide are interconvertible by simple acylation–deacylation reactions, either or both could be responsible for the cellular responses and, at the present time, this issue has not been clarified.

In summary, increasing the knowledge of the spectrum of bioactive products of sphingolipid turnover could add new perspectives to the understanding of cellular processes.

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This listing is incomplete due to space constraints, but will provide access to the original literature. We apologize to those whose work is only indirectly cited.

Letters or comments relating to this article would be received with interest by Pauline Rudd, Assistant to the Special Advisory Editor, R. A. Dwek.