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Enzymes of Sphingolipid Metabolism: From Modular to Integrative Signaling[†]

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ABSTRACT: Many enzymes of sphingolipid metabolism are regulated in response to extra- and intracellular stimuli and in turn serve as regulators of levels of bioactive lipids (such as sphingosine, ceramide, sphingosine 1-phosphate, and diacylglycerol), and as such, they serve a prototypical modular function in cell regulation. However, lipid metabolism is also closely interconnected in that a product of one enzyme serves as a substrate for another. Moreover, many cell stimuli regulate more than one of these enzymes, thus adding to the complexity of regulation of lipid metabolism. In this paper, we review the status of enzymes of sphingolipid metabolism in cell regulation and propose a role for these enzymes in integration of cell responses, a role that builds on the modular organization while also taking advantage of the complexity and interconnectedness of lipid metabolism, thus providing for a combinatorial mechanism of generating diversity in cell responses. This may be a general prototype for the involvement of metabolic pathways in cell regulation.

Eukaryotic cells are under a constant challenge to respond to their environment, monitor a myriad of intracellular functions, react to internal signals, and, in the case of multicellular organisms, react to messages from neighboring cells as well as distant tissues. At the cellular level, this is accomplished through a multitude of mechanisms that constitute what is now loosely termed "signal transduction" processes. Understanding the molecular (signaling) mechanisms that mediate and modulate these cellular processes is essential to comprehending the sophistication of eukaryotic biology and to intervening rationally and successfully in the various pathological conditions that arise from dysregulation of these processes.

In its simplest formulation, a signaling module requires an input signal, a transducer, and an output signal (Figure 1). Although several signaling pathways have been identified (such as those involving cyclases, kinases, and phospholipases), the shear complexity and diversity of the various functions and processes monitored by the cell suggests, at face value, the existence of an enormous number of distinct mechanisms of signal transduction. Therefore, the question arises as to how the cell can possess all the biochemical machinery required for these signaling pathways. Perhaps a significant component of the answer lies in lipid metabolism and other pathways of intermediary metabolism that could provide not only a plethora of "signaling" modules but also a combinatorial mechanism for generating a large number of responses.

Indeed, any regulated metabolic pathway may serve a modular signaling function (Figure 1) if it satisfies the following criteria: (1) existence of a regulated enzyme that responds to at least one specific input, (2) modulation of

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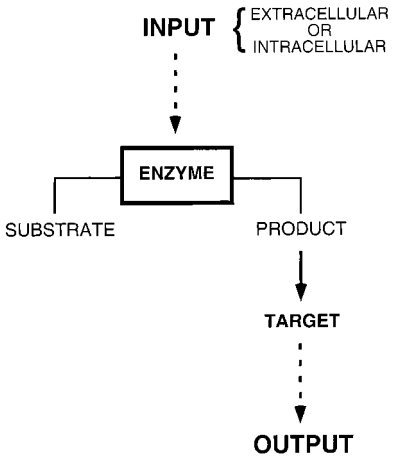

FIGURE 1: Scheme of function of metabolic enzymes in modular signaling. It is proposed that any metabolic enzyme can serve a signaling function if it responds to inputs (intracellular or extracellular) to generate a product that is specifically recognized by one or more targets.

levels of substrate and/or product which then serve as the output signals, and (3) existence of at least one target whose activity or function responds to changes in the levels of these output signals.

It is now well appreciated that several enzymes of lipid metabolism serve as signaling modules, and their products serve as bioeffectors. The best studied include phosphatidylinositol phospholipases C, phosphatidylinositol kinases, and phospholipases A_2 and D which catalyze distinct reactions in glycerolipid metabolism. More recently, sphingolipids have also emerged as a particularly rich source of bioactive molecules. There are an estimated 300−400 distinct species of sphingolipids. Importantly, every distinct species usually implies the existence of specific enzymes of formation and degradation, and each enzyme, if subject to regulation, may serve a potential role as a signaling module. Thus, these biochemical pathways allow for great versatility in sphingolipid-driven biology.

In addition to viewing enzymes of sphingolipid metabolism as isolated signaling modules, these pathways may serve more complex regulatory functions based on three additional features. First, these pathways are highly interconnected (Figure 2) with the product of one enzyme serving as a substrate for another. For example, ceramide formed from sphingomyelinase (SMase)[1] may either act directly or serve as a substrate for ceramidase, sphingomyelin synthase (SMS), or glucosylceramide synthase (GCS), and thus "converts" to either sphingosine, diacylglycerol (DAG), or cerebroside, respectively. These interconnections may then serve to link two or more different signaling modules. Second, these enzymes may be coordinately regulated in response to cellular stimuli. For example, tumor necrosis factor (TNF) may activate and/or inhibit several enzymes of sphingolipid metabolism. Third, many of these enzymes are compartmentalized, and given the hydrophobicity of many of their products, their actions may be topologically restricted to the site of enzyme activation and product generation.

In this paper, we will propose and discuss how these complexities in sphingolipid metabolism can provide a rich matrix for the integration of cell signaling and for the coordination of multiple responses to various stimuli.

Overview of Sphingolipid Metabolism

Prior to delving into sphingolipid-mediated cell regulation, it is profitable to acquire a working knowledge of the basic chemistry and biochemical pathways of sphingolipid metabolism since these provide the underpinning of eventual understanding of sphingolipid function. In mammalian cells, the route for sphingolipid biosynthesis starts in the endoplasmic reticulum with the condensation of palmitoyl-CoA and serine and proceeds in this compartment until the formation of ceramide through a series of reducing, acylating, and oxidizing reactions (Figure 2). Additional reactions take place in the Golgi apparatus, where sphingomyelin (SM) and complex sphingolipids (glycolipids, gangliosides, and sulfatides) are synthesized. SM is formed by the action of SMS, whereas the predominant initial enzyme in glycolipid synthesis is GCS. Glucosylceramide then serves as the precursor for the many glycolipids and gangliosides. Another metabolic reaction involves the phosphorylation of ceramide to form ceramide 1-phosphate.

The breakdown of complex sphingolipids proceeds by stepwise degradation through the action of several distinct hydrolases that sequentially trim the headgroups of complex sphingolipids, eventually resulting in the formation of ceramide.

Subsequent catabolism of ceramide results in the formation of sphingosine through deacylation by ceramidase. In turn, sphingosine may be phosphorylated into sphingosine 1-phosphate (S-1-P) by the action of sphingosine kinases. Alternatively, sphingosine may be salvaged by acylation through the action of ceramide synthase to form ceramide. S-1-P can also enter the salvage pathway through the action of a phosphatase that regenerates sphingosine, or it can be cleared through the action of a lyase that results in the formation of a fatty aldehyde and ethanolamine phosphate, both of which may then enter glycerolipid pathways of metabolism.

Bioactive Sphingolipids

Several sphingolipids have already joined the group of bioactive lipids, and several more are candidates for such roles. Following is a brief summary of the status of these molecules.

[1] Abbreviations: A-SMase, acid sphingomyelinase; CAPK, ceramide-activated protein kinase; CAPP, ceramide-activated protein phosphatases; $cPLA_2$, phospholipase A_2; DAG, diacylglycerol; DHS-1-P, dihydrosphingosine 1-phosphate; EDG, endothelial differentiation gene; EGF, epidermal growth factor; FAN, factor associated with neutral sphingomyelinase activation; GCS, glucosylceramide synthase; GSH, reduced glutathione; GSSG, oxidized glutathione; HEK293T cells, human embryonic kidney cells; IPC, inosytolphosphorylceramide; IPS-PLC, inositol phosphosphingolipid phospholipase C; KSR, kinase activator of ras; LCS, lactosylceramide synthase; MDR, multi-drug resistant; MIPC and MIP_2C, mannosylated forms of IPC; NO, nitric oxide; NSD, neutral sphingomyelinase activation domain; N-SMase, neutral sphingomyelinase; Ox LDL, oxidized low-density lipoprotein; PARP, poly(ADP-ribose) polymerase; PC-PLC, phosphatidylcholine-specific phospholipase C; PDGF, platelet-derived growth factor; PKC, protein kinase C; PI, phosphatidylinositol; S-1-P, sphingosine 1-phosphate; SM, sphingomyelin; SMase, sphingomyelinase; SMS, sphingomyelin synthase; SPHK, sphingosine kinase; SPT, serine palmitoyltransferase; TNF, tumor necrosis factor.

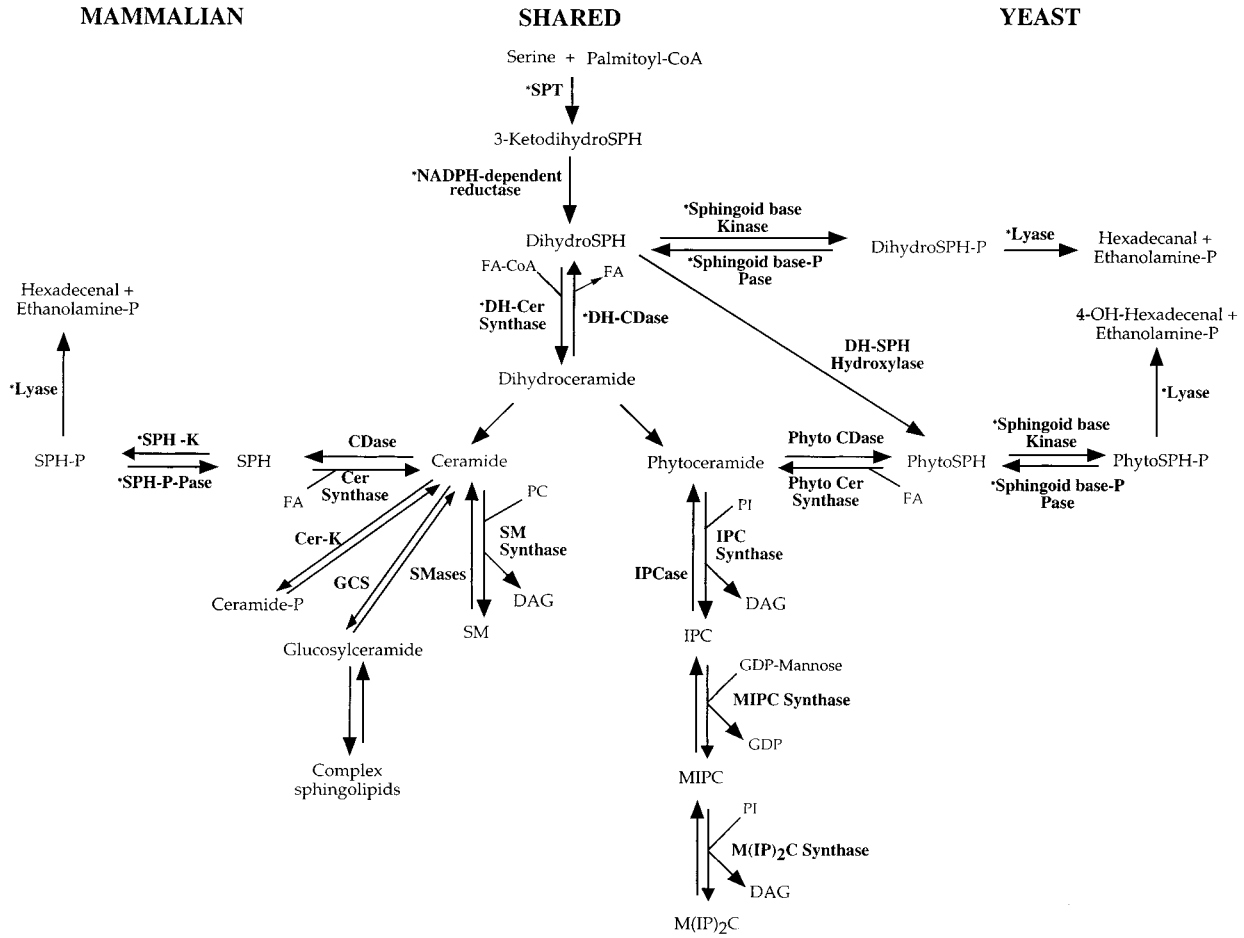

FIGURE 2: Sphingolipid metabolism in yeast and mammals. A schematic representation of the reactions involved in sphingolipid metabolism in yeast and mammals. The first reactions along the de novo biosynthetic pathway are common to yeast and mammals. From these shared reactions, these organisms evolved biochemical pathways that specifically characterize the two groups (i.e., the formation of complex sphingolipids, SM or IPC). Asterisks (*) denote enzymes that are present in both yeast and mammals. CDase, ceramidase; DAG, diacylglycerol; DH−CDase, dihydroceramidase; DH−CER, dihydroceramide; DH−SPH, dihydrosphingosine; FA, fatty acid; GCS, glucosylceramide synthase; IPC, inositol phosphorylceramide; K, kinase; MIPC, mannosylinositol phosphorylceramide; $M(IP)_2C$, mannosyldiinosytol phosphorylceramide; Pase, phosphatase; PC, phosphatidylcholine; PI, phosphatidylinositol; SM, sphingomyelin; SMases, sphingomyelinases; SPH, sphingosine; SPH−P, sphingosine 1-phosphate; SPT, serine palmitoyltransferase.

Sphingosine. Sphingosine is formed primarily from the breakdown of mammalian ceramide, and therefore, it appears to be strictly a catabolic product. Sphingosine is able to diffuse rapidly between cell membranes, unlike ceramide, which remains associated with the membrane. This property allows sphingosine to have effects in different cellular compartments. Initial studies led to the identification of inhibitory effects of sphingosine on protein kinase C (PKC) in vitro and in cells (*1*). Subsequent studies led to the discovery that sphingosine also inhibits phosphatidic acid phosphohydrolase while activating phospholipase D and DAG kinase (*2*−*4*). In concert, these actions appear to result in the shutdown of the DAG/PKC pathway while augmenting the levels of phosphatidic acid. Sphingosine has also been shown to affect multiple other targets, including activation of casein kinase 2 and p21-activated kinase (*5*, *6*). Sphingosine has also been shown to specifically activate a new class of kinases termed sphingosine-dependent kinases that phosphorylate members of the 14-3-3 class of proteins which play important roles as adapter proteins in signaling pathways (*7*). At the level of the cell, the actions of sphingosine are cell type specific with sphingosine exerting antimitogenic effects in many cells, but promitogenic effects in others (*8*). In most cases, a unifying theme has been the anti-phorbol ester/DAG effects of sphingosine, consistent with its in vitro actions. There has been a lack of significant insight into regulated formation of sphingosine as a physiologic bioactive molecule, although recent studies have shown that some agents such as PDGF induce transient increases in the levels of sphingosine, the significance of which has not been determined (*9*).

Phytosphingosine. Yeast and plants contain predominantly phytosphingosine as their main sphingoid base. Recent studies in *Saccharomyces cerevisiae* point to key functions of phytosphingosine in the heat stress response and in endocytosis (*10*). Heat stress (change in temperature from 25−30 to 39−42 °C) results in significant elevation in the levels of phytosphingosine (especially the C_{20} molecular species) (*11*, *12*). A combination of pharmacologic and genetic analysis shows that phytosphingosine (and dihydrosphingosine) is essential for activation of a ubiquitin-dependent pathway of degradation of nutrient permeases that occurs upon heat stress. Also, similar studies show that yeast sphingoid bases are necessary for the transient cell cycle arrest by which yeast respond to heat stress (*13*). In another line of studies, it was shown that defects in the formation of phytosphingosine result in defective endocytosis, and it was suggested that the sphingoid bases may act by either

activating protein kinases or inhibiting protein phosphatases (*14*). Thus, phytosphingosine is emerging as a bona fide signaling molecule in yeast.

Ceramide. In mammalian cells, many agents of stress induce transient and sustained elevations in the levels of ceramide with variable kinetics (*15*). These involve predominantly activation of sphingomyelinases and/or the de novo pathway of sphingolipid biosynthesis. Ongoing studies suggest important roles for the generated ceramide in coordinating the response of the cells to these stress stimuli and in directing the cells to the execution of specific programs of cell cycle arrest, differentiation, apoptosis, or senescence (*8*, *16*−*18*). In addition, ceramide (or metabolites) may regulate the production of inflammatory eicosanoids (*19*, *20*). At this point, the role of ceramide in apoptosis has been the most intensively studied. Results show that ceramide formation is intimately related to mitochondrial function in apoptosis such that ceramide accumulation occurs "upstream" of the mitochondrial phase of apoptosis, and in turn, ceramide regulates, and in many cases is necessary for, the execution phase of apoptosis involving mitochondria. These activities of ceramide have been reviewed recently (*16*).

At least four direct targets for ceramide have been identified, and these include ceramide-activated protein phosphatases (CAPP) (*21*), ceramide-activated protein kinase (CAPK) (*22*), cathepsin D (*23*), and PKCζ (*24*). CAPP have been shown to comprise the serine/threonine protein phosphatases PP1 and PP2A. Activation of CAPP by ceramide shows stereospecificity and a strict requirement for the 4−5 trans double bond of the sphingoid base, thus matching the biologic activity of ceramide as compared to dihydroceramide (*25*). Several substrates have been identified for CAPP, and these include PKCα, bcl-2, c-jun, PKB/Akt, and the retinoblastoma gene product (*26*−*30*). Thus, activation of CAPP by ceramide in vivo may mediate its effects through dephosphorylation and regulation of these and other substrates. On the other hand, CAPK was shown recently to be the same as KSR (kinase activator of ras), whose activation may mediate the effects of ceramide on the ERK Map kinases (*22*, *31*). There are two reports showing direct activation of PKCζ by ceramide, implicating PKCζ in mediating the effects of ceramide on SAPK (*24*, *32*). One recent study identified cathepsin D as a ceramide binding protein, and it was shown that ceramide, and to a lesser extent sphingosine, specifically activates this protease (*33*).

Sphingosine 1-Phosphate (S-1-P) and Dihydrosphingosine 1-Phosphate (DHS-1-P). S-1-P and DHS-1-P are the main phosphorylated sphingoid bases in mammalian cells. Various biological effectors have been shown to promote the biosynthesis of S-1-P, including growth factors (PDGF and EGF), cytokines (TNFα), and G-protein-coupled receptor agonists (fMLP) (*34*−*37*). PDGF and IL-1 have been shown to activate sphingomyelinases and ceramidases, thus resulting in the formation of sphingosine, upon which sphingosine kinase acts in forming S-1-P. Additionally, S-1-P has been shown to be a constituent of plasma where it is thought to derive from stores in platelets that are released into serum upon platelet activation.

Recent studies have shown that S-1-P binds to several members of the endothelial differentiation gene (EDG) G-protein-coupled receptor family (*38*−*40*). Interaction of S-1-P with members of the EDG receptor family leads to an array of cellular responses that includes proliferation, enhanced extracellular matrix assembly, stimulation of assembly of adherens junctions and actin stress fibers, and inhibition of apoptosis induced by either ceramide treatment or growth factor withdrawal (*38*, *41*−*44*). By contrast, in certain cells, including smooth muscle cells, S-1-P causes actin filament disassembly and inhibition of focal adhesion contact formation (*45*). Many of the aforementioned cellular responses are elicited through the Rho family of GTPases and the ERK/MAP kinase pathway (*38*, *46*−*50*). Like sphingosine, S-1-P is able to move rapidly between membranes, and it may function as an intracellular mediator in addition to its ability to serve as an extracellular effector. In this regard, it has been implicated in calcium mobilization and inhibition of caspases (*44*, *51*−*53*).

Phytosphingosine 1-Phosphate. Phytosphingosine 1-phosphate and DHS-1-P are the main phosphorylated bases found in yeast. They have been shown to accumulate transiently (10−20 min) following heat stress (*54*). Hyperaccumulation of these phosphorylated bases (and also S-1-P) inhibits growth of *S. cerevisiae*, but their physiologic function has not yet been determined.

Ceramide 1-Phosphate. Two published studies have demonstrated mitogenic activity of exogenously added ceramide 1-phosphate (*55*, *56*). The difficulty in delivering this molecule to intracellular compartments, which is identical to the situation with phosphatidic acid, has contributed to the lack of progress with these mediators.

Other Candidate Sphingolipid Mediators. Recently, glucosylceramide was shown to be associated with resistance to chemotherapy. In fact, accumulation of glucosylceramide is a characteristic of some multi-drug resistant (MDR) cells and tumors (melanoma and breast cancer) derived from patients who are less responsive to chemotherapy (*57*). Moreover, recent studies showed that maintenance of glucosylceramide levels in MDR cells is important in preventing apoptosis of these cells (*58*). Glucosylceramide itself may be an endogenous substrate for some members of the p-glycoprotein family of transporters, which have been closely associated with the MDR phenotype.

Regulated Enzymes of Sphingolipid Metabolism: The Backbone of Modular Signaling through Sphingolipids

Serine Palmitoyltransferase (SPT). SPT catalyzes the first committed step in the sphingolipid biosynthetic pathway through the condensation of L-serine with palmitoylcoenzyme A, yielding 3-ketodihydrosphingosine. SPT activity has been localized to the cytosolic side of the endoplasmic reticulum (*59*). Two gene products are required for formation of the SPT heterodimeric enzyme complex, designated LCB1 and LCB2, and these were first isolated in *S. cerevisiae* (*59*−*61*). Homologues of both genes have been identified in mice, hamsters, and humans (*63*−*65*). In yeast, a third protein, Tsc3p, has been shown to be required for optimal activity of LCB1 and LCB2 (*66*).

Deletion of LCB1 or LCB2 is lethal in yeast, and loss of activity also leads to loss of viability in mammalian cells. Recently, a temperature-sensitive mutant (lcb-100) of LCB1 was obtained as an endocytosis-defective gene, and studies with this strain disclosed a requirement for sphingolipids (most probably the free sphingoid bases) in regulating

endocytosis. Also, the lcb-100 strain displays important defects in the response of *S. cerevisiae* to heat stress, including loss of the ability to undergo a transient arrest in the cell cycle and loss of ubiquitination and degradation of nutrient permeases, both of which allow yeast cells to adapt to heat stress. These defects could be bypassed by the addition of phytosphingosine but not closely related sphingolipids, and epistasis analysis supports a role for the free sphingoid bases in these responses (*10*, *13*). Therefore, these studies are beginning to implicate SPT as a key regulated enzyme in the yeast stress response. Also, mutation of the LCB2 gene in *Drosophila* (lace) revealed that it is required for normal development, playing a role in organogenesis through suppression of apoptosis (*67*).

In mammalian cells, the de novo pathway of sphingolipid synthesis has been implicated in the generation of ceramide during apoptosis, independent of sphingomyelinase activation (*68*−*70*). Activation of this pathway has been associated with specific manifestations of apoptosis. For example, in Molt-4 cells, etoposide-induced ceramide is produced via activation of SPT and leads to disruption of membrane integrity (*70*). In vascular smooth muscle cells, stimulation of the type II angiotensin receptor leads to ceramide accumulation through the de novo pathway which in turns leads to caspase-induced poly(ADP-ribose) polymerase (PARP) proteolysis (*68*).

Sphingosine Kinase. Sphingosine kinase (SPHK) converts sphingosine to S-1-P. Two distinct SPHK genes have been identified in humans and mice: SPHK1 and SPHK2 (*71*−*73*). SPHK1 expression predominates in the lung, spleen, and liver, whereas SPHK2 expression predominates in the heart, kidney, and testis and is expressed to a greater extent than SPHK1 in the liver (*71*). In addition, other homologues have been identified in *Caenorhabditis elegans*, *S. cerevisiae*, *Schizosaccharomyces pombe*, and *Arabidopsis thaliana*. In *S. cerevisiae*, two genes, sphingoid long chain base kinases LCB4 and LCB5, have been identified whose products have sphingosine kinase activity (*74*).

Both SPHK1 and SPHK2 phosphorylate D-*erythro*-sphingosine with similar K_m values of 5 and 3.4 μM, respectively (*71*, *75*). D-*erythro*-Sphingosine is the preferred substrate for SPHK1, whereas SPHK2 prefers D-*erythro*-dihydrosphingosine (*71*). In addition, D,L-*threo*-dihydrosphingosine and phytosphingosine are both phosphorylated by SPHK2, but not by SPHK1. Neither SPHK1 nor SPHK2 phosphorylates other lipids such as C_2-ceramide, C_{16}-ceramide, diacyloglycerol, phosphatidylinositol, or *N*,*N*-dimethylsphingosine. These data indicate that SPHK1 and SPHK2 display specificity for the sphingoid base as a substrate.

Sphingosine kinase activity can be increased by an array of external stimuli, including treatment with oxidized low-density lipoprotein (Ox LDL) and growth factors such as PDGF, EGF, nerve growth factor, or TNFα. In addition, various antagonists and/or agonists of signaling pathways, such as PMA, forskolin, and the B subunit of cholera toxin, can stimulate SPHK. SPHK is also a substrate for PKC, and it binds to calmodulin. However, controversy exists as to whether its activation is calcium- and calmodulin-dependent (for a review, see ref *76*).

The physiological functions of SPHK1 and SPHK2 have not been fully defined. However, it has been proposed that SPHK1 may be responsible for many of the cell growth and survival properties attributed to S-1-P (*71*), whereas SPHK2 may be responsible for other effects of S-1-P on angiogenesis or allergic responses because of its unique pattern of expression in the embryo and adult as compared to SPHK1 (*71*).

Pitson et al. (*72*) have studied the effects of introducing a dominant negative mutant form of SPHK1 into human embryonic kidney cells (HEK293T cells). Their findings indicate that basal levels of S-1-P are unaffected, but that the dominant negative blocks activation by certain agonists of SPHK (e.g., TNFα, IL-1, and PMA).

Sphingomyelinases (SMases) and Inositol Phosphosphingolipid Phospholipase C (IPS-PLC). At least five major classes of SMases have been identified, based mainly on different pH profiles, cation requirements, and cellular localization. These include a lysosomal acid SMase (A-SMase), a cytosolic Zn^{2+}-dependent acid SMase (a product of the same A-SMase gene), a membrane-bound Mg^{2+}-dependent neutral SMase (N-SMase), a cytosolic Mg^{2+}-independent neutral SMase, and an alkaline SMase. Among these, the acid and the Mg^{2+}-dependent neutral SMases have received the greatest attention as potentially regulated enzymes.

A-SMase. The A-SMase has been purified (*77*) and cloned (*78*). It is localized in the endosomal/lysosomal compartment, and its biological role was established when its deficiency was found to be the cause of Niemann-Pick syndrome, a human autosomal-recessive lysosomal storage disease (*79*).

Subsequently, more specific roles as an important modulator of ceramide levels and of apoptosis have been proposed and disputed (*80*, *81*). The first proposed model for A-SMase activation was based on the positive effect of DAG, produced through the activation of a putative phosphatidylcholine-specific phospholipase C (PC-PLC), on A-SMase directly (*82*). On the basis of this model, the role of A-SMase in mediating some TNF (and later Fas)-induced effects (ceramide formation, cell death, and Nf-κB translocation) was suggested (*83*). A few points of concern about the validity of this hypothesis should be considered. First, the effects of cellular treatments with DAG or PMA are most often associated with cell proliferation rather than cell death, and they counteract ceramide-induced cytotoxic effects (*84*). Second, this model is critically dependent on the role of PC-PLC, a poorly characterized enzyme, and on its inhibition by D609, an effect that has not been documented. Third, the direct effects of DAG on A-SMase are much less potent than the effects of DAG on PKC, and they show little stereospecificity. Fourth, several studies have attempted to validate this model using cells from Niemann-Pick patients, but have been unsuccessful (for a review, see ref *80*).

More recently, a second model of regulation of A-SMase has been proposed involving the activation of the death domain of the TNF receptor (and also perhaps Fas). Upon recruitment of the apoptotic adapter proteins TRADD and FADD to the receptor, A-SMase is activated by an unknown mechanism (*85*). It is also undetermined how this activation contributes to apoptosis, but it has been suggested that lysosomally generated ceramide may directly activate cathepsin D (*33*). In non-apoptosis-related studies, both interleukin-1 and NGF have been shown to activate A-SMase and induce SM hydrolysis that appears to be localized to a caveolin-rich compartment of the plasma membrane (*86*, *87*).

The availability of A-SMase knockout mice has also provided insight into the possible role of A-SMase in cellular regulation. A protective effect in the knockout animals against Fas-induced hepatocyte apoptosis appears to be the most clear-cut result with little effect on lymphocyte apoptosis (*88*).

Clearly, much remains to be elucidated of the biochemical mechanisms involved in activation of A-SMase in response to agonists, where this occurs, and what it contributes to ceramide-mediated cell regulation.

N-SMase. N-SMases recently have been purified from bovine and rat brain (*89*, *90*), and three putative forms have been cloned (*91*−*93*). The first cloned enzyme resides in the ER and is most likely a lysoPAF phospholipase C and not a sphingomyelinase (*94*). The second enzyme is brain specific, and it resides in the Golgi. The third N-SMase was identified by expression cloning of a human kidney cDNA library. The latter two enzymes await further study.

Despite the lack of well-defined molecular and pharmacological tools for studying N-SMase, different potential mechanisms for its regulation have been proposed. According to one scenario, a domain in the TNF receptor termed NSD (neutral sphingomyelinase activation domain) is proposed to be involved in direct binding to FAN (factor associated with N-SMase activation) which in turn activates N-SMase by an unknown mechanism. Recent observations that TNF-induced activation of ERKs occurs without involvement of FAN (*95*) contradict an earlier report implicating FAN in ERK activation (*96*). Moreover, CD40-induced apoptosis in EBV-transformed lymphocytes has been shown to involve FAN-regulated activation of N-SMase (*97*). Thus, there is an emerging connection between TNF, FAN, and N-SMase which requires further study for elucidation of the mechanism of activation of N-SMase and its cellular functions.

Another line of investigation has demonstrated in vitro regulation of N-SMase by arachidonate (*98*), although the physiological significance of this direct activation has not been established. Cell studies suggest at least an indirect role for arachidonate in activation of N-SMase. In HL-60 and L929 cells, activation of phospholipase A_2 ($cPLA_2$) and accumulation of arachidonate preceded SM hydrolysis triggered by IFN-γ and TNF treatment (*99*, *100*). Also, treatment of these cells with arachidonate or with pharmacological activators of $cPLA_2$ induced N-SMase activation (*100*). Most importantly, cells lacking $cPLA_2$ failed to activate N-SMase and were resistant to apoptosis induced by TNF. Restoration of $cPLA_2$ by transfection enabled TNF to activate N-SMase and to induce cell death (*20*).

At a cellular level, TNF-induced activation of N-SMase involves the action of upstream caspases, but not the effector caspases, and is inhibited by the action of bcl-x but not bcl-2, the latter acting downstream of ceramide in the mitochondrial pathway of apoptosis (*101*, *102*).

Recent studies demonstrate a role for intracellular oxidation in the regulation of N-SMase (*103*−*106*). The action of TNF in breast cancer cells (*107*), rat primary astrocytes, oligodendrocytes, microglia, and C6 glial cells (*108*), hydrogen peroxide and hypoxia in PC12 cells (*106*, *109*), and hypoxia-reoxygenation of cardiac myocytes (*110*) cause a drop in the levels of reduced glutathione (GSH) and an increase in the levels of oxidized glutathione (GSSG). This has been shown to be related mechanistically to activation of N-SMase and ceramide formation. Therefore, it has been proposed that N-SMase is a sensor of oxidative stress, coupling intracelluar oxidation to ceramide formation (*107*).

IPS-PLC. The yeast IPS-PLC gene, ISC1, was recently identified by homology to the bacterial neutral SMase (*91*). The enzyme acts in vitro on sphingomyelin, but its natural substrates are the inosytolphosphorylceramide (IPC) and its mannosylated forms (MIPC and MIP_2C) (*111*). Deletion of this gene in *S. cerevisiae* has a growth defect phenotype, opening the way for further investigation into mechanisms of regulation and function.

Ceramidases. Ceramidase hydrolyzes ceramide into sphingosine and fatty acid, and in doing so, it is important in modulating levels of ceramide versus sphingosine with possible physiological consequences. There are at least three types of ceramidases, acid, alkaline, and neutral, based on the pH at which their activity is optimal.

Acid Ceramidase. Homologous genes encoding lysosomal acid ceramidase have been characterized in mice and humans (*112*, *113*). The preferred substrates for acid ceramidase are ceramides with unsaturated fatty acids and 12-carbon fatty acyl chains (*114*, *115*). Genetic impairment of its activity is the cause of Farber's disease, a human disorder characterized by ceramide accumulation and lipogranulomatosis. It is not known if this enzyme is regulated or participates in signaling functions.

Nonlysosomal Ceramidases. A neutral ceramidase was purified from *Pseudomonas aeroginosa*, and the gene has been cloned from this species. Homologous genes have been identified in mouse and in *A. thaliana* (*116*, *117*). A highly related enzyme was purified from rat brain, and the human homologue was cloned (*118*, *119*). The human enzyme, initially termed nonlysosomal ceramidase in recognition of its broad pH spectrum, was found to localize to mitochondria using cell fractionation as well as studies with a GFP fusion protein. This intriguing finding immediately suggests the presence of a mitochondrial pool of ceramide metabolism, and raises the possibility of participation of this enzyme in regulation of mitochondrial functions, especially apoptosis. The mitochondrial ceramidase shows high selectivity for D-*erythro*-ceramide as opposed to the precursor dihydroceramide, suggesting divergence of the catabolic pathways regulating these two molecules.

Neutral ceramidase activity in hepatocytes was found to be stimulated in response to IL-1β and appeared to be regulated via tyrosine phosphorylation, and perhaps was involved in the regulation of the expression of the cytochrome CYP2C11 (*120*).

Alkaline Ceramidase. Recently, Mao et al. (*121*, *122*) identified two gene products, YDC1 and YPC1, from *S. cerevisiae* as a dihydroceramidase and a phytoceramidase, respectively. These two enzymes were found to reside most likely in the ER. Along with the mammalian mitochondrial enzyme, this distinct substrate specificity and localization of the three enzymes suggests specific pathways for regulation of individual ceramide molecular species. The two yeast enzymes display heat stress phenotypes, further supporting an important role for yeast sphingolipids in the eukaryotic heat stress response.

Mammalian alkaline ceramidase activity has been shown to be stimulated by certain growth factors and/or cytokines (e.g., PDGF). It is thought that neutral and alkaline ceram-

idases are responsible, in part, for the mitogenesis induced by growth factors (i.e., PDGF) through production of sphingosine, which then may act on its own, and/or is converted to S-1-P (*9*).

Another emerging feature of some of these ceramidases is their ability to function in reverse, as CoA-independent ceramide synthases utilizing phytosphingosine/sphingosine and palmitate as substrates. Thus, YPC1 functions as phytoceramide synthase, whereas mitochondrial ceramidase functions as a ceramide synthase. Interestingly, these activities are not inhibited by fumonisin B1, thus providing for a distinct mechanism for ceramide synthesis.

Sphingomyelin Synthase and Inositol Phosphorylceramide Synthase-1. As with SMases, different SMS activities have been described in various cellular compartments, such as Golgi, plasma membrane, and mitochondria, but none of them has been purified and/or cloned. Importantly, SMS not only regulates ceramide and SM levels but also contributes to the formation of DAG. This dual regulation of ceramide and DAG levels raises the question of whether this enzyme might have a direct role in controlling cellular responses mediated by these bioactive lipids. Indeed, it has been shown that SMS activity was increased under conditions of active cellular proliferation, such as regenerating liver (*123*) and transformation (*124*). Moreover, SMS activity has been associated with Nf-κB activation (*125*). On the other hand, growth suppressor stimuli may turn off this enzyme; in one recent study, TNFα treatment of Kym-1 rabdomyosarcoma cells induced activation of SMases, but also caused inhibition of SMS, as shown by an in vitro assay (*126*), suggesting that inhibition of the enzyme contributes to the elevation of the ceramide level (and possibly a drop in the DAG level). IPC synthase is the yeast counterpart of the mammalian SMS, and it regulates the formation of IPC from ceramide and phosphatidylinositol (PI). Although the gene has been isolated (AUR1/IPC1) (*127*), the protein has not been purified, possibly due to the existence of a necessary accessory subunit. The use of a "leaky" galactose promoter in *Cryptococcus neoformans* has allowed up- and downregulation of this essential gene. From these studies, it was shown that downregulation of IPC synthase significantly impaired melanin formation, growth in acid pH, and growth in human macrophages (a model for human pathogenicity) (*128*). The availability of the IPC synthase gene and the simplicity of the yeast model offer prospects of identifying mechanisms of regulation of this enzyme that could also be applicable to the mammalian SMS.

Glucosylceramide Synthase and Lactosylceramide Synthase. The recent purification and cloning of GCS have represented an important contribution to the understanding of ceramide-mediated biology (*129*, *130*). TNFα has been shown to inhibit the activity of GCS as well as SMS, thus contributing to the accumulation of ceramide (*126*). Reciprocally, overexpression of GCS attenuated ceramide response to chemotherapeutic agents and significantly protected from the induction of cell death in response to these agents (*131*). Moreover, treatment with specific GCS inhibitors, such as D-t-PDMP or P4 (*58*), or downmodulation of GCS by antisense (*132*), resulted in accumulation of ceramide and enhancement of agonist-induced cytotoxic effects. These and related studies have led to the proposal that GCS is a key regulator of chemotherapy drug resistance and sensitivity, probably through the modulation of ceramide levels (*133*).

The second enzyme in the cerebroside biosynthetic pathway, lactosylceramide synthase (LCS), transfers a galactose from UDP-galactose to glucosylceramide. It has been proposed that activity of this enzyme might be associated with proliferative responses and activation of Nf-κB, particularly in atherosclerotic tissues, and LCS activity seems to be stimulated after treatment with oxidized LDL and proinflammatory cytokines, such as TNFα (*134*−*136*).

Other Potentially Regulated Enzymes of Ceramide Metabolism. Other enzymes that could potentially regulate ceramide levels include dihydroceramide synthase (also known as ceramide synthase), dihydroceramide desaturase, and ceramide kinase. Dihydroceramide synthase, the target of fumonisin B1, catalyzes the acylation of dihydrosphingosine, and it has been shown that the chemotherapeutic agent daunorubicin induces modest increases in its activity (*137*). Dihydroceramide desaturase has received some attention recently, and it was shown to be an NADPH-dependent enzyme (*138*). Little is known about its regulation, but it may be rate-limiting in generating ceramide from dihydroceramide. Ceramide kinase has been described in synaptic vesicles and in leukemia cells (*139*, *140*), and its product ceramide 1-phosphate has been detected in cells and has been suggested to exert mitogenic effects (*55*).

Complexity of Sphingolipid Metabolism: A Rich Network That Provides a Combinatorial Mechanism for Regulation by Bioactive Lipids

In addition to providing for multiple specific pathways of cell regulation, the complexity of sphingolipid metabolism provides for a rich matrix of interconnected pathways leading to the regulation of the levels of multiple bioactive sphingolipids. Moreover, in many cases, a cell agonist may coordinately regulate more than one enzyme, thus selectively channeling sphingolipid metabolism in specific directions. The following examples serve to highlight these possibilities.

(1) Studies by Nikolova-Karakashian et al. showed that interleukin-1 activates sphingomyelinase and also ceramidase when used at low concentrations, thus resulting in the formation of sphingosine which then regulates the expression of the cytochrome P450 (CYP2C11) (*120*). On the other hand, higher concentrations of interleukin-1 activated only sphingomyelinase without ceramidase, resulting in the accumulation of ceramide, which mediates the effects of interleukin-1 on induction of acid glycoprotein.

(2) Studies by Huwiler et al. showed that nitric oxide (NO) activated sphingomyelinase and inhibited ceramidase in mesangial cells, resulting in the accumulation of ceramide which was linked to apoptosis. However, the action of TNF in these cells resulted in activation of both sphingomyelinase and ceramidase, thus precluding the accumulation of ceramide, shunting the ceramide formed from sphingomyelin into sphingosine and S-1-P, and preventing the induction of apoptosis (*141*).

(3) Treatment of vascular SMC with oxidized LDL stimulated neutral sphingomyelinase, ceramidase, and SPHK, leading to mitogenesis (*142*). However, oxidized LDL treatment of endothelial cells induced accumulation of ceramide followed by apoptotic cell death (*143*).

(4) PDGF has been shown to activate ceramidase (*9*) and SPHK (*34*), which act on ceramide to result in the formation

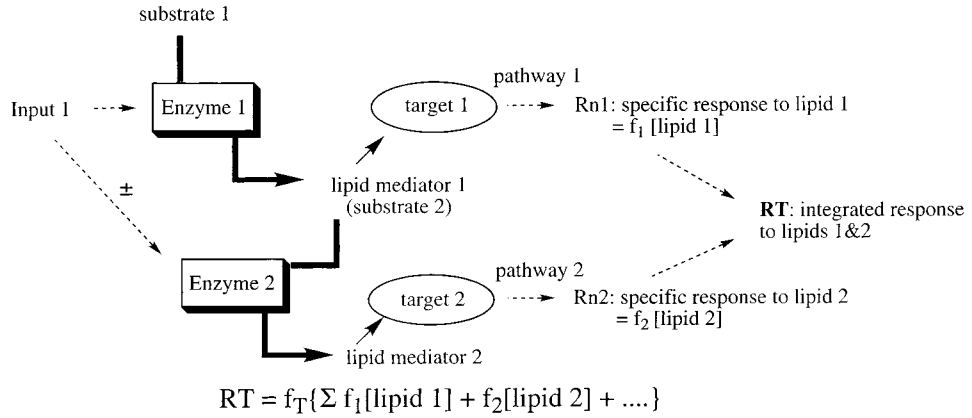

FIGURE 3: Enzymes of sphingolipid metabolism from modular to integrative signaling. We equate the production of any individual lipid mediator with at least one specific response [*R*(*n*)], and then assume that the magnitude of this response is dependent on the degree of accumulation of the lipid mediator. The overall response to any particular agonist (or set of agonists), *RT*, is then a function of the integrated sum of the individual responses; $RT = f_T[\sum R(\text{cer}) + R(\text{sphingosine}) + R(\text{DAG}) + R(\text{S-1-P}) + ...]$, or $RT = f_T(\sum f_1[\text{cer}] + f_2[\text{sphingosine}] + f_3[\text{DAG}] + f_4[\text{S-1-P}] + ...)$. If for each *f*[lipid], *f* can vary from 0 (no contribution of that lipid to the overall response) to 1 (maximal contribution), then the continuous variation of *f* values between 0 and 1 provides for an infinite combination of overall responses based on a rather limited set of biochemical outputs.

of S-1-P, which in turn was suggested to mediate the effects of PDGF on calcium mobilization and activation of ERKs.

(5) Transformed human diploid fibroblasts and hepatoma cells were shown to manifest a high level of a plasma membrane-related sphingomyelin synthase (*124*), an activity that was nearly absent in the wild-type fibroblasts and very low in normal liver cells. Treatment of these malignant and transformed cells with sphingomyelinase resulted in formation of ceramide which fed into SMS, thus resulting in the formation of DAG and Nf-κB activation (*125*). Therefore, in the presence of a high level of SMS, a ceramide signal is "converted" into a DAG signal.

Given the increasing number of examples, one could postulate three levels of function for each regulated enzyme of sphingolipid metabolism. (1) At the basic level, each regulated enzyme may function as a **module** in cell regulation through the control of formation of a specific output. (2) Many of these enzymes may also function as **switches** that shunt the response from one lipid to another. (3) At the most complex level, a network of regulated enzymes provides for a **combinatorial** mechanism for integrating cellular responses. This is illustrated in Figure 3, where the overall response to any particular agonist is a function of the integrated sum of the individual responses.

Conclusions: Sphingolipid Metabolism as a Prototype for Integrative Signaling and Cell Regulation

A remarkable picture is emerging whereby the complexity of sphingolipid metabolism serves to regulate the levels of multiple molecules, each with a distinct set of bioeffector functions. A necessary consequence of this complexity is that the cell is presented with a very convenient mechanism to "integrate" the ultimate consequences of changes in sphingolipid metabolism in response to agonists. In extreme cases, the cell response will be dominated by one metabolite over others (for example, profound changes in ceramide, or changes restricted to S-1-P). However, it is more likely that any agonist (or combination of agonists) would result in changes in several metabolites, and the ultimate outcome will depend on the overall balance of action of these pathways. This provides for a very rich mechanism for generating multiple outputs from a rather limited set of biochemical pathways through a process of coordinated integration of sphingolipid metabolism.

Another layer of complexity of sphingolipid metabolism arises from the topological restriction of distinct enzymatic pathways. As discussed, ceramidases and sphingomyelinases exist in at least three compartments. Since the lipid products of these reactions are most likely to remain at the site of generation (with the option of flipping for many of them), this compartmentalization not only speaks to the distinct topology of metabolism but also suggests distinct functions for bioactive lipids generated at distinct sites. It should also be noted that changes in the sphingolipid content of membranes may effect cellular properties such as membrane fluidity and the properties of certain proteins within the membrane (for a review, see ref *144*).

Finally, this emerging hypothesis on integration of sphingolipid signaling can be easily generalized to other aspects of lipid metabolism and intermediary metabolism in general. In the case of glycerolipids, the interconnections of DAG, phosphatidic acid, monoacylglycerol, arachidonate, and phosphatidylinositols provide for a similar rich metabolic network where individual lipids interact with distinct targets, and therefore mediate distinct responses. Again, integration of these responses would allow many more outputs than those allowed from the simple operation of distinct modules.

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