Sphingolipid Metabolism in the Regulation of Bioactive Molecules

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A living organism is an extraordinary complex system in which harmony and thus communication between parts is one of the most important goals to pursue. Communication within an organism occurs at different levels: intercellularly and intracellularly. In both cases one of the main targets involved in this process is the cellular membrane. From or through this compartment of one given cell, a wide variety of signals can be sent either to interact with other cellular units or to specifically direct the behavior of the same given cell. Important players in this communication system are not only the proteins present in the membrane but interestingly also the lipid counterpart. In this way the concept of lipids as bioactive molecules and not only as structural components of the membranes has evolved. At first, this concept was mainly applied to the glycerolipid class, such that the signal transduction pathways regulated by enzymes of glycerolipid metabolism are now well defined (1). More recently another class of lipids has been proposed to play a key role in the cellular communication system: the sphingolipids.

SPHINGOLIPIDS: STRUCTURE AND METABOLISM

Sphingosine and ceramide are the two structurally simplest molecules belonging to the sphingolipid family, from which all other complex sphingolipids derive (2). Sphingosine represents the sphingoid backbone, and ceramide carries the fatty acyl group in amide linkage to the sphingoid moiety. The wide variety of the naturally occurring sphingolipid species is due to the different chain length of either the sphingoid unit or the fatty acyl moiety, and to the nature of the functional group at the 1-position. For instance, sphingomyelin (SM) carries a phosphorylcholine moiety, the cerebroside subclass carries sugar residues (such as glucose and galactose), and the gangliosides contain acidic sialic acid residues in addition to the sugar units (3).

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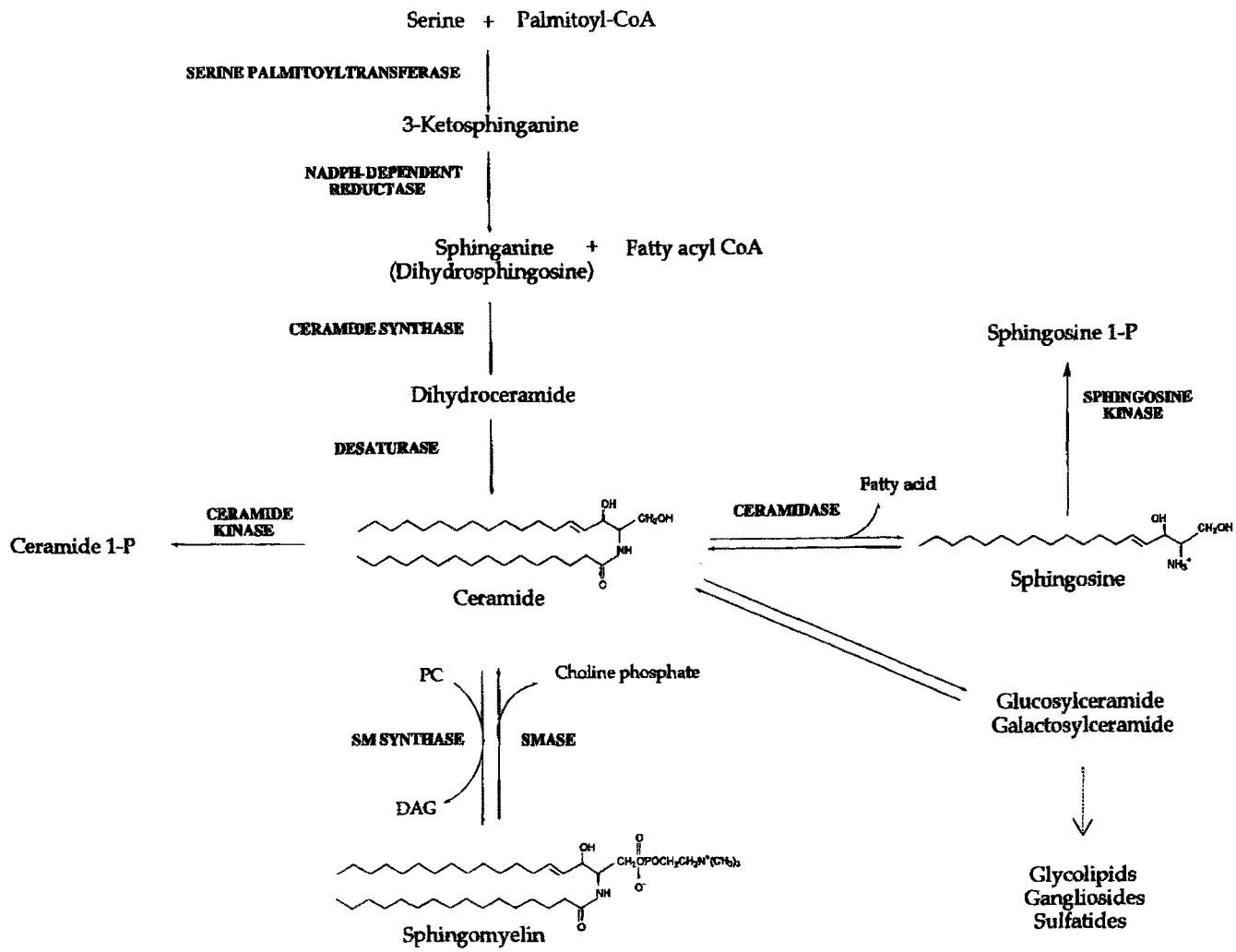
Abbreviations: A-SMase, a lysosomal acid SMase; DAG, diacylglycerol; GSH, glutathione; ICE, interleukin-converting enzyme; N-SMase, neutral magnesium-dependent; PC, phosphatidylcholine; PKC, protein kinase C; PLC, phospholipase C; Rb, retinoblastoma gene product; SM, sphingomyelin; SMase, sphingomyelinase; TNFα, tumor necrosis factor α.

Sphingolipid metabolism includes a series of biosynthetic and catabolic reactions in which ceramide plays a very central role (Scheme 1). The *de novo* synthesis starts with the condensation of palmitoyl-CoA with serine (forming 3-ketosphinganine) through the rate limiting action of serine palmitoyl transferase (3). Reducing and acylating reactions, through a NADPH-dependent reductase and ceramide synthase, then follow yielding ceramide formation probably by the final action of a dehydrogenase (4). At this point there are multiple fates of the newly synthesized ceramide. Ceramide can be converted into SM through the transfer of the choline phosphate group from phosphatidylcholine (PC) through the action of PC:ceramide phosphocholine transferase (SM synthase) (5,6). On the other hand, ceramide can be utilized as a precursor for cerebrosides and gangliosides through the sequential addition of carbohydrate units (from sugar nucleotide donors) and/or sialic acid residues (from CMP-*N*-acetylneuraminic acid donors) (7). Moreover, ceramide can be phosphorylated at the 1-position through the action of a distinct ceramide kinase yielding ceramide-1-P (8).

In sphingolipid catabolism clearly ceramide plays once again a crucial role. In fact, ceramide is the breakdown product of complex sphingolipids which are sequentially hydrolyzed in their head groups. Likewise, ceramide is the product of SM catabolism through the action of different sphingomyelinases (SMase), which hydrolyze the phosphocholine head group releasing choline phosphate (9). Ceramide, in turn, is the target of ceramidases which cleave off the fatty acyl chain, yielding sphingosine and its phosphorylated metabolite sphingosine-1-P (10).

SPHINGOLIPIDS AS SECOND MESSENGERS

As evidenced from the previous description, the web of sphingolipid metabolism is complex and leads to a large variety of molecules. In spite of this, it is only recently recognized that many of these metabolites have a role as bioactive molecules. The demonstration of inhibition of protein kinase C (PKC) activity by sphingosine was the first step in this direction (11). The discovery of SM hydrolysis and ceramide formation in response to certain stimuli represented a further move into the understanding of sphingolipid biology (12). Nowadays, significant effort is directed at the elucidation of the role of ceramide.



SCHEME 1. Shown is a schematic of the blueprint of sphingolipid metabolism focusing on the earlier steps leading to and from ceramide and on the steps that relate ceramide to sphingosine, sphingosine 1-phosphate, and DAG. Abbreviations: PC, phosphatidylcholine; DAG, diacylglycerol; SM, sphingomyelin.

sphingosine, and sphingosine 1-P as key regulators of these emerging signal transduction pathways.

Growth regulation by ceramide. Ceramide accumulates in response to several different inducers such as cytokines [tumor necrosis factor α (TNFα), interleukin β1 (ILβ1), interferon-γ (Infγ) and Fas], cytotoxic agents (chemotherapeutic agents, irradiation), and finally stressful conditions (such as serum deprivation and heat shock) (13). The general response to these stresses is a growth-suppressed condition which can be due either to cell cycle arrest or to apoptosis. The choice between the two fates is determined mostly by the cell type. Cellular treatments with exogenously added short-chain ceramide analogs (C2-, C6-ceramide) mimic the same biological effects observed when natural stress inducers are used. The effects of ceramide on cell cycle arrest were studied mostly in response to serum deprivation (14). This condition induced elevation of intracellular ceramide level with time, a progressive arrest of cell cycle in the G0-G1 phase and dephosphorylation of the retinoblastoma gene product (Rb). Treatment with exogenously added ceramides also induced dephosphorylation of Rb (15). On the other hand, ceramide-mediated apoptosis was studied upon treatment with several different agonists, in particular with TNFα and after addition of short-chain ceramide analogs. The latter treatment was able to induce DNA fragmentation similar to TNFα treatment (16). Also recent studies established the involvement of pro-interleukin-1 converting enzyme (ICE) family members upstream (such as FLICE) and downstream (such as caspase 3) to ceramide signaling (17). Interestingly, activation of the PKC pathway by treatment with phorbol esters seems to enhance viability and thus to counteract ceramide mediated apoptosis (16). Importantly, treatment with exogenous ceramide increased proliferation of quiescent Swiss 3T3 fibroblasts, indicating that eventually ceramide may positively regulate cell proliferation (18).

Regulation by sphingosine and sphingosine-1-P. Sphingosine was first discovered to be a PKC inhibitor (11), and in line with this observation it has been shown to inhibit cell growth (19). Moreover, sphingosine was implicated as endogenous me-

diator of apoptosis during phorbol ester-induced differentiation of HL-60 cells (20). On the other hand, sphingosine stimulated the proliferation of growth-arrested Swiss 3T3 (21) and Rat-1 fibroblasts (22). The mitogenic effects exerted by sphingosine may be due to its conversion to sphingosine-1-P (23). One of the mechanisms by which sphingosine and sphingosine-1-P may elicit mitogenic effects could be the induction of phosphatidic acid accumulation (19). This accumulation could be the result of diacylglycerol (DAG) kinase activation (24), inhibition of phosphatidic acid phosphohydrolase (25), and most importantly activation of phospholipase D (26).

Other metabolites. Other sphingolipid metabolites, such as ceramide 1-P (8) and GD3 gangliosides (27), seem to be also bioactive molecules in the regulation of cellular proliferation and/or apoptosis, but further investigations are in process.

The web of sphingolipid signaling. In addition to the general paradigm that ceramide negatively regulates cell growth, it is clear that there are several interrelated events, such as competing functions of ceramide, sphingosine, sphingosine-1-P, ceramide-1-P, gangliosides and DAG, which create a more complex picture. The emerging scenario is best understood as an intricate web of interconnected metabolic reactions, which modulate the relative intracellular concentrations of the several different species. These metabolic pathways may play a critical role in the fate of a signaling pathway by differential regulation of the key enzymes. So, in order to better understand the biological behavior of a given pathway as part of a more general picture, it becomes extremely important to study and seriously characterize each enzymatic activity and try to relate it to the cellular environment, in terms of inhibitors or activators.

ENZYMES OF SPHINGOLIPID SIGNALING

Until now, great efforts have been spent in order to better understand the biological functionality of the sphingolipid pathway. On the other hand, this approach cannot replace investigations directed to study the biochemical characteristics of the reactions themselves, and thus of their regulatory enzymes. Researchers normally find several obstacles when approaching the problem. First, sphingolipid metabolism is based on a number of interconnected reactions; different enzymes may share the same precursor or lead to the same product, possibly influencing each other. In this case it is difficult to discriminate between enzymatic activities. Second, very few enzymes of the pathway have been purified and cloned; thus most of the studies conducted *in vitro* use mixed preparations (homogenates or membrane pools) which may yield misleading results. The involvement of different enzymes regulating the levels of specific sphingolipid species in response to external stimuli is becoming more and more evident. In this report, we will be focusing on a few of them, such as ceramide synthase, sphingomyelinases (SMase), ceramidases, and SM synthase.

CERAMIDE SYNTHASE

Ceramide synthase catalyzes the acylation of long-chain sphingoid bases by transfer of a fatty acid from the CoA thioester (28). This activity is localized in the endoplasmic reticulum, with the catalytic site thought to face the cytosol and an *in vitro* pH optimum of 7.4 (29). The most popular approach followed to study the role and biological importance of ceramide synthase is the use of specific inhibitors. Since ceramide synthase is one of the early enzymes involved in sphingolipid *de novo* synthesis, eventual *in vivo* experimental manipulations of this activity may result in a general alteration of sphingolipid metabolism. One may predict that inhibition of ceramide synthase activity would lead either to sphinganine accumulation and/or loss of complex sphingolipids. In many cell types both free sphinganine and specific inhibition of sphingolipid metabolism are growth inhibitory and cytotoxic. It follows that results obtained by altering/inhibiting ceramide synthase activity may not be easy to interpret. Among the inhibitors, fumonisin B1 is the best studied (29). Treatment with fumonisin B1 mainly induces accumulation of sphinganine, which is then partially phosphorylated to sphinganine-1-P. The most rapid intracellular effect of fumonisin B1 treatment is sphinganine elevation rather than depletion of complex sphingolipids, but the relative magnitudes of these two effects vary between cell types (30). Fumonisin B1 treatment alters the behavior of cell-surface proteins and changes cell morphology, inhibits PKC, and induces Rb dephosphorylation and cell cycle arrest (29). On the other hand, fumonisin B1 was shown to induce cell proliferation in growth-arrested Swiss 3T3 cells (28). Moreover, fumonisins were recognized to play an important role in the induction of different diseases such as esophageal cancer, equine leukoencephalomalacia, and porcine pulmonary edema (31).

SMASE

SMase is a SM-specific phospholipase C (PLC)-type activity, hydrolyzing SM phosphodiester bond and yielding ceramide and phosphorylcholine. Evidence for the importance of SMase activity arose with the discovery of the so-called "sphingomyelin cycle." Several different inducers (TNFα, INFγ, IL-1β, nerve growth factor, Fas ligand, dexamethasone, and others) were found to cause SM hydrolysis in a variety of cell types (monocytes, lymphocytes, fibroblasts, glioma cells) (13). SM hydrolysis leads to ceramide production which mediates at least part of the biological effects exerted by these inducers. SM is then regenerated by the action of the SM synthase. So far, five types of SMases have been described: a lysosomal acid SMase (A-SMase), a plasma membrane-bound neutral magnesium-dependent (N-SMase), a cytosolic neutral magnesium-independent, a zinc-stimulated acid SMases and finally an alkaline SMase. At least two of these SMases, the A-SMase, and the N-SMase, may regulate ceramide intracellular levels (32).

N-SMase. The N-SMase activity can be detected in a variety of organs even if its distribution is not uniform. For example, in rat, the highest activity can be found in brain; less activity is found in testis, adrenal gland, liver, spleen, and kidney (9). Subcellular fractionation studies showed that N-SMase is a plasma-membrane bound enzyme with a possible intracellular

orientation (33). Its activity requires the presence of magnesium and its optimal pH values range between 6.0 and 8.5. Phospholipids (in particular phosphatidylserine (34)) and free arachidonic acid can stimulate N-SMase activity (35). *In vitro* (direct enzymatic assay) and *in vivo* (changes in SM and ceramide levels) measurements of N-SMase activity showed enzyme activation after cellular treatments with inducers of the SM cycle (9). The N-SMase time-course activation varies between cell lines, such that after TNFα treatment of monocytic U937 cells, human skin fibroblasts and mesangial cells a peak of activity is reached after few minutes (2–60 min), while in murine L929 fibro-blasts a significant increase is evident only after several hours (12 h) (13). Studies conducted using a potent ICE inhibitor (crm A) seem to suggest a possible role of this family of proteases in the events that lead from TNFα/receptor interaction to N-SMase activation (17). Recently another forwarding step toward a better understanding of the biochemical/biological N-SMase regulation has been made. Remarkably, glutathione (GSH), at concentrations similar to intracellular levels, was shown to actively modulate N-SMase activity (36). *In vitro* studies on a partially purified N-SMase showed that physiologic intracellular GSH concentrations (1–20 mM) were inhibitory of the enzymatic activity, whereas at lower GSH concentrations, N-SMase activity was recovered. The GSH inhibitory effect was not due to the sulfhydryl group as other general reducing agents used (dithiothreitol, β-mercaptoethanol) were ineffective. Instead, the γ-glutamyl-cysteine moiety of GSH seems to be required. Moreover, cellular treatment with L-buthionine-(*SR*)-sulfoximine, a GSH synthesis inhibitor, caused, as expected, intracellular GSH depletion and induced the SM cycle. Since GSH depletion is a general cellular phenomenon observed in response to stressful conditions, it can be an important mechanism of N-SMase activation, while under physiological conditions GSH would control N-SMase activity. GSH/N-SMase model is also a useful example of a regulatory system involving cross-talk between different cellular areas implicated in stress signaling. Finally, the recent reported cloning of the enzyme will be extremely helpful in the near future to get a better understanding of the processes just discussed (37).

A-SMase. A-SMase is distributed in all mammalian tissues analyzed, and compared to the N-SMase, A-SMase activity is much higher (except for the brain) (32). Its pH optimum sets around 5.0, and its activity localizes with the lysosomal fraction (9). A-SMase was the first of the SMases to be described, purified, and cloned (38). A-SMase defect was found to be the biochemical determinant of Type A and Type B Niemann-Pick disease, resulting in SM accumulation and causing neurological disorders (32). Divalent ions and chelating agents do not affect the activity of this enzyme, while dithiothreitol and AMP are inhibitors (9). On the other hand, ApoC-III, an apolipoprotein from human very low density lipoprotein and a group of proteins named saposins (sphingolipid activator proteins), in particular saposin D, stimulate A-SMase activity (9). The biological role of A-SMase, and in particular its importance in the regulation of the apoptotic signal, is still controversial. Schutze *et al.* (39) reported A-SMase activation after TNFα treatment: through the activation of a putative PC-specific PLC, TNFα induced DAG release, turning on A-SMase. A-SMase has been proposed also to mediate Fas-induced apoptosis (40). On the other hand, evidences against the involvement of A-SMase in the TNFα and IL-1β induced SM cycle were reported by other authors (41). Finally, A-SMase knock-out mice have reproduced a phenotype similar to the type A Niemann-Pick disease and they showed resistance to radiation-induced apoptosis (62).

CERAMIDASES

Ceramidase activity hydrolyzes ceramide into free sphingoid base (sphingosine) and fatty acid. Therefore, it may represent a very important tool in order to modulate the switch between ceramide- and sphingosine-regulated biology. In spite of this potential major role, not much is known about their biochemical and biological regulation even if some of the forms have been purified and cloned. In the literature, different forms of ceramidases have been reported, mainly based on different pH profiles.

Acid ceramidase. Acid ceramidase activity is assumed to be ubiquitous (rat brain, liver, kidney, and human skin fibroblasts, spleen, kidney, brain, leukocytes) and to play a housekeeping role being localized in the lysosomes (42). Its optimal pH ranges between 4.0 and 5.0. This type of activity was the first ceramidase to be purified and cloned (43). Acid ceramidase activity does not seem to be affected by phospholipid addition, while saposin D was shown to activate *in vivo* and less clearly *in vitro* (44). On the other hand, *N*-oleoylethanolamine is recognized as an acid ceramidase inhibitor even if its inhibitory effect is rather weak and at very high concentrations (44). Substrate specificity studies showed preference for ceramides with unsaturated fatty acids (45); moreover, the preferred fatty acyl chain would carry 12 carbons, while increasing or decreasing chain length would result in loss of activity (46). The main biological significance for acid ceramidase is its association with lipogranulomatosis or Farber's disease, evolving when this ceramidase activity is impaired (42). Interestingly, this kind of disease is also observed when normal saposin D is absent (47).

Alkaline ceramidase. Less is known about alkaline ceramidase. Its activity was reported in rat cerebellum and liver, in human fibroblasts and brain, in guinea pig skin, and porcine epidermis (44). It is a membrane-bound enzyme, possibly localized in the plasma membrane, with an *in vitro* pH optimum ranging between 8.0 and 9.0 (44). Nowadays, two alkaline ceramidases were purified from guinea pig skin (48): the first one (CDase I) purified to homogeneity with pH optimum around 7–10, the second (CDase II) partially purified with pH optimum around 8–9. Contrary to acid ceramidase, saposin D, as well as the A, B, and C forms, do not have any effect on alkaline activity (49). On the other hand, a ceramide analog (1*S*,2*R*)-D-erythro-2-(*N*-myristoylamino)-1-phenyl-1-propanol was shown to be a potent inhibitor of alkaline ceramidase in HL-60 cells (at lower concentrations than *N*-oleoylethanolamine), with no effect on acid ceramidase activity (50). The implication of alkaline ceramidase in mediating sphingolipid signaling became evident when the cellular response to growth factors ([Platelet Derived Growth

Factor (PDGF), Fibroblast Growth Factor (FDF), Epidermal Growth Factor (EDF)] was investigated in Swiss 3T3 and vascular smooth muscle cells (44). In this model, ceramide degradation and sphingosine accumulation accompanied the growth factor- induced proliferative effect. Induction of alkaline ceramidase by growth factors seems to be subsequent to tyrosine kinase phosphorylation (44). Cytokines (TNFα, IL-1β), on the other hand, are more directly involved in SMase induction and do not stimulate ceramidases activation.

Neutral ceramidase. Neutral ceramidase activity is poorly characterized from every point of view. Ceramidase activity, ranging in neutral pH, is reported in rat liver plasma membrane and microsomes and rat intestinal brush border membrane (44). One problem in studying neutral and alkaline ceramidases is the rather broad range of pH in which these enzymes work, so that it is difficult to discriminate between the two forms considering only this parameter. On the other hand, the high affinity shown by the neutral ceramidase toward very short- (C2) or long-chain ceramides (C16) represents a tool which can be efficiently used for this purpose (51). Until now, there are no reports on neutral ceramidase involvement in signal transduction events.

SM SYNTHASE

Mechanisms of *in vivo* SM biosynthesis have been somewhat controversial over the past years, and different biochemical routes have been proposed. At first, CDP-choline was suggested to be the donor of the phosphocholine moiety to ceramide (52); then, the acylation by stearoyl-CoA of sphingosylphosphorylcholine was considered (53). Finally, *in vitro* (5,6) and most importantly *in vivo* cellular labeling studies (6,54) established the existence of a preferred biochemical pathway which transfers the phosphorylcholine unit directly from PC to ceramide. This reaction is catalyzed by the PC:ceramide phosphocholine transferase (SM synthase) and leads to SM and DAG formation. SM synthase activity has been reported in mouse and rat kidney, lung, liver, and spleen and in monkey liver and heart (5,32,55); on the other hand, in the brain, SM synthesis may pass through phosphatidylethanolamine instead of PC (5). Subcellular fractionation studies characterized SM synthase as a membrane-bound enzyme with an intracellular orientation of the active site (56). In liver most of the activity seems to be localized in the Golgi apparatus (>80%), with the remainder mainly distributed between plasma membrane and endoplasmic reticulum (55). On the other hand, SM synthase localization in cellular models shows more variability. For instance, it was reported that in SV40-transformed fibroblasts (57), oligodendrocytes (58), and BHK cells (59), plasma membrane SM synthase shows a higher relative activity compared to the one that resides in the Golgi. *In vitro* studies indicated no strong dependency of the activity on the presence of divalent cations in the incubation mixture (6). The enzyme shows a pH optimum around 7.4 when Tris buffer is used and around 6.5 in the presence of imidazole buffer (5). SM synthase is one of the less-studied enzymes along the sphingolipid pathway, and little is known about its involvement in ceramide-mediated signal transduction events. On the other hand, because of the direct regulation of ceramide and DAG levels, its biological importance in directing a cell toward cell cycle progression or growth arrest is more than reasonable. Elevation of SM synthase activity (seven- to ninefold) was recently observed in malignant conditions, such as Morris hepatomas (60). Moreover, recent studies (60) showed that SV40-transformation induced a significant increase of SM synthase activity in human lung fibroblasts. *In vivo* metabolic labeling with a short-chain ceramide analog and *in vitro* measurements showed a twofold increase of SM synthase activity in SV40-transformed fibroblasts compared to the normal counterpart. Interestingly, when elevation of a specific pool of cellular ceramide, localized in the plasma membrane, was induced by bacterial SMase treatment, normal fibroblasts were completely unable to produce SM from it, while the SV40-transformed cells actively metabolized this ceramide, converting it back to SM. These results may suggest the possibility that SV40 transformation would induce a form of SM synthase that resides in the plasma membrane or in functional proximity to it. This would indicate that different forms of SM synthase may have different targets and eventually different biological effects (*de novo* synthesis for the enzymatic activity in the Golgi and signal transduction for the one in the plasma membrane). As a consequence of the elevated SM synthase activity in SV40-transformed cells, a decrease of the intracellular ceramide levels and an increase of DAG levels occurred. The regulation of intracellular ceramide/DAG ratio makes SM synthase an important "biostat" in the regulation of cell viability/cell death. Another important feature of this enzyme is its reported inhibition by the putative PC-PLC specific inhibitor, D609 (60). PC-PLC is thought to play an active role in signal transduction events regulating DAG formation (39). On the other hand, no genuine evidence of the real existence of the PC-PLC has been produced. Interestingly, SM synthase and PC-PLC share several biochemical and biological characteristics (DAG formation from PC, increased activity in transformed phenotype (61), inhibition by D609); thus, these considerations raise the question whether the biological events that until now have been attributed to the PC-PLC may be partially due to the SM synthase.

CONCLUSIONS

In conclusion, the study of signaling and cell regulation through ceramide has now evolved to the biochemical level by focusing on specific enzymes of ceramide metabolism. A general hypothesis can be presented whereby individual enzymes of ceramide metabolism serve as input points in the regulation of ceramide levels (Scheme 1). For example, activation of SMases or ceramide synthase would elevate ceramide levels and activate ceramide-induced responses. On the other hand, activation of enzymes of ceramide degradation or incorporation such as ceramidases or SM synthase decreases and attenuates ceramide levels. Also, as an important

corollary, some of these enzymes may play an additional fundamental role in interconverting lipid signals. For example, SM synthase has the capacity of interconverting a ceramide signal into a DAG signal, whereas ceramidases can transform a ceramide signal into a sphingosine or sphingosine-phosphate signal. This area of research promises great future insight into important areas of cell studies.

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