ARTICLE

Functions of Ceramide in Coordinating Cellular Responses to Stress

Yusuf A. Hannun

Sphingolipid metabolites participate in key events of signal transduction and cell regulation. In the sphingomyelin cycle, a number of extracellular agents and insults (such as tumor necrosis factor, Fas ligands, and chemotherapeutic agents) cause the activation of sphingomyelinases, which act on membrane sphingomyelin and release ceramide. Multiple experimental approaches suggest an important role for ceramide in regulating such diverse responses as cell cycle arrest, apoptosis, and cell senescence. In vitro, ceramide activates a serine-threonine protein phosphatase, and in cells it regulates protein phosphorylation as well as multiple downstream targets [such as interleukin converting enzyme (ICE)–like proteases, stress-activated protein kinases, and the retinoblastoma gene product] that mediate its distinct cellular effects. This spectrum of inducers of ceramide accumulation and the nature of ceramide-mediated responses suggest that ceramide is a key component of intracellular stress response pathways.

The paradigm of signaling through the glycerophospholipids (*1*) has taught us a critical lesson in lipid-mediated signal transduction and cell regulation: The structural complexity of membrane glycerophospholipids belies the role they subserve in signal transduction. Second messengers derived from precursor glycerophospholipids include diacylglycerol (DAG), inositol trisphosphate (IP_3), arachidonate, the eicosanoids, and platelet activating factor. Individual glycerophospholipids can be viewed as stores of potential information released in response to cellular stimuli through the activation of specific enzymes. Messages are then carried by the released lipid-derived products in the form of specific interactions of these products with their targets [such as protein kinase C (PKC) or the IP_3 receptor].

Sphingolipids, which are structurally even more complex than the glycerophospholipids, also participate in signal transduction. Sphingolipids have roles in the response to cell contact, as receptor components, as anchors for proteins, and as markers of tumor progression and cell differentiation (*2*). Defects in several sphingolipid hydrolases result in the various forms of inherited sphingolipidoses (*3*). Sphingolipids also have an essential role in cell viability. In both *Saccharomyces cerevisiae* and mammalian cells, mutations in the first enzyme of de novo sphingolipid biosynthesis (serine-palmitoyl transferase) result in abolition of sphingolipid formation and loss of viability that is reconstituted by replacement with sphingolipids (*4*). Several bacterial hemolysins and cytotoxins have been identified as sphingomyelinases, and toxic fungal metabolites (such as the fumonisins, the sphingofungins, and the australifungins) specifically target enzymes of sphingolipid metabolism (*5*); these findings further underscore the importance of sphingolipids in cell regulation.

Sphingosine, sphingosine-1-phosphate, and possibly other lysosphingolipids have potential roles in signal transduction (*2*, *6*). This role has been best defined, however, for sphingomyelin and ceramide. The following review focuses on recent developments in ceramide metabolism and physiology and their implications for aspects of cell biology such as apoptosis, growth suppression, and the stress response.

Generation, Kinetics, and Magnitude of the Ceramide Signal

A number of extracellular inducers of sphingomyelin hydrolysis, ceramide accumulation, or both have been identified. Among these inducers are 1,25-dihydroxyvitamin D_3, tumor necrosis factor–α (TNF-α), endotoxin, interferon-γ, interleukin-1 (IL-1), Fas ligands, CD28, dexamethasone, retinoic acid, progesterone, ionizing irradiation, chemotherapeutic agents, heat, and nerve growth factor (NGF) (*7*). Ceramide concentrations are also elevated in cells infected with human immunodeficiency virus (*8*) and in senescent fibroblasts (*9*).

The kinetics of ceramide formation in response to these inducers are complex and variable; reported responses range from seconds to hours, and the same inducer has generated very different ceramide responses in different studies. This range of findings has become a major source of confusion as to the possible relevant roles of ceramide in signal transduction and cell regulation. Figure 1 is an idealized conglomeration of the results of studies of ceramide accumulation in response to various agonists. From this scheme, at least three different categories of ceramide responses can be discerned.

Acute changes in ceramide concentration within seconds or within 1 to 2 min have been described primarily with TNF-α, ionizing radiation, and engagement of the Fas receptor (*10*). These changes may be indicative of a role for ceramide in mediating some of the very early responses to TNF-α, such as activation of nuclear factor κB (NF-κB). Such acute kinetics of ceramide are problematic for several reasons. First, the magnitude of the response has usually been modest, with increases on the order of 20 to 50% (Fig. 1). Second, these acute changes have not been detected in multiple other studies examining responses to TNF-α and

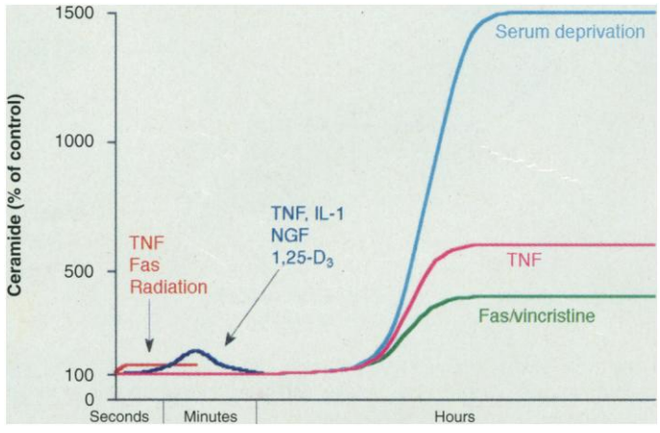

Fig. 1. Kinetics of ceramide formation. This is a composite scheme that illustrates three phases of ceramide formation (see text).

The author is in the Department of Medicine, Duke University Medical Center, Durham, NC 27710, USA.

Fas (*11*). Third, acute changes in ceramide (on the order of 50 to 100%) have been described that are caused by changes in culture conditions and exchange of media (*12*). Finally, the specificity of the ceramide effects implicated with these kinetics (for example, activation of NF-κB and Raf-1) has not been examined, which raises the distinct possibility that ceramide per se may not be the relevant lipid mediator in these responses.

Intermediate and reversible kinetics of ceramide accumulation have been best documented with IL-1, TNF-α, 1,25-dihydroxyvitamin D_3, NGF, and several neurotrophins (*13*) that cause accumulation of ceramide over 5 to 120 min in a reversible manner. The relevance of these responses—in which ceramide concentrations may double—is not readily apparent because they occur after the earliest responses to IL-1 and TNF-α, such as activation of NF-κB.

Several extracellular stimuli and agents induce prolonged and persistent accumulation of ceramide that occurs over a period of several hours (Fig. 1). Serum withdrawal in leukemia cells results in a 15-fold accumulation of ceramide over 24 to 48 hours, and TNF-α and Fas activation also induce a three- to eightfold increase in intracellular concentrations of ceramide over 12 to 24 hours (*11*, *14*). The magnitude of this response is more commensurate with cellular amounts of ceramides achieved when cells are exposed to exogenous short chain ceramides. This persistence of accumulation of ceramide raises the possibility of a reprogramming of cell function through a ceramide-activated pathway. Such a mechanism may be relevant to proposed roles for ceramide in growth suppression.

The sites of ceramide formation and the possible compartmentalization of signaling pools of sphingomyelin remain poorly understood. Sphingomyelin hydrolyzed in response to 1,25-dihydroxyvitamin D_3, NGF, or TNF-α is located in the plasma membrane (*15*), with several observations indicating localization on the inner leaflet. On the other hand, IL-1–induced sphingomyelin hydrolysis and ceramide formation colocalize with a caveolin-rich fraction. This pool of sphingomyelin resides on the outer leaflet of the plasma membrane (*16*). These studies underscore the possibility that distinct pools of sphingomyelin and ceramide exist and could participate in distinct pathways of cell regulation. No studies have examined the site of formation of the long and persistent phase of ceramide accumulation (Fig. 1).

Roles of Ceramide in Distinct Pathways of Cell Regulation

Recent discoveries have lent support to the principle that cells have intrinsic biochemical and molecular machinery that functions primarily to sense various forms of injury and insult and to execute appropriate programs of response. Mammalian cells respond to such stimuli primarily by undergoing cell cycle arrest to allow adequate time for repair of damage or by undergoing apoptosis (programmed cell death) if the damage is too severe and irreparable. It is also possible that other outcomes, such as terminal cell differentiation and senescence, represent a form of stress response.

Several lines of evidence suggest a role for ceramide in various forms of growth suppression and cell death. First, the spectrum of inducers of ceramide accumulation includes, and is perhaps limited to, most of the major inducers of apoptosis, terminal differentiation, or growth suppression. Growth factors do not appear to produce a similar response. Second, the changes in intracellular concentrations of ceramide in response to these extracellular agents precede the cellular effects of these agents on growth suppression. Third, treatment of cells with cell-permeable analogs of ceramide such as C2- and C6-ceramide has been shown to induce apoptosis, cell senescence, terminal differentiation, or cell cycle arrest in several cell types (*9*, *14*, *17*). These effects of exogenous ceramides are specific to D-*erythro*-ceramide; D-*erythro*-dihydroceramide lacks any such activity. Dihydroceramide is a naturally occurring ceramide that lacks the 4-5 trans double bond but retains the stereochemical configuration of D-*erythro*-ceramide, and its uptake and metabolism are very similar to those of D-*erythro*-ceramide (*18*). Fourth, the cellular concentrations of C2- and C6-ceramide in cells exposed to 1 to 10 μM C2- or C6-ceramide is approximately 10 to 100 pmol per nanomole of phospholipid, which is comparable to the concentrations of ceramide achieved after prolonged response to TNF-α or serum deprivation (Fig. 1). Finally, indirect manipulation of endogenous amounts of ceramide also supports a role for ceramide in mediating apoptosis and growth arrest. For example, the addition of precursor gangliosides, precursor sphingosine, bacterial sphingomyelinase (which generates ceramide at the plasma membrane), D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) (which inhibits further incorporation of ceramide into glycolipids), or D-*erythro*-2-(*N*-myristoylamino)-1-phenyl-1-propanol (D-MAPP) (which inhibits ceramide metabolism through ceramidase) results in accumulation of ceramide ranging from three to eight times the concentrations in unstimulated cells (*19*). These increased concentrations are similar to those observed in cells treated with TNF, Fas, or dexamethasone, or in cells deprived of serum. In all these cases, the addition of these agents is accompanied by apoptosis, cell cycle arrest, or both.

Ceramide, growth suppression, and cell cycle arrest. In several cell lines, ceramides and inducers of ceramide formation cause inhibition of thymidine uptake and induce a G_0/G_1 cell cycle arrest that is accompanied by early dephosphorylation of the retinoblastoma gene product (Rb) (*14*, *19*, *20*) (Fig. 2). Several lines of evidence suggest a necessary role for Rb in mediating cell cycle arrest in response to ceramide. Cells deficient in Rb (such as cell lines derived from retinoblastoma tissues) do not manifest cell cycle arrest in response to ceramide. Cells that do respond to ceramide become unresponsive when Rb is inactivated or sequestered by any of several Rb-binding proteins such as adenoviral E1a and large T of SV40. On the other hand, the absence of Rb does not appear to diminish the responsiveness of cells to the apoptotic effects of ceramide. Ceramide-independent pathways for the regulation of Rb also exist. For example, as

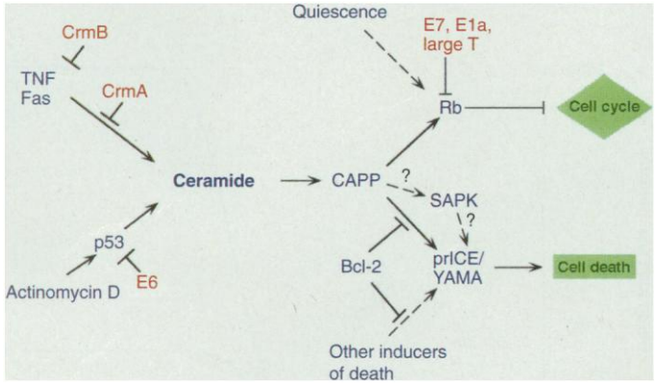

Fig. 2. Regulation of cell cycle arrest and apoptosis by ceramide. Various cytokines and extracellular agents (such as TNF and Fas) activate sphingomyelinases in a CrmA-inhibitable pathway. Other agents such as actinomycin D cause elevation of ceramide in a p53-dependent mechanism (*55*). The accumulated ceramide activates CAPP, which then can result in activation of Rb, which in turn mediates the effects of ceramide on cell cycle arrest. Alternatively, ceramide can activate proteases of the prICE/YAMA family, resulting in apoptosis. Various viral proteins (indicated in red) appear to target several key components of this pathway.

fibroblasts or MCF-7 breast cancer cells enter quiescence, Rb becomes dephosphorylated without concomitant changes in ceramide (Fig. 2). Thus, ceramide may be involved preferentially in stress-induced dephosphorylation of Rb.

In addition, ceramide suppresses the expression of the c-*myc* protooncogene by interfering with transcription elongation (*21*). Ceramide also inhibits the cellular activation of phospholipase D (*22*). Because phospholipase D and its immediate and sequential products (phosphatidic acid and DAG, respectively) are implicated as either mitogenic or viability factors, these results indicate that ceramide not only activates growth suppressor programs but may also interfere with proliferative signaling pathways.

Ceramide, proteases, Bcl-2, and the apoptotic response. In several malignant and nonmalignant cell lines, ceramide rapidly and specifically induces apoptosis while closely related lipids remain inactive (*17, 23*). Ceramide activates proteases (*24*) of the interleukin converting enzyme (ICE) family, especially prICE/YAMA/CPP32, the protease responsible for cleavage of poly(adenosine diphosphate–ribose) polymerase (PARP). Activation of prICE by ceramide and induction of apoptosis are inhibited by overexpression of Bcl-2 (*25, 26*), which suggests that Bcl-2 functions downstream of ceramide. On the other hand, overexpression of Bcl-2 does not reduce the amounts of ceramide produced in response to extracellular agents (*25*) (Fig. 2). The effects of ceramide on prICE can be dissociated from activation of Rb, and vice versa (*25*); similarly, activation of PKC antagonizes the effects of ceramide on apoptosis but not on cell cycle arrest. Therefore, ceramide may relay a stress signal, whereby the specific cellular outcome (apoptosis or cell cycle arrest) appears to be determined by additional downstream modulators (such as Rb, Bcl-2, proteases, and PKC). Indeed, in some cell lines, ceramide may actually protect from apoptosis (*27*), possibly by preferentially steering cells into cell cycle arrest.

Other effects of ceramide. Although ceramide has been reported to activate the ERK1 and ERK2 members of the mitogen-activated protein (MAP) kinase family (*28*), the balance of current evidence suggests that ceramide may be more specifically coupled to stress-activated protein kinases (SAPK) and may even inhibit ERK1 and ERK2 (*29*). Addition of exogenous ceramides or sphingomyelinase to cells induces SAPK-dependent transcriptional activity through activation of c-Jun. A dominant negative mutant of SEK1, the protein kinase that phosphorylates and activates SAPK, interferes with ceramide-induced apoptosis (*30*), which suggests that SAPK functions downstream of ceramide in the cell death pathway. The relation of SAPK to Rb and death proteases (such as prICE/CPP32) has not yet been determined.

Ceramide has an uncertain role in inducing the activity of NF-κB. TNF-α may induce activation of an acidic sphingomyelinase, and the resulting ceramide has been suggested to activate NF-κB (*31*). Exogenous ceramides have for the most part been shown not to induce nuclear translocation and activation of NF-κB (*32*). In the only studies to show such an effect, the degree of stimulation of NF-κB by ceramide was small relative to that observed with TNF-α (*33*). Also, ceramide appears not to induce transcription of NF-κB–dependent genes (*29*). Other studies have also dissociated the ceramide response from NF-κB activation (*32, 34*). For example, in cells treated with TNF-α, ceramide formation is not detected until after the induction of NF-κB. Treatment of cells with PDMP, which increases intracellular concentrations of ceramide, does not cause activation of NF-κB. Also, SR33557, an inhibitor of acid sphingomyelinase, does not inhibit TNF-induced activation of NF-κB. Finally, in Niemann-Pick fibroblast cells that lack acid sphingomyelinase, NF-κB is still activated in response to TNF-α and the effects of TNF-α on growth are preserved (*35*).

Ceramide may have a role in the regulation of protein secretion (*36*). Exogenously applied C6-ceramide accumulates in the Golgi apparatus and interferes with the "constitutive" secretion of proteins, apolipoproteins, and triacylglycerol. The macrolide brefeldin A (BFA) induces sphingomyelin hydrolysis and mimics many of the cellular effects of exogenous ceramides, and exogenous ceramides bypass resistance to BFA. The effects of ceramide on protein secretion are opposed by okadaic acid and by phorbol ester activators of PKC, which suggests that protein secretion is under opposite regulation by modulators of protein kinases and phosphatases.

Other effects of ceramides include induction of differentiation of a number of cell types, including HL-60, glioma, and neuroblastoma cells (*13, 37*). Ceramide induces the transcription of cyclooxygenases 1 and 2 (COX) and αB-crystallin, a heat shock protein (*13, 38*). Ceramides have also been reported to inhibit esterification of cholesterol and to inhibit cytochrome P450 (*39*). In frog oocytes, ceramide triggers meiotic cell cycle progression (*40*).

Exogenous ceramides also stimulate the formation of prostaglandins and the release of IL-6 (*41*). The relative potency of various forms of ceramide in inducing these proinflammatory effects does not match that for the apoptotic effects (for example, D-*threo*-ceramide, which is an unnatural analog, is the most potent ceramide in inducing IL-6 secretion). This raises the possibility that molecules other than D-*erythro*-ceramide are the physiologic mediators of these effects.

Mechanisms of Ceramide Action

Biologically relevant and direct targets of ceramide should be activated by ceramide in vitro, and they should mediate the most proximal effects of ceramide in cells. There are three potential candidates for direct targets of ceramide action.

Ceramide-activated protein phosphatase (CAPP). In vitro, ceramide activates a serine-threonine protein phosphatase. This phosphatase is related to the PP2A family of phosphatases because it copurifies with the heterotrimeric form of PP2A and is inhibited potently by okadaic acid in vitro (*42*). It is also activated by ceramides with various N-linked acyl groups, but is not activated by other sphingolipids or neutral lipids. Studies in *S. cerevisiae* demonstrate that yeast CAPP is composed of the catalytic subunit encoded by *SIT4* and the regulatory subunits encoded by *CDC55* and *TPD3* (*43*).

Several lines of evidence suggest a role for CAPP in mediating at least some of the cellular activities of ceramide (*21, 42–44*). First, some of ceramide's effects on cells (such as apoptosis and down-regulation of the c-*myc* protooncogene) are inhibited by low concentrations of okadaic acid. Second, CAPP is activated in vitro by ceramide but not by dihydroceramide, which is inactive in eliciting ceramide effects on cells. Third, studies in *S. cerevisiae* show that yeast cells deficient in the various subunits of CAPP become resistant to the effects of ceramide. Further studies will be required to determine the relevant physiologic substrates for CAPP.

PKC ζ. Ceramide induces phosphorylation of PKC ζ in cells, and it activates the enzyme in vitro (*45*).

Ceramide-activated protein kinase (CAPK). CAPK is a membrane-associated kinase with a substrate specificity for serine or threonine in proximity to proline (*46*). Treatment of cells with sphingosine (but not C2-ceramide) results in a unique phosphorylation of Thr^{669} on the epidermal growth factor (EGF) receptor, and C8-ceramide mimics the effects of sphingosine and EGF (*47*). In vitro, ceramide does not activate this kinase in a partially purified preparation, which raises the possibility that this kinase is not directly regulated by ceramide (*48*).

Regulation of Ceramide Formation

There are several possible sources of ceramide that accumulates in response to ex-

tracellular stimuli and agents of injury.

Sphingomyelinases. Current studies point to sphingomyelin as the major precursor for ceramide and to sphingomyelinase as the major enzyme responsible for ceramide generation. At least three distinct sphingomyelinases are implicated in distinct pathways. In HL-60 cells, 1,25-dihydroxyvitamin D_3 causes the activation of a Mg^{2+}-independent sphingomyelinase, which is active at neutral pH. This enzyme has been purified, and its cytosolic localization and Mg^{2+} independence distinguish it from the Mg^{2+}-dependent membrane sphingomyelinase (*49*).

A Mg^{2+}-dependent membrane neutral sphingomyelinase is activated in response to TNF-α or serum deprivation (*14*). The temporal profile of its activation coincides with the major and delayed phase of ceramide accumulation, which suggests that it may participate in mediating the apoptotic and antiproliferative activities of TNF-α, Fas, 1-β-D-arabinofuranosylcytosine (Ara C), and other inducers of apoptosis. In HL-60 cells, arachidonate causes accumulation of ceramide and activation of the Mg^{2+}-dependent sphingomyelinase in cell-free extracts (*50*). Because TNF-α activates phospholipase A2 (*51*), these studies may indicate that arachidonate (or one of its many products) is coupled to activation of neutral sphingomyelinase.

In addition, proteases are implicated in a pathway leading from TNF-α to the activation of sphingomyelinase (*14, 52*). Overexpression of CrmA (a viral protein that functions as a protease inhibitor with high affinity to ICE and related proteases) inhibits ceramide formation in response to TNF-α and protects cells from the cytotoxic action of TNF-α but not from that of ceramide. Reaper, a *Drosophila* gene product that causes cell death, results in ceramide formation that is abrogated by pharmacological inhibitors of ICE-like proteases. Acid sphingomyelinase has been proposed as a mediator of some of the activities of TNF-α, especially in the regulation of NF-κB (*31*) as discussed in the previous section.

De novo synthesis. The de novo synthesis of ceramide is stimulated by retinoic acid, by the exchange of medium in tissue culture, and by the chemotherapeutic agent daunorubicin (*12, 53*). However, daunorubicin induces a biphasic response in sphingomyelin hydrolysis and ceramide generation, and fumonisin B, an inhibitor of ceramide synthase, does not inhibit ceramide formation or apoptosis in response to daunorubicin (*54*).

Function of Ceramide as a Biostat

Unlike adenosine 3′,5′-monophosphate (cyclic AMP), IP_3, phosphatidylinositol-3,4,5-trisphosphate (PIP_3), and many of the eicosanoids, ceramide and DAG do not function solely in signal transduction. They are critical components in the intermediary metabolism of sphingolipids and glycerophospholipids. Also, the most pronounced changes in the amounts of ceramide and DAG occur over longer time frames than those seen with cyclic AMP, IP_3, or PIP_3. Therefore, ceramide may function more as a component of a "biostat" that measures and initiates responses to cellular stress, much as a thermostat measures and regulates temperature. For example, cellular concentrations of ceramide are increased by systemic stress (such as caused by TNF) or cell injury (such as from heat or chemotherapeutic agents). The cell then responds to these changes by undergoing apoptosis or growth arrest.

This concept of lipid biostats also offers a solution to the paradoxical dual function of ceramide and DAG as intermediary metabolites as well as second messengers and bioeffector molecules. Multiple enzymes are capable of regulating ceramide concentrations through distinct metabolic pathways (Fig. 3), and these individual enzymes may be activated by distinct stresses or extracellular agents. These enzymes may then serve to integrate the effects of several stimuli as a consequence of the regulation of ceramide concentrations. The ceramide concentrations would then reflect the effects of several stimuli and would serve as a gauge of the overall amount of stress or injury to which the cell has been exposed.

Studies of ceramide regulation and function, and more general studies of apoptosis and growth suppression, are beginning to define regulated pathways that ultimately function to deal with systemic as well as cellular stress and injury that may lead to cell cycle arrest, terminal differentiation, senescence, or apoptosis. Additional tools, such as inhibitors of specific enzymes of ceramide generation, are required to substantiate the hypotheses raised here. Understanding of these pathways may provide a basis for development of therapies to control cancer and inflammation.

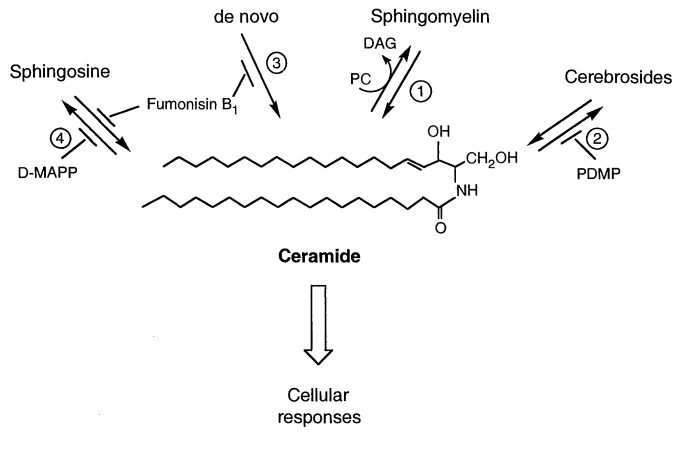

Fig. 3. Proposed role for ceramide and ceramide metabolism in the regulation of growth suppression. Ceramide plays a key role in sphingolipid metabolism, both as a key intermediate in sphingolipid metabolism and as a penultimate product in sphingolipid degradation. Various enzymes such as sphingomyelinases (1), de novo pathways of ceramide formation (3), cerebroside synthase (2), and ceramidase (4) may contribute to the regulation of ceramide concentrations. Therefore, it is proposed that various enzymes involved in ceramide metabolism can be potentially regulated in response to distinct classes of agents. The common effect of these pathways would be the accumulation of ceramide, which would then function as a biostat and launch various aspects of ceramide-mediated biology such as cell cycle arrest, cell senescence, or apoptosis.

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RESEARCH ARTICLE

Structure of Staphylococcal α-Hemolysin, a Heptameric Transmembrane Pore

Langzhou Song,* Michael R. Hobaugh,* Christopher Shustak, Stephen Cheley, Hagan Bayley, J. Eric Gouaux†

The structure of the *Staphylococcus aureus* α-hemolysin pore has been determined to 1.9 Å resolution. Contained within the mushroom-shaped homo-oligomeric heptamer is a solvent-filled channel, 100 Å in length, that runs along the sevenfold axis and ranges from 14 Å to 46 Å in diameter. The lytic, transmembrane domain comprises the lower half of a 14-strand antiparallel β barrel, to which each protomer contributes two β strands, each 65 Å long. The interior of the β barrel is primarily hydrophilic, and the exterior has a hydrophobic belt 28 Å wide. The structure proves the heptameric subunit stoichiometry of the α-hemolysin oligomer, shows that a glycine-rich and solvent-exposed region of a water-soluble protein can self-assemble to form a transmembrane pore of defined structure, and provides insight into the principles of membrane interaction and transport activity of β barrel pore-forming toxins.

The α-hemolysin (αHL) of the human pathogen *Staphylococcus aureus* is secreted as a 33.2-kD water-soluble monomer that binds to rabbit erythrocytes and human platelets, erythrocytes, monocytes, lymphocytes, and endothelial cells (*1*). Membrane-bound monomers assemble to form 232.4-kD heptameric transmembrane pores (*2*). The sensitivity of cells to αHL ranges from human erythrocytes, which require solution