Chapter 5

Tales and Mysteries of the Enigmatic Sphingomyelin Synthase Family

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Abstract

In the last five years tremendous progress has been made toward the understanding of the mechanisms that govern sphingomyelin (SM) synthesis in animal cells. In line with the complexity of most biological processes, also in the case of SM biosynthesis, the more we learn the more enigmatic and finely tuned the system appears. Therefore with this review we aim first, at highlighting the most significant discoveries that advanced our knowledge and understanding of SM biosynthesis, starting from the discovery of SM to the identification of the enzymes responsible for its production; and second, at discussing old and new riddles that such discoveries pose to current investigators.

Sphingomyelin Biosynthesis: An Historical Perspective

Initial Milestones

Sphingomyelin (SM) was first isolated by the German biochemist Thudichum in 1884 and its name derived from both the enigmatic and novel nature of its chemical structure (in the Greek mythology the sphinx is a monster that posed a riddle) and the tissue where it was isolated from (myelin).[1] In spite of the initial suggestion that SM might have a specific role in neural function, later studies showed that SM is present in all mammalian tissues as well as lipoproteins. SM is indeed one of the most abundant phospholipids and, in cells, it forms a concentration gradient along the secretory pathway with the highest concentration in the plasma membrane (where it accumulates in the exoplasmic leaflet).

SM is composed of a ceramide module and a phosphocholine (P-choline) moiety bound to the primary hydroxyl group (Fig. 1). It was first proposed by Sribney and Kennedy[2] that CDP-choline was the donor of the P-choline headgroup of SM, similarly to the reaction that leads to the biosynthesis of phasphatidylcholine(PC), known as the "Kennedy pathway".[3] In their experimental conditions, it was found that ceramide with the nonnatural threo configuration was a better substrate as compared to the naturally occurring erythro. In 1965, an alternative reaction for SM biosynthesis was proposed by Roscoe Brady and colleagues.[4] In this case, evidence was provided to show that, in rat brain preparations, the synthesis of SM could also occur from acylation of lyso-SM by stearoyl-CoA and later found that both erythro or threo-lyso-SM were equally active as lipid acceptors in the reaction.[5] Shortly after, Fujino et al[6] demonstrated that, by twitching the protocol for the preparation of the incubation mixture, also erythro- as well as threo-ceramide was recognized as acceptor of the P-choline group from CDP-choline to form SM according to the Kennedy reaction, even if these results were later questioned.[7] In 1972 a novel reaction for the synthesis of SM was proposed

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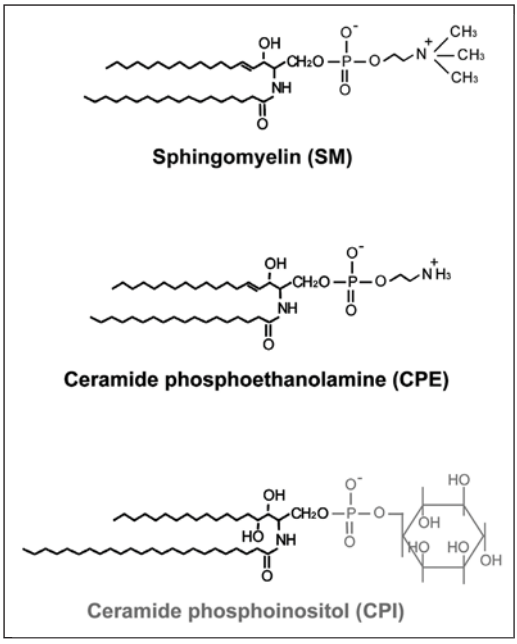

Figure 1. Schematic of the biochemical structures of SM, CPE and CPI. In black is represented the basic ceramide module common to the three molecules and in color is represented each specific head-group. The ceramide module of CPI is more often characterized by long and very long chain fatty acid and the presence of an hydroxy group in position 4 of the sphingoid backbone. A color version of this image is available at www.landesbioscience.com/curie.

by Diringer et al which identified PC as the donor of the P-choline, by using pulse-chase labeling techniques with ^{32}P-labeled phosphorus and ^{3}H-choline and looking at the kinetics by which the radioactivity associated with PC or SM was changing in growing SV40 transformed mouse fibroblasts.[8] Finally, in 1974 Ullman and Radin conclusively demonstrated in vitro that PC is indeed the donor of the P-choline for synthesis of SM in mouse liver preparations.[9] They found that natural erythro-ceramide was the preferred substrate, that the reaction did not need addition of cations and that it was characterized by an optimal neutral pH. In addition to liver, the reaction occurred in preparations from kidney, lung, spleen and heart but it was strikingly absent in brain, suggesting the existence of an alternative route for SM synthesis in this organ. Ever since, the scientific community settled on PC as the donor of the P-choline moiety of SM, with few exceptions,[10] such that SMS is also referred to as the phosphatidylcholine:ceramide cholinephosphotransferase (Fig. 2).

Localization of SM Synthase Activity in Cells

In the early studies addressing the biochemical characterization of the P-choline donor for SMS activity, the source of the enzyme was predominantly microsomal preparations, thus no major information on the subcellular localization of SMS activity was provided, except for the fact that it was tightly membrane-bound. Later on it was suggested that, in mouse SV40-transformed fibroblasts, rat liver, or 3T3-L1 fibroblasts most of the activity resided in the plasma membrane.[11-13] SMS activity at the Golgi was later detected also in the same mouse SV40-transformed fibroblasts and in Chinese hamster lung fibroblasts and epithelial Madin-Darby canine kidney cells.[14-17] In order to clarify the issue of subcellular localization of SM synthesis, a thorough study was performed in 1990 by Futerman and coworkers using the well-characterized protocol for subcellular fractionation of rat liver and by paying particular attention to inhibit the activity of the SM-metabolizing enzyme

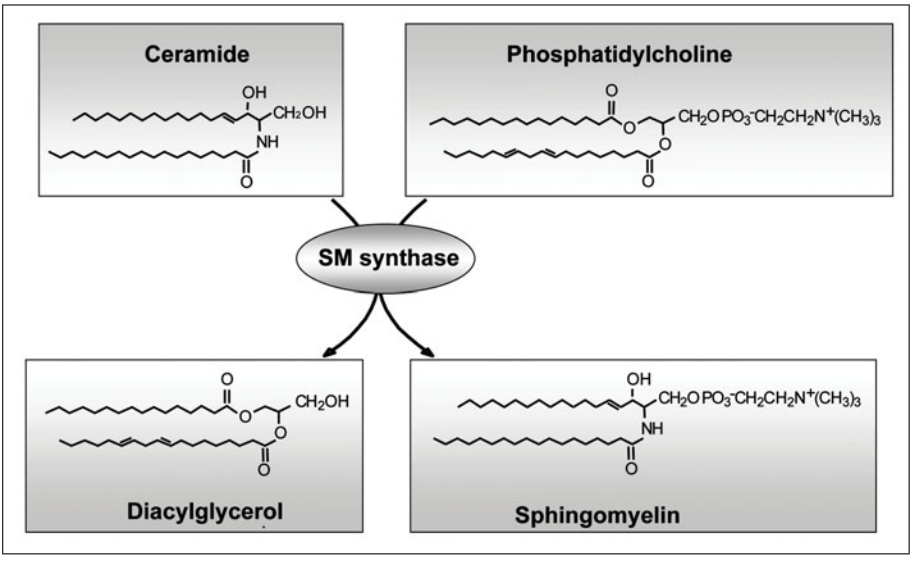

Figure 2. Schematic of the biochemical reaction catalyzed by SM synthase.

neutral sphingomyelinase by addition of metal chelators.[18] In this study it was shown that most of the SMS activity resided in the cis-medial enriched Golgi (75%) (with a luminal orientation of the catalytic site) and minor activity was detected in the trans enriched Golgi (15%) and plasma membrane (~10%) fractions. Similarly, Jeckel and coworkers confirmed enrichment of the SMS in enriched cis Golgi apparatus from rat liver.[19,20] Confirmation of the Golgi as the main site of SM biosynthesis came also from studies using inhibitors of vesicular trafficking (low temperatures or pharmacological agents). In this case, no appearance of newly synthesized SM on the plasma membrane could be detected, suggesting that the main site for SM synthesis resided in the cell and that vesicular trafficking was required for SM delivery to the plasma membrane.[16,17] To complicate matters, the idea that, in BHK cells, SM destined to the plasma membrane is synthesized in recycling endosomes was also put forward[21-23] but later unequivocally disproved.[24] Also, SMS activity seemed absent from an intermediate ER-Golgi compartment (ERGIC) and, also in the Vero cells employed in this study, it was mostly enriched in the Golgi fraction.[25] In 1998, the use of preparative free-solution isotachophoresis (FS-ITP) allowed separation of enzymatically active Golgi subfractions from rat liver isolated Golgi apparatus.[26] SMS activity was concentrated in the cis/medial fractions where the specific activity was the highest and where the ceramide was mostly concentrated. On the other hand, newly synthesized SM seemed to be transferred to the trans Golgi fractions where SM mass was most abundant. Shortly after, the question whether significant SMS activity could be present in the trans Golgi network (TGN) was raised by Allan and coworkers[27] using BHK cells infected with Semiliki Forest virus. It was postulated that this viral infection promoted incorporation of viral proteins into the TGN causing a significant increase in the density of these membranes. It was shown that, after infection, the shifted peak, which most likely contained trans Golgi network membranes, carried most of the SMS activity and it was enriched in SM whereas the profile of glucosylceramide synthase activity remained unchanged. The confusion about the sub-cellular localization of SMS within the Golgi apparatus found partial resolution when a study by Sadeghlar et al[28] showed that different cells might present a different distribution of SMS activity along this compartment. In fact, in primary neurons and neuroblastoma cells SM synthesis seemed to be primarily localized in late compartments of the Golgi apparatus, probably the TGN whereas in fibroblasts most of the activity resided in the early/mid Golgi. Finally, in rat

Sertoli cells most of SMS activity was found associated with the trans-Golgi cisternae and only a minor fraction was present in the early Golgi and plasma membrane.

Discovery of a Ceramide Transfer Protein with a Key Role in SM Biosynthesis

Additional clues on the localization and mechanism of SM synthesis came more recently with the identification of a ceramide transfer protein (CERT). In an attempt to identify the enzyme(s) responsible for SM biosynthesis, Hanada and coworkers set up a screen for CHO mutant cells searching for clones resistant to lysenin, a SM-directed cytolysin.[29] One of the mutant clones, LY-A showed a defect in SM biosynthesis without any appreciable impairment in the SMS enzymatic activity per se or in the synthesis of the precursors of the reaction, namely ceramide and PC.[30] By exploiting the hypersensitivity of LY-A cells to agents that extract cholesterol such as methyl-β-cyclodextrin (MCD), the authors searched for cDNAs that were able to revert the MCD-sensitivity and restore SM biosynthesis. This approach yielded a cDNA coding for CERT. Two splice variants of CERT exist, a long less represented variant, identical to the Goodpasture antigen-binding protein (GPBP) and a shorter more represented variant, missing 26 amino acids and identical to GPBPΔ26.[31] Both were found to mediate intermembrane transfer of ceramide.[30] CERT is characterized by three different domains: (i) a FFAT motif which favors CERT approximation to the ER, the site of ceramide synthesis, through the binding to the ER resident membrane protein vamp associated protein (VAP)[32]; (ii) a pleckstrin homology (PH) domain that binds to phosphatidylinositol-4-monophosphate (PI4P) and targets CERT to the Golgi/trans Golgi network[30]; (iii) a START domain that facilitates the extraction of ceramide from a donor membrane and its subsequent transfer to an acceptor membrane.[33]

The nonvesicular transfer of ceramide from the ER to the Golgi requires ATP.[34,35] The ATP-dependent step for ceramide trafficking might be attributable to the synthesis of PI4P in the Golgi. Alternatively, ATP may allow the recycling of CERT after its binding to PI4P or VAP or it could energize an ATP-dependent translocase that moves ceramide from the cytosolic side of the Golgi to the luminal side where the catalytic domain of SMS is located.[36] The spatial organization of the CERT-mediated transfer of ceramide between the ER and Golgi is not clear. The over-expression of a green fluorescent protein (GFP) tagged-CERT in CHO cells showed its localization at the cis/medial Golgi apparatus pointing to the early Golgi as the site for ceramide transfer.[30] Interestingly though, ER-trans Golgi contact sites exist[37,38] and therefore it is plausible that the transfer of ceramide from the ER to the Golgi could also occur at these sites of contact. This would also imply that a pool of SMS should reside at the trans Golgi.

Even though CERT regulates a main route for the transport of ceramide from the ER to the Golgi, it has been recently demonstrated that, in glioma cells, an additional vescicular mechanism of ceramide transport, sensitive to nitric oxide, is active alongside the CERT-mediated transport.[39] Whether the ceramide transported through this route can reach alternative pools of SMS in the Golgi apparatus (i.e., early Golgi) is a fascinating still open question.

Alternative Pathways of SM Biosynthesis and Analogous Reactions

The observation that brain, in spite of containing large amounts of SM, lacked any obvious SMS activity led to speculations on the existence of an alternative pathway of SM biosynthesis.[9] A transferase reaction similar to the one catalyzed by SMS was described in which phosphatidylethanolamine (PE) rather than PC is used as head group donor, yielding the SM analogue ceramide phosphoethanolamine (CPE)[40] (Fig. 2). Subsequent stepwise methylation reactions would then add three additional methyl groups to the phosphoethanolamine head group, producing SM. Indeed Malgat et al provided evidence for the existence of such a pathway in brain and liver microsomes and plasma membranes.[41] Interestingly, it was shown that in addition to CPE, both tissues were also able to synthesize SM when endogenous PC (and not dipalmitoylcholine) was utilized as substrate and the active site of CPE synthase was oriented versus the luminal side of the membrane bilayer.[42] CPE synthase activity was also observed in ram spermatozoa, mice synaptic vesicles and sciatic nerve.[43,44] Although the existence of CPE synthase has been proven, its contribution to SM formation or the function of CPE itself are still unresolved questions.

Another complex sphingolipid analogue of SM is the ceramide phosphoinositol (CPI). CPI is found in yeast and plants where no SM is present and the reaction leading to its synthesis is virtually identical to the one catalyzed by SMS, except that the head group donor molecule is phosphatydylinositol (PI) instead of PC.[45,46] Importantly, neither can mammalian SMS catalyze the CPI synthase reaction nor can CPI synthase produce SM. On the other hand, some parasitic protozoa have the ability to synthesize CPI, SM and CPE, depending on the phase of the life cycle.[47] Indeed, it has been recently demonstrated that during the procyclic phase of *Trypanosoma brucei* both CPI and SM can be detected whereas during the bloodstream stage, SM and CPE are produced instead by the *TbSLS1-4* gene product. This raises the question of whether, even in mammalian cells, a single transferase can catalyze the production of both SM and CPE.

Physicochemical Properties of SM

SM comprises a heterogeneous group of molecules because of the different fatty acyl chains linked with amide bond to the sphingoid backbone and for the nature of the sphingoid backbone itself.[48] The large majority of SM carries saturated fatty acids (ranging from C14 to C26 in length) and sphingosine as the sphingoid backbone. Alternatively, dihydrosphingosine (sphinganine) or 4-hydroxysphinganine (phytosphingosine) could account for the sphingoid backbone and unsaturated and/or branched fatty acids could also be found.[49,50]

Even though structurally similar, SM and phosphatidylcholine (PC) differ significantly for a number of features that determine the higher melting temperature of the first. In fact, the predominant presence of saturated fatty acids and the potential intermolecular hydrogen binding, via the amide bond and the free hydroxyl group at position 3, favor a high-density packing of SM, which increases the compactness and impermeability of the membrane.[51-53] According to the umbrella model,[54] the preferential mixing of sterols with SM is caused by shielding of the nonpolar sterol molecule by the phosphocholine head group of SM. Collectively these physicochemical features are believed to influence the lateral organization of cellular membranes. The lipid raft hypothesis postulates the existence of SM/sterol-enriched microdomains in the plasma membrane that serve as dynamic platforms for the clustering of membrane proteins with a role in signal transduction, membrane trafficking and cell adhesion.[55]

The Multigenic Sphingomyelin Synthase (SMS) Family

SMS Cloning Strategies

Initial studies indicated that mammalian SM synthases are membrane-bound enzymes that readily lose activity following solubilization with detergent.[12] This feature complicated their identification by classical biochemical approaches. Purification of a soluble SM synthase released by *Pseudomonas aeruginosa*[56] provided no clues on the identity of the mammalian enzyme. Efforts to isolate SM synthase mutants by screening CHO cells for resistance to a SM-directed cytolysin led to the identification of CERT (see above).[29,30]

A complementary approach was based on structural information available for the enzyme catalyzing synthesis of CPI in yeast. CPI production requires the product of the *AUR1* gene,[45] a protein containing the C2 and C3 active site motifs characteristic for members of the lipid phosphate phosphatase (LPP) superfamily.[57] A database search for novel sequences encoding integral membrane proteins containing the C2 and C3 domains common to Aur1p and LPPs identified three families of candidate SM synthase (*CSS*) genes with homologues in multiple animal species[58] (Fig. 3). Several members of each family were cloned and analyzed for their ability to mediate SM synthesis upon heterologous expression in yeast. One family, the CSS3 family, was found to contain multiple members with SM synthase activity. A detailed analysis of two human members, SMS1 and SMS2, revealed that they met all criteria previously assigned to mammalian SM synthase. First, their expression proved sufficient to support SM synthesis in yeast, an organism lacking endogenous SM synthase activity. Second, both proteins function as bidirectional lipid cholinephosphotransferases capable of converting PC and ceramide into SM and diacylglycerol (DAG) and vice versa. Third, the proteins reside in cellular organelles where SM synthesis is known to occur, namely in

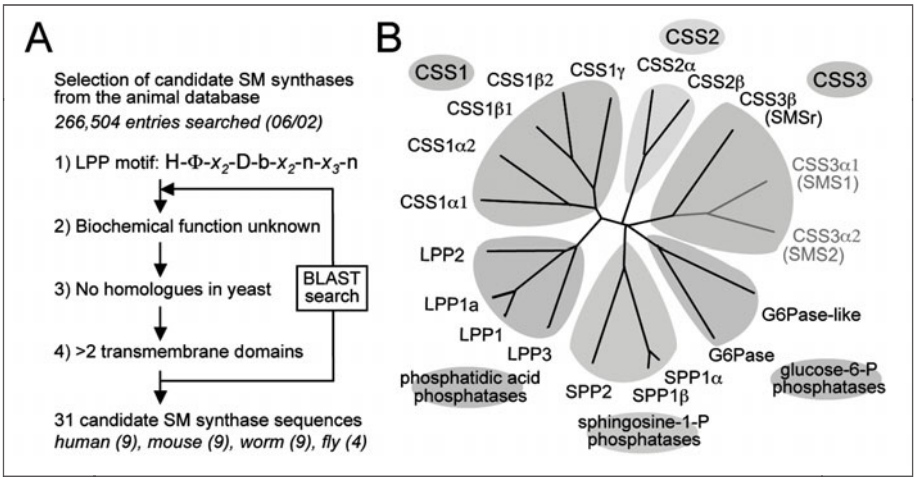

Figure 3. Selection and phylogenetic analysis of candidate SM synthases. A) Animal entries in SwissProt/TrEMBL were searched for the presence of a sequence motif shared by LPPs and Aur1p proteins and then further selected on the basis of three additional criteria, as indicated. B) Phylogenetic tree of human candidate SM synthases (CSS) and previously characterized members of the human LPP superfamily. Figure reproduced from Huitema et al, 2004.

the trans-Golgi (SMS1 and SMS2) and plasma membrane (SMS2). Finally, SMS1 and SMS2 share sequence motifs containing putative active site residues with Aur1p and LPPs that are facing the exoplasmic leaflet, the side of the membrane where SM synthesis is known to occur.

Taking advantage of a mouse lymphoid cell line with strongly reduced SM synthase activity and SM levels, a subsequent study reported the expression cloning of *SMS1* as a gene able to restore SM levels in these cells.[59] Due to a decreased SM content of the plasma membrane, these lymphoid cells are highly susceptible to the toxic effects of methyl-β-cyclodextrin (MβCD), a compound used to deplete cholesterol from cellular membranes. *SMS1* was recovered from a human cDNA expression library on the basis of its ability to restore MβCD resistance. Importantly, this work showed that SMS1 corresponds to a major SM synthase activity in mammalian cells with a critical role in sustaining cell growth (see also below).

Interestingly, at the time of the identification of SMS1 as a major SMS, *SMS1* was already annotated as Hmob33 (human medulla oblongata 33) or MOB, a gene with a predominant brain expression mapping on chromosome 10.[60] Structural and functional analysis of its transcripts predicted regulation at both transcriptional and translational level.[60,61] Subsequently, several alternative spliced products of *SMS1* were found both in human cerebellum and in the mouse[62] suggesting complex regulation for *SMS1* expression.

Structural Organization and Reaction Chemistry of SMS Family Members

Members of the SMS family share a common membrane topology with LPPs.[57,58] Both groups of enzymes contain a six times membrane-spanning core domain with the termini facing the cytosol and the putative C2 and C3 active site residues facing the exoplasmic leaflet (Fig. 4). The active site includes a catalytic triad of histidine and aspartate residues previously implicated in LPP-mediated hydrolysis of lipid phosphate esters.[63] Thus, it is likely that SMS family members utilize a reaction chemistry similar to that described for LPPs to catalyze the choline phosphotransferase reaction. This is predicted to occur via a two-step process, involving: (i) a nucleophilic attack on the lipid-phosphate ester bond in PC by the histidine in C3 assisted by the conserved aspartate in this motif, resulting in formation of a choline phosphohistidine intermediate and the release of

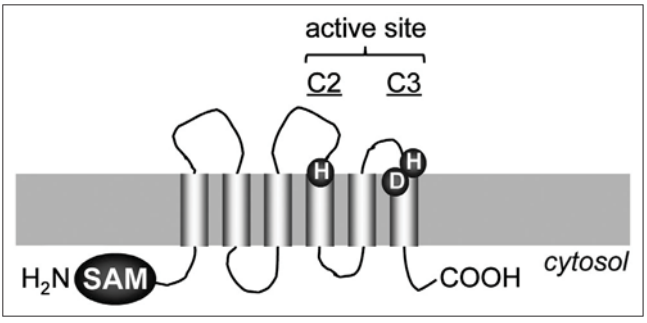

Figure 4. Predicted membrane topology of SMS family members. Putative active site residues in LPP sequence motifs C2 and C3 are highlighted in red and the sterile alpha motif or SAM domain in blue. A color version of this image is available at www.landesbioscience.com/curie.

DAG; (ii) a nucleophilic attack on the choline phosphohistidine intermediate by the oxygen of the ceramide hydroxyl group assisted by the histidine in C2, resulting in transfer to the sphingoid base and the release of SM. Consistent with this model, mutation of one of the histidine or aspartate residues that make up the catalytic triad is sufficient to abolish sphingomyelin synthase activity of SMS1 and SMS2 without affecting their subcellular distribution.[64]

A remarkable difference between the two SMS isoforms is that SMS1, but not SMS2, contains a N-terminal Sterile Alpha Motif or SAM domain.[58,60] SAM domains have been shown to homo- and hetero-oligomerize, forming multiple self-association architectures. They can also associate with various non-SAM domain-containing proteins and appear to possess the ability to bind RNA.[65] The function of the SAM domain in SMS1 is still unknown. Fusion of this domain to the N-terminus of SMS2 does not affect the subcellular distribution of SMS2, nor does its removal from SMS1 lead to redistribution of that enzyme.[64] Hence, it appears that the SAM domain is not involved in targeting SMS1 to the Golgi apparatus.

SMS Family Members Display Striking Variations in Substrate Specificity

The SMS family displays a multiplicity of SMS genes in essentially all organisms generating SM.[58,66] In addition to SMS1 and SMS2 genes, the mammalian genome contains a third, SMS-related (SMSr) gene that is highly conserved, from humans and worms to the fruit fly, *Drosophila melanogaster*. Interestingly, *Drosophila* lacks SMS1 and SMS2 homologues and does not synthesize SM. Instead, this organism produces the SM analogue CPE as a major membrane constituent.[67,68] Mammals also produce small amounts of CPE. Two CPE synthase activities have been described in mammalian cells, one associated with the ER and the other one associated with the plasma membrane.[41,42] As PE serves as the headgroup donor for both activities, the enzymes involved can be classified as PE:ceramide ethanolaminephosphotransferases. Consequently, SM and CPE biosynthesis in mammals share common reaction chemistry. As SMS1, SMS2 and SMSr are structurally related and share the C2 and C3 active site residues, SMSr provides an attractive candidate for the elusive CPE synthase.[58] Indeed, we recently demonstrated that *Drosophila* and human SMSr proteins catalyze EPC biosynthesis and, contrary to SMS1 and SMS2, localize to the ER.[69] SMSr thus qualifies as the ER-resident CPE synthase originally described by Malgat et al.[41,42]

Remarkably, we have recently found that SMSr produces only trace amounts of CPE and that bulk production of CPE in *Drosophila* S2 cells requires a different enzyme. This second, insect-specific CPE synthase uses CDP-ethanolamine instead of PE as headgroup donor,[69] analogous to the ethanolaminephosphotransferases of the Kennedy pathway.[70] This implies that, contrary to SM synthesis in mammals, bulk production of CPE in insects occurs in the cytosolic leaflet of the membrane. As insects require CERT for efficient CPE production,[68] the CDP-ethanolamine-dependent CPE synthase likely resides in the Golgi apparatus. Its identity remains to be established.

Since the plasma membrane-associated CPE synthase activity in mammalian cells shares the same subcellular distribution as SMS2, we recently reinvestigated the substrate specificities of all three mammalian SMS family members. This revealed that, while SMS1 and SMSr are monofunctional SM and CPE synthases, respectively, SMS2 is a bifunctional enzyme producing both SM and CPE.[71] Thus, SMS2 likely accounts for the plasma membrane-resident CPE synthase described previously.[41,42] These recent findings demonstrate an unexpected diversity in substrate specificity among mammalian SMS family members. Interestingly, characterization of a trypanosome sphingolipid synthase family that is orthologous to the *Leishmania* CPI synthase revealed an enzyme with dual SM and EPC synthase activity.[47]

Differential Expression of SMS1 and SMS2

Whereas SMS1 and SMS2 are expressed to a similar level in most human and murine tissues,[58,62] they seem to be differentially expressed in various cell lines. Interestingly, expression of both SMS1 and SMS2 has been observed in all adherent cell lines analyzed so far (breast cancer MCF-7 cells, cervical cancer HeLa cells, hepatocellular carcinoma HepG2 cells, colon cancer $CaCo_2$ cells, human lung fibroblasts, human epithelial HEK-293 cells, mouse melanoma MEB4, mouse fibroblasts) whereas expression of SMS2 seems to be very low or absent in the majority of suspension cell lines (S49, WR19L/Fas, Ramos and U937 lymphoma cells, HL-60, Molt-4 and K562 leukemia cells and primary human B, T and monocytic cells)[58,62,72-74] and Luberto, unpublished observations). Since SMS2 is in part localized at the plasma membrane, with its catalytic site oriented toward the outside of the cell and it is expressed mainly in adherent cells, it may serve a specialized function in cell-cell or cell to matrix interactions.

Cellular Functions of SMS Family Members

SMS1 and SMS2 as Regulators of SM Homeostasis and Receptor-Mediated Signaling

The accumulation of SM in the exoplasmic leaflet of the plasma membrane together with its high-density packing and affinity for cholesterol (see above) implies a vital role in the barrier function of the plasma membrane.[49-53] Recent evidence has further enriched this model supporting a role for SM in the homeostasis of lipid microdomains at the plasma membrane, often sites of receptor-mediated signaling. Taking advantage of the identification of SMS1 as bona fide SM synthase, the role of SM in the activation of Fas signaling was investigated by Miyaji et al.[75] Comparing SMS1 deficient cells (with over expressed Fas receptor) with cells in which expression of SMS1 was restored, the authors provide evidence for a critical role of SM in Fas-mediated signaling by enabling the formation of the death-inducing signaling complex (DISC) and consequently allowing caspase activation and production of ceramide at the plasma membrane. Subsequently, the involvement of SMS1 in the maintenance of raft homeostasis has been postulated in S49 mouse lymphoma cells.[73] A variant S49 cell line resistant to apoptosis induced by alkyl-lysophospholipids (ALP) turned out to be deficient in SM synthesis due to down-regulation of SMS1 expression. Since ALP appears to be internalized via raft-dependent endocytosis, it was suggested that SM depletion perturbs the internalization of ALP in the resistant S49 variant. Indeed, down-regulation of SMS1 in the parental S49 cells mimicked the resistant phenotype observed in the S49 variant cell line, i.e., a diminished raft-dependent uptake of ALP and ALP-induced apoptosis.

More recently, involvement of SMS2 in the maintenance of SM levels in plasma membrane lipid microdomains was determined in HEK 293 cells.[72] In these cells, it was shown that siRNA-mediated down-regulation of either SMS1 or SMS2 induced a significant reduction of SM content in detergent-resistant fractions. Confirmation of the involvement of SMS2 in the physiological maintenance of plasma membrane SM has come from studies using macrophages from SMS2 knock out (KO) mice.[76] Macrophages from SMS2 KO mice showed diminished recruitment of the Toll Like Receptor 4-MD2 complex on the cell surface in response to lypopolysaccharide (LPS) treatment, consistent with the reduction of LPS-mediated apoptosis

observed in THP-1-derived macrophages after siRNA-mediated down-regulation of SMS1 or SMS2.[77] Likewise, siRNA-mediated down-regulation of SMS2 in HEK 293 cells partly inhibited recruitment of the tumor necrosis factor (TNF) α receptor 1 to detergent-resistant microdomains in response to stimulation with TNF, decreasing TNF-mediated down-stream signaling. These observations complement the fact that over-expression of SMS1 or SMS2 in Chinese hamster ovary (CHO) cells increased the number of detergent-insoluble microdomains and TNF-induced apoptosis.[77] In both the SMS2 KO macrophages and HEK 293 cells, NF-κB activation was one of the down-stream signaling events that were inhibited upon loss or down-regulation of SMS2, thus supporting an early report in which a link between stimulation of SM synthesis and nuclear translocation of NF-κB was established.[78] SMS1 has been also implicated in raft-dependent activation of T-cell receptor in Jurkat cells.[79] By using Jurkat cells stably expressing shRNA targeting human SMS1, the authors established a cell line with impaired SM synthesis and levels in the plasma membrane. Stimulation of T-cell activation with CD3 revealed impaired expression of CD69 (early marker of leukocyte activation), adhesion, proliferation and TCR clustering and translocation to lipid rafts.

Finally, regulation of cellular SM has been observed upon modulation of either SMS1 or SMS2 in HeLa cells.[74,80] Over-expression of either SMS1 or SMS2 resulted in a net increment of basal SM mass.[74] Moreover, expression of either SMS1 or SMS2 favored comparable SM resynthesis after hydrolysis of plasma membrane SM induced by treatment with bacterial sphingomyelinase.[74] Since in HeLa cells, SMS1 localizes exclusively at the Golgi and SMS2 localizes at the Golgi and plasma membrane, these observations indicate that the unique plasma membrane localization of SMS2 did not specifically facilitate SM resynthesis from plasma membrane derived-ceramide, thus suggesting that the pool of SMS2 in the Golgi may be of significant activity. In support of this conclusion, over-expression of either SMS1 or SMS2 in HeLa cells comparably increased de novo SM biosynthesis, known to occur in the Golgi,[74] whereas down-regulation of either one significantly inhibited it in HeLa,[74,80] Huh,[72] HEK 293 cells and macrophages isolated from KO mice.[76] In HeLa cells down-regulation of either SMS1 or SMS2 inhibited TNF-mediated NF-κB activation by altering plasma membrane receptor activation, similarly to HEK 293 cells (Luberto and Marimuthu, unpublished observations).

SMS1 and SMS2 as Regulators of Lipid-Based Signaling

Given the enzymatic activity of SMS and the biological relevance of its substrates and products of the reaction, similarly to what discussed for SM, significant effort has been directed at determining the contribution of SMS1 and SMS2 to the homeostasis of ceramide and DAG levels in mammalian cells and their role in the cellular functions mediated by these bioactive lipids. Modulation of SMS1 and/or SMS2 expression has been achieved by gene over-expression or by their down-regulation using silencing RNA or by *SMS2* gene KO.

The effect of over expression of SMS1 on ceramide levels was first reported in Jurkat cells after stable transfection.[81] In these cells, enhanced SMS1 levels caused a general increase of sphingolipids in resting cells. On the other hand, over expression of SMS1 prevented accumulation of ceramide and dihydroceramides following photodamage, as compared to vector control cells, even though no SM accumulation could be observed. In this system over expression of SMS1 prevented apoptosis associated to photodamage. On the other hand, in wild type Jurkat cells, siRNA-mediated down regulation of SMS1 enhanced accumulation of ceramides, dihydroceramides and sphingosine following photodamage, with a concomitant enhancement of apoptosis.[82] Similarly, increased apoptosis was also observed after photodamage when SMS2 was down-regulated. A comparable correlation between SMS1 expression and cell death was observed in yeast.[83] In fact mouse *SMS1* was identified as a gene able to rescue yeast cells from cell death induced by expression of the pro-apoptotic Bcl-2 family member, Bax and other cytotoxic stimuli such as hydrogen peroxide, osmotic stress and elevated temperature. Since expression of *SMS1* would favor growth of yeast cells in the presence of either short-chain ceramide analogues or phytosphingosine and a mouse splice variant of *SMS1* missing the key catalytic residues did not rescue bax-induced cytotoxicity,[62] it was

speculated that the SMS1-mediated effect was due to metabolism of stress-inducing intermediates of sphingolipid metabolism, even though no lipid analysis was reported in these studies.

Similar to Jurkat cells,[81] over-expression of SMS1 or SMS2 in CHO cells caused an increase of ceramide levels.[77] An increase of ceramide levels was also observed upon down-regulation of either SMS1 or SMS2 in HeLa and Huh cells,[66,72,74] suggesting that changes in ceramide levels due to modulation of SMSs might be differently regulated depending on the specific cellular context.

Whereas the levels of PC do not seem to change following modulation of SMSs,[66,72,74,77] altering the expression of SMS1 or SMS2 exerts diverse responses on DAG levels. In CHO cells over-expression of SMS1 or SMS2 induced a significant accumulation of total DAG.[77] On the other hand, in HeLa cells down-regulation of either SMS1 or SMS2 did not alter total DAG levels[66,74] and in Huh7, SMSs down-regulation caused a tendency to decrease that was not significant.[72] Therefore, also in the case of DAG as for ceramide, cells react differently to modulation of DAG levels by SMS activity and that may have to do with the bioactivity of these lipids. Importantly, even though HeLa cells did not show a change in total DAG levels, a local decrease of DAG could be probed at the level of the Golgi when SMSs were down-regulated and SM synthesis stimulated.[74] These observations suggest that first, SMSs at the Golgi are able to produce DAG and that in resting cells, there are in place systems that effectively prevent large oscillations of DAG due to alteration of SMS activities. When the equilibrium of the system is tilted by enhanced availability of ceramide for SMS1 or SMS2 in the Golgi, then the metabolism of DAG produced in this organelle is not adequate and DAG might accumulate in this compartment. As also eloquently discussed by Richard Pagano,[84] a number of metabolic pathways could hypothetically regulate the level of SMS-produced DAG at the Golgi: (1) DAG produced at the Golgi could recycle back to the endoplasmic reticulum where it is utilized for a new round of PC biosynthesis; (2) DAG could be utilized in the Golgi itself to produce a new molecule of PC; and (3) DAG could be readily degraded through the action of cytoplasmic lipases or metabolized into other molecules, such as phosphatydic acid. Interestingly, stimulation of sphingolipid synthesis in HeLa cells led to translocation of the DAG-binding protein protein kinase D (PKD) to the Golgi, possibly through a SMS-mediated mechanism.[85] Indeed, with the identification of SMSs, it was later shown that down-regulation of SMS1 or SMS2 inhibited translocation of PKD to the Golgi induced by stimulation of SM synthesis, thus reinforcing the notion that if accumulated, the DAG produced by SMSs at the Golgi is biologically functional.[74] Since PKD is involved in regulation of the secretory pathway from the trans Golgi network, these observations may implicate SMS-derived DAG as potential regulator of the budding of secretory vesicles from the Golgi apparatus to the plasma membrane.

Conclusion

Over the last few years a wealth of novel insight into the dynamics of SM biosynthesis has emerged and yet a large number of still unresolved questions are ahead of us. For example, the enzymes responsible for SM biosynthesis in mammals belong to a conserved protein family whose members display striking differences in substrate specificity. This SMS family contains both single and dual activity enzymes, with SMS1 being a monofunctional SM synthase, SMSr a monofunctional CPE synthase and SMS2 a bifunctional enzyme with both SM and CPE synthase activity. These results, combined with the observation that each enzyme mainly resides in a different organelle along the secretory pathway, establishes an unexpected level of complexity in the organization of sphingolipid biosynthesis in mammals. What is the significance of SMSr-mediated CPE biosynthesis in the ER? Does this enzyme play a role in controlling ceramide-induced stress pathways that originate from the ER? The relative extent of cell surface-associated SM and CPE synthase activity mediated by the bifunctional enzyme SMS2 will be controlled, at least in part, by the availability of its substrates PC and PE. In healthy mammalian cells, PE is largely confined to the cytosolic leaflet of the PM whereas PC in the exoplasmic leaflet should be plentiful. However, when PM lipid asymmetry is dissipated, for example during apoptosis, the higher proportion of PE in the exoplasmic leaflet would stimulate SMS2-mediated CPE production. This raises the intriguing possibility that transbilayer lipid arrangement may have a direct impact on the activity

of SMS2 as a negative regulator of ceramide signaling at the PM. Moreover, SMS1 and a pool of SMS2 are both localized to the Golgi.[58,66,72,74,76] Why is this organelle equipped with two different SMS enzymes? What is the relevance of the reverse activity of SMS enzymes? Does it really take place in cells? Is the activity of SMS enzymes merely regulated by the reciprocal local concentrations of their substrates and products or are there other levels of regulation? And what is the fate and function of DAG molecules produced by either SMS1 or SMS2?

Several reports have described a complex regulation of SMS activity and SM, ceramide and DAG in the nucleus.[86-88] SMS activity has been detected in the nuclear envelope and associated with the chromatin.[89] Interestingly though, it seems that these two activities might have opposite regulation during proliferation and apoptosis. In fact, during proliferation, chromatin SMS decreases whereas the one localized in the nuclear envelope increases, while an opposite profile has been observed during apoptosis.[90,91] The changes in chromatin-associated SMS might regulate accessibility of DNA for transcription by regulation of DNA associated SM, similarly to what observed in the case of double stranded RNA.[92] Surprisingly, neither SMS1 nor SMS2 localize to the nucleus and so the identities of the nuclear SMS activities remain to be established.

Finally, it has been proposed that SMS activity might be responsible for the activity of the elusive PC-specific phospholipase C (PC-PLC), an enzyme potentially involved in the regulation of DAG but still of unknown identity.[78] Whether SMS1 or SMS2 are also responsible for carrying out the reaction proposed for PC-PLC, perhaps under specific conditions such as poor ceramide availability, remains to be established. In the near future, we hope to find answers to some of these questions.

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