The ins and outs of sphingolipid synthesis

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Sphingolipids are ubiquitous components of eukaryotic cell membranes, where they play important roles in intracellular signaling and in membrane structure. Even though the biochemical pathway of sphingolipid synthesis and its compartmentalization between the endoplasmic reticulum and Golgi apparatus have been known for many years, the molecular identity of the enzymes in this pathway has only recently been elucidated. Here, we summarize progress in the identification and characterization of the enzymes, the transport of ceramide from the endoplasmic reticulum to the Golgi apparatus, and discuss how regulating the synthesis of sphingolipids might impact upon their functions.

Introduction
The past few years have seen an upsurge of interest in sphingolipids (SLs) – membrane lipids containing a ceramide backbone – resulting in a vast increase in our understanding of the roles that they play in signaling events and in membrane lipid rafts. In addition, there has been remarkable progress recently in identifying the enzymes involved in the de novo synthesis of SLs, and a more-or-less comprehensive picture of the route taken by a SL can now be delineated from initiation of its biosynthesis on the cytosolic leaftet of the endoplasmic reticulum (ER), through its transport from the ER to the Golgi apparatus, and its metabolism in the Golgi apparatus.

Previous reviews have focused on a particular enzyme [1], transport step [2,3], or a particular aspect of SL function [4,5], whereas the current review presents an overview of all steps of SL synthesis and transport through the early compartments of the secretory pathway. To this end, we systematically discuss the enzymes of the SL synthetic pathway (Figure 1), what is known about the topology of the reactions, and the mechanisms of transport of ceramide from the ER to the Golgi apparatus.

Sphingolipid structure, synthesis and transport
SLs consist of three main structural elements (see insert of Figure 2). The basic building block of a SL is the sphingoid long-chain base (lcB), normally sphingosine, sphinganine (dihydrosphingosine) or 4-hydroxysphinganine (phytosphingosine). A fatty acid is attached to carbon-2 (C-2) of the lcB via an amide bond, yielding ceramide, and attachment of hydrophilic head groups to the OH-group at C-1 yields complex SLs. The head group can be a sugar, in the case of glycosphingolipids (GSLs), or phosphorylcholine in the case of sphingomyelin (SM). At least five different lcBs are known in mammalian cells, >20 species of fatty acid (varying in chain length, degree of saturation, and degree of hydroxylation) can be attached to the lcB, and hundreds of different carbohydrate structures have been described in GSLs. The possible relevance of this complexity is discussed elsewhere [6], but a central question in SL cell biology is how the synthesis of these different SL species is regulated, and how changing their levels, through regulation of de novo synthesis, might impact upon the various cellular events in which SLs play key roles.

Unlike synthesis of the other major membrane lipids, sterols and glycerolipids, which takes place mainly in the ER, the initial steps of SL synthesis take place in the ER but later steps take place for the most part in the Golgi apparatus; moreover, specific steps of SL synthesis occur on opposite surfaces of these organelles (lumenal versus cytosolic). Of the 11 enzymes in the SL biosynthetic pathway discussed below, eight are located in the ER and three in the Golgi apparatus; of these, the active site of most appears to face the cytoplasm, but some face the lumen. This implies significant transbilayer and interbilayer movement of SLs during the biosynthetic process, and, as these lipids do not appear, on the whole, to spontaneously undergo such movements [2], facilitated mechanisms are probably involved in their transport. The reasons for these complex topological relationships are not entirely clear, although, in some cases, such as SM and GSLs, synthesis in the Golgi lumen can be rationalized by the topological equivalence of the lumenal leaftet of the Golgi apparatus with the outer (extracellular) leaftet of the plasma membrane, where SM and GSLs mainly reside. By contrast, the reason that glucosylceramide (GlcCer) is synthesized on the cytosolic leaftet of the Golgi apparatus, whereas all other GSLs are synthesized on the lumenal leaftet of the ER or Golgi apparatus, is less easy to rationalize.

We now discuss each of the SL biosynthetic enzymes in their order of action during SL trafficking along the biosynthetic pathway. A relatively simple biochemical overview of SL biosynthesis in given in Figure 1, and another level of complexity is introduced in Figure 2, in which the location and topology of each of these reactions in the ER and Golgi apparatus, together with the transport mechanisms of SLs, is shown.

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Serine palmitoyl transferase (SPT) (Reaction i)

SL synthesis begins with the conversion of serine and fatty acyl CoA into 3-ketosphinganine, CoA and CO₂ by serine palmitoyl transferase (SPT) (Figure 1). The predominant fatty acid used in mammals and yeast is palmitoyl CoA, but stearoyl CoA can also be used. SPT belongs to a family of pyridoxal 5'-phosphate-dependent α-oxoamine synthases, and is composed of a heterodimer of two similar, but non-identical, subunits, named Lcb1p (long chain base 1) and Lcb2p, which were first identified in yeast by genetic methods [7,8].

Eukaryotic SPTs are membrane bound, with the active site facing the cytoplasm (Figure 2). Studies of the mammalian enzyme originally suggested a single-pass transmembrane protein, with the C-terminus in the cytoplasm [9], but these data are not inconsistent with recent studies in yeast suggesting that the enzyme actually traverses the membrane three times [10], as also predicted for an Arabidopsis plant SPT [11]. These data suggest that the SPT1 and SPT2 subunits of the SPT heterodimer might interact in the cytosol, as well as within the membrane and/or the lumen of the ER [10].

In addition to Lcb1p and Lcb2p, another small protein, Tsc3p, binds to yeast SPT and is required for optimal enzyme activity and for cell growth at 37°C [12]. The growth requirement could be due to a need for increased sphingolipid synthesis at the higher temperature. However Tsc3p is not indispensable for SPT activity, and dominant mutants in LCB2 have been identified that eliminate the need for TSC3 [13], suggesting that Tsc3p is a regulator of SPT. No similar subunit has been detected thus far in other cell types.

3-Ketosphinganine reductase (3KSR) (Reaction ii)

3-Ketosphinganine reductase (3KSR) reduces 3-ketosphinganine to dihydroceramide in an NADPH-dependent manner. Yeast 3KSR is predicted to have two transmembrane domains near the C-terminus, but the human ortholog, follicular lymphoma variant translocation-1 (FVT-1), has an additional transmembrane domain near the N-terminus [14]. The catalytic site is sensitive to proteases in intact microsomes and thus probably exposed to the cytosol [15]. Thus, no topological problems exist for the first two enzymes of the pathway as SPT uses soluble precursors to form 3-ketosphinganine on the cytoplasmic leaflet of the ER, and the active site of 3KSR also faces the cytosol.

Sphingosine kinase (SK) (Reaction iii)

Subsequent to its formation by 3KSR, sphinganine can either be acylated by dihydroceramide synthase (DH CerS, see Reaction vi below), or phosphorylated by sphingosine kinase (SK) to form sphingosine 1-phosphate (S1P), an important bioactive lipid mediator [16]. SKs were originally identified in yeast as products of two genes, LCB4 and LCB5, required for the efficient utilization of exogenously added sphingoid bases [17]. Lcb4p is principally required for this process and is found on the cytoplasmic face of internal membranes, including the ER, Golgi and, probably, endosomes [18,19]. Two mammalian isoforms, SK1 and SK2, have been identified that differ in their substrate specificity and possibly also in other functional aspects [20]. SK1 seems to be a soluble enzyme that translocates to membranes upon interaction with phosphatidic acid, which binds near to the C-terminus of the enzyme [21]. SK2 has been reported to have a nuclear targeting signal, although the relevance of this is currently unknown [22].

S1P phosphatase (SPP) (Reaction iv) and S1P lyase (SPL) (Reaction v)

Once formed in the cytoplasm, S1P can be degraded by either of two enzymes, S1P phosphatase (SPP), which removes the phosphate head group from sphinganine, or S1P lyase (SPL), which yields the cleavage products, hexadecanal and ethanolamine phosphate, the major exit
route of metabolites from the SL biosynthetic pathway. Similar to many other components of this pathway, two SPP isozymes, which both contain a typical lipid phosphatase motif, were initially identified in yeast and, more recently, in mammals [16]. The major yeast SPP, Lcb3p, is an ER integral membrane protein that probably spans the membrane eight or nine times, with its active site proposed to be on the luminal side of the ER [23]. Gene fusions with invertase were used to determine the topology of the enzyme and, although the data seem to be internally consistent, it is impossible to know whether the topology predictions are entirely accurate because the methodology used leads to the loss of enzyme activity. The active site of SPL, which is also an integral ER membrane protein, faces the cytoplasm [24].

**Dihydroceramide synthase (DHCerS) (Reaction vi)**

Dihydroceramide synthase (DHCerS) acylates sphinganine to form dihydroceramide, but can also acylate sphingosine to ceramide; sphinganine is produced through the biosynthetic pathway, and sphingosine mainly through SL degradation [25]. In contrast to yeast, whose SLs contain only one kind of fatty acid; mammalian (dihydro)ceramides contain a wide spectrum of fatty acids [6,25], and it was formerly assumed that this was due to a lack of specificity of DHCerS with respect to the use of fatty-acyl CoAs. However, recent studies have shown that this is not the case. Rather, there is a family of mammalian DHCerS genes, and each displays a remarkably high specificity in utilizing acyl CoAs (Figure 3).

Clues to the identity of DHCerS were originally obtained in yeast, in which it was shown that the two proteins Lag1p and Lac1p were required for the synthesis of the very long-chain (C26) ceramides found in yeast [26,27]. Subsequent to this, tomato (asc1) [28] and mammalian [29] homologs were identified, some of which contain a Hox domain (Figure 3), a transcription factor involved in developmental regulation; although there is currently no evidence to support this possibility, it would be surprising if the Hox domain did not play an important role in the regulation of this enzyme.

**Figure 2.** The sites and topology of sphingolipid (SL) metabolism in the endoplasmic reticulum (ER) and Golgi apparatus. Enzymes, transporters and steps of SL synthesis or transport are abbreviated as in the text and numbered as in Figure 1: SPT, serine palmitoyl transferase; 3KSR, 3-ketosphinganine reductase; SK, sphingosine kinase; SPP, sphingosine 1-phosphate phosphatase; SPL, sphingosine 1-phosphate lyase; DHCerS, dihydroceramide synthase; DHCD, dihydroceramide desaturase; GaIT, UDP-galactosylceramide galactosyltransferase; CERT, ceramide transfer protein; SMG, SM synthase; GCS, GlcCer synthase; GClflp, GlcCer flippase; GaIT1, LacCer synthase. FAH and ACS1p are not shown. Solid arrows indicate enzymatic reactions, and dashed arrows indicate transport steps. The topologies of the reactions, where known, are shown in green circles, and steps for which the topology is not known, or the protein not unambiguously identified, as green rectangles. The number of transmembrane domains of integral membrane proteins is shown when it has been determined or predicted, together with the putative cytosolic or luminal loops, but is not to scale. Likewise, the C- and N-termini of enzymes for which the topology has been established are indicated, and not shown for those for which the topology is still unclear. The insert shows the structure of ceramide, and is color-coded according to the SLs shown in the scheme: Blue, sphingoid long chain base; thin blue line, 3-OH (as in 3-ketosphinganine); black, fatty acid; white line, 4,5-double bond in sphingoid base; black circle and line, sugar head group (i.e. glucose, galactose, or GlcGal in the case of LacCer); purple rectangle, phosphorylcholine (as in SM); P, phosphate. Further details regarding each step are given in the text.
role in one or other aspect of the regulation of SL synthesis, perhaps similar to the role of the sterol regulatory element binding protein (SREBP), in which a transcriptional activation domain is released by proteolysis when sterol levels are low [30]. Quite unexpectedly, overexpression of one of the homologs, upstream of growth and differentiation factor 1 (UO1G1), increased the amount of dihydroceramide containing only one type of fatty acid, namely stearic acid [29]. Other family members were then identified [30, 31] recently renamed as LASS genes (Longevity assurance gene homologs) (Figure 3), and overexpression of two of these [32] also led to an increased amount of dihydroceramide synthesis, with a distinct fatty acid profile. Thus, to date, three of the six mammalian LASS genes have been characterized, and each synthesizes dihydroceramide containing distinct fatty acids (Figure 3). When some of these genes were expressed in yeast defective in LAG1 and LAC1, they were able to reconstitute ceramide synthesis [33].

Ceramide synthase was recently purified in active form from yeast, and a new subunit of the enzyme, Lip1p, was identified [34]. Lip1p is a single-span ER membrane protein required for ceramide synthesis activity in vivo and in vitro. No mammalian homolog of Lip1p has been found, and it is not known whether mammalian LASS homologs require additional subunits. Although (dihydro) ceramide synthesis has been assumed to occur on the cytosolic surface of the ER [2], based on protease protection experiments [15], the Lip1p regions required for (dihydro) ceramide synthesis are localized within the ER membrane or lumen. Topology predictions suggest that Lag1p and the LASS proteins span the ER membrane multiple (seven or eight) times, but the topology of the catalytic site of DHCRs has not been unambiguously identified.

Interestingly, sphinganine can be phosphorylated by SK on the cytoplasmic leaflet (Reaction iii), and, when exogenously added sphinganine is fed to cells or incubated in vitro with ER membranes in yeast, it is phosphorylated by SK (Lcb4p) [18, 35] before its acylation, and then dephosphorylated by SPP (Lcb3p), presumably on the luminal side of the ER membrane, as discussed above. Therefore, as endogenous sphinganine is produced on the cytosolic face and is acylated without being first metabolized to SPP, either DHCR5 can utilize sphinganine pools generated on either side of the ER membrane or the hypothesis for the topology of the SPP is incorrect. The rationale for the phosphorylation/dephosphorylation of exogenously added sphinganine is uncertain, but it is probably used to differentiate sphinganine arising from different pools, presumably for regulation of SL synthesis or signaling.

**Fatty acid 2-hydroxylase (FAH) and acyl-CoA-binding protein (Acb1p)**

Two other proteins involved in fatty acid supply and modification are important for SL synthesis, at least in yeast. In yeast, most SLs are hydroxylated on the 2 position of the fatty acid by a process requiring the ceramide 2-hydroxylase gene (FAH1). The protein encoded by this gene, Fah1p, has a consensus desaturase/hydroxylase motif and a cytochrome b5 domain, and membrane topology predictions suggest that these motifs are on the cytoplasmic side of the ER membrane. A human ortholog encodes a 2-hydroxylase that can work on free fatty acids, probably of various chain lengths, whereas the yeast enzyme appears to work on fatty acids only once they are attached to ceramide [36]. The essential fatty acyl CoA binding protein, Acb1p, is also required for efficient ceramide synthesis in yeast [37], and the mammalian Acb1p homolog is essential for the viability of mammalian tissue-culture cells [38]. Acb1p is most likely essential for efficient delivery or presentation of very long-chain fatty acids to the ceramide synthase in yeast.

**Dihydroceramide desaturase (DHCD)**

The enzyme responsible for the formation of the 4,5-double bond of the sphingoid lcb, dihydroceramide desaturase (DHCD), was identified by a bioinformatics approach [39], and its identity was subsequently confirmed by expression studies. Protease digestion studies and its being part of a cytochrome b5 electron transport system suggest that the active site of this enzyme faces the cytosol [40], which is consistent with topology predictions implying three transmembrane domains [41, 42].

**UDP-galactose:ceramide galactosyltransferase (CGalT)**

Once formed in the ER, ceramide can be removed from the ER by active transport mechanisms (see below), or metabolized to galactosylceramide (GalCer). The enzyme responsible for this reaction, UDP-galactose:ceramide galactosyltransferase (CGalT), is located in the ER, and is a type 1 membrane protein, with the N-terminus and active site in the lumen [43]. The UDP-galactose transporter, which is required for GalCer synthesis, might form a molecular complex with CGalT [44].

**SM synthase (SMS)**

Sphingomyelin synthase (SMS) transfers the phosphorylcholine head group from phosphatidylcholine to ceramide,
to yield SM and diacylglycerol. A major advance over the past year was the molecular identification of SMS. Based on a functional cloning strategy in yeast [45], and an expression cloning strategy in WR19L cells [46], two SM synthases were identified, which have different locations; SMS1 is in the Golgi apparatus and SMS2 on the PM. The active site of SMS1 faces the lumen of the Golgi apparatus, and topology predictions suggest that this protein spans the membrane four [46] or six [45] times, with the N- and C-terminus facing the cytosol.

**GlcCer synthase (GCS) (Reaction x)**
Glucosylceramide synthase (GCS) was first identified in 1996 [47], and subsequent work demonstrated that it is a type III membrane protein, with the C-terminus located in the lumen of the Golgi apparatus. Its active site faces the cytosol, which is consistent with biochemical analyses performed before the enzyme was cloned [1]. Drosophila [48] and plant [49] homologs of GCS have also been identified. Expression of the Drosophila homolog in GM95 cells (mammalian cells deficient in GCS) restores GlcCer synthesis and the synthesis of downstream complex GSLs, but the Drosophila enzyme is surprisingly localized to both the Golgi and the ER [48].

For both SMS and GCS, ceramide must be transported from the ER to the Golgi apparatus. One of the most exciting developments in this field over the past couple of years was the identification of a protein, CERT, which mediates the transport of ceramide from the ER to the Golgi apparatus in a non-vesicular manner [50]. CERT is a cytoplasmic protein with a phosphatidylinositol-4-monophosphate-binding domain and a putative domain for catalysing lipid transfer. This protein specifically extracts ceramide from phospholipid bilayers and shows very little activity towards other related SLs or glycerolipids. CERT also extracts dihydroceramide but displays a relatively restricted fatty acid specificity, efficiently mediating the transfer of ceramides containing C14–C20 fatty acids but is less efficient towards ceramide containing longer-chain fatty acids [51]. CERT is involved in the delivery of ceramide for SM synthesis [52], but not for GlcCer synthesis, which seems to depend on the supply of ceramide through a distinct ATP- or cytosol-independent (or less dependent) pathway [53]. Thus, at least two mechanisms exist for delivering ceramide from the ER to the Golgi apparatus.

Once delivered to the Golgi apparatus, ceramide needs to be translocated to the lumen for SM synthesis. Studies with ceramide analogs suggest that its rate of spontaneous transbilayer movement is significantly quicker than that of structurally related lipids [2], but a direct measurement of the rate of transbilayer movement of natural, long-acyl-chain ceramide is not available. However, recent studies have demonstrated that the asymmetric addition of ceramide to one side of a lipid bilayer promotes transbilayer movement [54,55]. These observations are likely to result in re-evaluation of traditional views concerning lipid bilayer stability and imply that changes in the lipid composition itself, without the need for membrane proteins, might be sufficient to drive lipid transbilayer movement.

Although spontaneous transbilayer movement or ‘scrambling’ might be sufficient to account for ceramide flip-flop, it is definitely not sufficient to account for GlcCer translocation. GCS is unique among glycosyltransferases in that its active site faces the cytosol, and thus GlcCer must be translocated across the Golgi membrane for subsequent metabolism to lactosylceramide (LacCer) by GalT1 (Reaction xi), which is found on the luminal leaflet of the Golgi apparatus [56,57]. Biophysical studies demonstrate a rapid, protease-sensitive transbilayer movement of GlcCer in rat liver ER and Golgi membranes, whereas transbilayer movement is much slower at the PM [58]. This movement occurs in both directions (i.e. from the cytosolic leaflet to the luminal leaflet, and vice versa), implying that GlcCer exists with a symmetric distribution in the Golgi apparatus. As no significant transbilayer movement of LacCer occurs [58], the metabolism of GlcCer to LacCer on the luminal leaflet would act to trap LacCer on this surface and make it available for subsequent glycosylation steps.

There is some controversy about the molecular identification of the flippase responsible for transbilayer movement of GlcCer [59]. It has been suggested that the multiple drug resistance pump, MDR1, is the GlcCer flippase in the Golgi apparatus, but other proteins might also be involved. However, inhibition of MDR1 by cyclosporin A inhibits LacCer and globotriaosyl ceramide synthesis, but not ganglioside synthesis [59]. This suggests that neutral and acid GSLs are synthesized from distinct precursor GSL pools. Nothing is currently known about putative distinct GlcCer pools in the Golgi apparatus.

**Moving forward**
The molecular identification of most of the components in the SL synthetic pathway is a major achievement and illustrates that the description of a biochemical pathway by itself (as in Figure 1) is not sufficient to understand, or even to ask the correct questions, about how a pathway is regulated; for this, the cellular context must also be known (as in Figure 2). With these tools in hand, it is now possible to turn to questions that, to date, have been inaccessible. Central among these is why the location and topology of SL synthesis is so complex, with different reactions occurring in either the ER or Golgi apparatus, and with distinct topologies. One way to address this would be to alter the location of specific enzymes, and then determine the effect, first, on levels of SL synthesis, and then on downstream events in which they are involved. For instance, what would happen if SMS was targeted to the ER or if DHCerS was targeted to the Golgi apparatus? In the case of the latter, how would the activities of SMS and GCS be altered by alleviating the need for ceramide transport through either CERT or vesicular transport? Would DHCerS be accessible to the same pool(s) of sphinganine if it were located in the Golgi apparatus? How would this impact upon the supply of sphinganine for SK? Although answers to this type of question would not by themselves provide an unambiguous answer to the question of why the cellular pathways of SL synthesis are so complex, it would allow us to begin to unravel the relationships between the different enzymes, and the
relationships between different substrate pools, a required first step in addressing this question.

Does the description of the location and topology of the enzymes in the GSL biosynthetic pathway also provide clues as to how this pathway is regulated? Intuitively, the answer to this question is 'yes', but, when pressed for specifics, it becomes harder to provide firm answers as to how regulation might be achieved. Clearly, multiple levels of regulation exist, which would not be the case were the enzymes all located in the same compartment and with the same topology. For instance, altering the activity of CERT would directly impact upon SM synthesis and would therefore change the balance between GSL and SM levels. Likewise, altering the activity of the GlcCer flippase, or of ceramide transbilayer movement, would have major consequences for their supply to upstream enzymes. Little experimental evidence exists at present for these modes of regulation, but it seems inevitable that discoveries concerning the regulation of each step are just around the corner, particularly in the light of recent studies showing that de novo synthesis of SLs, particularly ceramide, is regulated in various signaling paradigms. One candidate for this kind of regulation is DHcerS, which is the only enzyme in which multiple isoforms (the LASS genes) exist (Figure 3); this presumably indicates that the type of fatty acid added to the sphingoid long-chain base is of great importance in the intracellular signaling pathways in which ceramide is involved, and evidence is slowing accumulating that this is indeed the case [6]. Different LASS genes display different tissue distributions, and the presence of a Hox domain in five of the mammalian homologs potentially provides an important regulatory mechanism.

Concluding remarks

In summary, the molecular identification of the enzymes involved in SL synthesis allows both the formulation of relevant questions, mainly concerning regulation, and will also provide the tools to answer them. The availability of yeast mutants and the possibility of up- or down-regulating expression of mammalian homologs of the enzymes will permit new approaches to study the function of SLs. Thus, this is the beginning of an exciting new era in the study and discovery of the intricacies of SL function.

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