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Biochimica et Biophysica Acta 1486 (2000) 145–170

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Review

Sphingolipid transport in eukaryotic cells

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Received 27 October 1999; received in revised form 9 November 1999; accepted 9 November 1999

Abstract

Sphingolipids constitute a sizeable fraction of the membrane lipids in all eukaryotes and are indispensable for eukaryotic life. First of all, the involvement of sphingolipids in organizing the lateral domain structure of membranes appears essential for processes like protein sorting and membrane signaling. In addition, recognition events between complex glycosphingolipids and glycoproteins are thought to be required for tissue differentiation in higher eukaryotes and for other specific cell interactions. Finally, upon certain stimuli like stress or receptor activation, sphingolipids give rise to a variety of second messengers with effects on cellular homeostasis. All sphingolipid actions are governed by their local concentration. The intricate control of their intracellular topology by the proteins responsible for their synthesis, hydrolysis and intracellular transport is the topic of this review.

Keywords: Sphingomyelin; Inositolsphingolipid; Glycosphingolipid; Glycolipid; Translocation; Animal cell; Yeast

1. Introduction

Eukaryotic cells contain multiple membrane-bound compartments whose specialized functions require distinct protein and lipid compositions. Assembly of the correct mixture of proteins and lipids into each compartment requires a three-step process involving (1) the biosynthesis of proteins and lipids, (2) selective transport of proteins and lipids across the compartmental bilayer (intracompartmental transport), and (3) selective transport of proteins and lipids between different compartments (intercompartmental transport; see Fig. 1). Experimental work over the last 15 years has brought us detailed knowledge concerning the molecular mechanisms responsible for intra- and intercompartmental protein transport. In contrast, our insight into how lipids are sorted and moved within cells is far more limited. This is partially due to the fact that the trafficking rules that apply to lipids are less absolute than those for proteins, and therefore more difficult to translate into molecular terms. On the other hand, it is becoming increasingly clear that directional transport of at least some protein and lipid species is controlled by intricate sorting machineries. A striking example are the sphingolipid–cholesterol microdomains, called

Abbreviations: Cer, ceramide; CGlcT, ceramide glucosyltransferase; GalCer, galactosylceramide; GalNAc, *N*-acetylgalactosamine; GlcCer, glucosylceramide; GPI, glycosylphosphatidylinositol; IPC, inositolphosphoceramide; MIPC, mannosylinositolphosphoceramide; $M(IP)_2C$, inositolphospho-mannosylinositolphosphoceramide; SM, sphingomyelin; TGN, *trans*-Golgi network; Glycosphingolipid designation according to the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature [1], using the Svennerholm abbreviations for gangliosides and using sulfatide to indicate HSO_3-3 Gal β1-1 Cer

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1388-1981/00/$ – see front matter
PII: S1388-1981(00)00054-8

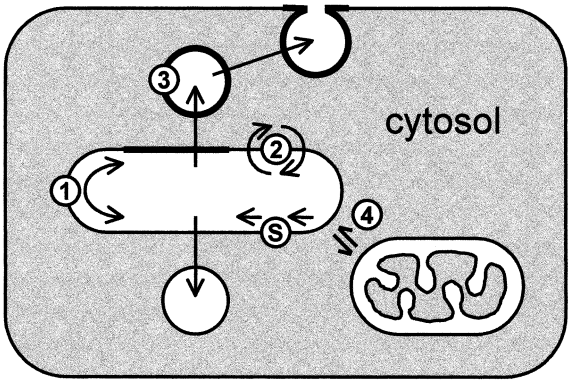

Fig. 1. Mechanisms of lipid transport. Membrane lipids diffuse laterally (lateral diffusion: 1) and transversely (transbilayer translocation: 2) in the membrane of each compartment, in this case for example the TGN (S: synthesis). Since lipids form the membrane matrix they are an integral part of vesicular carriers (vesicular traffic: 3) to, for example, the plasma membrane. Sorting along this pathway involves local changes in surface density at the sites of vesicle budding. At the same time, lipid molecules may exchange as monomers between the cytosolic membrane surfaces of all cellular compartments (monomeric transfer: 4). The latter process is especially relevant for organelles that are not connected by vesicular traffic like mitochondria and peroxisomes. Processes 1 and 2 are intracompartmental, 3 and 4 intercompartmental. The differences in lipid composition between organelles and between the two bilayer leaflets require specificity in the various transport processes. The question is, what molecular interactions are responsible for the specificity?

rafts, that have initially been proposed to function as sorting devices for protein and lipid transport from the Golgi to the apical cell surface in polarized epithelial cells [2]. Since then, an increasing number of cell biological and biochemical studies has indicated that rafts (1) are not only employed by polarized-epithelial cells, but also by many other cell types, including lower eukaryotes such as yeast, (2) occur at diverse intracellular locations and (3) play a role in cellular processes other than membrane trafficking alone. For example, at the plasma membrane their presence has important functional consequences for the activation of signaling cascades (reviewed in [3–5]). At the basis of raft formation lies the intrinsic tendency of sphingolipids to cluster with cholesterol in the plane of the membrane. Further progress in our understanding of raft dynamics and function in cells will therefore critically depend on our knowledge concerning the characteristics of its most elementary components, the sphingolipids.

Sphingolipids, comprising all lipids carrying a long-chain sphingoid base as their structural backbone (Fig. 2), are essential for eukaryotic life under natural conditions [6–8]. Although their roles in membrane structure and organization have been appreciated for some time, members of this diverse group of lipids are now also emerging as a novel class of signaling molecules that affect important cellular processes such as cell growth and survival (reviewed in [9–11]). How this multitude of actions comes into existence is poorly understood, but obviously depends on the type of headgroup and fatty chains of each particular sphingolipid involved. As sphingolipids are enriched in the outer leaflet of plasma membranes, but fulfill functions at a number of other locations in the cell as well, it will be important to determine how the appropriate distribution of sphingolipids over the various subcellular membrane surfaces is maintained. For this, it will be necessary to define the cellular sites of sphingolipid synthesis and hydrolysis. Also, it is necessary to determine the quantitative contributions of the various pathways of sphingolipid transport and the mechanisms that are responsible for the specificity in these pathways towards sphingolipids. Finally, in order to map the cellular sphingolipid economy, it is relevant to find out how synthesis, hydrolysis and transport along the various pathways are coordinately regulated in time and under different physiological conditions. Here, we explore what has been learned to date with respect to the issues raised above, and try to get a feeling for how sphingolipid metabolism and organization are integrated in eukaryotic life.

2. Possible mechanisms of intracellular sphingolipid transport

Because of their hydrophobic chain(s), sphingolipids are part of the lipid matrix of membranes. They rapidly diffuse in membranes with diffusion coefficients that are 10–100-fold larger than those of most membrane proteins: 10^{-8}–10^{-9} cm^2/s, which means that they cover an area of 0.1–1 $\mu m^2/s$ in a cell of typically 10–20 μm in each dimension (intracompartmental; Fig. 1: 1). They will thus participate in vesicular membrane traffic (intercompartmental; Fig. 1: 3). Since sphingolipids are synthesized in

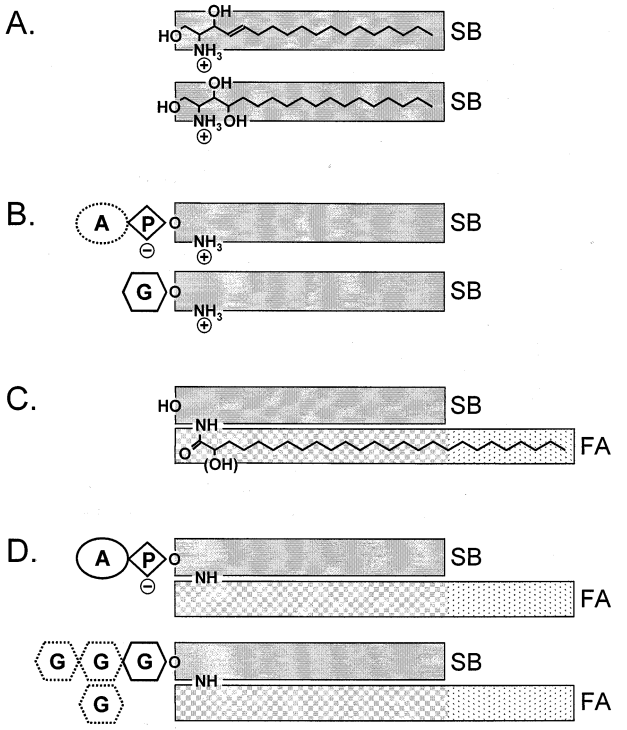

Fig. 2. Schematic structure of sphingolipids. All sphingolipids contain a sphingoid base, SB, an amino alcohol of typically 18–20 carbon atoms, as their basic structural motif (A). The saturated C_{18} form is referred to as sphinganine, the form with a double bond between C_4 and C_5 as sphingosine, and the saturated form with an additional hydroxyl at C_4 as phytosphingosine [1]. The sphingoid base may contain a polar headgroup, as in sphingosine-1-phosphate (B), or in lysosphingolipids where A represents choline or inositol, and G a carbohydrate such as glucose in the case of glucopsychosine. When *N*-acylated with a fatty acid, FA, with a typical length of 18–26 carbons, the molecule is termed ceramide (Cer; C). Some, but not all, fatty acid adducts are hydroxylated as in (C). The bulk of the cellular sphingolipids contains a polar headgroup (D). In mammals the headgroup is phosphocholine in sphingomyelin (SM), and a carbohydrate in glycosphingolipids. The carbohydrate can be glucose in glucosylceramide (GlcCer), galactose in galactosylceramide (GalCer), or may be complex, mostly based on glucose. In plants and yeast, the headgroup is phosphoinositol (inositolphosphoceramide, IPC), often glycosylated and otherwise extended, which in yeast results in mannosyl-inositolphosphoceramide (MIPC), and inositolphosphomannosyl-inositolphosphoceramide ($M(IP)_2C$). However, a multitude of other headgroups have been identified in the various eukaryotes and even in some bacteria [79,156]

the endoplasmic reticulum (ER) and the Golgi but are enriched in the plasma membrane, they must be subject to extensive intracellular sorting. Since sphingolipid sorting and protein sorting coevolved in evolution, their sorting may occur at the same sites, and the mechanisms by which proteins and sphingolipids are segregated may be interdependent. In both cases sorting of the molecule involves a local change in its surface density at a site of vesicle budding.

Major sites for protein sorting are situated at the exit sites of the organelles lining the secretory pathway and the endocytotic recycling pathway. In the ER, proteins are sorted at the transitional elements [12–15]. In the Golgi, protein sorting occurs at the *cis*-exit back to the ER [12], at the rims of each cisterna [16–20], and at the *trans*-Golgi network (TGN) back into the Golgi stack, to the plasma membrane (domains in epithelial cells), to secretory granules, and to the elements of the endocytotic pathway [21–24]. Proteins are sorted into various endocytotic pathways to the endosomes [25], and, in the endosomes, back to the plasma membrane, to the TGN, and to later compartments in the pathway like lysosomes in mammals and the vacuole in yeast [26,27].

Membrane lipids only span half the membrane and they are much smaller than proteins. For that reason, various possibilities must be considered for lipid transport that are generally irrelevant for membrane proteins. First of all, lipids can move between the two monolayers of the membrane lipid bilayer, a process termed transbilayer translocation or flip-flop, where 'flip' has been coined for the translocation towards the cytosolic membrane leaflet (Fig. 1: 2). In model lipid membranes this process is generally slow for the lipids that carry a polar headgroup (Fig. 2B,D) but more rapid for lipids without one (Fig. 2A,C). In cells, the process may be facilitated or even driven by proteins termed flippases or translocators. A second mechanism of transport that is mostly irrelevant in the case of membrane proteins is desorption of the lipid from the membrane into the aqueous phase and insertion into a different membrane (Fig. 1: 4). This process is slow for the sphingolipids carrying two long fatty chains (Fig. 2C,D) but relatively rapid for single chain sphingolipids (Fig. 2A,B, or sphingolipids carrying short acyl chains like C6, see Section 5.2). Monomeric exchange can be facilitated by soluble lipid transfer proteins, or by the existence of close contacts between membranes, like those between ER and mitochondria [28–31]. Monomeric exchange as a transport mechanism

is only relevant between cytosolic membrane leaflets. So far, there is no evidence that compartments exchange lipids via hemi-fusion, whereby lipids would diffuse from the one compartment to the other via a continuous cytosolic membrane leaflet.

3. The subcellular distribution of sphingolipids

3.1. Sphingomyelin

Most data on the distribution of sphingolipids over the various cellular organelles have been derived from cell fractionation studies on rat liver. These have been mostly limited to the phosphosphingolipid sphingomyelin (SM), by far the most abundant sphingolipid in this tissue. Generally, SM was found enriched in the plasma membrane as compared to the ER with an intermediate concentration in the Golgi. Whereas the SM concentration as a percentage of total phospholipid was 2–4% in the ER, it was found to be 15–23% in the plasma membrane [32–35]. Mitochondria were found to contain 1–2% SM [33,34]. Lysosomes were found to contain amounts similar to or higher than plasma membranes [33,36]. Unfortunately, the values reported in the earlier studies cannot be taken too absolutely, as endosomes were not recognized as discrete membranes until the early 1980s, and thus contaminated the isolated organelles. Endosomes were found to contain SM concentrations at least as high as found in plasma membranes [37,38]. A loss of the distinct SM distribution over the various organelles has been reported for fetal liver and fast-growing hepatomas. Under neoplastic conditions, a remarkable increase was observed in the SM concentration of the mitochondria [39–42].

Unusually high concentrations of SM have been reported for the apical plasma membrane domain of epithelial cells isolated from the kidney cortex of rats and dogs. While the basolateral phospholipids contained 19% SM, this was 36% for the apical domain [43–46]. ''Percent of phospholipid'' is not a direct reflection of the concentration or surface density of the lipid in the membrane since this term does not take into account the presence of non-phosphate containing lipids like cholesterol and glycosphingolipids [47]. However, the glycosphingolipid content of brush border from rat renal cortex is very low (4 μg/mg protein [48]) as compared to apical membranes from intestinal cells (300 μg/mg protein [49,50]), which contain equimolar amounts of glycosphingolipids, phospholipids, and cholesterol.

The distribution of SM across the plasma membrane was first addressed by the application of exogenous bacterial sphingomyelinase to erythrocytes [51]. According to this assay, 80–85% of the SM resided in the external, non-cytosolic leaflet of the bilayer. Similar data obtained on a membrane virus that budded from the plasma membrane of BHK cells and on isolated plasma membrane vesicles have been interpreted as an indication that SM is highly enriched in the non-cytosolic plasma membrane leaflet of nucleated cells [52–54]. Sphingomyelinase has also been applied directly to nucleated cells, mostly in the context of lipid transport studies (see Section 6.1). In some cases, plasma membranes were isolated from the treated cells to allow for a calculation of lipid asymmetry, which in all cases assigned most SM to the non-cytosolic surface [55–57]. In line with this, sphingomyelinase assigned 80% of the SM to the lumenal, non-cytosolic surface of chromaffin granules [58]. Some forms of cellular signaling involve hydrolysis of SM by an endogenous neutral sphingomyelinase [9,59,60]. The enzyme [60] probably resides on the cytosolic surface of the plasma membrane. Evidence, partially obtained using exogenous sphingomyelinase, has assigned the 'signaling' SM pool to the cytosolic leaflet of the plasma membrane [61–63].

In erythrocyte membranes, the sphingomyelinase-resistant SM pool contained shorter fatty acids [64]. This could imply that the inner leaflet SM contains shorter fatty acids. Alternatively, all SM could be accessible to the sphingomyelinase, the enzyme action could be incomplete (as reported for other phospholipases C, see [65]), and it could display a preference for long-chain SM. Actually, some observations suggest that the sphingomyelinase assay may not be generally applicable. Sphingomyelinase treatment of isolated intestinal brush border membranes yielded higher values for the accessible SM than did an assay based on a lipid transfer protein [66]. In addition, the sphingomyelinase assay applied to a membrane virus [53] yielded higher values that the original study on the lipid asymmetry of BHK cell-derived virus, which used a kinetic assessment of the SM hydrolysis [67].

The possibility exists that the conversion of the bilayer-forming SM to the non-bilayer-forming ceramide (Cer) may disrupt the transbilayer orientation of the lipids when the fraction of SM in the leaflet that is accessible to the sphingomyelinase is too high [68], as has been found to be the case for phospholipases C [65,69].

Of the cellular SM, 90% has been assigned to the plasma membrane in human fibroblasts [70]. This estimation seems high in the light of stereometric measurements which indicated that the surface ratio of endocytotic membranes/plasma membrane is 0.25 in fibroblastic BHK cells [71]. These membranes contain similar concentrations of SM [37,38]. Sphingomyelinase generally hydrolyzes some 60–65% of the total cellular SM when applied at 37°C for 1 h. Glutaraldehyde fixation did not change this number suggesting that replenishment of the surface SM by recycling SM did not occur under sphingomyelinase conditions [72]. The fact that similar numbers were obtained when sphingomyelinase was applied at the reduced temperature of 15°C, where vesicular traffic is essentially blocked, confirms that most of the cellular SM is situated in the plasma membrane [73,74]. Notably, signaling events that involve endogenous hydrolysis of SM will, locally and temporarily, reduce the concentration of SM.

SM is constitutively degraded in the lysosomes. SM accumulates in the lysosomes when the acid sphingomyelinase is inhibited, as in patients suffering from Niemann–Pick disease type A and B which are due to mutations in the acid sphingomyelinase [75,76]. Interestingly, Niemann–Pick type C disease is characterized by accumulation of SM but the actual defect is in intracellular cholesterol transport [77,78].

3.2. Inositolsphingolipids

Instead of SM, a large number of eukaryotic organisms (including plants, fungi and protozoa) contain inositolphosphoceramide (IPC) and its more complex, glycosylated derivatives [79]. In yeast, where this class of sphingolipids has been subject to extensive investigations, three major species are found: IPC, mannosyl-IPC (MIPC), and inositolphospho-mannosyl-IPC ($M(IP)_2C$). $M(IP)_2C$, the most complex sphingolipid found in yeast, accounts for 75% of the mass of total sphingolipids [80]. IPC, MIPC and $M(IP)_2C$ are primarily located in the yeast plasma membrane, where they constitute some 30% of total phospholipid [81]. Upon closer inspection [82], considerable amounts of IPC were found both in the Golgi and in the vacuole (the yeast equivalent of lysosomes). Trace amounts of IPC were found in mitochondria but none has been detected in lipid particles. Nuclei were not examined. No data are available on the distribution of the IPCs over the two leaflets of the plasma membrane bilayer. Interestingly, in yeast the glycosylphosphatidylinositol anchor by which some proteins (GPI proteins) are attached to the non-cytosolic leaflet of secretory membranes is modified by the replacement of the diacylglycerol by Cer [83]. This remodeling is carried out by an as yet unidentified enzyme(s) which resides in the ER and the Golgi [84].

3.3. Glycosphingolipids

Glycosphingolipids have been found to be exposed on the cell surface by a number of techniques. Galactose lipids were assayed by chemical oxidation using periodate [85], and by enzymatic oxidation using galactose oxidase [86–89]. The presence of ganglioside neuraminic acid on the cell surface was addressed using periodate oxidation [90–92] and by exogenously added neuraminidase [90,93,94]. The presence of numerous different glycosphingolipids on cell surfaces has been demonstrated using binding proteins that express preferential binding to specific glycosphingolipids. Examples are antibodies, toxins, and lectins. The general picture from this work is that the majority of the glycosphingolipids is situated in the plasma membrane and is facing outwards (reviewed in [68]). Whereas glycosphingolipids usually constitute a minor fraction of the plasma membrane lipids (several mol% of total lipid), particularly high concentrations of glycosphingolipids have been reported for the apical plasma membrane domain of epithelial cells from intestine [49,50,95] and urinary bladder [96], which contained double the glycosphingolipid concentration (30–40 mol% of total lipid) of the basolateral surface domain.

It is known that intracellular pools of glycosphingolipids exist in glycosphingolipid storage diseases (see [75]), and that glucosylceramide (GlcCer) is

stored in lamellar bodies in keratinocytes as a precursor for extracellular skin Cers [97,98], but it has become clear that intracellular pools of sphingolipids are a more general phenomenon [99–102]. Sphingolipids have been colocalized with cytoskeletal elements using fluorescence microscopy, but the necessity of using detergent has complicated the interpretations [103–106]. A major technical improvement was the application of the freeze-substitution technology to the immuno-gold electron microscopic (EM) localization of glycosphingolipids. Forssman glycolipid, IV^3-α-*N*-acetylgalactosamine (GalNAc)-Gb_4Cer, was localized by an antibody to the plasma membrane but was found in high concentrations in endocytotic compartments [107]. While small but distinct concentrations of Forssman glycolipid were observed over the Golgi and the ER, no gold label was observed over mitochondria and peroxisomes. As for SM, a highly increased content of the ganglioside GD1b was reported for the mitochondria of a malignant hepatoma [108]. It will be interesting to see a confirmation of these biochemical data by immuno-EM. By the same technique it was found that the ganglioside GM1, labeled by cholera toxin, was not distributed uniformly on the cell surface but was concentrated four-fold in caveolae [109].

From their presence on the cell surface, the non-cytosolic leaflet of the plasma membrane, glycosphingolipids would be expected to reside on the lumenal surface of the compartments along the secretory and endocytotic pathways. However, newly synthesized GlcCer has been localized on the cytosolic surface of the Golgi [110]. In addition, proteins have been isolated from cytosol that can interact with (specific) glycosphingolipids (Section 6.2), which leaves open the possibility that a fraction of the glycosphingolipids is oriented towards the cytosol [111–114].

3.4. Other sphingolipids

Only few data are available on the intracellular distribution of sphingoid bases or their phosphorylated forms, and Cers. The Cer concentration was reported to be twice as high in plasma membrane as in the ER of rat liver [35], while 10–15% of the total Cer pool was estimated to reside in the plasma membrane of CHO cells [115]. Obviously, Cer is produced in the biosynthetic route, but also by hydrolysis. Constitutive hydrolysis of SM, glycosphingolipids, and Cer occurs in the lysosomes. In the absence of acid ceramidase, Cer accumulates in the lysosomes (Farber disease; see [116,117]). Cer can also be produced during signal transduction via the SM cycle, or by the non-lysosomal glucosylceramidase, presumably at the plasma membrane (Section 4.1). Clearly, the relative amounts of Cer and SM in the signaling compartment will change when cell stimulation activates this pathway [9,59].

4. Topology of sphingolipid synthesis and hydrolysis

4.1. Sphingoid bases and ceramide

The first committed step in sphingolipid synthesis is the condensation of palmitoyl-CoA and serine to 3-ketosphinganine [1]. This then acts as a precursor for the sphingolipids sphinganine and phytosphingosine [118,119], both long-chain amino alcohols (Fig. 2). The sphingoid bases are converted to Cer by *N*-acylation. Only then the sphinganine chain can be desaturated to sphingosine [120,121]. Cellular sphingosine is therefore the product of hydrolysis of Cer or higher sphingolipids. In yeast (but to some extent also in mammals) sphinganine is hydroxylated to phytosphingosine before *N*-acylation. Cer assembly and the introductory reactions occur on the cytosolic surface of the ER (Fig. 3) [122]. Alternatively, Cer is produced from hydrolysis of SM and glycosphingolipids. Constitutive hydrolysis occurs in the lysosomes by acid sphingomyelinase and cerebrosidases. In addition, Cer is produced by a neutral sphingomyelinase, probably at the plasma membrane (see [60]), or by a non-lysosomal glucosylceramidase [123]. Cer itself is degraded in the lysosomes by an acid ceramidase [124,125].

Sphingosine, phytosphingosine, and sphinganine can be phosphorylated [126–128]. The enzymes were found to be partially membrane-associated, although none of them contains a conserved membrane localization signal [128–131]. The phosphorylation serves as a first step in the degradative pathway. It is followed by the activity of a lyase on the cytosolic surface of the ER membrane which converts phosphorylated long-chain bases into ethanolamine phosphate and a fatty aldehyde [132–134]. In

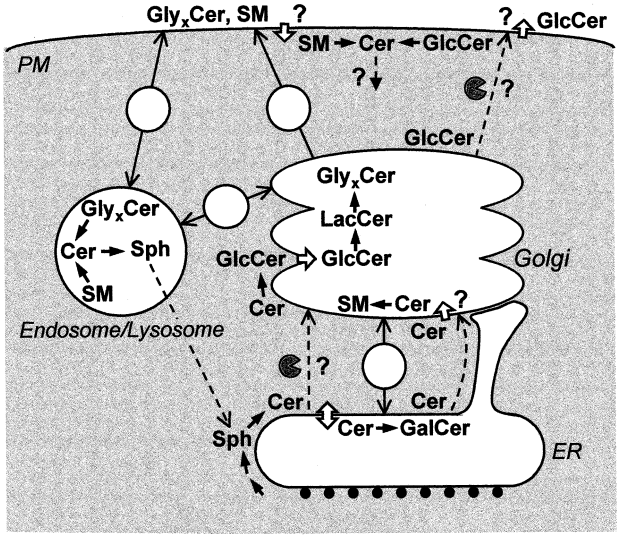

Fig. 3. Comprehensive view of intracellular sphingolipid transport. Sphinganine or phytosphingosine (Sph) in the ER is *N*-acylated to ceramide (Cer). Cer is transported by vesicular traffic and/or an undefined non-vesicular pathway (?) to the *cis*-aspect of the Golgi, drawn here as a single compartment. Cer can cross the ER membrane and, in some cell types, be converted to GalCer. GalCer can then follow the vesicular pathways. On the cytosolic surface of the Golgi, Cer is converted to GlcCer. GlcCer, at least partially, translocates towards the lumenal leaflet of the Golgi membrane, where it can be galactosylated to LacCer and further glycosylated (Gly$_x$Cer). After translocation across the membrane of the ER or the Golgi, Cer on the lumenal side of the Golgi can be converted to SM. All lumenal lipids can follow the vesicular transport steps to the plasma membrane (PM) and into the endocytotic recycling pathway. During recycling small quantities of the sphingolipids end up in the lysosome (drawn here as part of the endosome), where they are degraded to Cer and (phyto)sphingosine (Sph). This base can diffuse as a monomer across the lysosomal membrane and the cytosol to the ER for reutilization. GlcCer on the cytosolic surface of the Golgi can be transported by vesicular or monomeric (protein-mediated?) transport to the cytosolic surface of the plasma membrane. There it can be translocated to the outer leaflet (by a multidrug transporter), or it can be degraded by the non-lysosomal glucosylceramidase. SM on the cell surface may be translocated inwards (by the scramblase?) and be degraded by the neutral sphingomyelinase. The fate of the resulting Cer is unclear. The preferential incorporation of sphingolipids in the vesicular pathway from ER and Golgi to the plasma membrane is not indicated. This preferential incorporation may be mediated by sphingolipid rafts on the lumenal surface, while anterograde transport of Cer and GlcCer may also be mediated by rafts on the cytosolic surface.

addition, the sphingoid base phosphates appear to be important signaling molecules (see [11,135]). The molecules can be inactivated by the lyase or by a specific phosphatase [136–139].

4.2. Sphingomyelin

In mammals, Cer can be converted to SM by the transfer of a phosphocholine moiety from phosphatidylcholine yielding SM plus diacylglycerol. The topology of this reaction, whether it occurs in the Golgi, on the plasma membrane, or in endosomes, has long been a matter of debate. This was partially due to the fact that these membranes could not be reliably separated in the initial studies. The present evidence suggests that Cer from the ER is transported to the Golgi and is converted to SM on the lumenal surface of the membrane of the *cis*- and *medial* Golgi ([140–142]; Fig. 3). In line with this, brefeldin A, a drug that makes the Golgi complex fuse with the ER [143], relocated SM synthase activity to the mixed ER–Golgi compartment in CHO cells [74]. However, no relocation was observed in hepatocyte-derived HepG2 cells [144], which may indicate that SM synthase in hepatocytes is situated in the *trans*-Golgi or TGN. This may also be the case in neurons, where SM synthesis was reduced by brefeldin A [145].

At the same time, part of the SM synthase activity was assigned to the plasma membrane [140] and a SM synthase activity has been found on the surface of epithelial cells, where it is exclusively present on the basolateral surface [146]. This activity has now been observed on the surface of many cell types including polarized [147] and non-polarized cells [74,148]. In a direct experiment to settle an ongoing discussion [149] endosomes contained no sphingomyelin synthase activity [148]. The activity on the cell surface may be relevant for the removal of Cer produced by signaling through the SM cycle. Importantly, this would result in the production of the signaling lipid diacylglycerol at the plasma membrane. Neither of the SM synthases has been cloned so far.

4.3. Glycosphingolipids

In the first step in the synthesis of complex glycosphingolipids in mammalian cells, Cer from the ER is glucosylated by the Cer glucosyltransferase (CGlcT) ([150]; Fig. 3). CGlcT has its active center on the cytosolic surface of the early Golgi [110,142,151]. However, part of the activity is ex-

pressed in a hitherto undefined intracellular site, which has been proposed to be pre-Golgi or *trans*-Golgi [142,151]. No CGlcT was found in the ER–Golgi intermediate compartment in Vero cells [152]. In the lumen of the Golgi [153], GlcCer is galactosylated to LacCer [154]. GlcCer thus occurs on both the cytosolic and non-cytosolic surface of cellular membranes. It is degraded in the lysosomes by the glucocerebrosidase [155], or at a different location by the non-lysosomal glucosylceramidase [123].

LacCer in the lumen of the Golgi can serve as a substrate for glycosyltransferases, each of which gives rise to a trihexosylceramide that is the precursor of a specific series of glycosphingolipids assembled by the stepwise addition of carbohydrate residues [156]. Most cells synthesize the ganglioside GM3, by the transfer of sialic acid to LacCer [157–159], but in addition make one other trihexosylceramide. An interesting question is then how the flux into the two pathways is regulated [160].

Cell fractionation experiments have suggested that the sequential glycosyltransferases display concentration gradients along the *cis–trans* axis of the Golgi, with the highest concentration of the early acting transferases in the *cis*-Golgi (including the trihexosylceramide synthases) and the late acting transferases in the *trans*-Golgi and TGN [161–163]. Using similar methodology, a recent study assigned all lumenal transferases to the late Golgi, but with considerable overlap with the early Golgi [164]. Also here, the pharmacologic agent brefeldin A has been applied to discriminate the glycosyltransferases in the Golgi stack from those in the TGN. Glycosyltransferases that were still active in new glycosphingolipid synthesis after brefeldin treatment were supposedly transported back to the ER indicating their original localization in the Golgi stack, while those that no longer acted on newly synthesized glycosphingolipids were supposed to be located in the TGN [145,165–167]. Similarly, monensin, which causes a block in membrane traffic between the *cis-medial* and *trans*-Golgi, possibly assigns the transferases that are still active in the synthesis of new glycosphingolipids [168–171], but no longer act on exogenously added glycosphingolipids [172], to the *cis*-medial Golgi [173]. These studies generally confirmed the presence of early acting and the absence of late acting transferases in the proximal Golgi. A tagged GalNAc transferase was not limited to the *trans*-Golgi [174] in contrast to predictions from brefeldin studies [145,165]. Whether or not this represents a transfection artefact has been critically discussed [173,175]. The physiological significance of the distribution of the transferases in the Golgi is as yet unclear.

Besides GlcCer, certain mammalian cells, especially myelin-forming cells but in man also epithelial cells, also synthesize galactosylceramide (GalCer) by means of the ceramide galactosyltransferase ([176, 177]; Fig. 3). This enzyme belongs to the family of the glucuronosyltransferases [178,179]. Biochemical and morphological evidence has confirmed its localization on the lumenal surface of the ER [180]. It displays a strong preference for Cers carrying a 2-hydroxy fatty acid [181,182]. GalCer can be sulfated by a sulfotransferase in the lumen of the Golgi [183,184].

4.4. Inositolsphingolipids

The production of IPC in yeast involves the transfer of the headgroup from phosphatidylinositol to phytoceramide, a reaction yielding diacylglycerol as a side product [119]. The IPC synthase, or an essential subunit thereof, is encoded by the AUR1 gene [185]. The AUR1 gene product forms the target of several potent antifungal agents and its homologs have now been identified in a wide variety of fungi [186]. The protein has recently been located to the yeast Golgi (T. Levine, S. Munro, in press). Whether IPC synthesis occurs in the lumen or on the cytosolic surface of the Golgi remains to be established. Subsequent mannosylation of IPC to yield MIPC most likely occurs in the Golgi lumen [187,188]. In the Golgi, MIPC is further converted into $M(IP)_2C$ by an inositolphosphotransferase. This reaction resembles the one yielding IPC and requires the IPT1 gene whose product exhibits a striking structural similarity to that of the AUR1 gene [189].

5. Intracompartmental sphingolipid transport

5.1. Lateral diffusion

Sphingolipids diffuse laterally in the plane of the membrane [190]. However, this diffusion does not

result in a random distribution of sphingolipids over the membrane surface. Numerous indications have been presented that the sphingolipid domains that have been demonstrated in model membranes containing sphingolipid/glycerophospholipid mixtures also occur in natural membranes [191,192]. Although there are still major uncertainties concerning the composition, organization, size, lifetime and transbilayer arrangement of the domains, it is clear that sphingolipids segregate with cholesterol into patches, that these patches are less fluid than the bulk of the membrane, and that the patches, by a mechanism that is essentially not understood, are capable of interacting with certain proteins on both sides of the membrane. In the non-cytosolic leaflet these are the GPI proteins [192,193], on the cytosolic side proteins that carry two or more saturated fatty acyl chains [194]. Also transmembrane proteins have been found to interact with these domains, due to multiple acylation: the hemagglutinin of influenza virus in infected or transfected cells [194,195], or due to its *O*-glycosylation: sucrase-isomaltase [196], or for unknown reasons: MAL [197,198], ephrin B1 [199].

It was originally believed that sphingolipids and GPI proteins are clustered in large surface domains called caveolae. These are characterized by a protein coat on their cytosolic surface, consisting of the cholesterol binding protein caveolin [109,200,201]. However, it was soon realized that clusters, as defined by detergent insolubility [192,193], are also present in cells that do not contain caveolae [202,203], and that GPI proteins and glycosphingolipids are not present in large patches unless clustering is induced [204–207]. The latest evidence supports the idea that GPI proteins, (glyco)sphingolipids and cholesterol are aggregated in small clusters [208–210], and that oligomerization of these clusters or 'rafts' has functional consequences in, for example, activating signaling cascades [211–214]. In this respect, the hydrolysis of SM by the neutral sphingomyelinase may not generate a signaling ceramide, instead hydrolysis of 30–50% of the plasma membrane SM may just upset the raft balance of the plasma membrane. In contrast to the small size of sphingolipid rafts in normal plasma membranes, the apical surface of epithelial cells, notably that of intestinal epithelial cells, is essentially covered by sphingolipids and cholesterol [2]. It must therefore be one big raft [215].

5.2. Transbilayer translocation

The sphingoid bases, sphinganine, sphingosine, and phytosphingosine, and Cers essentially lack a polar headgroup (Fig. 2). This predicts that they translocate across cellular membranes spontaneously within seconds, as had been reported for diacylglycerol [216]. The rate of translocation across a phosphatidylcholine bilayer has been measured for diacylglycerol and Cer carrying a hydrophobic C_5-DMB moiety as the *sn*-2-acyl and *N*-acyl, respectively. While C_5-DMB–diacylglycerol translocated with a half-time of some 70 ms, this was 22 min for the Cer [217]. The possibility therefore exists that an asymmetric distribution of Cer in a biomembrane is maintained sufficiently long for it to act as a substrate for an enzyme on one side of the membrane without becoming available to an enzyme on the opposite surface. This possibility is supported by evidence suggesting that cholesterol, which possesses a hydroxy moiety as the polar headgroup just like Cer, directly or indirectly needs an ATP-driven transporter for translocation from the cytosolic to the non-cytosolic leaflet of the plasma membrane [218–221]. In contrast, C_6-NBD–Cer, C_6-Cers and Cers with two truncated chains rapidly cross the plasma membrane and the Golgi membrane because after addition to the outside of cells they are readily converted to SM in the Golgi [222–227], even at low temperature. This is also the case for exogenously added sphingoid bases, which reach the intracellular sites of Cer synthesis [228]. The phosphate in sphingoid phosphates prevents spontaneous translocation across lipid bilayers on a time scale of hours. If rapid transbilayer translocation would occur, this must be either protein-mediated or the pertinent cellular membrane does not have the properties of a lipid bilayer (which has been proposed for the ER, see [65]).

Bai and Pagano [217] also studied the half-times for translocation of C_5-DMB–SM and C_5-DMB–phosphatidylcholine in liposomes, which were calculated to be 3 and 7 h, respectively. After synthesis in the lumenal leaflet of the Golgi, an SM analog with two truncated chains and C_6-NBD–SM were found to be restricted to the lumenal leaflet of the Golgi membrane [142,225,226,229], implying that SM could not translocate from the non-cytosolic to the cyto-

solic leaflet. This has indeed been observed for spin-labeled and C_6-NBD analogs of SM in numerous studies on erythrocytes (see [230]), while also the reported difference in SM species between the outer and inner leaflets of the erythrocyte membrane could support the virtual absence of SM flip [64]. No evidence for short-chain SM flip towards the cytosolic leaflet of the plasma membrane was found in fibroblasts nor in the apical or basolateral plasma membrane domain of epithelial cells [231,232]. Similarly, no SM flip across plasma membranes or endocytotic membranes has been noted for C_6-NBD–SM in studies on endocytotic recycling [233–240].

However, this absence of SM flip in the plasma membrane may temporarily change when (specific) cells are activated. First of all, an activity has been noted in erythrocytes and blood platelets that scrambles the asymmetric distribution of the phospholipids in the plasma membrane, and an abundantly expressed 'scramblase' has been purified and its cDNA cloned [241,242]. Evidence has been presented that scramblase action flips SM to the cytosolic surface [243], whereas others have not observed this [244]. As a result of some stimuli SM must reach the cytosolic leaflet of the plasma membrane to serve as a substrate for the neutral sphingomyelinase (see Section 3.1). This could be mediated by the activation of the scramblase, but, alternatively, SM could flip across the membrane in a more proximal part of the secretory pathway. Indeed, evidence has been provided for a bidirectional translocation of phospholipids across the ER membrane, whereby SM translocation was slower than that of the glycerophospholipids [245,246]. Whether this rapid flip-flop is due to a 'flippase' or to non-bilayer structures in the ER membrane has been debated for years [65]. A flip of C_6-NBD–SM from the lumenal to the cytoplasmic leaflet of the ER has also been inferred from studies using brefeldin A to fuse the Golgi with the ER. Under those conditions newly synthesized C_6-NBD–SM was able to leave the mixed ER–Golgi compartment in the absence of vesicular traffic [74,147]. These studies demonstrated that plasma membranes contain proteins that can translocate (flop) C_6-NBD–SM from the cytosolic surface to the outer surface of the plasma membrane, and have identified the major multidrug transporters MDR1 P-glycoprotein and MRP1 as lipid translocators [74,147,247]. Whether these proteins can actually translocate natural SM, and whether this is of physiological relevance is an open question.

As for phospholipids, the spontaneous transbilayer translocation of glycosphingolipids across lipid bilayers is thought to be slow. However, GlcCer, the precursor of the complex glycosphingolipids, is synthesized on the cytosolic surface of the Golgi and conversion to LacCer occurs on the opposite side of the Golgi membrane, in the Golgi lumen. In experiments on isolated Golgi membranes, newly synthesized LacCer and more complex glycosphingolipids did not translocate to the cytosolic surface. The data suggest that an energy-independent GlcCer translocator is present in the Golgi membrane [153,164,229]. Studies on transport of short-chain analogs of GlcCer to the cell surface have demonstrated that the multidrug transporters that transport C_6-NBD–SM, MDR1 P-glycoprotein and MRP1 translocate GlcCer analogs as well [74,147,247]. Although MDR1 was found to translocate a variety of short-chain lipids, its absence or presence has been selectively correlated with the concentration of natural GlcCer [248,249], which supports the notion that it is a real GlcCer translocase or floppase (R. Raggers, D. Sillence, G. van Meer, submitted). Its physiological function is as yet unclear. C_6-NBD–GlcCer can translocate from the outer leaflet to the cytosolic leaflet of the plasma membrane of fibroblasts [238]. However, studies in many other cell types have provided no evidence for such inward translocation [235,239,240,250–252].

The other monohexosyl glycosphingolipid, GalCer, is synthesized in the lumen of the ER (see Section 4.3). In isolated ER, newly synthesized GalCer carrying a C_6-hydroxy fatty acid rapidly became accessible on the cytosolic surface. When added to Golgi membranes from MDCK cells, this GalCer analog was galactosylated to GalGalCer and sulfated to S-GalCer, sulfatide. Both products were inaccessible to bovine serum albumin in the medium, implying that GalCer translocated to the Golgi lumen and that the products were unable to translocate back out [229]. A small fraction of the complex glycosphingolipids, like Forssman glycolipid, is present in the ER and the nuclear membrane with which it is continuous. The question whether these complex glycosphingo-

lipids can translocate across the ER membrane to its cytosolic surface has not been addressed.

6. Intercompartmental sphingolipid transport

6.1. Vesicular pathways

Cer is synthesized in the ER. In some cells, part of the Cer is converted to GalCer in the ER. However, Cer must be transported to the Golgi for synthesis of GlcCer and SM, or IPC (Fig. 3). Some evidence has been presented that Cer would not be present in the transport vesicles that leave the ER for the Golgi [283], and that Cer is still transported in the absence of vesicular traffic. For that reason Cer transport will be addressed in Section 6.2. GalCer, though synthesized in the ER, occurs in high concentration on the cell surface of myelin-producing cells and of many epithelial cells [2]. Again, there is no reason why GalCer would not follow the vesicular traffic from the ER to the Golgi, but it is unclear whether there is any GalCer aggregation at the site of ER vesicle budding, or that such reactions only start in the Golgi, as they may require a certain threshold concentration of sphingolipids. A similar question concerns the complex glycosphingolipids in the ER: a small but significant fraction of Forssman glycolipid was found in the ER/nuclear membrane [107]. It seems necessary that these complex lipids, and SM, must be continuously removed from the ER, but it is as yet unclear how this works.

On the cytosolic surface of the Golgi, part of Cer is glucosylated to GlcCer. From here, GlcCer may follow three pathways, none of which can be excluded at present: (1) It can translocate across the Golgi membrane, evidence for which has been found in vitro (Section 5.2), and can be (partially?) converted to higher glycosphingolipids. GlcCer and the higher glycosphingolipids may then leave the Golgi on the lumenal side of transport vesicles; (2) GlcCer may leave the Golgi on the cytosolic surface of secretory vesicles, and subsequently undergo translocation to the outer leaflet of the plasma membrane (by MDR1 P-glycoprotein? Section 5.2). Alternatively, along the retrograde pathway, GlcCer might be translocated across the ER membrane, after which it would have a fate similar to newly synthesized GalCer; (3) GlcCer might leave the cytosolic surface of Golgi membrane by desorption from the membrane and monomeric exchange with another organelle's cytosolic surface (Section 6.2). Good evidence has been presented that higher glycosphingolipids follow a vesicular transport pathway from their site of assembly in the lumen of the Golgi to the plasma membrane. This was demonstrated for the early ganglioside GM3 [92], and for the late ganglioside GD1a [91].

In the lumen of the Golgi, Cer is converted to SM. SM does not translocate to the cytosolic surface (Section 5.2). Besides the logic that lumenal sphingolipids can only be shuttled between organelles via the lumenal side of transport vesicles, all evidence supports traffic on the lumenal surface of carrier vesicles as the major path of SM transport in the cell. After synthesis in the Golgi lumen, SM is transported to the plasma membrane. Transport was severely reduced in mitotic cells where vesicular transport is known to be inhibited [74]. In various cell lines, transport of radiolabeled SM to the plasma membrane, as measured by accessibility to exogenous sphingomyelinase or by cell fractionation, was inhibited by pretreating the cells with brefeldin A [74,253–255], which fuses the Golgi stack to the ER and blocks vesicular traffic of proteins from the Golgi to the plasma membrane. In the case where brefeldin did not inhibit transport, in hepatocytes [73], as stated above, the SM synthase appears to be situated beyond the brefeldin block [144]. After synthesis of an SM with two shortened chains, it was found inside transport vesicles [225,226]. Transport depended on cytosolic proteins and ATP, was inhibited by GTPγS, and transport of this SM and of C_6-NBD–SM was strongly temperature-dependent with complete inhibition below 10–15°C, the temperature where vesicular traffic of proteins is blocked [225,252,256]. Transport of C_6-NBD–SM was inhibited in mitotic cells [257]. Brefeldin could not be used as an assay for vesicular traffic, as, in this case, the short-chain SM escapes from the fused ER–Golgi by monomeric transport [74,147]. Experiments performed with late secretory mutants in yeast revealed that, like SM and the complex glycosphingolipids in mammalian cells, the inositol sphingolipids depend on vesicular traffic for reaching the plasma membrane [258].

As the sphingolipids are synthesized in organelles along the secretory pathway, and as they diffuse laterally in the membrane, they should flow along this pathway in both directions and randomly distribute over the connected organelles unless they are restricted. The fact that sphingolipids after synthesis in the Golgi are enriched in the plasma membrane, and are hardly present in the ER, suggests just such a restriction. The sphingolipids would thus be preferentially included into vesicles directed in the anterograde direction or would be prevented from entering the opposite, retrograde pathway (Fig. 1). Both possibilities are the consequence of the same phenomenon, the clustering of sphingolipids (and cholesterol) that was discussed earlier. Of course, clustering by itself would not result in directionality in a vesicular pathway. Since the targeting of transport vesicles depends on the presence of specific targeting molecules, notably a specific SNARE (see [259,260]), the sphingolipid domains are predicted to incorporate a particular molecule of this family.

Originally, the argument was proposed that, to maintain the enrichment of glycosphingolipids on the apical plasma membrane domain of epithelial cells, glycosphingolipid domains must exit in the lumenal leaflet of the TGN and that they must be specifically included into transport vesicles with an apical destination [2,224]. Whereas in the *cis*-Golgi sphingolipids would have to be segregated from glycerolipids, in the TGN glycosphingolipids would be segregated from SM and glycerophospholipids which are basolaterally enriched in these cells [261]. The actual lipid composition of the domains must therefore change with the overall change in lipid composition along the secretory pathway. Although the mechanism of sphingolipid domain-mediated sorting has by no means been solved, the first proteins that preferentially interact with sphingolipid/cholesterol domains and may be part of the sorting machinery have now been identified [3]. Amongst these are the first parts of the SNARE machinery that provide targeting specificity to the apical transport vesicles [262]. Furthermore, it has become clear that a number of membrane proteins for their proper targeting depend on sphingolipids and cholesterol. Amongst these are GPI proteins, in kidney epithelial cells [263] and in yeast [264], which also displays typical sphingolipid domains [265], and membrane-spanning proteins like the hemagglutinin of influenza virus [195,266,267] and sucrase-isomaltase [196].

Vesicular traffic does not stop at the plasma membrane. Endocytotic traffic of C_6-NBD–SM has been followed in various cell types. In these studies, C_6-NBD–SM displayed the kinetics of bulk membrane transport as defined by the transferrin receptor [236,237], and while most SM recycled, a small fraction ended up in the lysosomes for degradation [233–235]. From the cell surface, also C_6-NBD–GlcCer and C_6-NBD–GalCer followed vesicular recycling in a number of studies [235,239,240,250–252]. Sorting during endocytotic recycling was observed between C_6-NBD–GlcCer and C_6-NBD–SM [235,239,240]. This was then interpreted as reflecting lateral segregation in the lumenal membrane leaflet of an intracellular recycling compartment. However, it would be somewhat unexpected if C_6-NBD–lipid sorting via domains would accurately reflect sorting of their natural counterparts because the necessary aggregation of the sphingolipids largely depends on the lipid backbone [192,192], which is dramatically distorted in C_6-NBD–lipids [268]. The original finding of C_6-NBD–lipid sorting in epithelial cells [224] later turned out not to be due to lateral lipid segregation in the TGN, but to preferential transport of C_6-NBD–GlcCer across the apical plasma membrane domain by a multidrug transporter whereas C_6-NBD–SM was transported to both surfaces by vesicular transport [74,147]. No sorting between C_6-NBD–GlcCer and C_6-NBD–GalCer was observed in the transcytotic pathway in kidney epithelial cells [252]. Actually, in this study C_6-NBD–GlcCer was shown to be an excellent marker for bulk membrane transport along the transcytotic pathway. It has been used as such in studying endocytosis [269]. In one study, C_6-NBD–GlcCer escaped vesicular endocytosis by an unknown mechanism [238].

The glycosphingolipids GM1 and Gb_3 can act as receptors for the bacterial toxins cholera toxin (see [90]) and Shiga toxin [270], respectively. These toxins need to reach the ER, where they translocate across the membrane to become active in the cytosol. Endocytotic transport of the toxin–glycosphingolipid complex has provided information on vesicular transport pathways from sphingolipid domains on the plasma membrane to the ER [271–275]. Retrograde transport of the complexes may be driven by

protein sequences in the toxin [276–279], and the transport efficiency of free glycosphingolipids to the ER is unclear. Finally, vesicular traffic of sphingolipids can be expected to display the full complement of properties that characterize vesicular transport of proteins, like dependence on energy and cytosolic proteins (coats, SNAREs, cytoskeleton), which are not reviewed in detail here.

6.2. Monomeric exchange

Sphinganine, sphingosine, and phytosphingosine are single-chain lipids that probably exchange readily between membranes through the aqueous phase in monomeric form. Even sphingoid bases carrying a shortened acyl chain rapidly transfer between membranes [146,217,222]. Short-chain Cers have been used to characterize sphingolipid synthesis and transport in numerous studies since Lipsky and Pagano [222]. Because exogenous sphingoid bases rapidly translocate across membranes, they can efficiently reach the site of Cer synthesis [280]. In order to prevent cellular loss of sphingoid bases it seems required that they are efficiently trapped in the ER by metabolism. This is the more relevant as it was observed that most of the sphingoid bases used for the synthesis of ceramide may actually be derived from sphingolipid hydrolysis in the lysosomes [281]. Phosphorylated sphingoid bases, like sphingosine-1-phosphate, will exchange as monomers very rapidly. However, in combination with a low rate of transbilayer translocation this may still allow a physical separation between two signaling pools: (1) exogenous sphingosine-1-phosphate, signaling at the outside of the plasma membrane, and (2) a sphingosine-1-phosphate pool freely exchanging between the cytosolic surfaces of all organelles and exerting intracellular second messenger functions. The second pool is expected to overlap with the pool destined for degradation as the responsible lyase has been assigned to the cytosolic surface of the ER [132].

While Cer is produced on the cytosolic surface of the ER, its conversion into GlcCer takes place on the cytosolic surface of the Golgi apparatus and its conversion into SM in the Golgi lumen. The mechanism(s) by which Cer is moved from the ER to the sites of GlcCer and SM production in the Golgi is unclear. ER-derived transport vesicles enriched in Cer fused with Golgi membranes in an *N*-ethylmaleimide-sensitive manner under cell-free conditions [282]. In another cell-free system, the opposite conclusion was reached, namely that Cer is largely excluded from ER-derived transport vesicles and can reach the Golgi via a non-vesicular pathway which is ATP-independent and *N*-ethylmaleimide-insensitive [283]. Consistent with the existence of a non-vesicular pathway for Cer transport, the conversion of Cer into SM and GlcCer continued in mitotic HeLa cells, in which transport of newly synthesized membrane proteins is blocked [284]. However, with respect to the latter study, it should be noted that the issue of whether the disassembly of the Golgi complex during mitosis involves its fusion with the ER or not remains to be settled [285].

In intact and streptolysin O-permeabilized cells, the de novo synthesis of GlcCer from metabolically labeled Cer was sustained under various conditions that abolished ER-to-Golgi trafficking of VSV-G protein [286]. On the basis of these results, the authors concluded that Cer transfer from ER to Golgi does not depend on vesicular carriers that mediate protein transport. However, in the same assays the de novo synthesis of SM from metabolically labeled Cer was found to be both energy- and temperature-dependent. In agreement with this, genetic evidence was recently provided for the existence of an ATP-dependent trafficking pathway of Cer from the ER to the site of SM synthesis in the Golgi [287]. Here, the authors took advantage of an SM-directed cytolysin to isolate CHO cell mutants with specific defects in SM synthesis. One of the mutant strains, LY-A, displayed a specific defect in the conversion of Cer into SM which was unrelated to alterations in enzymatic activities responsible for SM synthesis or breakdown. Pulse-chase experiments with a fluorescent Cer (C_5-DMB–Cer) revealed that in wild-type cells C_5-DMB–Cer was redistributed from intracellular membranes to the Golgi in an ATP-dependent fashion, and that LY-A cells were defective in this energy-dependent redistribution. On the other hand, glycosphingolipid production and ER-to-Golgi trafficking of GPI-anchored or transmembrane proteins in LY-A cells appeared to be normal. The authors proposed that the delivery of Cer from the ER to the sites of sphingolipid synthesis in the Golgi is mediated by two pathways: an ATP-independent, non-vesicular path-

way for glycosphingolipid synthesis and an ATP-dependent pathway for SM production. Several possibilities can be put forward to explain the preferential ATP-dependent delivery of Cer to the site of SM synthesis: (1) Cer might reach the Golgi primarily by a non-vesicular pathway involving Cer-specific transfer proteins and/or close contacts between ER and Golgi membranes (see [288]). In that case, Cer might require ATP-stimulated translocation across the Golgi membrane in order to reach the site of SM synthesis. Flip-flop of natural Cer may be slow [217], and from the new data on a potential ATP-dependent cholesterol translocator (Section 5.2), it seems possible that Cer has to be pumped against a concentration gradient, and that a higher concentration in the cytosolic leaflet would be maintained by preferential interactions, maybe even by some sort of domain organization on the cytosolic surface; (2) SM synthesis might occur in a sub-Golgi compartment distal to that involved in GlcCer synthesis (see Section 4.2). In this scenario, SM synthesis requires not only ER-to-Golgi, but also intra-Golgi transport of Cer, the latter being ATP-dependent; (3) There might be multiple, independent vesicular transport pathways from the ER to the Golgi, one of which is exclusive for Cer.

The existence of a non-classical vesicular transport mechanism for Cer can also be surmised from studies with early secretory mutants in yeast. In this organism, the production of IPC was found to be largely independent of genes whose products control protein trafficking from the ER to the Golgi [289]. As IPC synthase has recently been located to the Golgi (Section 4.4), a tentative interpretation of the data is that newly synthesized Cer in yeast can reach the Golgi independently of the machinery responsible for vesicular ER-to-Golgi transport of proteins. However, it is important to realize that many Golgi-resident proteins, including Golgi enzymes, have been shown to recycle to the ER [290,291] and may accumulate there in significant amounts when membrane transport out of the ER is blocked (for example, see [19]). If the same holds true for enzymes responsible for sphingolipid assembly in the Golgi, then some of the experimental data discussed above may require a reinterpretation. Clearly, additional studies are needed to establish the precise nature of the pathway(s) used for intercompartmental Cer transport. Identification of the gene affected in the LY-A mutant strain could provide an important step forward in defining the molecular mechanism(s) underlying the intracellular trafficking of Cer.

After conversion of Cer to its various sphingolipid products, only GlcCer is present on the cytosolic surface of the Golgi (Section 3.3). Possibly, also GalCer has access to the cytosolic surface of the ER and thereby other membranes along the secretory pathway (Section 5.2). This orientation allows desorption into the cytosol and monomeric transport to other organelles. Indeed, evidence has been presented that GlcCer (and not SM) still reached the plasma membrane when vesicular traffic was inhibited by brefeldin A [255]. Because the spontaneous exchange of GlcCer, SM, and other glycosphingolipids through the water phase is very slow (half-time of days [292]), transfer would be expected to occur at sites of close membrane contact [293] or it should be stimulated by the presence of glycosphingolipid transfer proteins. Such a protein has been isolated from cytosol [294,295], and its cDNA has been cloned [296]. It is capable of transferring a variety of glycosphingolipids between membranes in vitro. Its function in vivo is unclear. Other proteins that stimulate monomeric glycosphingolipid transfer in vitro have been found to be involved in lysosomal glycosphingolipid hydrolysis (see [297]), and not intercompartmental transport.

Under some conditions, SM has been found in mitochondria (Section 3.1). Since no vesicular transport occurs to the mitochondria, the mechanism of transport of SM to the mitochondria was most likely monomeric. It will be interesting why SM is available for monomeric transfer under those conditions and whether there is a function for an SM transfer protein [298,299].

7. Perspectives

Evidence from the past few years has strengthened the notion that sphingolipids are particularly relevant for the cellular physiology by their property to cluster with cholesterol in the plane of the membrane. These clusters, called microdomains or rafts, seem most important for organizing signaling events at the plasma membrane and for sorting and targeting

membrane proteins and lipids to their proper destinations along the cell's vesicular highways. In that way, sphingolipids can be viewed as vital parts of the machinery by which a cell ensures the proper functioning of its secretory and endocytic organelles, including the plasma membrane. It has now become clear from the many different types of sphingolipids, each with its own specific physical properties, and from the multitude of proteins involved in their metabolism and transport, that there is more to sphingolipids than just their property to segregate from glycerophospholipids in liposomes. When thinking about raft function in the various organelles, each with its own typical lipid and protein composition, it is clear that we completely lack insight into the fine tuning of the raft system.

Presently, exciting progress is being made with the identification and localization of protein factors involved in the regulation of raft assembly and trafficking. These are first of all the biosynthetic and hydrolytic enzymes of sphingolipid metabolism, some of which have been found at quite unexpected locations. Moreover, the first translocators have been identified that flip sphingolipids from one leaflet of the bilayer, across into the opposite leaflet, although at present it is fully unclear how such translocations are connected to the raft principle. Also the first targeting factors have been identified for one specific raft-mediated transport step, namely that between the TGN and the apical membrane of epithelial cells. Still, the genes encoding major synthetic enzymes (e.g. the ceramide and sphingomyelin synthases), hydrolytic enzymes (e.g. the non-lysosomal glucosylceramidase), and translocators, (e.g. the Golgi glucosylceramide importer) have escaped molecular cloning. Other proteins, for example those that are able to specifically bind sphingomyelin or monohexosylceramides, have been identified and sequenced, but their physiological functions remain enigmatic.

In the next phase of unraveling sphingolipid physiology, one of the main challenges will be to define the parameters of the raft framework that must be regulated (size, i.e. degree of aggregation, and molecular composition, which will have to be different for each organelle and each membrane leaflet), to identify the effectors of the regulation (components regulating sphingolipid metabolism and flow), and the factors that govern their activity. A subsequent question will then be, what are the sensors that govern the regulatory activities? Probably, we will have to wait until the regulatory loops have been characterized before we obtain insight into the really important functions of sphingolipids. Only recently have we learned that even eliminating a subclass of sphingolipids in mammals, namely the glucosphingolipids, is embryonically lethal [300]. In order to understand this, it seems wise to start with unraveling sphingolipid physiology at the level of a single cell. For this purpose, yeast provides an attractive model system, as it will most likely be the first organism in which the full set of genes involved in sphingolipid metabolism is known. When combined with DNA microarray technology, this information would create a unique starting point to determine how sphingolipid metabolism is integrated with other cellular processes that are fundamental for eukaryotic life, like cell growth, membrane trafficking and the capacity to cope with environmental stress. Lessons learned from yeast will provide a useful base from which to work our way up to the multicellular organisms that are genetically amenable like *Drosophila* and *Caenorhabditis elegans*, an approach that has already started to disclose new elements of raft physiology [301,302]. However, let us be modest. The mere fact that sphingolipid variety coevolved with the organisms over many millions of years should tell us that we still have a long way to go before we will be able to appreciate the full detail of their beneficial effects for human life.

Acknowledgements

We dedicate this work to Mara, daughter of our friend and colleague Thomas Pomorski and Ines Günther, born August 14, 1999. We are grateful to all our friends and colleagues for sharing their newest data and for critical assessment of our statements, and to the editors for encouragement.

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