The Organizing Potential of Sphingolipids in Intracellular Membrane Transport

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Holthuis, Joost C. M., Thomas Pomorski, René J. Ragger, Hein Sprong, and Gerrit van Meer. The Organizing Potential of Sphingolipids in Intracellular Membrane Transport. Physiol Rev 81: 1689–1723, 2001.—Eukaryotes are characterized by endomembranes that are connected by vesicular transport along secretory and endocytic pathways. The compositional differences between the various cellular membranes are maintained by sorting events, and it has long been believed that sorting is based solely on protein-protein interactions. However, the central sorting station along the secretory pathway is the Golgi apparatus, and this is the site of synthesis of the sphingolipids. Sphingolipids are essential for eukaryotic life, and this review ascribes the sorting power of the Golgi to its capability to act as a distillation apparatus for sphingolipids and cholesterol. As Golgi cisternae mature, ongoing sphingolipid synthesis attracts endoplasmic reticulum-derived cholesterol and drives a fluid-fluid lipid phase separation that segregates sphingolipids and sterols from unsaturated glycerolipids into lateral domains. While sphingolipid domains move forward, unsaturated glycerolipids are retrieved by recycling vesicles budding from the sphingolipid-poor environment. We hypothesize that by this mechanism, the composition of the sphingolipid domains, and the surrounding membrane changes along the cis-trans axis. At the same time the membrane thickens. These features are recognized by a number of membrane proteins that as a consequence of partitioning between domain and environment follow the domains but can enter recycling vesicles at any stage of the pathway. The interplay between protein- and lipid-mediated sorting is discussed.
I. INTRODUCTION

Sphingolipids are typically found in eukaryotic cells where they comprise a small but vital fraction (10–20%) of the membrane lipids. Their lipidic part consists of a sphingoid base, a straight-chain amino alcohol of 18–20 carbon atoms, which normally carries a long saturated fatty acid amide bonded to the amino group at the C2 position (Fig. 1). Based on the type of headgroup attached to the C1, sphingolipids are classified as phosphosphingolipids or glycosphingolipids. The phosphosphingolipids sphingomyelin (SM) in animals and inositol phosphoceramide (IPC) in plants and fungi carry the polar headgroups phosphocholine and phosphoinositol, just like the major glycerolipids phosphatidylcholine (PC) and phosphatidylinositol (PI). In glycosphingolipids, the headgroup can contain a variety of monosaccharides linked by various types of glycosidic bonds. Since the discovery of the sphingolipids more than 100 years ago by Thudichum (376), their special lipid backbone and their bewildering structural heterogeneity have fascinated biologists, biochemists, and biophysicists alike.

![Common lipids in fungi and various animals.](http://physrev.physiology.org/)

**Mammals**

a. 

b. 

c. 

d. 

**Insects**

i. 

j. 

k. 

l. 

**Nematodes**

e. 

f. 

g. 

h. 

**Fungi**

m. 

n. 

o. 

FIG. 1. Common lipids in fungi and various animals. a, Sphingomyelin (SM); b, glucosylceramide (GlcCer), and a derived glycosphingolipid, e.g., GM1; c, glycerolipids; d, cholesterol; e, SM, f, glycosphingolipid of the arthuro series; g, see b, h, sterol; i and j, see f, k, see b; l, sterol; m, M(IP)_2C; n, see c, o, ergosterol. Fat print indicates the sphingoid bases in the sphingolipids a, b, e, f, i, j, and m; glycerol in the glycerolipids c, g, k, and n; and the sterols d, h, l, and o. A, head group: choline, ethanolamine, serine, or inositol; C, choline; E, ethanolamine; G, glucose; Ino, inositol; M, mannose; P, phosphate (note that insects and nematodes are sterol auxotrophs).
The major mission of the sphingolipid field is to understand the specific functions of these lipids in eukaryotic organisms and to evaluate their significance both for the functioning of individual cells and the organism as a whole. This is an arduous task, not in the least because, unlike glycerolipids and sterols, sphingolipids display a striking structural variation between distinct organisms, and even between different tissues and cell types within one organism. Moreover, sphingolipids are no longer viewed as primarily inert structural components of cellular membranes, but also increasingly recognized as diverse and dynamic regulators of a multitude of cellular processes. An important development in this respect has been the realization that sphingoid bases, ceramides, and other intermediates of sphingolipid metabolism act as signaling molecules in mediating cell cycle control, stress responses, and apoptosis (75, 127, 356). In addition, considerable attention has been drawn to the concept that sphingolipids drive the lateral differentiation of cellular membranes into a mosaic of areas with unique molecular compositions. In essence, it has been postulated that a differential miscibility of sphingolipids, glycerolipids, and sterols triggers the formation of lateral lipid assemblies, termed microdomains or rafts, that acquire specific functions by concentrating or excluding specific membrane proteins (341). Rafts are now believed to serve as platforms for various cellular events including polarized protein sorting, signal transduction, and cell adhesion (35, 118, 178, 305, 340).

Sphingolipids are essential to sustain eukaryotic life. Whereas glycosphingolipids are indispensable for the development of complex multicellular organisms, phosphosphingolipids fulfill a vital function at a more fundamental level, namely, in the growth and survival of individual cells. This requirement for phosphosphingolipids appears to be a conserved feature of eukaryotic cells, as it is found both in animal cells and in yeast. Hence, it is not unlikely that the different sphingolipids synthesized in the various eukaryotic cell types serve a common function. If so, one would expect sphingolipids to share essential features that determine their interactions with other cellular components. If there is such a unifying principle, what could it be?

This review serves to highlight some remarkable aspects of sphingolipids that we believe represent important guidelines for unraveling their vital function in eukaryotic cells. One of these is the striking observation that in yeast the requirement for sphingolipids can be bypassed by a suppressor mutation that enables yeast to synthesize a novel set of glycerolipids whose structural appearance and predicted biophysical properties closely mimic those of the sphingolipids (74, 193, 281). As discussed in section II, this would indicate that some structural function, rather than a signaling one, accounts for the sphingolipid requirement in cell growth. A comparison of sphingolipid structures from evolutionary distinct organisms (see sect. III) indicates that, despite considerable chemical differences, the biophysical properties held responsible for microdomain formation have been well preserved. Indeed, counterparts of the microdomains analyzed in mammalian cell membranes appear to exist in flies, worms, and even in yeast (see sect. IV).

A further striking aspect of sphingolipids is that their assembly in eukaryotic cells is spatially separated from that of glycerolipids and sterols. Whereas the latter are produced in the endoplasmic reticulum (ER), sphingolipid synthesis occurs primarily in the Golgi complex (see sect. V). The Golgi has been well established as a polarized sorting and processing station that is situated right at the intersection of two major circuits of intracellular membrane trafficking. One circuit interconnects the cis-side of the Golgi with the ER; the other one the trans-side with the plasma membrane. The ER and plasma membrane are engaged in fundamentally distinct cellular processes, as reflected by the dramatic differences found in the molecular composition and biophysical properties of their bilayers. In section VI we consider the possibility that sphingolipids evolved as an integral and essential part of the Golgi machinery responsible for establishing the compositional and functional differences between the ER and the plasma membrane.

II. WHY ARE SPHINGOLIPIDS ESSENTIAL FOR EUKARYOTIC LIFE?

A. Sphingolipid Requirements at the Cellular Level

The early steps in sphingolipid synthesis, up to the formation of sphingoid bases and ceramides, are essentially the same in all eukaryotes. It is in the subsequent reactions, when ceramide is converted to complex sphingolipids, that major species- and cell type-specific differences become apparent. Yeast, for example, is equipped with a single biosynthetic pathway that serves to convert ceramide into only a handful of inositol-containing phosphosphingolipids. Animals, on the other hand, evolved three independent pathways that can operate simultaneously to produce both phosphosphingolipids and hundreds of different types of glycosphingolipids.2 The divergence in headgroup structures found in glycosphingolipids

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1 It should be noted that the name raft implies that this would be a small domain floating in a sea of other lipids. However, in the many cases where the relative sizes of the sphingolipid-rich and glycerolphospholipid-rich environments are not known the term domain would be less suggestive.

2 Glycosphingolipid designation is according to the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature (53), using the Svennerholm abbreviations for gangliosides and using sulfatide to indicate HSO3 -3 Gal β1–1 Cer.
accompanies functional differences between the various cell types of an animal. Whereas the synthesis of at least some glycosphingolipid species appears essential for the viability of animal organisms (see sect. II B), as a lipid class they are dispensable for the growth and survival of animal cells in culture. This is not the case for phosphosphingolipids. Phosphosphingolipid synthesis is required for cell growth and survival, in yeast as well as in mammals (the only organisms in which this has been studied so far). Yeast and Chinese hamster ovary cell mutants lacking the first committed enzyme in sphingolipid biosynthesis, serine palmitoyl-transferase (Fig. 2), die in the absence of externally added sphingoid base (123, 411). The mammalian mutant could be rescued by exogenous SM, but not by glucosylceramide (GlcCer), the precursor of higher glycosphingolipids (121, 123). It therefore appears that SM, and not GlcCer (or higher glycosphingolipids), fulfills a vital role in mammalian cells. Indeed, mammalian cell lines unable to synthesize GlcCer are viable and proliferate (145). The alternative monoglycosylceramide, galactosylceramide (GalCer), is not required for cell survival either, since it is absent from most mammalian cell types including the GlcCer-negative cells (145).

Collectively, these observations raise two major questions. What vital function do phosphosphingolipids serve in the cell? And for what purpose do animal cells create so many different types of glycosphingolipids in addition, given that, in principle, they can live without them? Complete answers to these questions are still lacking. However, experimental work over the last decade has indicated that cells exploit both chemical and biophysical characteristics of sphingolipids to accomplish some of their most fundamental tasks. Before exploring which of these characteristics account for the sphingolipid requirement in cell growth and sur-

**FIG. 2.** Pathways of ceramide synthesis. Known metabolic intermediates, substrates, and enzymes are indicated. Names of genes whose products are required for specific steps in yeast ceramide synthesis are shown in italics and discussed in section V A. Note that it remains to be established whether C4-hydroxylation occurs on the free sphingoid base (sphinganine) or on ceramide.
vival, we will first turn our attention to what is known about the sphingolipid requirements of complex multicellular organisms.

B. Sphingolipid Requirements at the Organismal Level

Gene knock-out studies in mice are starting to reveal the physiological significance of sphingolipids in mammals. First of all, in addition to phosphosphingolipids being indispensable for cell proliferation (see sect. II A), glycosphingolipid production is essential for development and differentiation. Mice lacking GlcCer and all complex glycosphingolipids due to disruption of the gene encoding ceramide:glucosyltransferase were found to be embryonically lethal at the gastrulation stage just after formation of the primitive germ layers (421). After ectopic transplantation, glycosphingolipid-deficient embryonic stem cells were able to differentiate into endodermal, mesodermal, and ectodermal derivatives but failed to give rise to mature, well-differentiated tissues.

Knock-out mice have also been generated without a functional ceramide:galactosyltransferase (27, 58), the enzyme that synthesizes GalCer, which is the precursor of only a few other lipids like sulfatide (HSO$_3^-$GalCer). These mice live, but male mice were unable to breed, which reflects a function of galactolipids in spermatogenesis (95). In addition, such mice displayed compromised nerve function (27, 58), a finding consistent with the putative insulatory function of the bulk quantities of GalCer and sulfatide found in myelin, the plasma membrane of oligodendrocytes, and Schwann cells. High concentrations of GlcCer (murine) and/or GalCer (bovine, human) and derivatives have also been found in the apical plasma membrane domain of epithelial cells lining the gastrointestinal and part of the urogenital tract (341). Here, the glycosphingolipids are thought to provide mechanical stability and, especially in the gut, protection against harmful, hydrolytic enzymes such as phospholipases.

In contrast to GlcCer and GalCer, complex glycosphingolipids are mostly present on cell surfaces in low quantities only. Their importance for development and differentiation is supported first of all by the observation that knock-out mice being unable to synthesize the complex sialoglycosphingolipids. Of the double knock-out mice being unable to synthesize glycosphingolipids beyond the simple ganglioside GM$_3$ (Fig. 3), 50% died within 13 wk (158). Mice unable to synthesize glycosphingolipids beyond GM$_3$ and GD$_3$ displayed axonal degeneration and decreased myelination (332, 371). As a possible explanation, it has been proposed that glycosphingolipids are involved in specific recognition events between cells and between cells and matrix via their specific carbohydrate moiety, and in the modulation of plasma membrane signal processing. The latter may occur via specific glycosphingolipid-protein interactions (119) or via organizing functions of the glycosphingolipids in signaling domains (118, 202).

It should be noted that not only sphingolipid production, but also the proper removal of sphingolipids, is of physiological significance for an organism. The absence of hydrolytic enzymes down to ceramidase or cofactors like sphingolipid activator proteins from the lysosomes results in lysosomal storage of sphingolipids with characteristic and often severe pathologies in humans (167, 171, 186, 260). Strategies devised to cure the symptoms of these diseases now include enzyme replacement and gene therapy (22, 29, 323, 435) and prevention of accumulation by administrating an inhibitor of glycosphingolipid synthesis (153, 287).
C. Do Sphingolipids Exert a Vital Signaling Function?

An impressive number of studies have implicated sphingolipids in virtually all aspects of cellular signaling (reviewed in Refs. 75, 118, 127, 356). First, sphingolipids serve as ligands for receptors present on neighboring cells (or in the matrix) to trigger various types of cell behavior (growth, adhesion, differentiation, migration). Second, sphingolipids influence properties of receptors on the same cell via specific lipid-protein interactions, thereby changing the cellular responsiveness to external stimuli (119). Third, sphingolipids modulate signaling by their ability to assemble both receptors and their downstream effectors (e.g., Src family kinases, G proteins) in specialized plasma membrane microdomains, known as rafts and caveolae (7, 36, 118, 147, 202, 340). Finally, sphingolipid bases, ceramides, and their phosphorylated derivatives act as signaling molecules in the regulation of membrane trafficking, cell growth, cell death, and the ability of cells to cope with environmental stress (13, 152, 346, 356). Especially this last paradigm has attracted much attention in the recent literature. Although ceramide-activated protein kinases and phosphatases have been implicated in transmitting sphingolipid-derived signals (126, 431), the mechanisms by which ceramide pathways operate have not been elucidated (138, 139, 170, 409). Recent work has shown that ongoing synthesis of sphingoid bases forms a prerequisite for the internalization step of endocytosis in yeast (429). It appears that sphingoid base levels help control the relative activities of specific protein kinases and phosphatases whose downstream targets are elements of the endocytic machinery and/or actin cytoskeleton (93). Another exciting development in the field is the emergence of sphingosine-1-phosphate as a prototype of a new class of lipid signaling molecules that function not only as intracellular second messengers, but also as extracellular ligands for cell surface receptors (356). In support of the extracellular ligand receptor, several closely related transmembrane receptors have recently been identified as putative sphingosine-1-phosphate receptors in mammals (6, 191).

Sphingolipid signaling pathways have been found to operate in many different cell types, from mammals down to yeast (74). The impressive array of cellular processes that appears to be regulated by these pathways would provide a logical explanation for the observed lethality of sphingolipid-deficient mutant cells and organisms. However, studies in yeast have demonstrated that the putative signaling function of its sphingolipids is dispensable for cell growth and survival, although only under nonstressed conditions. A mutant strain lacking sphingolipids has been isolated upon suppression of a genetic defect in sphingoid base synthesis. This suppression is due to a mutation in the SLC1 gene, believed to encode a fatty acyltransferase (261). The suppressor mutation enables cells to produce a novel set of glycerolipids that mimic sphingolipid structures, both with respect to their headgroup and fatty acyl chain composition (193). The novel lipids identified were phosphatidylinositol (PI), mannosyl-PI, and inositol-P-(mannosyl-PI), all containing a C26 fatty acid in the sn-2 position of the glycerol moiety. Normally the C26 fatty acid is not found in yeast glycerolipids, but only in the sphingolipids (Fig. 1) and in the lipid backbone of some glycosylphosphatidylinositol (GPI)-anchored proteins. When exposed to extremes of pH or temperature, the suppressor mutant fails to grow unless provided with externally added phytosphingosine (74, 193, 281). These and other observations (76) show that yeast requires sphingolipids to build up a proper stress response. In contrast, the essential function of sphingolipids in growth and survival under normal conditions can be taken over by the novel glycerolipids, and is, apparently, structural.

D. Sphingolipids and the Spatial Organization of Cells

Clearly, sphingolipids are not just a reservoir of signaling molecules; they also contribute to vital properties of cellular membranes. Studies of their physical behavior (see sect. iii) have provided thorough insights in the basis of how sphingolipids and cholesterol induce lateral segregation of membrane components (see sect. iv). However, to understand the functional implications, we will have to define the consequences of this lateral organization for activities in and on the membrane. With what other molecules do sphingolipids interact, and for what processes are these interactions relevant? If we want to learn how sphingolipid-mediated processes are integrated in the physiology of the cell, we will also need to know how these processes are regulated at the level of the sphingolipids. What rules govern their interactions at the biophysical level, and what determines their concentration in the various cellular membranes in time? First insights have been obtained from the localization of the subcellular sites of sphingolipid synthesis and hydrolysis, and from studying their mechanisms of transport (see sect. v). Because metabolism and transport are mediated by enzymes and transporters, regulation of these processes must be exerted at the level of the proteins and the genes by which they are encoded. The available data suggest a pivotal role for sphingolipids in the operation of the Golgi complex, the central sorting station in the delivery of cargo, and membrane components to their proper destinations (see sect. vi). So far, it was believed that sorting processes were governed exclusively by information in the molecular structure of proteins. We now start to realize that sphingolipids produced in the Golgi

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may well form indispensable parts of the organelle’s sorting machinery. Our challenge is to find out how the machine works.

III. SPHINGOLIPID STRUCTURE AND BIOPHYSICAL PROPERTIES

A. A Concise Inventory of Sphingolipid Structure in Distinct Eukaryotic Life Forms

The key structural features by which sphingolipids can be distinguished from glycerolipids and sterols are outlined in Figure 1. First of all, the backbone of sphingolipids is a sphingoid base. With respect to molecular conformation, the sphingoid base is structurally equivalent to the glycerol and the sn-1 acyl chain in a typical glycerolipid. Saturated sn-1 chains of 16 or 18 carbons (C16:0, C18:0) predominate in most eukaryotic glycerolipids. The prevalent sphingoid base found in mammalian sphingolipids is sphingosine with a chain length of 18 carbons. With respect to molecular conformation, the sphingoid base is structurally equivalent to the glycerol and the sn-1 acyl chain in a typical glycerolipid. Saturated sn-1 chains of 16 or 18 carbons (C16:0, C18:0) predominate in most eukaryotic glycerolipids. The prevalent sphingoid base found in mammalian sphingolipids is sphingosine with a chain length of 18 carbons and a trans-double bond between carbons 4 and 5. However, over 60 different species of sphingoid bases have been described with alkyl chain lengths varying from 14 to 22 carbon atoms, different degrees of saturation (the saturated form of sphingosine is called sphinganine (53)) and hydroxylation (154). The saturated and 4-hydroxylated form of sphingosine is generally referred to as phytosphingosine because it is the common sphingoid base found in sphingolipids of plants and fungi. In vertebrates, high levels (even up to 70%) of phytosphingosine-based sphingolipids have been found in kidney and intestine (8, 28, 113, 155, 266). Sphingolipids in insects are based on a C14 sphingoid base rather than the C18 backbone generally found in mammals, plants, and fungi (192, 304, 414). Sphingolipids of nematodes are unusual in that they are based entirely on a C16 sphingosine methylated at C15 (55, 105).

The amino group at the second carbon of the sphingoid base serves to attach a fatty acid in amide linkage. The N-acylated sphingoid base ceramide is the precursor of all membrane sphingolipids. The trivial name of ceramides based on sphinganine is dihydroceramide. The sphingoid C1 hydroxyl group in ceramide is used as the attachment site for a polar head group. Based on their head groups, sphingolipids are often grouped into phosphosphingolipids and glycosphingolipids. However, these categories are not mutually exclusive; plants and fungi, for example, add phosphoinositol to phytoceramide to generate IPC, and this head group can be further decorated with one or more monosaccharides. Sphingolipids with inositol phosphate-containing head groups are also common in protozoans (192). The main phosphophingolipid in mammals and nematodes, SM, carries a phosphocholine (12, 55). Mammals also produce small quantities of ethanolaminephosphorylceramide (213). The latter is the principal phosphosphingolipid found in insects (304, 414). Tremendous diversity exists among the carbohydrate head groups of glycosphingolipids (Fig. 3), which may contain as many as 30 glycosyl residues per lipid. A glycosphingolipid is termed ganglioside if one or more of its sugar residues is a sialic acid. Gangliosides are particularly abundant in the central nervous system of higher organisms (240). Whereas sphingolipids display a striking organism- and cell type-dependent variation in head group composition, this is generally not the case for the glycerolipids. In most eukaryotic cells, glycerolipids utilize phosphate ester-linked choline, serine, ethanolamine, and inositol to achieve diversity.

One additional feature that makes sphingolipids different from glycerolipids is their fatty acyl chain. Typically, the amide-linked fatty acyl chains in sphingolipids are long and saturated. Often they are hydroxylated at the α-position. In mammals, SM typically consists of two types, roughly half containing C16:0 and C18:0 and the other half C22:0, C24:0, and C24:1 (12, 156). The glycosphingolipids have similar fatty acids, but, depending on the tissue, a large fraction (up to 70%) may consist of the α-hydroxylated form of each of these fatty acids (28, 268). The acyl chains in the sn-2 position of mammalian glycerophospholipids, on the other hand, are mostly shorter, (poly)unsaturated (e.g., C18:1, C18:2, or C20:4) and not hydroxylated. As illustrated in Figure 1, this striking difference in length, saturation, and hydroxylation status between fatty acyl chains of sphingolipids and glycerolipids has also been observed in Caenorhabditis elegans (55), Drosophila (304), and yeast. In yeast sphingolipids, C26:0 and hydroxylated C26:0 are the major fatty acids (192, 411). Interestingly, the prevalence of monounsaturated species of aminophospholipids at the expense of diunsaturated species in the plasma membrane of wild-type yeast is reversed in elo3 mutant cells that accumulate C22:0 fatty acid-containing sphingolipids instead of the normal C26:0 fatty acid-containing ones (269, 325). This observation indicates that the structural differences between the fatty acid chains of sphingolipids and glycerolipids serve important biological functions.

We conclude that, despite the considerable variation in chemical composition between lipids of distinct organisms, the structural features by which sphingolipids can be distinguished from glycerolipids can be discriminated from glycerolipids have been preserved from vertebrates down to flies, worms, and fungi. Moreover, when these differences are undermined by muta-

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3 Skin contains sphingolipids carrying very-long-chain (C28-C34) fatty acids that are hydroxylated at the α-position. After having been esterified to linoleic acid and hydrolyzed, the α-hydroxy group serves to couple the sphingolipid, first glucosylceramide and later ceramide, to proteins on the cell surface (79, 412) where they are essential for the permeability barrier of the skin (17).
tion, organisms will exploit lipid-remodeling mechanisms to recreate structural diversity (193, 325). This presents us with the question, What would be the functional significance of these structural differences for eukaryotic life?

B. Biophysical Differences Between Sphingolipids and Glycerolipids

The interfacial region that links the nonpolar hydrocarbon region to the polar headgroup provides sphingolipids and glycerolipids with different biophysical properties. In sphingolipids, this region contains the 2-amide and 3-hydroxy groups, often supplemented with additional hydroxyls at the sphingoid C4 and fatty acid C2, and with further hydroxyls on the carbohydrates. These groups can function both as hydrogen bond donors and acceptors and are thought to participate in extensive inter- and intramolecular hydrogen bonding of the sphingolipids, whereas glycerolipids have only hydrogen bond-accepting properties in this part of the molecule (278). This property allows sphingolipids but not glycerolipids to associate with themselves in the plane of the membrane into a highly flexible hydrogen-bonded network (23; see sect. ivf). In membranes exposed to physical and chemical stress as in the apical membranes in kidney and intestine and in microorganisms, the sphingolipids exhibit an increased number of hydroxyl groups. This property is believed to improve the stability and decrease the permeability of those surface membranes (278). Although this may be generally true, under certain conditions the loss of a specific hydroxyl was found to increase membrane stability in yeast (82).

The lipid tails of natural sphingolipids are more tightly packed than those of the most abundant phosphoglycerolipid, monounsaturated PC. The surface area of GlcCer and GalCer at a surface pressure of 30 mN/m, typical of plasma membranes, is 0.40 nm² (212), versus 0.63 nm² for stearoyl-oleoyl PC (71). The fatty chains of sphingolipids are therefore more extended, and consequently the thinner and taller sphingolipids form up to 30% thicker membranes than unsaturated phospholipids.

Second, sphingolipids contact their neighbors along a greater and flatter surface, which results in a dramatic increase in the van der Waals attraction between neighboring sphingolipid molecules. Van der Waals interactions have also been held responsible for the strong binding between the sphingolipids and cholesterol with its rigid and flat-cylindrical steroid backbone (235). A preferential binding of cholesterol to sphingolipids, in particular to SM, has been observed in many biophysical studies (23). Interestingly, the surface areas of disaturated PC (0.41–0.44 nm²; Refs. 71, 212) and PS (0.40–0.44 nm²; Ref. 72) are close to those of sphingolipids, and a preferential interaction of cholesterol with diC16:0 PC (less than or equal to that with SM; Refs. 181, 208) over mono-unsaturated PC has been observed (208). This illustrates the possibility that disaturated phospholipids may resemble sphingolipids in their hydrophobic interactions with other membrane components. This is especially relevant for the cholesterol-rich plasma membrane, where 40% of the PC and 75% of the PS are disaturated, while disaturated species, sphingolipids, and cholesterol are virtually absent from the ER (160).

The sphingoid chain extends less far into the membrane core than the N-linked fatty acid, a disparity not found in glycerolipids (Fig. 1). It has been argued that the difference in length between the two chains leads to packing defects (voids) in the membrane interior and that these defects may be complemented via interdigitation of two sphingolipids in the opposed bilayer leaflets (23, 44, 324). This may be an example of how lipids in one bilayer leaflet may couple to lipids in the opposite leaflet (see sect. vif). Interestingly, also cholesterol molecules in the two opposed bilayer leaflets may interact by forming transmembrane cholesterol dimers (129, 205, 250).

IV. SPHINGOLIPIDS AND THE LATERAL ORGANIZATION OF BIOLOGICAL MEMBRANES

A. Ordered Sphingolipid Domains in Model Membranes

At low temperatures, bilayers of a single phospholipid or sphingolipid exist in a frozen state. Above a melting temperature \( T_m \) characteristic of each lipid, the bilayer enters a phase in which the lipid acyl chains are fluid and disordered (50). The frozen state is referred to as the gel, solid-ordered \( (s_o) \), or \( L_o \) phase. The fluid phase is

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4 The distance between the head groups in a double layer of C18:0 SM in the presence of cholesterol was reported to be 46–48 Å (232, 300), whereas this was 40 Å for C16:0-C18:1 PC in the presence and 35 Å in the absence of cholesterol (202, 300).

5 The \( cis \) double bond in C24:1, which in its 2-hydroxylated form or its nonhydroxylated form is found in a significant fraction (up to 40%) of the sphingolipids in mammalian myelin (89) and intestine (28), is located at C15, deep in the bilayer, where it does not disrupt the close packing arrangement of the sphingolipids (204) and the sphingolipid-cholesterol interaction (296). This contrasts with the C9 \( cis \) double bond in the C2 fatty acids of the glycerolipids.
termined the liquid-crystalline, liquid disordered (l\textsubscript{o}), or l\textsubscript{d} phase (173, 319). Sphingolipids (especially glycosphingolipids) display a much higher T\textsubscript{m} than glycerolipids, due to their denser packing, which in turn is due to the saturation of their fatty chains and to the fact that they are more prone to intermolecular hydrogen bonding. While the phase transition for the typical mono-unsaturated PCs C16:0-C18:1 PC and C18:0-C18:1 PC occurs at 3 and 7°C, this temperature is 42°C for diC16:0 PC, 45°C for C18:0 SM, but 85°C for C18:0 GlcCer and GalCer (see Refs. 172, 173). Studies with model membranes consisting of binary mixtures of lipids with different T\textsubscript{m} revealed that at temperatures between the T\textsubscript{m} of the two lipids, a cooperative phase separation can occur, resulting in the coexistence of gel and l\textsubscript{d} phase. The physiological significance of this type of phase separation in eukaryotes is questionable, mainly because membranes that contain high amounts of lipids with T\textsubscript{m} values at or above room temperature, like the plasma membrane (160), also contain high levels of cholesterol. Cholesterol at concentrations of 30–50 mol\% abolishes the gel-liquid crystalline phase transition (179, 319).

Remarkably, a phase separation between two fluid phases has been described in model membranes containing binary mixtures of a high-T\textsubscript{m} lipid (saturated-chain lipid) and cholesterol (299). At temperatures above the T\textsubscript{m} of the lipid, with increasing cholesterol concentrations a liquid-ordered (l\textsubscript{o}) phase can separate from the liquid-disordered (l\textsubscript{d}) phase. Above a threshold concentration of cholesterol, only the l\textsubscript{o} phase exists, independent of the temperature. For diC16:0 PC, this occurs at 30 mol\% cholesterol (319). Acyl chains of lipids in the l\textsubscript{o} phase have properties that are intermediate between those of the gel and l\textsubscript{d} phases; they are extended and ordered as in the gel phase but are laterally mobile in the bilayer as in the l\textsubscript{d} phase. Also in bilayers of more complex composition the coexistence of l\textsubscript{o} and l\textsubscript{d} phases depends on the cholesterol concentration (reviewed in detail by Refs. 37, 40), and three fluid phases can coexist, of which two are l\textsubscript{o} phases (294). The phase separation is especially pronounced when the T\textsubscript{m} of the lipids is greatly different, like in the case of PCs of different chain length (338). Disaturated phospholipids and sphingolipids would preferentially partition into the l\textsubscript{o} phase, which as a consequence would contain more cholesterol, whereas (poly)unsaturated lipids would prefer the l\textsubscript{d} phase. Using a fluorescence quenching assay, Ahmed et al. (1) found that cholesterol induces the formation of a SM-enriched l\textsubscript{o} phase at 37°C in a mixture of SM and monounsaturated PC. Mixtures of GlcCer, GalCer, and monounsaturated PC were found to be significantly inhomogeneous at 37°C, regardless of whether cholesterol was absent or present at concentrations at which the lateral separation in mixtures of SM and PC is abolished. Hence, glycosphingolipids appear to have a stronger tendency than SM to segregate from glycerolipids into an l\textsubscript{o} phase (337).\

Ceramides at low concentrations occur as single monomers in a membrane, whereas at high concentrations they partition into hydrophobic ceramide droplets in between the two leaflets of the bilayer (404). At intermediate concentrations (~10 mol\%, Ref. 141), the ceramides can aggregate to form a ceramide domain. The higher the affinity between ceramides, e.g., long acyl chain containing hydroxylated ceramides of yeast versus short acyl chain and sphingosine containing ceramides of mammals (204), the lower the ceramide concentration needed for segregation.

In a membrane with coexisting l\textsubscript{o} and l\textsubscript{d} phases, the distribution of each lipid species over the two phases is not governed solely by its relative affinity for the lipids in either phase. A different energy parameter is the long-range order of the lipids. According to the superlattice view (350, 372), lipids tend to be regularly distributed. Cholesterol with its different shape and smaller surface area imposes a steric strain on the alkyl chain matrix. To minimize the strain, cholesterol will adopt a regular lateral distribution, which yields a so-called superlattice. In a particular phospholipid mixture, only a defined set of cholesterol concentrations fits a superlattice, and this has been experimentally confirmed (349). At any given cholesterol concentration, a membrane would thus consist of domains displaying one of the allowed, minimum-energy superlattice organizations. A particular lipid will be arranged according to some superlattice pattern whether it is present in an l\textsubscript{o} or in an l\textsubscript{d} phase. Still, individual molecules will rapidly exchange positions, whereby the rate of exchange, and thus lateral diffusion, will be higher in the l\textsubscript{d} phase. The degree to which each lipid type fits the superlattice of one phase will contribute to its partitioning between the two phases.

B. Evidence for the Existence of Ordered Sphingolipid Domains in Cellular Membranes

An overwhelming number of studies in artificial bilayers have demonstrated that lipids have a strong self-organizing capacity; lipid immiscibility can drive phase separation and give rise to domains (used in the meaning of “environments”) with unique lipid compositions and biophysical properties. The current evidence does support the existence of such phase-separated lipid domains.

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6 Phase separation will only occur above a critical concentration of a lipid in the membrane. In analogy to the aggregation of detergent into micelles above the critical micellar concentration (CMC), the concentration above which a lipid aggregates in an environment of other lipids may be called the critical domain concentration (CDC). Just as the CMC depends on the molecular characteristics of the detergent, the CDC depends on the molecular characteristics of the aggregating lipid. The CDC also depends on the lipid matrix within which the aggregate forms. Furthermore, it depends on the presence of similarly aggregating lipids, just like detergents in water coaggregate with other detergents.
in cellular membranes. However, many uncertainties remain concerning their composition, size, and dynamics and concerning their location. In which of the subcellular membranes do they occur? Do they exist on both sides of the membrane, and if so, do domains on the one surface colocalize with domains on the opposite surface? Are domains the cause of variations in biomembrane thickness? Finally, a most relevant question: by what molecular interactions can membrane proteins discriminate between different lipid environments (69), and what is the contribution of membrane proteins to the properties of the domains?

1. Early evidence for lipid lateral heterogeneity

The fluid-mosaic models of biomembranes in which proteins freely float around in an oily lipid bilayer (148, 343) marked a breakthrough in the thinking about membrane structure and function. However, already at that time, evidence became available suggesting that lipids and proteins were not randomly distributed in the membrane. For the lipids, phase separations had been demonstrated in the plasma membrane of Acholeplasma by calorimetry (383) and by freeze-fracture electron microscopy (403). Electron spin resonance studies suggested lipid phase heterogeneity in the ER (234, 364), and subsequently, a variety of techniques suggested the same for the plasma membrane of mammalian cells (157). Ideas on long-range order in the organization of lipids and proteins and the occurrence of microdomains were then formulated (148).

2. From macrodomain in epithelial plasma membrane to microdomain in the trans-Golgi network

A unique type of sphingolipid (macro)domain organization exists in the plasma membrane of epithelial cells, where originally an accumulation of glycosphingolipids was reported for the apical plasma membrane domain of intestinal epithelial cells (92). Later work (30, 81, 159) demonstrated a two- to fourfold enrichment of glycosphingolipids in the apical versus the basolateral domain of the continuous plasma membrane of these cells. Similar differences have been reported for urinary bladder epithelium (369) and, indirectly, for Madin-Darby canine kidney (MDCK) cells derived from the distal nephron (341). Apical membranes from kidney cortex, which contain low levels of glycosphingolipids (358), contained an exceptionally high level of SM (35% compared with 13% in basolateral membranes; Refs. 48, 137, 247, 330, 402). These differences reside in the outer leaflet of the plasma membrane where they are maintained by the presence of a barrier to lipid diffusion, the tight junctions (reviewed in Ref. 341). Similar surface domains have been proposed to exist in sperm (reviewed in Refs. 14, 102), and a lipid-phase separation has been held responsible for their maintenance. Based on sphingolipid transport experiments (398), it was subsequently postulated that the sphingolipid-rich apical microdomains originate from sphingolipid- and cholesterol-enriched microdomains in trans-Golgi membranes (339, 341, 395). These would then act as a sorting device for the delivery of apically directed cell surface components in polarized epithelial cells (see sect. vi).

3. Resistance to detergent extraction

The first candidate proteins of sphingolipid membrane domains were the proteins that are anchored in the noncytoplasmic bilayer leaflet of apical membranes by a GPI anchor (201). These proteins were found to be targeted to the apical cell surface, obviously via some interaction in the luminal leaflet of the Golgi membrane (34, 200). At the same time it was observed that influenza virus hemagglutinin, which is targeted to the apical membrane of infected epithelial cells, became partially resistant to extraction by Triton X-100 (TX-100) in the cold during transport to the surface (344), and it was proposed that the protein may complex with glycosphingolipids, as glycosphingolipids had been shown to be TX-100 insoluble as well. Progress in the field was tremendously accelerated when it was realized that not only hemagglutinin and sphingolipids but also the GPI proteins show resistance to extraction by detergent at low temperature, that this behavior might reflect the presence of these components in GPI protein/sphingolipid microdomains in the membrane, and that detergent insolubility might be an experimental tool to study the domains (38). The method consisted of the addition of 1% TX-100 to cells on ice for 20 min, homogenization of the cells, addition of sucrose to 40%, and flotation in a 30%-5% linear sucrose gradient without detergent. A GPI protein was found to float to a density of 1,081 g/ml, and closer inspection showed that the GPI protein was present in closed membrane vesicles that were now resistant to TX-100 (detergent-resistant mem-

7 Although GM1 was found to be exclusively localized in axons of hippocampal neurons in culture (188), this was not confirmed in independent studies in the same system (335). Also, the presence of a lipid diffusion barrier between axon and dendrites (165) has been contested (99).

8 With an SM content of 8–12% in the total Golgi (90, 160) and with all SM in the luminal leaflet, 16–24% of that surface might be covered by a sphingolipid microdomain. The expected enrichment of SM in the TGN to plasma membrane levels (16–19%), the presence of cholesterol, and participation of disaturated PC (9% of total Golgi lipids; Ref. 160) increases the relative area of the TGN luminal surface covered by the microdomain to numbers above 40%. In this sense, the term microdomain is misleading.

9 A density of 1,081 g/ml equals 20% sucrose (wt/vol), 21.6% sucrose (wt/vol), or 0.63 M sucrose.
branes; DRMs). The low-density vesicles contained some 10% of the total cellular lipids: all of the SM, 50% of the glycosphingolipids, 25% of the cellular cholesterol, but only 5% of the glycerolipids (38). Subsequent work on model membranes has suggested that SM is detergent insoluble only when it was present in an \( l_0 \) liquid-ordered phase before the addition of the detergent (1), supporting the idea that DRMs are derived from preexisting \( l_0 \) phase domains. The power of the DRM isolation method to study the structure and function of domains was demonstrated by the finding in the original paper (38) that the GPI proteins became detergent insoluble at 20 min after synthesis, which suggested that the proteins became insoluble upon entering the Golgi where they are thought to come into contact with the sphingolipids (see sect. vi). The method has been applied to identify proteins involved in the transport pathway from the trans-Golgi network (TGN) to the apical domain (180, 304). Evidence has been reported for the coexistence in the same membrane of domains with different (GPI) protein compositions and different physical properties (211, 307). The same was reported for myelin where TX-100 and CHAPS yielded DRMs of different protein compositions (342). SM-enriched DRMs have been isolated from nuclei, where they were assumed to represent the inner nuclear membrane (18).

DRMs have been isolated from the plasma membrane, suggesting the existence of phase-separated sphingolipid/cholesterol domains on the cell surface where they would compartmentalize, modulate, and integrate signaling events by providing sites for the assembly of components involved in signal transduction (202, 340). Exciting new developments in the field are the regulation, by ligand binding, of the association with the domain of signaling receptors and of the Src family kinases that transduce the signals into the cell. The latter kinases are anchored to the cytoplasmic bilayer leaflet by two saturated acyl chains, whereas in contrast proteins anchored by the branched isoprenyl groups are not recovered in DRMs (237). This supports the idea that an ordered lipid environment is present on the cytoplasmic surface of the sphingolipid rafts. Some of the DRMs from the plasma membrane were derived from caveolae, morphologically well-established flasklike invaginations of the plasma membrane (7, 37). Caveolae are defined by the presence on the cytosolic surface of the palmitoylated, cholesterol-binding protein caveolin (7, 256). Multiple palmitoylation of caveolin was found to be required for its interaction with dually acylated G proteins (103), but not for binding to caveolae (78). Caveolin (VIP21) was also localized to the Golgi (177, 207), where it is supposed to be involved in membrane transport. DRMs have been isolated from many different mammalian cell types, but also from Dictyostelium (304), Dicytostelium (419), yeast (11, 174), and protozoans (430).

Obviously, the detergent extraction method has its limitations. It cannot be concluded that the organization of the lipids (and proteins) in the resulting DRMs reflects their organization in the cellular membrane of origin in full detail, because some rearrangement may have occurred as a consequence of low temperature, detergent addition, and detergent dilution. Low temperature favors formation of ordered domains, while detergent will insert into the membrane and influence the partitioning of the various lipids. Notably, the size of the original sphingolipid domains cannot be assessed by detergent extraction, because separate domains within the same membrane may coalesce due to removal of the surrounding glycerolipids. Because the bulk of the sphingolipids has been localized to the noncytoplasmic surface of cellular membranes (see sect. vB), one other uncertainty is the composition of the lipids on the cytosolic surface facing the sphingolipid domain and their behavior during detergent extraction (discussed in detail in Ref. 36). Various groups have published methods to isolate caveolae without the use of detergent (326, 348, 351, 361). Whereas three groups set out by isolating plasma membranes, the fourth group (351) followed the detergent extraction method but without detergent. Cells were homogenized and sonicated, and the membranes were floated up in a sucrose gradient. Membranes at the 35%/5% sucrose interface were separated from membranes at the 45/35% sucrose interface and were designated "purified caveolae membranes." It will be interesting to see how these protocols will complement the DRM method.

11 Although also hemagglutinin became detergent insoluble after 20 min (344), this may have a different reason. The insolubility of hemagglutinin requires its triple palmitoylation (sites), and the organelle where palmitoylation takes place has not been established (237). So insolubility 20 min after synthesis might be due either to contact with sphingolipids in the Golgi or to palmitoylation in the Golgi.

12 However, 60–80% of the GalCer and cholesterol in myelin were resistant to each detergent, showing that at least part of the GalCer was resistant to both detergents. One possible explanation in that there were three different domains: one domain containing TX-100-resistant proteins, one containing CHAPS-resistant proteins, and a third domain resistant to both detergents and containing some 40% of the GalCer. Alternatively, there was only one type of domain, but TX-100 and CHAPS selectively extracted certain proteins.

13 According to common cell fractionation experience, the 35%/5% sucrose interface should contain most of the Golgi, early endosomes, and plasma membrane. The fraction at the 45%/35% sucrose interface typically contains ER and lysosomes, while the alkaline carbonate used for homogenization should cause a large part of the ER to float up to the 35%/5% interface as well. Without any determination of the flotation of these membranes, the use of the term purified caveolae membranes is optimistic, if not naive.
4. Microscopy on fixed cells

Independent evidence for clustering of glycosphingolipids on the cell surface has been obtained by microscopy. A first approach has utilized glycosphingolipid-binding proteins, which were visualized by a fluorescent or electron-dense tag or by a secondary labeled protein. The major problem in such studies is that lipids cannot be fixed at their original location. Artificial clustering was induced when the binding of a primary antibody to globoside (Gb₄Cer) or Forssman glycosphingolipid (IV²⁻α-GalNAc-Gb₄Cer) was followed by labeling with a dimeric secondary antibody, tetrameric protein A, or when multimeric complexes were used of the primary or secondary ligand to ferritin or colloidal gold (47, 96, 377). Clustering of the ganglioside GM1 was observed when it was labeled with pentameric cholera toxin B subunit by itself (9) or conjugated with gold into a multimeric complex (276), or when a biotinylated GM1 was labeled with anti-biotin-gold (246). The latter studies were performed on freeze-substituted samples in which redistribution seems rather unlikely. In an independent approach, labeling of glycosphingolipids with a primary antibody was followed by fixation before the addition of the secondary antibody-gold complex, a condition that had been shown to prevent redistribution of Forssman glycosphingolipid (47, 96). Clusters of GM3 were still observed in one study (354), while clustering of a number of sphingolipids in caveolae was no longer observed under these stringent conditions (96). Still, a local enrichment of the ganglioside GM1 in caveolae was found by a postembedding labeling protocol using cholera toxin where redistribution could be excluded (276). In cells transfected with influenza virus hemagglutinin or GPI proteins, the distribution of these proteins overlapped with that of GM1 (128). Clustering of GPI proteins has been observed by microscopy under a variety of conditions (7). However, in many cases clustering was induced by the protocol used. A major point of concern in most microscopic studies on lipid and lipid-linked molecules is the use of multimeric reporter ligands without proper controls.

5. Optical studies on living cells

Already in the early 1980s, measurements on the behavior of fluorescent probes in biomembranes supported the concept of lipid domains in membranes (157), and microscopy was performed on living cells using fluorescent lipids (357). In the latter study, the redistribution of GM1 by cholera toxin caused cocapping of the unrelated ganglioside GM3, which suggested that the headgroup-labeled GM1 and GM3 were associated by lipid-lipid interactions. Much more recently, a major breakthrough in the field has been the application to living cells of novel high-resolution optical techniques, like resonance energy transfer between fluorescent membrane molecules, single particle tracking, two-dimension scanning resistance, and single dye tracing. A number of these studies support the existence of locations on the cell surface that are enriched in GPI proteins and glycosphingolipids, and, in addition, of areas with enhanced resistance to lateral diffusion that are preferred by some but not by other probes. One controversial issue is the size of the domains. What is their diameter? Whereas the detergent-extraction studies yielded DRM vesicles with a diameter of 0.1–1 μm (39), suggesting a diameter size of 200–2,000 nm, this was a few hundred nanometers for the small regions to which a GPI protein and GM1 were found to be confined in particle tracking studies (290, 331). The newest single particle tracking measurements on these domains suggest that they are even smaller with a diameter of 50 nm (roughly 3,500 lipids), exist for more than 1 min, and comprise <50 proteins (289). Chemical cross-linking and fluorescence resonance energy transfer to measure GPI-protein interactions led to estimates of 70 nm (94, 401). In contrast to these data, no clustering of a GPI protein was observed on the apical surface of MDCK cells (162). In addition, a comparison between various GPI proteins on various cell surfaces did not provide evidence for the occurrence of a sizeable fraction of the GPI proteins as stable clusters (163). The authors explained the discrepancies with the earlier work, by concluding that lipid rafts either exist only as transiently stabilized structures or, if stable, comprise at most a minor fraction of the cell surface.

Here, it becomes relevant to discuss the area of the membrane covered by rafts. SM constitutes ~20% of the plasma membrane phospholipids. If, as generally believed, SM is located in the outer leaflet, it covers 40% of the surface. When saturated phospholipids and chole-

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14 When sections of freeze-substituted A431 cells were labeled with cholera toxin B-gold, clusters were observed in uncoated invaginations of the plasma membrane. It seems unlikely that clustering was induced by the multimeric ligand as lipids most likely do not diffuse on the surface of the section which in this case consists of Lowicryl polymer. This is different in frozen sections, where during the labeling of the thawed sections lipids are free to diffuse in the membranes. In the freeze-substituted cells, GM1 was also found in small vesicles in the cytosol in close approximation with the patches on the plasma membrane. Because the vesicle profiles were not in contact with the plasma membrane during the labeling of the section, a higher concentration of GM1 in these vesicles must have been present already in the living cell. In the cell, the vesicle membranes are thought to be continuous with the plasma membrane or to be derived from the plasma membrane by endocytosis. In both cases, the results demonstrate that GM1 is enriched in subdomains of the plasma membrane of these A431 cells.

15 It should be noted that each gold particle possessed many binding sites against the GPI protein (antibodies conjugated to gold) and to GM1 (cholera toxin B subunits conjugated to gold). In the case of the GPI protein, the number of binding sites did not affect the results. Concerning GM1, cholera toxin has been shown to reduce its solubility in detergent, which implies that binding of the pentavalent toxin changes the phase behavior of GM1 (117).
terol are added to this figure, one would expect most of the plasma membrane to be covered by a liquid-ordered domain. In a special case, the apical surface of epithelial cells of kidney and intestine, the surface is completely covered by sphingolipids (341). This supports the idea that the apical membrane of kidney cells, like MDCK, is one big raft (162). Clustering on apical surfaces may therefore not occur (162). When it occurs, it may imply that there are different types of rafts (211, 307, 342). Interestingly, when beads attached to a GPI protein (major histocompatibility complex class I) were scanned over the surface of hepatoma cells by laser tweezers, the beads experienced areas of increased resistance of 100 nm diameter, that were independent of the actin skeleton and covered an area of <10% of the surface, which is similar to caveolae (370). The physicochemical basis of the existence of multiple types of rafts remains to be resolved but may involve cholesterol-induced domains (294) and caveolae (7), both of which are very stable. In contrast to measurements on beads attached to diC16:0 phosphatidylethanolamine (PE) where no indications for areas of higher resistance were observed (370), in tracings of single fluorescent molecules on muscle cells, the lipid analog Cy5-diC14:0-PE, but not Cy5-diC18:1-PE (which were assumed to represent lipids preferring areas of lower and higher fluidity, respectively), was 100-fold enriched in domains of ~0.7 μm, that were stable for several minutes (329). No ultrastructural correlate for such domains has been found.

In line with the model of GPI protein sorting in epithelial cells via sphingolipid domains (201, 341), a GPI protein was found by various techniques to be clustered during transport to the apical MDCK cell surface, after which it diffused over the apical surface (125). However, in contrast to inhibition of sphingolipid synthesis (233), cholesterol depletion did not change polarity of cell surface delivery of the GPI protein (124). Cholesterol depletion did inhibit transport of hemagglutinin to the apical face delivery of the GPI protein (124). Cholesterol depletion did not change polarity of cell surface delivery of the GPI protein (124). Cholesterol depletion did inhibit transport of hemagglutinin to the apical face. The product of SPT, 3-ketosphinganine, is reduced to sphinganine (also called dihydrosphingosine), which in yeast requires the TSC10 gene product (15). Reactions up to the formation of sphinganine appear to be the same from mammals down to yeast. Sphinganine in mammals is N-acylated by ceramide synthase to form dihydroceramide, which is rapidly desaturated to give ceramide; a 4,5-trans-double bond is introduced in the sphinganine moiety to form ceramide containing sphingosine as its long-chain base (241). In yeast, but probably also in mammals, sphinganine is hydroxylated to form 4-hydroxy-sphinganine, commonly named phytosphingosine. This reaction requires the SYR2 gene product and, unlike earlier enzymatic steps in the biosynthetic pathway, is not essential for cell growth in yeast (116). Amidic linkage of a fatty acid to phytosphingosine yields phytoceramide, the ceramide found in most fungal and plant sphingolipids and abundant in some mammalian tissues (see sect. m4). Ceramide synthesis is believed to take place on the cytosolic face of the ER (220). In mammals, this reaction can be inhibited by fumonisins (405), while australifungin effectively blocks ceramide synthesis in yeast (216). So far, no single acyl CoA-dependent ceramide synthase has been purified or cloned. However, the alkaline ceramidases encoded by the yeast genes YPC1 and YDC1 possess a reverse, acyl CoA-independent ceramide synthase activity that is insensitive to classical ceramide synthesis inhibitors (222, 223). The very-long-chain (20+ carbon) fatty acids found in (phyto)ceramides are formed by ER membrane-bound fatty acid elongation systems whose components belong to a evolutionary conserved family of transmembrane proteins (269, 382, 383). Disruption of the corresponding genes in yeast reduces the cellular sphingolipid levels (269) and induces pleiotropic phenotypes such as bud site localization defects, changes in plasma membrane H+-ATPase levels, and resistance to sterol synthesis inhibitors (104, 303). The fatty acid moiety in ceramides can be mono- or dihydroxylated. In yeast, the first hydroxylation step occurs in the ER and requires SCS7, a gene structurally related to the SUR2 gene (116). The second hydroxylation takes place in the Golgi and requires the Golgi copper transporter encoded by the CCC2 gene (16).

V. SPHINGOLIPID ASSEMBLY AND TRANSPORT

A. Enzymes of Sphingolipid Metabolism and Their Topology

1. Ceramide synthesis

Sphingolipid synthesis in all eukaryotes starts in the ER with the condensation of L-serine with palmitoyl coenzyme A, a reaction catalyzed by serine palmitoyl-transferase (SPT) and yielding 3-ketosphinganine (Fig. 2). SPT forms the target of several potent natural inhibitors that include sphingofungin (436), lipoxamycin (214), myriocin (245), and viridiofungins (217). Genetic studies in yeast revealed that at least two genes, LCB1 and LCB2, are required for SPT activity, suggesting that the enzyme is composed of at least two distinct proteins (43, 258). This was confirmed when the proteins encoded by mammalian homologs of these genes were characterized and found to be part of a functional enzyme complex (121, 122). The product of SPT, 3-ketosphinganine, is reduced to sphinganine (also called dihydrosphingosine), which in yeast requires the TSC10 gene product (15). Reactions up to the formation of sphinganine appear to be the same from mammals down to yeast. Sphinganine in mammals is N-acylated by ceramide synthase to form dihydroceramide, which is rapidly desaturated to give ceramide; a 4,5-trans-double bond is introduced in the sphinganine moiety to form ceramide containing sphingosine as its long-chain base (241). In yeast, but probably also in mammals, sphinganine is hydroxylated to form 4-hydroxy-sphinganine, commonly named phytosphingosine. This reaction requires the SYR2 gene product and, unlike earlier enzymatic steps in the biosynthetic pathway, is not essential for cell growth in yeast (116). Amidic linkage of a fatty acid to phytosphingosine yields phytoceramide, the ceramide found in most fungal and plant sphingolipids and abundant in some mammalian tissues (see sect. m4). Ceramide synthesis is believed to take place on the cytosolic face of the ER (220). In mammals, this reaction can be inhibited by fumonisins (405), while australifungin effectively blocks ceramide synthesis in yeast (216). So far, no single acyl CoA-dependent ceramide synthase has been purified or cloned. However, the alkaline ceramidases encoded by the yeast genes YPC1 and YDC1 possess a reverse, acyl CoA-independent ceramide synthase activity that is insensitive to classical ceramide synthesis inhibitors (222, 223). The very-long-chain (20+ carbon) fatty acids found in (phyto)ceramides are formed by ER membrane-bound fatty acid elongation systems whose components belong to a evolutionary conserved family of transmembrane proteins (269, 382, 383). Disruption of the corresponding genes in yeast reduces the cellular sphingolipid levels (269) and induces pleiotropic phenotypes such as bud site localization defects, changes in plasma membrane H+-ATPase levels, and resistance to sterol synthesis inhibitors (104, 303). The fatty acid moiety in ceramides can be mono- or dihydroxylated. In yeast, the first hydroxylation step occurs in the ER and requires SCS7, a gene structurally related to the SUR2 gene (116). The second hydroxylation takes place in the Golgi and requires the Golgi copper transporter encoded by the CCC2 gene (16).
medial Golgi and involves the transfer of phosphocholine from PC to ceramide, yielding diacylglycerol as a (potentially important) side product (101, 150). A second SM synthase activity has been located on the cell surface (392). Two SM synthases with different properties have also been found in the intra-erythrocyte stage of _Plasmodium_ (120). The SM synthases remain to be identified. The ceramide:glucosyltransferase involved in GlcCer production is located on the cytosolic surface of the early Golgi (45, 65, 100, 151, 224). Whereas the GlcCer synthase in mammals is encoded by a single gene, three GlcCer synthase analogs have been identified in the _C. elegans_ genome (144). The physiological significance of this multiplicity remains to be solved. By an unknown mechanism, GlcCer is translocated to the Golgi lumen where it can be trapped by galactosylation to Galβ1–4GlcCer (LacCer; Refs. 45, 183, 267). Various series of complex glycosphingolipids can then be generated by stepwise addition of sugars to LacCer, whereby the first sugar and its glycosidic linkage determine the name of the series (53), e.g., Galα1–4: globo- or Gb, Galα1–3: isoglobo or IgB, GlcNAcβ1–3: lacto or Lc (Fig. 3). The ganglio series (or Gg) is based on GalNAcβ1–4LacCer. The ganglio series also comprises the simple gangliosides NeuAcα1–3:LacCer, well-known under the Svennerholm nomenclature as GM3, GD3, and GT3, respectively (53). Glycosphingolipids in the various series may be fucosylated or sulfated. LacCer synthesis and all further conversions occur in the lumen of the Golgi and require import of the necessary sugar nucleotides (45, 183; reviewed in Ref. 209). With the use of subcellular fractionation, a sequential distribution over the Golgi was observed for glycosphingolipid glycosyltransferases (143, 380, 381), with a significant overlap in distributions. A later paper (184) assigned the synthesis of LacCer, GM3, and GM2 to the trans-Golgi and the TGN, whereby a considerable fraction of the LacCer and GM3 synthases localized to the cis-Golgi. Pharmacological studies, using the drugs brefeldin A (308, 333, 384, 427) and monensin (244, 316, 317, 385) to discriminate enzymes of the Golgi stack from those in the TGN, localized GM2 synthase (GalNAc-transferase) and two galactosyltransferases of complex glycosphingolipid synthesis to the TGN (see, however, Ref. 426). The same conclusion was reached in a study on mitotic cells (62).

Arthropods and mollusks transfer a mannose onto GlcCer and further extend this chain in the arthro (At) or the mollu (Mu) series. In some mammalian tissues, notably myelin, but in humans also the epithelia of the gastrointestinal and urogenital tracts, ceramide is mainly glycosylated to GalCer by the ceramide:galactosyltransferase, an enzyme situated on the luminal face of the ER (328, 359, 360). The enzyme displays a preference for ceramides containing a 2-hydroxylated fatty acid which are abundant in these tissues. GalCer can be further galactosylated and/or sulfated (or sialylated) in the Golgi lumen (45, 183, 373).

3. _Synthesis of inositol sphingolipids_.

In contrast to animals, all fungi and plants studied so far, as well as several protozoa, add inositol phosphate to phytoceramide to form IPC (192). The biosynthetic route of inositol sphingolipids in yeast has been studied in great detail. Yeast produces only three types of inositol sphingolipids: IPC, mannosyl α1–2IPC; MIPC, and inositol-1-P-6 mannosyl α1–2IPC or mannoinositol-P₂-ceramide: M(IP)₂C. The IPC synthase, or an essential subunit thereof, is encoded by the _AUR1_ gene (259). _AUR1_ homologs have been identified in a wide variety of fungi (132, 176). IPC activity is effectively blocked by the antifungal agents aureobasidin A (259), khaferfungin (219), and rustumycin (218). Because IPC synthase activity is essential and not found in mammals, it provides an ideal target for therapeutic drugs to fight pathogenic fungi in immunocompromised individuals. IPC synthesis in yeast was previously thought to occur in the ER. This idea was based on the fact that the formation of IPC from ER-derived ceramide and PI continues under conditions when ER-to-Golgi vesicular transport is blocked in temperature-sensitive secretion (sec) mutants (292). However, fluorescence microscopy and membrane fractionation experiments have recently shown that both the _AUR1_ gene product and IPC synthase activity are located in the Golgi (195). This discrepancy in results remains to be clarified but could be explained if the IPC synthase would constitutively cycle between the ER and the Golgi, a feature inherent of several Golgi-based proteins (60, 146, 210). An alternative explanation could be that ceramide made in the ER can reach the Golgi by a vesicle-independent transport mechanism (see sect. vc).

Whereas the sidedness of IPC production is unknown, mannosylation to form MIPC most likely occurs in the lumen of the Golgi. The latter reaction requires at least three genes: _SUR1, VRG4_, and _CSG2_. _SUR1_ most likely encodes a mannosyltransferase (16), whereas _VRG4_ is required for GDP-mannose transport into the Golgi lumen (68). _CSG2_ encodes a member of the major facilitator superfamily, and its role in MIPC production is unclear (432). The final and most abundant sphingolipid in yeast, M(IP)₂C, is formed by transfer of inositol phosphate from PI onto MIPC and requires a protein encoded by the _IPT1_ gene (77). This reaction resembles the one that yields IPC. Accordingly, the _IPT1_ and _AUR1_ gene products exhibit a striking structural similarity.

4. _Sphingolipid hydrolysis and signaling sphingolipids_.

The major pathway along which sphingolipids are degraded is removal of the head group and subsequent hydrolysis of ceramide to sphingoid base and free fatty acid. The breakdown products are then further metabolized or reutilized. Glycosphingolipids are degraded via a

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complex cascade of glycosidases and activator proteins or saposins in the lysosomes, and defects in any of the steps result in lysosomal storage diseases (171). A nonsoluble glucocerebrosidase activity has been found (400). It appears to be active on the cytosolic surface of a cellular membrane, possibly the plasma membrane. GlcCer is the only glycosphingolipid that is synthesized on the cytosolic surface of the Golgi. The enzyme may be involved in regulating GlcCer availability on cytosolic surfaces (R. Raggers and G. van Meer, personal communication). SM is degraded by a lysosomal acid sphingomyelinase (327) in the lysosomal lumen. Alternatively, the acid sphingomyelinase and neutral sphingomyelinases (51, 85, 140, 378) have been invoked as the enzymes that generate ceramide in signal transduction on the cytosolic surface of cellular membranes (see sect. xC). Sphingomyelinase activity has also been reported for yeast (84) and requires ISC1, a gene whose product is structurally related to neutral sphingomyelinases (320). The ISC1-encoded protein displays phospholipase C activity toward SM, IPC, MIPC, and M(IP)2C, but not to PI, PC, or lyso-PC, indicating that it functions as ainositol phosphoglycophospholipid-specific phospholipase C in yeast (320). Where in the cell and on which side of the membrane Isc1p-mediated hydrolysis of sphingolipids occurs is unclear. Whether yeast contains additional enzymes for breaking down its sphingolipids remains to be established. Ceramides are broken down by a number of ceramidase activities that are classified as acidic, neutral, or alkaline, based on their pH optimum. Acidic ceramidase is localized in lysosomes and its gene has been cloned from human (166). Two alkaline ceramidases associated with the ER have been identified in yeast (222, 223).

Although the bulk of sphingoid bases and ceramides is incorporated into sphingolipids, cells do contain small quantities of free sphingoid bases and ceramides, including some phosphorylated derivatives; these are newly synthesized or derived from sphingolipid breakdown and are generally (re)utilized for sphingolipid synthesis in ER and Golgi. Sphingosine-1-phosphate and sphinganine-1-phosphate are special in the sense that they are the final substrates in sphingolipid hydrolysis, being converted by a lysase to ethanolamine phosphate and a C16 aldehyde (315, 399, 433). At the same time, sphingoid long-chain base-1-phosphates are important intra- and intercellular second messengers (see sect. xC), and their concentration is tightly regulated by the combination of kinases (167, 186, 260), the lysase, and a phosphatase (149, 215, 221, 399).

**B. Subcellular Distribution and Topology of Sphingolipids**

A sphingolipid gradient exists along the organelles of the secretory pathway. Although primarily assembled in the Golgi, sphingolipids are enriched in the plasma membrane and endocytic membranes of cells, whereas only low amounts are found in the ER. This has been demonstrated both in yeast (131, 280) and in animal cells (59, 90, 160, 302). Although over 90% of the cellular SM was assigned to the plasma membrane by a cell fractionation approach (182), some 60% of the cellular SM has been routinely found in the plasma membrane by an assay based on exogenous sphingomyelinase (5, 297, 334, 347, 389). Very high concentrations of glycosphingolipids (30–40% of total membrane lipids) have been found in the apical plasma membrane domain of epithelial cells (see sect. vB) and in myelin, a specialized plasma membrane domain of Schwann cells and oligodendrocytes (306). By electron microscopy, the complex glycosphingolipid Forssman antigen was found absent from mitochondria and peroxisomes, low in ER and Golgi and enriched in plasma membrane and endocytic structures (387). Although generally enriched in the plasma membrane and related membranes, there are differences with respect to the distribution of individual sphingolipid classes between organelles (231) or between apical and basolateral plasma membrane domains (159, 358). The intracellular distribution of ceramides, sphingoid bases, and their 1-phosphates is less clear.

The accessibility of glycosphingolipids and SM to reagents, antibodies, and enzymes on the cell surface has led to the general belief that sphingolipids are primarily situated in the noncytoplasmic leaflet of cellular membranes. This is in line with their site of synthesis that is on the luminal aspect of the Golgi (for GalCer on the luminal aspect of the ER). Only GlcCer is synthesized on the cytosolic surface of the Golgi (see sect. vA). The best evidence seems available for SM. In the original studies on erythrocytes, bacterial sphingomyelinase hydrolyzed 80–85% of the SM (404), suggesting that the bulk of the SM is situated in the outer, noncytoplasmic leaflet. A similar conclusion can be drawn from the accessibility of 60% of the SM to sphingomyelinase in intact nucleated cells (5, 297, 334, 347, 389), where a significant fraction of the remaining 40% can be expected to be present in intracellular membranes like endosomes and Golgi. Indeed, no SM was found accessible to sphingomyelinase in microsomal membranes (263, 264), and ≤20% was hydrolyzed in isolated chromaffin granules (42), in which cases the cytosolic surface is exposed. Only little quantitative data are available on the transbilayer distribution of gly-

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16 A similar SM hydrolysis of 50–70% was observed when sphingomyelinase was applied after glutaraldehyde fixation (5) or at 15°C (334, 389), conditions under which there is no vesicular traffic between the plasma membrane and the endosomes/Golgi. Still, it should be realized that the sphingomyelinase method is an invasive technique; it modifies the membrane under study and may not be free from artifacts (336).
cosphingolipids. From studies on GalCer in myelin (199), GM3 in membrane viruses (365, 367), and GM3 and GD1a in a number of cells (242) it has been concluded that the bulk of these lipids is situated in the noncytoplasmic surface of the plasma membrane (for discussion of the methodology, see Ref. 336).

Still, pools of sphingolipids may exist on the cytosolic surface of membranes. This is especially true for GlcCer, which after synthesis is initially present in the cytosolic leaflet of the Golgi membrane (65). A cytosolic protein that can interact with GlcCer has been isolated from cytosol and its gene cloned (198). Other cytosolic proteins have been found to be capable of interacting with complex glycosphingolipids (49, 54, 134, 135, 243, 352, 353), whereas also glycosphingolipids have been colocalized with cytoskeletal elements (106, 107, 318). This may suggest that also complex glycosphingolipids are present in cytosolic surfaces. This could be a consequence of lipid mixing caused by fission and fusion events during membrane traffic, or of transbilayer equilibration of a small sphingolipid fraction reaching the ER (see below).

C. Sphingolipid Transport and Sorting

1. Concepts

After synthesis, sphingolipids can move around the cell in various ways. Intracellular transport processes are fast (minutes) compared with sphingolipid turnover (many hours). So, to maintain the differences in sphingolipid concentration between cellular membranes, there must be specificity in sphingolipid transport. We recently discussed sphingolipid transport in a separate review (397). The present paper focuses on the specificity in sphingolipid transport and the involvement of sphingolipids in sorting other membrane components.

When situated in a membrane, sphingolipids first of all can diffuse as monomers in four directions. If we do not take into account the motions of the entire molecule that do not result in transport, like the rotation around their longitudinal axis and the wobble (279), sphingolipids can diffuse laterally in the two-dimensional plane of the membrane; they can diffuse out of the membrane into the aqueous phase, and they can flip across the membrane into the opposite lipid monolayer. Of these movements, only diffusion out of the membrane may result in transport between cellular organelles. The second mechanism of lipid transfer in cells is by the vesicular transport pathways that connect most cellular organelles. Finally, lipids may be transported between organelles via transient contacts between the membranes of the two organelles.19

The word sorting is used to indicate the process by which the cell generates the differences in protein and lipid composition between two membranes, starting from a membrane where these components were mixed. Because the intracellular traffic is practically a closed system for membrane components, the term generates is equivalent to the term maintains. Where transport between membranes occurs by aqueous diffusion of a certain component as monomers, sorting requires a different affinity of this component for the two membranes. This may concern the affinity of the component for other membrane components, or, theoretically, the aqueous diffusion could be made unidirectional by transfer proteins.20

In vesicular transport pathways, bidirectional transport of vesicles of random composition would result in mixing of all components and dissipation of differences between the two compartments. In this case sorting requires preferential inclusion of a specific component in the budding vesicle in at least one of the two compartments. This means lateral concentration of this component and locating the site of higher concentration to the site of vesicle budding.

2. Monomeric transport through the cytosol

The rate of monomeric diffusion of a sphingolipid between two membranes strongly depends on its physical structure. The smaller the hydrophobic part, and the larger or more polar the hydrophilic part, the higher the rate of exchange. Sphingoid bases and their phosphory-

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17 Because the cells had been treated with acetone and/or TX-100 before antibody addition, it is possible that these lipids were originally present on the luminal side of cytosolic vesicles or vacuoles.

18 Diffusion into the opposite leaflet of the bilayer without a change in the longitudinal orientation of the molecule, by which the molecule merely dips deeper into the membrane, is followed by movement back to its original position, without consequences for transport. Only transversal diffusion plus rotation around one of its short axes, by which the head group changes orientation, flips the molecule into the respective membrane leaflet. The degree of wobble experienced by a lipid in a certain membrane is clearly one parameter that determines the flip rate. Other parameters are the size and polarity of the polar head group and the dielectric constant of the membrane interior.

19 Membranes may come into close proximity, which stimulates monomeric exchange though the aqueous phase by enhancing the desorption from the membrane (treated in detail in Ref. 39). The cytosolic monolayers of the two organelles may transiently become continuous (hemi-fusion), or transient fusion between the apposed membranes may occur. The last possibility can be considered a special case of vesicular traffic.

20 In an in vitro study in a mixture of ER and plasma membrane, cholesterol preferentially partitioned into the plasma membrane, which is thought to be due to its high affinity for the sphingolipids abundant in plasma membranes (408; for a review see Ref. 203). On the other hand, transfer proteins might pick up a lipid from one membrane and deliver it to another membrane after being modified by, e.g., phosphorylation at that target membrane. The activated protein would then leave the membrane empty, be inactivated at the donor membrane, and start a new round of delivery. No experimental support for this mechanism exists.
The aqueous phase. Both GlcCer and GalCer can be re-
moved from the cytosolic surface of the Golgi to the Golgi
lumen where they can be utilized for higher glycosphin-
golipid synthesis. This process is apparently mediated by
a non-energy-consuming translocator (45, 183). In addition,
GlcCer is actively translocated from the cytosolic
surface of the plasma membrane to the outer bilayer
leaflet by the multidrug transporter MDR1 P-glycoprotein
(390; Raggers and van Meer, personal communication).
Translocation across the ER membrane may not be lim-
ited to GalCer, since this event is probably mediated by a
nonspecific translocator (46, 133). Although only a small
fraction of the sphingolipids is in the ER, these molecules
may have access to the cytosolic surface and transfer
through the aqueous phase. It is presently unclear
whether the cell contains translocators to flip them back
to the noncytoplasmic surface (295). Finally, also a small
fraction of the SM may reside in the cytosolic leaflet (see
above). Under some conditions SM may flow into the
cytosolic leaflet of the plasma membrane due to the acti-
vation of a scramblase (295, 434). It is unclear what is its
fate: transfer to other organelles via a transfer protein in
the cytosol (73), removal by a translocator like MDR1
P-glycoprotein (390), or hydrolysis by a cytosolic neutral
sphingomyelinase. In cancer cells, a remarkable increase
in SM content has been observed in microsomes (ER) and
mitochondria (20, 21, 142, 301). Because mitochondria are
thought to receive lipids from the ER by a monomeric
transfer process only, SM may have reached the mito-
chondria via translocation across the ER membrane fol-
lowed by monomeric transport, maybe via sites of ER-
mitochondrial contact (see Ref. 66).

3. Vesicular traffic

The organelles along the exocytic and endocytic
routes are connected through a series of fusion and fission
events. Essentially, membrane vesicles bud from one
compartment and fuse to the next, while at the same time
there is a vesicular pathway in the opposite direction. As
a variation on this theme, both at the level of the Golgi
and that of the endosomes, the complete organelle may
move forward in a maturation process, while generating
vesicles that follow the opposite direction (discussed in
detail in sect. vi). The rate of the process is sufficiently
high that, if each budding vesicle would randomly reflect
the composition of the membrane of origin, complete
mixing of all membrane components could be expected
on a time scale of hours.21 Such mixing is not observed,
and the different cellular membranes are capable of main-

21 Calculations on fibroblasts suggested that per hour a surface
area is endocytosed equivalent to the plasma membrane (112). An area
equivalent to five times their surface area would pass through the
endosomes per hour. Endocytic uptake of 50% of the plasma membrane
surface area per hour was measured for the apical and the basolateral
plasma membrane domain of kidney epithelial MDCKII cells (386).
taining their unique protein and lipid compositions. For proteins, the underlying process is clearly not ongoing local synthesis and hydrolysis, and this is also true for lipids. The enrichment of sphingolipids in the plasma membrane compared with the ER is not due to synthesis of these lipids in the plasma membrane nor to hydrolysis of sphingolipids in the ER. As a consequence, there must be specificity in vesicular transport; membrane components must be sorted. As defined above, this implies that at each budding event, specific membrane components must be included or excluded from the budding vesicle. In the exocytic pathway, the sphingolipids are preferentially included in membranes that travel in the forward direction and/or excluded from transport in the retrograde direction.

Because SM and all glycosphingolipids with the exception of GlcCer are synthesized on the luminal surface of the Golgi, sphingolipid domains would form in the luminal leaflet of the Golgi membrane (398). From the preferential interaction between cholesterol and sphingolipids (see sect. vi), it was then suggested that sorting of the sphingolipids could be the driving force for sorting of cholesterol along the exocytic pathway (395). Obviously, the presence of cholesterol may be a prerequisite for segregation to occur. To convert the lateral segregation into a sorting event whereby sphingolipids and cholesterol are sorted to the plasma membrane, minimally one of the two domains must be selectively included into a budding vesicle. Vesicle budding along the exocytic pathway is mediated by protein coats on the cytosolic surface. Therefore, sorting via lipid domains requires the recruitment of a specific set of coat components to one domain. Because the coat proteins are recruited on the cytosolic surface while the domains reside on the opposite, luminal surface, there must be components that recognize both. These could be proteins that span the membrane like SNAREs (discussed in sect. vi). Alternatively, or in addition, lipid segregation may also occur in the cytosolic surface, and a liquid-ordered domain on the luminal surface may colocalize with a similar domain on the cytosolic surface. Evidence for such a mechanism may be found in the fact that at least one type of lipid on the cytosolic surface of the plasma membrane, phosphatidylserine (PS), is highly saturated compared with the same lipid class in the ER of rat liver (160) and yeast (325). One additional indication that the lipid environment on the cytosolic leaflet opposed to a sphingolipid domain in the noncytoplasmic leaflet may be special comes from studies on sphingolipid domains involved in signaling in the plasma membrane (see sect. viiB). By various techniques, the coat protein caveolin and signaling proteins that are anchored to the cytosolic surface via saturated fatty acids were found to colocalize with the domains in the opposite noncytoplasmic leaflet (202, 237).

VI. SPHINGOLIPIDS AND THE CREATION OF SELECTIVITY IN INTRACELLULAR MEMBRANE TRANSPORT

A. The Golgi: a Central Sorting Device on the Exocytic and Endocytic Pathways

Remarkably, sphingolipid synthesis in eukaryotic cells primarily takes place in the Golgi, spatially separated from glycerolipid and sterol production in the ER (see sect. vA). To better understand the biological significance of this arrangement, the first two parts of this section serve to describe the elementary features of the Golgi and to discuss how the organelle is organized to perform its vital function in the cell.

The Golgi occupies a key position in the secretory membrane system of cells. Arguably its most fundamental task is to act as a filter between the glycerolipid-rich ER and sterol/sphingolipid-rich plasma membrane. A filter function is reflected by the organelle’s unique morphological appearance: a stack of flattened cisternae flanked by two tubular networks that serve as the polarized entry and exit faces (Fig. 4). The number of cisternae within the stack can vary from 3 to >20, depending on cell type (248). The multicisternal nature of the Golgi is thought to allow repeated opportunities for sorting out ER-based and Golgi-resident components from constituents to be delivered to the plasma membrane or elsewhere in the cell.

The cis-Golgi network, also called ER-Golgi intermediate compartment, forms the site where ER-derived cargo is received in a process that involves the formation and fusion of vesicular tubular clusters. It also provides a major site for the budding of vesicles mediating retrograde transport of selected proteins and lipids from the Golgi back to the ER. This retrograde flow serves to maintain the surface area of the ER in the face of extensive membrane outflow into the secretory pathway (415), to return escaped ER resident proteins (284), and to recycle membrane machinery involved in ER to Golgi

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22 In principle, domains of GlcCer may also exist on the cytosolic surface of cellular membranes. In cells that synthesize GalCer, sphingolipid domains may already form in the luminal leaflet of the ER, where GalCer is synthesized (328, 350, 360). As discussed in section viiB, the possibility exists that ceramide in the ER forms a domain, especially in yeast with its long-chain hydroxylated ceramides (252). Because short-chain analogs of Cer and GalCer readily translocate across the ER membrane (45), domains of these lipids may be situated in either membrane leaflet.

23 While of the PS in the plasma membrane, which is exclusively present in the cytosolic leaflet, at least 75% was found to be disaturated, in the ER at least 50% of the PS contained two unsaturated fatty acids. For comparison, the major species of cellular PC contains a saturated C16 or C18 at the sn-1 position and an unsaturated fatty acid at the sn-2 position of the glycerol.
transport (196, 310). As cargo moves through the stack, it is modified by Golgi-associated processing enzymes. These enzymes, which include numerous glycosidases and glycosyltransferases, are generally not distributed evenly between the cisternae, but often found in the order in which they act on their substrates (see sect. V A).24 An advantage of this compartmental organization is that cargo can be exposed to an ordered array of processing steps, allowing the cell to generate highly complex glycoproteins and glycosphingolipids. Positioned at the trans-side of the Golgi stack is the TGN. Here, processed cargo is sorted, packaged into distinct vesicles (or larger membrane carriers), and shipped to various destinations. These post-Golgi destinations include the cell surface (either the apical or basolateral surface of epithelial cells), secretory storage granules, and the various compartments of the endosomal/lysosomal system. The TGN not only serves as a major branching point in the secretory pathway but also forms the major site where the secretory and endocytic pathways become interconnected. This interconnection enables cells to balance membrane flow between the pathways and to maintain the proper composition of their surfaces and intracellular organelles.

B. A Maturing View on Golgi Organization

A fundamental problem currently being addressed in cell biology is how the Golgi is organized to perform its vital tasks and how its functional integrity is maintained

24 It should be noted that this intra-Golgi separation is not precise because the enzymes are generally spread over several cisternae, displaying considerable overlap in their distributions (reviewed in Ref. 109). Therefore, different Golgi cisternae have unique mixtures of enzymes rather than different sets of enzymes. Moreover, the distribution of a given enzyme can vary between different cell types. The basis for this plasticity is unknown but may reflect an underlying dynamic behavior of Golgi membranes, as discussed further below.
subcellular locations. Movement of cargo is achieved exclusively by transport vesicles that pinch off from one cisterna to the next, while Golgi resident enzymes stay put in the appropriate cisternae. This idea was largely based on results obtained with a cell-free system in which isolated Golgi membranes were used to measure the sequential processing of cargo by Golgi-associated enzymes (309, 312). These studies offered key insights into the intragolgi transport machinery. They allowed the identification of a cytoplasmic coat protein complex, termed COPI, whose regulated assembly on Golgi membranes was found to promote the formation of fusion-competent vesicles containing secretory cargo (270, 275). In the light of these data, it was assumed that COPI-coated vesicles mediate transport of secretory cargo through sequential Golgi compartments in a cis-to-trans (or anterograde) direction.

However, this view was challenged by the discovery that COPI plays a crucial role in retrograde vesicular transport. This notion emerged when COPI subunits were found to bind the d lysine retrieval signal on the cytoplasmic tails of ER membrane proteins (64). Moreover, mutations in COPI blocked retrieval of these proteins from the Golgi complex (194). Consistent with a function of COPI in retrograde transport, quantitative immunoelectron microscopy revealed that COPI-coated tips at the CGN are enriched for recycling membrane proteins whereas secretory cargo prevailed at COPI-negative regions (228). The finding that COPI vesicles contain Golgi enzymes and serve as their transport intermediates in vitro (185, 197, 206) offered an alternative explanation for the results previously obtained with the Golgi cell-free transport assay; instead of moving secretory cargo forward, COPI may serve to retrieve transport machinery and Golgi enzymes to cisternae containing cargo to be modified. These novel data predicted a prominent role for COPI-mediated retrograde traffic in Golgi function and maintenance. Support for this prediction came when movement of green fluorescent protein (GFP)-tagged Golgi enzymes was followed with fluorescence video microscopy. In contrast to previous assumptions (265), it was found that Golgi enzymes are highly mobile (61), undergo substantial recycling within Golgi stacks (418), and redistribute into the ER when export from this compartment is blocked (60, 368, 428).

The existence of recycling pathways both within and from the Golgi stack together with the unsolved problem of how COPI vesicles, the only type of vesicles known to mediate intra-Golgi transport, would contribute to vectorial transport of secretory cargo led to a renewed interest into an old idea, namely, that the Golgi is a collection of maturing compartments (111). This “cisternal progression” or “maturation” model arose from previous observations that large cargo complexes produced by certain cell types (e.g., algal scales, procollagen) move through the Golgi stack without entering transport vesicles or leaving the cisternae (25, 238). According to the model, cisternae are continuously formed de novo by fusion of ER-derived vesicles (314), pass through the stack as they mature, and eventually disintegrate at the level of the TGN. While secretory cargo stays put in cisternae, Golgi enzymes are delivered at the appropriate time and in the appropriate order by recycling vesicles so that each cis terna matures into the next (reviewed in Refs. 4, 110, 285).

On the basis of the currently available data, evidence in support of cisternal maturation cannot preclude the possibility of coexisting anterograde vesicular transport, and vice versa. In fact, there is reason to believe that both mechanisms operate simultaneously (286). First, procollagen aggregates appear to traverse the Golgi stack at a much slower pace (hours) than other cargo proteins such as VSV-G (10–20 min; Refs. 24, 25). This finding suggests that cisternal maturation is too slow to account for all anterograde cargo transport. Second, Golgi proteins involved in vesicle targeting, like SNAREs, are typically distributed over multiple cisternae across the stack (130, 272). Because there are no known components of the targeting machinery that can specify a single cisterna, it is hard to envision how an exclusive unidirectional movement of COPI vesicles, whether forward or backward, can be achieved. On the basis of these notions, it has been postulated that COPI vesicles may “percolate” up and down the stack in a bidirectional fashion, allowing a rapid

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25 Coat proteins are believed to have a dual function in membrane trafficking, serving both to shape a transport vesicle and to select, by direct or indirect interactions, the desired set of cargo molecules. Among the cargo molecules recruited by these vesicle coats are components that specify the docking and fusion of transport vesicles with their appropriate target organelles. These include members of the SNARE family of membrane proteins (311). Coat recruitment, cargo selection, and the subsequent fission and fusion of transport vesicles is coordinated by distinct families of GTPases. Hence, coat proteins function together with targeting and fusion components to form so-called “vesicle sorting machines” by which specific types of cargo can be moved from one destination to the next. Cells have a variety of coat proteins, allowing different species of vesicles to depart from various subcellular locations.

26 A role for COPI in bidirectional vesicular transport has been postulated based on the observation that pancreatic β-cells contain two distinct populations of Golgi-associated COPI vesicles: one containing recycling components and the other one secretory cargo (274). However, because the concentration of secretory cargo found in the second vesicle population was comparable to that in the cisternae, it is unclear how these vesicles would contribute to vectorial transport through the stack. Moreover, the model leaves unresolved by what mechanism proteins bearing d lysine retrieval signals can be excluded from anterograde COPI vesicles while being included in retrograde ones.
equilibration of plasma membrane and soluble secretory cargo between adjacent cisternae (272, 286). Percollating vesicles (or transient tubular connections; Ref. 410) would give rise to a “fast track” by which cargo can flow across the entire Golgi stack. Larger cargo structures and other molecules that cannot enter COPI vesicles (or tubules) would then transit the stack at the slower pace of cisternal maturation.

In view of these coexisting modes of transport, how does the Golgi ensure efficient vectorial movement of cell surface-destined proteins and lipids, while maintaining at the same time an asymmetric arrangement of its processing enzymes? Without a mechanism that regulates the partitioning of cargo molecules and Golgi residents into COPI vesicles, a rapid interchange would result in complete uniformity and undermine directionality of transport. In the next section, we discuss how the assembly of sphingolipids in Golgi cisternae and their impact on the lateral organization of other membrane molecules may provide a physical basis exploited by the Golgi to segregate forward-moving cargo from recycling components, and once this is accomplished, to organize specialized TGN export sites used for polarized secretion.

C. Sphingolipids: Integral Parts of the Golgi-Based Sorting Machinery?

1. Segregating forward-moving cargo from recycling components

After assembly in the Golgi, sphingolipids accumulate in the plasma membrane (see sect. vB). Their long and saturated fatty acyl chains, capacity for hydrogen bonding, and association with sterols is thought to promote the compactness and thickness, and hence the impermeability of the cell’s outer bilayer (see sect. wB). Compared with the plasma membrane, the ER contains much lower concentrations of sphingolipids (396), despite extensive bidirectional membrane trafficking at the ER-Golgi interface (415). Hence, a mechanism must exist by which the Golgi prevents backflow of sphingolipids to the ER and promotes their concentration toward the trans-site where membrane carriers depart for the plasma membrane. This could in principle be achieved if sphingolipids were prevented from leaving the cisternae in which they are made. The result would be a gradual rise in their concentration as cisternae mature. Such a scenario is in line with the recent finding that COPI vesicles contain significantly less sphingolipids and cholesterol than their parental Golgi membranes (41). Indeed, a segregation of sphingolipids and cholesterol from COPI vesicles could explain why the interleaflet clear space of these vesicles appears thinner than that of the cisternal membranes from which they bud (273).

How are sphingolipids and sterols segregated from COPI vesicles? Two distinct mechanisms can be envisaged: 1) segregation may occur because the coat machinery influences the lipid composition at the bud site. According to this scheme, sorting could result from preferential interactions of certain lipids with the membrane-proximal surfaces of the coat proteins and/or the transmembrane segments of selected proteins (e.g., COPI coat receptors; Ref. 31). Alternatively, the COPI coat machinery might influence the lipid composition at the bud site by imposing a high curvature onto the vesicle membrane. This would result in a preferential exclusion of those lipid species that contribute to the rigidity of flat bilayers (e.g., sphingolipids, sterols, and saturated glycerolipids). In this respect, it is of interest to note that COPI assembly on liposomes is negatively influenced by acyl chain saturation.27 2) Segregation may occur because the coat machinery is specifically recruited to lipid domains in the Golgi bilayer. Sphingolipids have a strong intrinsic tendency to segregate from unsaturated glycerolipids and display a high affinity for cholesterol (see sect. iv). Hence, sphingolipids synthesized in Golgi cisternae may trigger a phase separation, thus creating domains enriched in unsaturated glycerolipids that function as donor sites for COPI vesicle biogenesis versus sphingolipid- and sterol-enriched domains that do not. There is evidence that such lipid subdomains exist in early Golgi cisternae (108).

Of the two lipid-sorting mechanisms described above, the latter has the particular appeal that it is inherently self-organizing, thus escaping the requirement for proteins that to trigger a lateral segregation of lipids need themselves to be retained in place. It is tempting to speculate that ongoing sphingolipid synthesis in the Golgi acts as a sink for ER-synthesized cholesterol and that a phase separation is nucleated once these lipids have reached a critical concentration in the cisternal bilayer (see sect. iv).

Regardless of the actual mechanism of lipid segregation, the exclusion of sphingolipids and sterols from COPI vesicles would cause their progressive accumulation in the maturing cisternae. As a result, anterograde transport of sphingolipids and cholesterol would be coupled to a gradual depletion of unsaturated glycerolipids along the secretory pathway. A sphingolipid/sterol concentration gradient would form across the Golgi stack (26, 56, 57, 271), causing an increase in bilayer thickness toward the

27 In a chemically defined in vitro assay, the assembly of COPI coats on synthetic liposomes was found to be strongly affected by acyl chain saturation; dC18:1 PC/dC18:1 PE vesicles, which contain unsaturated fatty acyl chains, allowed coat recruitment, whereas C16:0-C18:1 PC/C16:0-C18:1 PE vesicles, carrying one saturated and one unsaturated fatty acid, did not (355).
TGN (114; Fig. 4). Preferential interactions between lipids and transmembrane proteins with the best matching hydrophobic length has been recognized as a potential mechanism for protein sorting in the Golgi (32, 255). This idea emerged with the observation that the transmembrane domains (TMDs) of Golgi-resident proteins are on average five residues shorter than those of plasma membrane proteins (32). This phenomenon is well conserved among eukaryotic cells, from mammals (32) to yeast (195). It has been postulated that due to their shorter TMDs, Golgi-resident proteins would be excluded from the thicker sphingolipid/sterol-enriched membrane regions destined for the cell surface and hence be retained in the Golgi (32, 255). In support of this idea, lengthening the TMDs of several Golgi enzymes results in their movement to the plasma membrane (229, 253, 254), while shortening the TMD of a plasma membrane protein causes its accumulation in the Golgi (60). In addition, the ER residency of several membrane proteins can be disrupted by elongating their TMDs (282, 422). TMD length has also been recognized as a critical parameter in the sorting of SNAREs (298), membrane proteins whose specific interactions contribute significantly to the specificity of vesicle docking and fusion (236, 311). Particularly intriguing in this respect is the finding that the requirement of exocytic v-SNAREs (vesicular SNAREs) for secretion in yeast can be bypassed by genetic mutations in enzymes responsible for the elongation of the long-chain fatty acids found in ceramide and sphingolipids (67).

Apart from being short, some Golgi TMDs display an unusual high content of the bulky residue phenylalanine, a feature that may well promote a segregation from the ordered sphingolipid/sterol-rich membrane regions (32). Oligomerization into multi-envelope complexes (265) provides another factor that could influence phase behavior of Golgi enzymes. Collectively, the variation in such properties would offer considerable scope for establishing different steady-state distributions of enzymes within the Golgi stack. Bidirectional transport mediated by percolating COPI vesicles or transient tubular connections would allow an enzyme to transiently explore the entire stack until it finds a cisterna whose lipid composition suits its TMD (283). Ongoing sphingolipid synthesis and depletion of unsaturated glycerolipids will gradually remodel the lipid composition of the cisternal bilayer to that of the plasma membrane, and eventually drive all Golgi-resident proteins, including the sphingolipid-synthesizing enzymes themselves, into recycling COPI vesicles.

 Likewise, plasma membrane proteins whose rapid diffusion through the stack is mediated by percolating vesicles or tubules could achieve the desired directionality in transport if their membrane anchors would favor cisternal bilayers that have become thicker and more organized due to a progressive accumulation of sphingolipids and sterols. Indeed, GPI-anchored proteins have a high affinity for glycosphingolipid/cholesterol-rich domains (see sect. nB), a feature thought to be required for their efficient transport from the Golgi to the cell surface (233, 345). The same has been reported for influenza virus hemagglutinin (see sect. nB; Ref. 161).

The notions that sorting in the Golgi depends on transitions in bilayer thickness and that these transitions are the result of sphingolipid-induced phase separations provide a compelling explanation for why structural features render sphingolipids essential for the viability of cells; they form an integral part of the mechanisms by which cells generate the compositional and functional differences between their plasma membrane and internal organelles.

2. Organizing specialized export sites for polarized secretion

The TGN is a major branching point in secretory traffic where apical and basolateral cell surface compo-
ponents are segregated and delivered via separate transport routes. Although previously viewed as a special feature of epithelial cells, it has become clear that apical and basolateral delivery pathways operate in both polarized and nonpolarized cell types (257, 425). However, only in epithelial cells, they would result in macroscopic domains laterally separated by a diffusion barrier at the tight junction. Recent studies have indicated that the establishment of cell polarity is initiated by signaling pathways that are responsive to cell-cell and cell-matrix contacts (423). This would allow a molecular definition of contacting and noncontacting cell surfaces (the precursors of the basolateral and apical membrane domains, respectively). The next step would then involve a localized assembly of cytoskeletal elements and a positioning of the TGN relative to the spatial cues. Finally, the cell surface polarity that was initially defined by the spatial cues would be reinforced by targeted delivery of apical and basolateral cell surface components that are constitutively sorted in the TGN.32

As discussed in the previous section, the enrichment of sphingolipids and saturated glycerolipids along the secretory pathway could be explained if COP1 vesicles budding from the Golgi cisternae would select thinner, unsaturated lipid-enriched membrane regions. Still an additional level of lipid sorting must be invoked to explain the difference in lipid composition between the apical and basolateral surface of epithelial cells. The basolateral cell surface has a composition very similar to that of the plasma membrane of nonpolarized cells, but the apical surface is highly enriched in sphingolipids, either glycosphingolipids or SM (see sect. vB; Ref. 341). It therefore appears that superimposed on the lateral segregation of sphingolipids from unsaturated glycerolipids throughout the Golgi stack, a second lateral segregation in the TGN membrane would occur. This would give rise to domains highly enriched in glycosphingolipids or SM and with targeting information for the apical surface, and domains with a more moderate enrichment in sphingolipids that would contain basolateral information (395). The segregation of apical from basolateral lipids could imply the existence of three phases in the TGN membrane. However, a simpler mechanism would be extension to the TGN of the segregation of the two fluid lipid phases occurring in the Golgi stack. Depletion of unsaturated lipids and ongoing sphingolipid synthesis would result in a gradual change in the composition of both subdomains, culminating in an apical domain and a basolateral domain at the TGN.

It is believed that apical protein sorting relies on the formation of glycosphingolipid and cholesterol-enriched domains in the luminal leaflet of the TGN (341, 398). This idea is supported by the following observations: 1) GPI proteins use their lipid anchors as apical sorting determinants (34, 200), while sorting information in at least two apically expressed transmembrane proteins (influenza hemagglutinin and neuraminidase) resides in their transmembrane segments (175, 322); 2) influenza hemagglutinin and a GPI protein acquire detergent resistance in the Golgi during biosynthetic transport (38, 86); and 3) inhibition of sphingolipid synthesis or deprivation of cholesterol causes a reduction in detergent insolubility and randomizes the cell surface distribution of influenza hemagglutinin and a GPI protein without affecting sorting of basolateral proteins (161, 233). A lipid-based sorting mechanism similar to that reported for the apical delivery of proteins in epithelial cells has been suggested to mediate axonal delivery of proteins in neurons (80, 188, 189).

In addition, it appears that carbohydrates in glycoproteins also provide apical sorting information (88). Several secretory and membrane proteins have been reported to rely on their N- or O-glycans for apical delivery (3, 115, 321), including GPI-anchored proteins (19). The mechanism of carbohydrate-mediated apical sorting is unclear (see Ref. 306). One potential mechanism involves recognition of N-glycans by sorting lectins such as VIP36 (87, 420), which is thought to partition into glycosphingolipid/cholesterol-rich domains in the TGN along with other apical membrane proteins. In summary, protein sorting to the apical membrane is variable and largely depends on targeting signals present in the luminal domain or membrane anchor.

In contrast, basolateral sorting is typically mediated by discrete targeting signals that are confined to the cytoplasmic tails of transmembrane proteins. These signals often consist of tyrosine- or dileucine-based amino acid motifs and resemble the signals that mediate endocytosis and protein sorting to endosomes and lysosomes. It has been well established that interactions between tyrosine- and leucine-based sorting signals and AP coats are responsible for selective cargo uptake into TGN- and plasma membrane-derived clathrin-coated vesicles (239). Basolateral sorting is most likely based on a similar coat-mediated mechanism. An epithelial cell type-specific isoform of the AP-1 coat complex, designated AP-1B, has recently been identified and found to play a critical role in the basolateral targeting of a number of membrane proteins (91). Still, AP-1B-independent mechanisms for basolateral sorting must exist as epithelial cells are capable of correctly delivering some basolateral membrane proteins in the absence of AP-1B (313). Moreover, AP-1B is not expressed in hepatocytes and neurons, yet these cell

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32 It should be noted that a fraction of the apical proteins are transported to the apical surface via the basolateral surface in some cells (187, 230), but not in other cells (413). Interestingly, the polarity of apical and basolateral proteins may alter with the physiological state of a cell (2). In retinal pigmented epithelial cells, developmental switches control apical and basolateral trafficking of a number of proteins (225).
types rely on basolateral-type targeting signals for transport to their sinusoidal and somatodendritic surfaces, respectively (416, 424). A novel, ubiquitously expressed adaptor protein complex, AP-4, localizes to the TGN (70) and may be involved in basolateral sorting as well, but this remains to be shown.

Interestingly, there appears to be a hierarchical read-out of basolateral and apical sorting signals. Basolateral sorting signals are usually dominant; influenza hemagglutinin equipped with a tyrosine-based sorting signal is converted from an apical to a basolateral protein (33). On the other hand, inactivation of basolateral signals does not always lead to apical delivery, even if the protein is glycocyalted. It has been suggested that some basolateral membrane proteins are sorted by exclusion from glycosphingolipid rafts; such a scenario may apply to a number of proteins whose efficient basolateral distribution is achieved in the absence of an active basolateral sorting signal (233, 423).

3. Apical sorting by retention?

In contrast to the high-affinity protein-protein interactions involved in the formation of basolateral vesicles, apical sorting seems largely based on a cooperativity of weak interactions between lipids, proteins, and sugars that occur predominantly within the bilayer domain or lumen of the TGN. Therefore, a role for vesicular coats in the formation of apical transport carriers has remained rather elusive. The production of apical transport vesicles has been proposed to involve VIP21/caveolin, a membrane protein with high affinity for cholesterol (256). It was suggested that oligomerization and intercalation of VIP21/caveolin into the glycosphingolipid/cholesterol-enriched domains would cause the bilayer to bend into apical transport vesicles (277). However, evidence in support of this hypothesis remains indirect. An alternative possibility is that the inclusion of basolateral molecules into coated vesicles budding from the TGN provides the primary means by which apical and basolateral components are physically segregated into different membrane carriers. Interestingly, correlative light and electron microscopic studies have indicated that the membrane carriers involved in transport from the Golgi to the plasma membrane are not only small vesicles, but also larger tubulovesicular structures (136, 379), sometimes even as large as half a Golgi cisternea (288). It appears that these structures arise by a controlled drawing out and progressive breakdown of TGN material from the Golgi complex according to a mechanism that bears similarities with the formation and maturation of secretory storage granules in neuroendocrine cells (10, 288). An intriguing possibility would be that apical transport intermediates are formed by means of a progressive maturation of TGN membranes via AP-dependent removal of endosomal/lysosomal and basolateral material (Fig. 4). Such a mechanism would escape the requirement of a specific apical coat complex as it would represent a terminal step in the cisternal maturation process. Analogous to the mechanism of cargo sorting in secretory storage granules (374), sorting of apical cargo may rely on a "sorting by retention" principle, based on the coaggregative properties of the apical proteins and membrane lipids. To become a functional transport intermediate, a maturing apical container must recruit components of the docking and fusion machinery. Specific members of the SNARE family (TI-VAMP and syntaxin 3) have been identified on apical membrane carriers and were shown to play a critical role in membrane trafficking from the TGN to the apical cell surface (180). The MAL/VIP17 proteolipid may represent another part of the apical transport machinery (227, 291), but its mode of action remains to be clarified. All these components occurred in detergent-resistant membrane fractions along with protein cargo destined for the apical cell surface, suggesting that both apical cargo and transport machinery are subject to the same lipid-based sorting mechanism.

VII. CONCLUDING REMARKS

A. Sphingolipids and Golgi Maturation

Above, we have worked out the concept that a separation between two fluid phases of lipids lies at the heart of the sorting potential of the Golgi complex. This phase separation would be based on the differential miscibility of sphingolipids, glycerolipids, and sterols. An essential feature of the model is that the Golgi membrane matures along the cis-trans axis, based on cisternal progression, a gradual change in sphingolipid composition by biosynthetic reactions, and continuous retrieval of the most fluid lipids. As a consequence, the Golgi membrane as a whole becomes less fluid and thickens, resulting in a sequential loss of Golgi-resident enzymes and transport machinery to recycling pathways that run from the cis-Golgi to the ER and from within the Golgi stack to more proximal cisternae. After distillation, a membrane persists that is highly enriched in sphingolipids, cholesterol, and selected proteins and forms the prototype "apical" membrane carrier. The ordered array of sphingolipid synthetic enzymes that extends from the ER to the trans-Golgi seems an ideal device to gradually convert the highly fluid and flexible ER bilayer into a rigid and robust plasma membrane. In fact, it may form an integral part of the mechanistic framework by which the compartmental organization of the secretory pathway is established.
B. Domains of Saturated Lipids on the Cytosolic Surface

Because SM and all glycosphingolipids with the exception of GlcCer are synthesized on the luminal surface of the Golgi, sphingolipid domains would form in the luminal leaflet of the Golgi membrane (398). We have recently obtained evidence that the pool of GlcCer in the cytosolic leaflet is regulated by the combination of MDR1 P-glycoprotein, a GlcCer translocator, and a nonlysosomal glucocerebrosidase (390). In addition, our studies show that glycosphingolipid synthesis is required for an intact AP-3 pathway to melanosomes (H. Sprong, P. van der Sluijs, and G. van Meer, personal communication). The data suggest that GlcCer on the cytosolic surface is involved in the recruitment of specific coat proteins at the level of the TGN. This could be by a direct interaction of GlcCer with a cytosolic protein (complex). Alternatively, GlcCer could form (or be part of) a domain on the cytosolic surface.

Evidence for lipid phase separation in the cytosolic bilayer leaflet of Golgi membranes may be found in the fact that at least one type of lipid on the cytosolic surface of the plasma membrane is highly saturated compared with the same lipid class in the ER of rat liver (160) and yeast (325). Such a domain would mature along with the luminal sphingolipid/cholesterol domain. One additional indication that the lipid environment on the cytosolic leaflet opposed to a sphingolipid domain in the noncytoplasmic leaflet may be special comes from studies on sphingolipid domains involved in signaling in the plasma membrane (see sect. nB). By various techniques, the coat protein caveolin and signaling proteins that are anchored to the cytosolic surface via saturated fatty acids colocalize with the domains in the opposite noncytoplasmic leaflet (202, 237). Whether the opposed domains would be connected via transmembrane lipid-lipid interactions (see sect. nB) or via membrane-spanning proteins is an open issue.

C. Sphingolipid Domains as a General Theme in Cellular Membrane Traffic

Lipid segregation does not necessarily start in the cis-Golgi. As discussed in section nB, the possibility exists that ceramides in the ER, especially in yeast with its long-chain hydroxylated ceramides, occur in domains (252). In cells that synthesize GalCer, this sphingolipid may organize in domains already in the luminal leaflet of the ER, where it is synthesized (328, 359, 360). Short-chain analogs of Cer and GalCer (45) and other (sphingo)lipids (133) readily translocate across the ER membrane. If true for natural lipids, domains of these lipids may be situated in either membrane leaflet.

Moreover, the ultimate formation of an “apical” sphingolipid/cholesterol remnant in the TGN is a final stage in Golgi maturation but clearly is not a final stage in cellular membrane transport. Membrane recycles from the plasma membrane via endosomes to the TGN, and evidence for lipid-based sorting has been found in the endocytic recycling pathway (reviewed in Ref. 397). Although, unfortunately, essentially all evidence has been obtained using fluorescent analogs of membrane lipids, still it leads to the conclusion that certain lipid molecules can be sorted in this pathway. In short, fluorescent sphingolipids are concentrated during the first step(s) of endocytosis (52, 407). In some cells, fluorescent GlcCer segregates from fluorescent SM during recycling from the plasma membrane (168, 393, 394). However, such sorting was not observed in different cell types (386), and it cannot be assumed that these lipid analogs strictly monitor vesicular transport processes (226). Independent evidence for lipid sorting via phase separation was obtained by studying transport of lipid probes of different chain length and saturation (251). The data in the latter study suggest that lipids with longer or more saturated chains are preferentially transported to late endosomes versus the recycling endosome. Furthermore, cholesterol had a dramatic effect on endocytic lipid sorting (293). Because endosome maturation has many parallels with the maturation of Golgi cisternae, a sphingolipid-driven sorting device may also be central to the endocytic pathway. Because sphingolipid synthesis is limited to the Golgi (see sect. nA; Ref. 391), and all components are present at the start of the endosomal maturation process, in the plasma membrane, the endosomal lipid maturation can be considered to be an extension of the Golgi system.

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