Sphingolipid trafficking – sorted out?

Gerrit van Meer and Koert N. J. Burger

Studies of intracellular membrane traffic have traditionally focused on the protein components of membranes, but what about lipids? Recent findings have drawn attention to the transport of one type of lipid, the sphingolipids. Their unique physical properties may allow them to aggregate into microdomains in membranes that concentrate sphingolipids into specific transport pathways. Gerrit van Meer and Koert Burger consider here the routes of sphingolipid biosynthesis and transport, and the role of proteins in their targeting. The following article by Deborah Brown turns the tables to review the evidence suggesting that sphingolipid domains are important in specific targeting of GPI-anchored proteins to the plasma membrane.

Sphingolipids are found almost exclusively in eukaryotic cells, where they are restricted to the plasma membrane and to a limited number of intracellular organelles. Their overall structure is similar to that of the glycerolipids, i.e. both have two long hydrocarbon chains and a polar head group (Fig. 1). However, one of the hydrocarbon chains in the sphingolipids is provided by the sphingoid base itself, which also carries the head group. This lipid structure, excluding the polar head group, is called a ceramide. It confers important physicochemical properties on the sphingolipids. The abundant phosphosphingolipid sphingomyelin (SM) carries a phosphorylcholine head group, and is the counterpart of the omnipresent phosphoglycerolipid phosphatidylcholine (PC)[1]. In glycosphingolipids the head group consists of one out of some 250 distinct carbohydrate structures[2].

The sphingolipids are abundant on the cell surface, and the past few years have witnessed dramatic developments in our understanding of sphingolipid function in cell surface recognition and signalling[1,2]. In addition, the sphingolipids have been proposed to play a major role in the sorting events that occur during intracellular membrane transport (Ref. 3; and see the accompanying review by Deborah Brown), whereas hardly any evidence is available on the behaviour of glycerolipids in these processes. Various excellent reviews have appeared on the intracellular transport of sphingolipids[4–6]. The aim of this review is to reappraise the current state of affairs of lipid sorting.

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Supply of ceramide for sphingolipid synthesis

We now know that the biosynthesis of the ceramide precursor up to the formation of dihydroceramide occurs at the cytosolic surface of the endoplasmic reticulum (ER)[7]; however, it is not clear where the introduction of the double bond to yield ceramide takes place. Ceramide is expected to have access to both bilayer leaflets because its apolar character should allow rapid translocation across membranes. In agreement with this, externally added short-chain ceramide analogues rapidly translocate across the plasma membrane and reach the intracellular organelles of intact, living cells even at low temperatures that slow down vesicular traffic[8].

The transfer of head groups onto ceramide by SM-synthase to give SM and by glucosyltransferase to give glucosylceramide (GlcCer) (Fig. 2) occur in the *cis* Golgi complex[9–12]. The synthesis of galactosylceramide (GalCer) has also been assigned to the Golgi complex[13]. By contrast to the terminal glycosyltransferases that may utilize both glycolipids and glycoproteins[14], the transferases that are responsible for the initial glycosylation events appear to be specific.

Ceramide can reach its main site of metabolic conversion in the Golgi complex simply by equilibration via the vesicular transport pathway between the ER and Golgi complex (Fig. 3). Since the amount of membrane that recycles between the ER and the Golgi complex far exceeds the vesicular traffic from the Golgi complex onwards, a low steady-state level of ceramide in the ER would be sufficient to guarantee considerable sphingolipid synthesis in the *cis* Golgi complex.

Vesicular transport of SM to the cell surface

Evidence for the mode of transport of SM comes mainly from indirect sources. First, the resistance of SM synthesis in Golgi fractions to protease added to the cytoplasmic surface has led to the conclusion that SM-synthase is oriented towards the Golgi lumen[9,11,12]. Second, the absence of SM from the cytosolic surface of organelles in cellular subfractions and in permeabilized cells[12,15–18] suggests transport to the cell surface[8] could only be by carrier vesicles, and not by monomer exchange through the cytoplasm. Third, SM does not reach the surface during mitosis, when vesicular traffic stops[19]. (Box 1 describes how transport of lipids to the cell surface can be measured.)

From the Golgi complex, SM is transported via direct pathways to both the apical and basolateral cell surface of epithelial cells[20,21]. It is not clear whether SM might also follow the mannose-6-phosphate receptor pathway from the *trans* Golgi network (TGN) to the (late) endosomes and then to the cell surface (Fig. 3), but studies of protein secretion suggest this pathway is of minor import-

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ance compared with the direct pathway to the cell surface.

Translocation of GlcCer into the Golgi lumen

GlcCer, the simple glycosphingolipid that serves as the precursor for most complex glycosphingolipids, is synthesized in the *cis* Golgi complex, although cell fractionation studies have also assigned a significant part of the glucosyltransferase activity to some other, possibly pre-Golgi, compartment[10,12]. Glucosyltransferase is sensitive to proteases and labelling reagents under conditions where these act on the cytosolic surface of the Golgi complex exclusively[10,12,22,23], and newly synthesized GlcCer is accessible from the cytosol[12,22]. These findings suggest GlcCer is synthesized in the cytosolic leaflet. Evidence has been presented suggesting that the next step in the synthesis of complex glycolipids, LacCer (Gal-GlcCer) synthesis, may also occur on the cytosolic face of the Golgi complex isolated from rat liver[23]. However, this result is countered by the finding that mutants of CHO cells that possess an intact galactosyltransferase, Gal-T I (Fig. 2), but lack the translocator for transfer of the activated sugar UDP-Gal into the Golgi lumen, displayed strongly reduced levels of LacCer[24]. The next step of ganglioside synthesis, the addition of sialic acid to LacCer to produce GM_3 by sialyltransferase I occurs at the luminal surface of the Golgi complex[23,25]. The overall conclusion from these studies is that translocation of GlcCer into the Golgi lumen appears to be required for complex glycosphingolipid synthesis to occur. Whether or not complex glycosphingolipids can translocate back to the cytosolic surface, where they could bind to specific cytosolic proteins[26,27], remains an important issue to be resolved.

All the glycosyltransferases from rat liver that are required for GM_3 production, and also the sialyltransferase II, which converts GM_3 into GD_3, cofractionate with *cis* Golgi markers on sucrose gradients[28–30]. This suggests that both the synthesis and translocation of GlcCer must occur in or before the *cis* Golgi complex. Nonetheless, whereas ceramide and GlcCer are synthesized concurrently, the synthesis of LacCer lags behind by 5–6 min and that of GM_3 by 11–12 min[31], suggesting that these events occur in separate compartments connected by discrete transport steps. In the presence of brefeldin A (BFA), which causes redistribution of enzymes from the Golgi complex, but not the TGN, to the ER[32], the glycosphingolipids GlcCer, LacCer, GM_3 and GD_3 accumulate, showing that the respective enzymes still have access to their endogenous substrates and therefore have probably returned to the ER. The next enzyme in ganglioside biosynthesis, GalNAc-transferase, which converts LacCer, GM_3 and GD_3 into GA_2, GM_2 and GD_2, respectively, is no longer active in the presence of BFA[33,34] and therefore may be located beyond the BFA block, in the late Golgi complex. On sucrose gradients the enzyme fractionated between *cis* and *trans* Golgi markers, whereas the later-acting sialyltransferase IV and V colocalized with markers for the *trans* Golgi complex[28–30]. These data confirm the idea that, like the glycoprotein glycosyltransferases, the lipid glycosyltransferases in the Golgi complex are located in the *cis–trans* direction in the order in which they act.

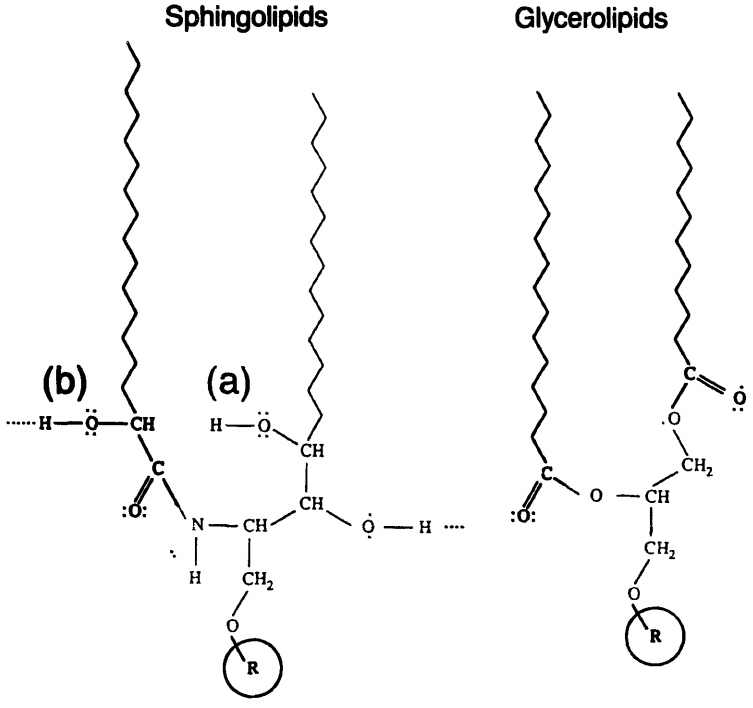


FIGURE 1

The ceramide backbone of the sphingolipids (left) is the entire lipid structure, excluding the polar head group (R). The sphingoid base of the sphingolipid is shown in blue. The glycerol backbone of the glycerolipids (right) is also shown in blue. The lipid structure shown for the glycerolipids, excluding the polar head group (R), is diacylglycerol. By contrast to the glycerol backbone of glycerolipids, the ceramide backbone of sphingolipids can function as a donor in hydrogen bond formation (indicated by dotted lines). The ceramide backbone shown here is particularly suited for this purpose since it contains an extra hydroxyl group (a) on the sphingosine (phytosphingosine) and a hydroxyl group (b) on the fatty acid (α-hydroxy fatty acid)[46]. The latter is rare in SM and the phosphoglycerolipids. In phospholipids, such as SM and PC, R stands for phosphate esterified to a base (e.g. choline), whereas in the glycolipids it represents a carbohydrate (chain). The length of the fatty acyl chains, but not that of the sphingoid base, is variable. Usually the acyl chains contain 16–20 carbon atoms in glycerolipids and 18–26 carbon atoms in the sphingolipids.

The fact that GlcCer is synthesized on the cytosolic surface of the Golgi complex raises the possibility that transport through the cytosol may be mediated by transfer proteins[35]. Unfortunately, the crucial question of whether or not GlcCer is present in the cytosolic membrane leaflet of organelles other than the Golgi complex has not been solved. Evidence is, however, available on the transport of GlcCer to the cell surface. After intracellular synthesis, short-chain GlcCer analogues reached the exoplasmic leaflet of the plasma membrane (which is topologically equivalent to the luminal leaflet of intracellular compartments)[8,15,20,21], and current data indicate that, like SM, GlcCer is transported by a vesicular mechanism. For example, like SM, a

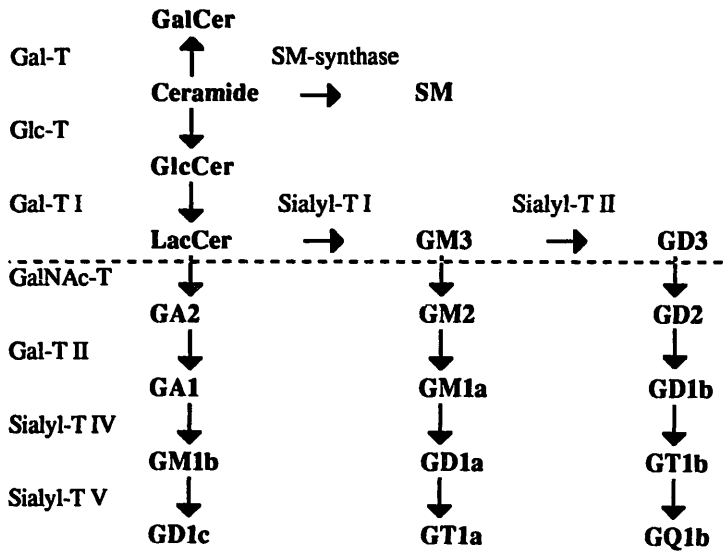


FIGURE 2

Scheme of enzymatic reactions involved in the synthesis of a number of sphingolipids discussed in the text. Terminology for the sialic-acid-containing glycosphingolipids, the gangliosides, is that of Svennerholm[30,33,34]. Reactions below the dotted line are inhibited in the presence of BFA[33,34]. Sialyltransferases I–IV have alternatively been named SAT-1 to SAT-4, and the galactosyltransferase producing GalCer has also been termed GalT-1, and consequently the galactosyltransferases I and II have also been termed GalT-2 and GalT-3 (Ref. 49). T, transferase.

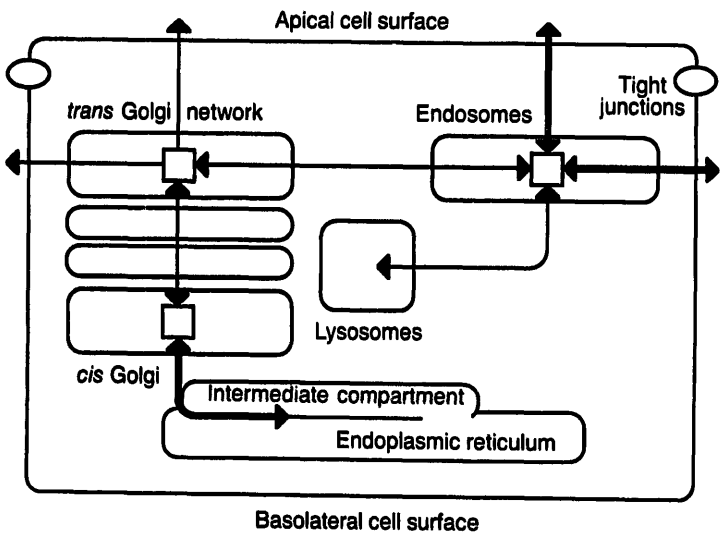


FIGURE 3

Schematic view of the vesicular transport pathways connecting the various organelles of the vacuolar system in polarized epithelial cells, as established by studies of protein transport. The two plasma membrane domains are separated by tight junctions that act as a barrier to lipid diffusion in the outer leaflet of the plasma membrane bilayer[3,37]. The discrimination between apical and basolateral endosomes has been omitted for reasons of simplicity. The compartments have been drawn roughly to scale except for the ER, which should measure about three times the surface area of the Golgi complex[47]. The thickness of the arrows emphasizes the size of the recycling pathway between the ER and Golgi complex relative to forward transport to the plasma membrane (50% versus 1% of the ER surface area per 10 min in fibroblasts[47,48]), and between endosomes and the plasma membrane (an area equivalent to the complete plasma membrane surface is internalized and recycled each hour[47]) relative to the other pathways shown. Sorting occurs at each intersection of transport routes.

short-chain GlcCer did not reach the cell surface of mitotic CHO cells[19]. In addition, transport of both lipids in fibroblasts was inhibited to a similar extent by monensin, an ionophore that interferes with protein transport through the Golgi complex[8], and in epithelial cells was inhibited by lowering the temperature[20,21]. Transport to both apical and basolateral epithelial cell surfaces was affected to the same extent (Box 1).

Additional evidence that GlcCer transport, at least to the apical cell surface of epithelial MDCK cells, is vesicular comes from a study in which the basolateral plasma membrane was permeabilized to allow a scavenger of short-chain lipids, bovine serum albumin (BSA), into the cytosol[17]. In this experiment, addition of BSA to the basolateral side of the permeabilized cells depleted a significant fraction of short-chain GlcCer from the Golgi complex[17,21]. Remarkably, the presence of BSA in the cytosol did not reduce delivery of short-chain GlcCer to the apical surface, indicating that transport probably did not occur via exchange of monomers through the cytosol. In addition, the lipid was found to reside in the lumen of transport vesicles. Also, our observation that depolymerization of microtubules with nocodazole reduces the efficiency of apical transport of both short-chain GlcCer and SM about twofold (G. van Meer, unpublished) supports the conclusion that translocation of GlcCer occurs in or before the TGN, and possibly as early as the *cis* Golgi complex; GlcCer subsequently follows the vesicular pathway to the cell surface.

Sphingolipids are sorted continuously

A vesicular or tubular return pathway from the *cis* Golgi complex to the ER/intermediate compartment has now been firmly established, but the ER and the intermediate compartment have been found to contain very low levels of sphingolipids[3,36]. If SM and GlcCer synthesis occur in the *cis* Golgi complex[9–12], these lipids must be excluded from the return pathway in a sphingolipid sorting event. (See Box 2 for a discussion of sorting.) Sphingolipid sorting may involve clustering of sphingolipids into a microdomain that excludes glycerolipids[3]. The microdomain would be selectively incorporated into anterograde transport vesicles and/or excluded from retrograde transport. Sphingolipids may cluster due to their tendency to form intermolecular hydrogen bonds (Fig. 1). The event could repeat itself in each subsequent Golgi cisterna, thereby enhancing the sorting efficiency of the system. However, the extent of return traffic through the Golgi stack remains uncertain and an alternative explanation for the absence of equilibration of, for example, SM with the ER could be location of SM-synthase distal to a unidirectional transport step.

The time for delivery of newly synthesized short-chain sphingolipids to the surface of CHO fibroblasts is 5 min for GlcCer and 14 min for SM[15]. This short delivery time for GlcCer is very similar to that observed for the native ganglioside GM_3 in

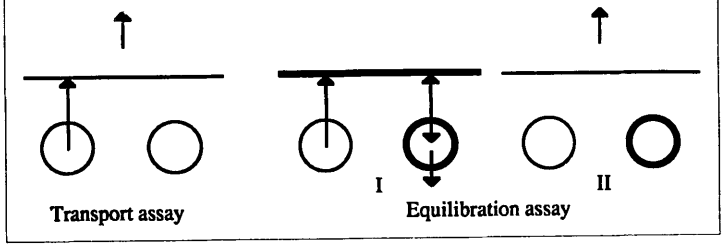
the same cell line[31]. In HepG2 cells the half-times for surface delivery of short-chain GlcCer and SM are 10 and 30 min, respectively (G. van Meer, unpublished). A similar difference was observed in intestinal epithelial cells[20], but not in MDCK cells[21] or fibroblasts[8]. Faster arrival of GlcCer at the surface could be explained by location of the GlcCer translocation event distal to SM-synthase[15]. Alternatively, GlcCer could be sorted more efficiently than SM. Indeed, due to its carbohydrate moiety, GlcCer has a greater capacity for hydrogen bonding than SM.

Sorting of newly synthesized GlcCer from SM has been observed in polarized epithelial cells[20,21]. In intestinal epithelial cells[37], the apical surface contains a much higher concentration of glycosphingolipids than the basolateral surface – it is almost completely covered by them. By contrast, the basolateral plasma membrane has a two- to fourfold higher concentration of SM and PC. A variety of newly synthesized short-chain analogues of GlcCer reached the two surfaces with an apical:basolateral ratio that was three- to ninefold higher than that of the corresponding SM[20], implying sorting of GlcCer from SM (see Box 2). This enrichment of glycosphingolipids in the apical pathway over SM would be sufficient to generate the lipid differences observed between the two surfaces *in vivo*. Based on the assumption that both GlcCer and SM travel through the TGN, a sorting mechanism was proposed[21,37]. In this model, GlcCer clusters in the luminal leaflet of the TGN membrane and forms a domain relatively poor in SM and the major basolateral phospholipid PC. This domain subsequently buds into a vesicle with an apical destination.

A third sphingolipid sorting event has been observed in studies of lipid endocytosis. After endocytosis, short-chain SM recycles to the cell surface with a half-time of 40 min, transport to the lysosomes being some 20-fold slower than recycling. The TGN appears not to play a significant part in SM recycling[4,38]. By contrast, a fraction of the complex gangliosides GM_1 and GM_2 (Fig. 2) reaches the Golgi complex after endocytosis and is subsequently used for GD_{1a} synthesis, but this takes place on a timescale of days[5]. Similar experiments have also shown that some GlcCer derived from endocytosed LacCer is re-glycosylated to LacCer[39], suggesting that GlcCer in the endocytic pathway may even reach the *cis* Golgi complex. More convincingly, after only 1 h at 37°C as much as 10% of Shiga toxin that was endocytosed after binding to a glycolipid (globotriasylceramide) on the cell surface was recovered in the Golgi complex, possibly also reflecting transport of the glycolipid[40]. Furthermore, in undifferentiated HT29 intestinal cells, endocytosed short-chain GlcCer accumulates in the Golgi complex, while the corresponding SM, GalCer and LacCer analogues do not[38]. In these latter experiments, data were provided to exclude sphingolipid degradation and re-utilization of ceramide that had earlier been observed in fibroblasts[5], and it was proposed that sorting had occurred in the early endosome. In order to be sorted, the Shiga toxin–globotriasylceramide complex and GlcCer must have concentrated into a domain in the luminal leaflet of the endosomal membrane, a domain probably destined for the TGN (Fig. 3).

BOX 1 – ASSAYS FOR LIPID TRANSPORT TO THE CELL SURFACE

Two types of assays have been employed to measure transport of newly synthesized sphingolipids to the cell surface. The first, the transport assay, makes use of artificial short-chain lipid analogues, which is an inherent drawback. Upon arrival at the surface these lipids either spontaneously partition into the medium or they can be depleted by liposomes or BSA. Surface delivery can be monitored continuously during the experiment, while endocytosis is prevented[15–17,20,21]. In the second type of assay, the equilibration assay, radiolabelled but otherwise natural lipid analogues are allowed to be transported to the surface for a given time period (I), after which transport is blocked by cooling or fixing the cells. The presence of the lipid on the cell surface is subsequently assayed (II) by an enzymatic or chemical assay[31,50] or by cell fractionation[39]. The drawback of this method is that it does not monitor surface arrival *per se*, but rather the amount at the surface at a specific time-point, which is the product of two processes: cell surface delivery and endocytic equilibration. Short-chain analogues can also be allowed to be endocytosed during a transport incubation. Afterwards the analogues still residing at the cell surface can be 'back-exchanged' to a suitable acceptor in the cold[8,19]. Because lipid is withdrawn from the recycling route by transport to the lysosomes, eventually no labelled lipid will be found at the cell surface if an equilibration assay is used. By contrast, all the lipid delivered to the surface will be recovered in the medium in a transport assay. The different approaches give different information, which must be interpreted accordingly.

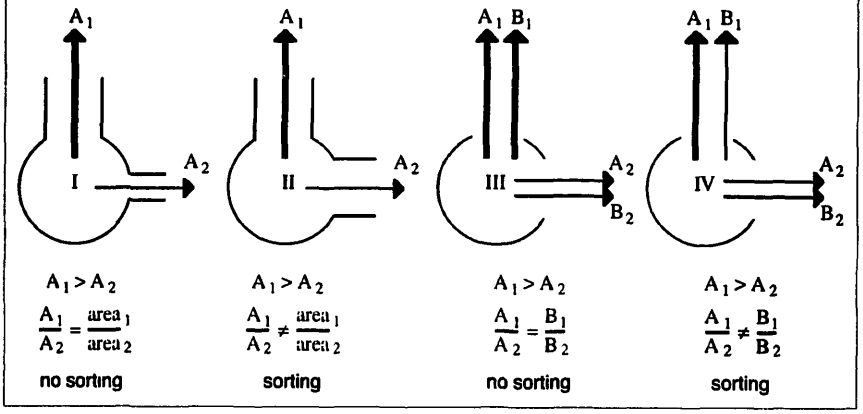


Proteins and lipids: sorting and sorted

A common theme in the sorting of membrane components is their concentration at a site where vesicles bud and leave with a defined destination. The structure of sphingolipids and their behaviour in mixed-lipid model membrane systems suggest that concentration into a microdomain could be driven by intermolecular hydrogen bond formation; other lipids, notably PC, would then be excluded. This process would be expected to be more efficient for GlcCer than for SM, and the specific properties of each compartment, such as their lipid composition and luminal ion concentrations, should influence the degree to which microdomain formation occurs. Thus, in the *cis* Golgi complex even SM may efficiently partition into a sphingolipid microdomain, whereas the conditions for domain formation in the TGN and endosomes may be less favourable. Here, a domain of glycosphingolipids may still form but SM may no longer efficiently interact with it. Despite the attraction of models invoking sphingolipid microdomains in intracellular sorting, it should be noted that proof that such microdomains are a biological reality is still lacking.

BOX 2 – PATHWAY SIZES AND SORTING

The fact that more of membrane component A is transported in direction 1 than in direction 2 ($A_1 > A_2$) does not necessarily imply that A is sorted. A is not sorted when at the branching point of the pathway it partitions into the outgoing pathways 1 and 2 with the same ratio as the area of membrane flowing through those pathways: in other words, when the surface density of A is the same in both pathways ($A_1/area_1 = A_2/area_2$; I). A is sorted only when the surface density is higher in one of the pathways ($A_1/area_1 \neq A_2/area_2$; II). Mostly, the areas of membrane flowing through particular pathways are unknown. In that case, surface densities cannot be calculated and it is impossible to establish sorting by measuring transport of one component only. One way to get around this difficulty is to study sorting of one component from another[20,21,38]. Sorting is not observed when A and B partition into the outgoing pathways with the same ratio (III), but sorting is observed when A is concentrated over B in one of the pathways ($A_1/B_1 \neq A_2/B_2$; IV).



Components to be sorted must interact with the structural determinants that guide a transport vesicle to its membrane of destination. Since this requires information on the cytoplasmic surface of the vesicle, sorting of a luminal sphingolipid domain must involve a transmembrane protein, the putative 'sorter' protein[37]. It is inevitable that proteins are involved in sorting lipids, although it remains unclear by what physicochemical mechanism the sorter protein would recognize and anchor a lipid domain. But are lipids involved in protein sorting? All proposed sites of sphingolipid sorting have already been established as sites of membrane protein sorting. Furthermore, one example has now been demonstrated where sorting of proteins correlates with that of lipids and where we can at least start to think of a sorting mechanism involving specific lipid–protein interactions. This concerns the GPI-anchored proteins that are attached to the membrane by a glycosylphosphatidylinositol anchor (see accompanying review by Deborah Brown). GPI-anchored proteins are virtually confined to the apical cell surface in MDCK cells and it has been suggested that their glycolipid tail would aggregate them into the same microdomain as GlcCer[41]; hydrogen bonding between the carbohydrates would have to provide the driving force as their diacylglycerol backbone does not possess this property. In support of this idea, we have recently observed that in thyroid FRT cells, which display a reversed polarity of GPI-anchored proteins, GlcCer polarity is also reversed: compared with SM, GlcCer is preferentially targeted to the basolateral domain (C. Zurzolo, W. van 't Hof, G. van Meer and E. Rodriguez-Boulan, unpublished). In this context, it is interesting that both GPI-anchored proteins and sphingolipids are highly resistant to detergent extraction in the cold, whereas the bulk of the glycerolipids are not. Newly synthesized GPI-anchored proteins become resistant to detergent only upon entering the Golgi complex, which fits with the idea that this is caused by an interaction of the GPI-anchored proteins with SM and/or glycosphingolipids[42].

A major challenge will now be to define similar correlations between individual transmembrane proteins and sphingolipids with respect to their intracellular traffic, sorting and physicochemical properties. Cell-free transport systems may prove valuable in this task. For example, transport of LacCer into a sialyltransferase-I-containing compartment was found to have the same requirements as the vesicular transport of the vesicular stomatitis virus (VSV) G glycoprotein[43]. Another approach is to interfere with sphingolipid synthesis or sorting and then study the effects on transport and sorting of proteins. An inhibitor of GlcCer synthesis retarded transport of the VSV G protein through the Golgi complex, although only at concentrations where SM synthesis was also reduced[44]. Along these lines, we have recently found two agents, monensin and BFA, that abolish sphingolipid sorting in epithelial cells without drastic effects on surface delivery of the lipids (G. van Meer and W. van 't Hof, unpublished).

The intracellular transport pathways of proteins and sphingolipids clearly need to be defined in more detail. In the case of the sphingolipids this should include their distribution among cellular organelles and the exact location of their biosynthetic and translocation events. In the light of these questions, the recently developed procedure to immunolocalize lipids at the electron microscopy level may prove useful, particularly as this method has the potential to provide quantitative information[45]. The relative importance of the various lipid transport pathways will need to be determined, as will the physicochemical behaviour of the individual lipid species and their complex mixtures in the various cellular membranes. The lipid-synthesizing enzymes and translocators must be purified and characterized, and finally the components of the sorting machinery must be elucidated. This constitutes a formidable task. However, we are sure that the results will justify the labour.

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