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Minireview

Intracellular lipid heterogeneity caused by topology of synthesis and specificity in transport. Example: sphingolipids

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Abstract The differences in lipid composition between intracellular membranes cannot be adequately explained by local synthesis and degradation. Especially in the case of sphingolipids, which are synthesized in the Golgi complex but enriched on the cell surface and in endocytotic organelles, there is evidence for a cellular machinery that preferentially shuttles these lipids in vesicles to the cell surface. The machinery appears to involve the formation of domains of sphingolipid and cholesterol in the lumenal leaflet of Golgi membranes. Several pieces of evidence suggest that the selective anterograde transport of plasma membrane proteins may be mechanistically related to the sphingolipid domains.

Key words: Glycosphingolipid; Sphingomyelin; Ceramide; Diacylglycerol; Sorting

1. Membrane composition and function

The membranes of eukaryotic cells separate the cytosol from the external environment and from the lumen of the various organelles. Thereby they compartmentalize the cellular metabolism. The difference in function between organelles is reflected by a difference in the chemical composition of their membranes, first of all in the protein complement. It is well-recognized that the membrane proteins are essential for maintaining the unique chemical conditions inside organelles by selective translocation of molecules across membranes. In addition, many enzymatic reactions in organelles occur by specific membrane proteins on the lumenal surface of their membrane. A special case is the role of receptors in recognition events on the cell surface. Correlations between differences in lipid composition and function have so far been observed especially for the lumenal, exoplasmic leaflet of membranes. The clearest case are the glycosphingolipids (GSL) which are enriched in the exoplasmic leaflet of the plasma membrane where they protect [1] and take part in recognition events and signal transduction [2]. In general, the enrichment of sphingolipids and cholesterol in the distal compartments of the exocytotic pathway and of the endocytotic pathway (Fig. 1) provides the exoplasmic surface of these membranes with a high chemical and mechanical stability [3].

It becomes increasingly clear that membranes (and the cytoskeleton) serve as scaffolds to organize chemical events in the cytosol as well. Also here a need for specific lipids has been documented. Besides proteins, lipids modulate the recruitment of cytosolic proteins to specific membranes and regulate their activity. Mostly, this involves changes in lipid composition on a time-scale from seconds to minutes. Prime example is the inositol-lipid cycle on the inner surface of the plasma membrane [4,5], but evidence exists for the more general nature of this phenomenon (see below).

To understand the lipid heterogeneity between cellular membranes, the sites of synthesis, modification and degradation must be defined for the major membrane lipids and the rates of these processes at the various sites must be determined. As for proteins it will become clear that the metabolic reactions by themselves can not explain the lipid compositional differences. The solution must be found in the selective nature of intracellular lipid traffic.

2. Lipid transport pathways

Most organelles in the cell are connected by vesicular transport (Fig. 1). Vesicles bud from one compartment, traverse the cytosol and fuse with a second compartment. Targeting appears to be exquisitely controlled [6]. Besides carrying membrane proteins, the vesicles transport membrane lipids from both the cytosolic and lumenal leaflet of the membrane, whereby the transmembrane orientation of the lipids is maintained through the various steps of the process (Fig. 2, '1'). For example, GSL synthesized in the Golgi lumen after a vesicular transport step will be delivered to the cell surface with a halftime of some 20 min [7].

In contrast to membrane proteins, membrane lipids can also be transported by monomeric exchange through the aqueous phase. This exchange can only occur between cytosolic leaflets of cellular membranes (Fig. 2, '2'). It is the only pathway by which lipids can be transported to organelles that are not connected to vesicular transport routes, like mitochondria and peroxisomes. As an example, the glycerophospholipid phosphatidylcholine (PC), after synthesis at the cytosolic face of the ER, reaches the plasma membrane within minutes [8] and equilibrates with PC in the outer mitochondrial membrane [9]. Because spontaneous exchange is a slow process for most membrane lipids ($t_{1/2}$ = hours to weeks [10]), PC exchange may have been accelerated by proteins like the PC- and the phosphatidylinositol/PC-specific transfer protein [11]. These proteins have the potential to equilibrate lipids between donor and acceptor vesicles in vitro. However, whether they are able to support net transport of phospholipids and whether they do so under biological conditions is still unclear. Monomeric exchange may be

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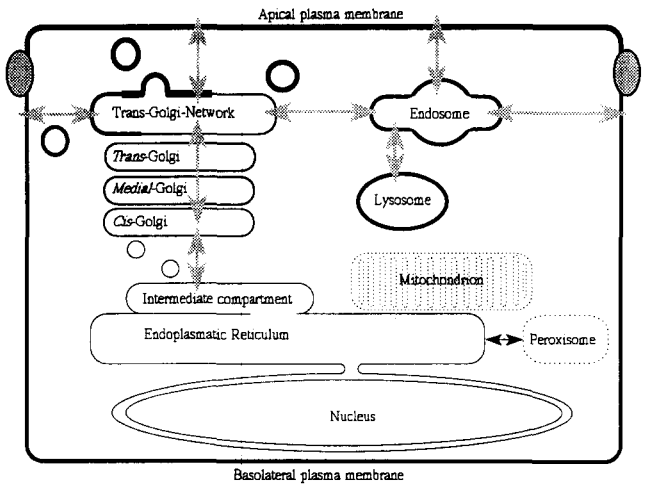

Fig. 1. Cellular organelles connected by vesicular traffic. Dotted lines indicate organelles not connected by this type of transport, but by exchange of monomers through the cytosol. Thickness of the lines reflects the concentration of (glyco)sphingolipids and cholesterol.

limited to specialized areas of close 'contact' between the two lipid bilayers, as has been argued for mitochondria and ER [12,13]. The non-specific lipid-transfer protein nsL-TP is thought not to be present in the cytosol [14].

When membranes of different lipid composition are continuous, a relevant lipid transport process is lateral diffusion (0.1–1 $\mu m^2/s$). Occasionally, membrane continuities between organelles have been proposed [15] but it is generally held that intracellular organelles are separate entities. In the case of the two plasma membrane domains of epithelial cells, which have widely different lipid composition, the structure separating them, the tight junction, was found to inhibit diffusion in the exoplasmic leaflet where the differences reside [16]. So it would seem that lateral diffusion is most relevant for equilibrating lipids within one bilayer leaflet of an organelle (Fig. 2, '3') or between subdomains of an organelle like the ER and nuclear membrane (Fig. 1). However, it should be realized that areas of different lipid composition have been found in model membranes and are thought to exist in biomembranes [17,18], which puts a restriction on lateral diffusion.

The two bilayer leaflets of biomembranes may have different lipid compositions. This is especially clear for the plasma membrane, where sphingolipids and PC are generally enriched in the exoplasmic and the aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) in the cytoplasmic bilayer leaflet [19]. The rates of transmembrane translocation are essential for lipid traffic: From the lumen of an organelle, a lipid can reach the lumen of another organelle, or the cell surface by vesicular transport but also by flipping across the membrane followed by exchange and a reverse translocation (Fig. 2, '4'). The importance of the two routes depends on the rates of the individual processes. Because in this process the polar headgroup of the membrane lipids has to traverse the hydrophobic bilayer interior, the spontaneous 'flip-flop' of lipids with a large or charged headgroup, glycolipids and phospholipids, is very slow ($t_{1/2}$ of many hours or days), whereas molecules like cholesterol, diacylglycerol (DAG) and ceramide flip in seconds. Partially due to this rapid flip, the transbilayer distribution of cholesterol is still controversial. While preferential interaction of cholesterol with sphingolipids would predict a location in the exoplasmic leaflet of the plasma membrane [20], experimental evidence has suggested the opposite [21]. The rate of flip-flop in the ER has been found to be high for PC [22] and for other phospholipids ($t_{1/2} < 30$ min) [23], and the presence of an energy-independent flippase has been proposed. In the plasma membrane, PS and PE are actively translocated towards the cytosolic side by the 'aminophospholipid translocase' [24].

In order to understand the differences in lipid composition between cellular membranes, we now have to find specificity in the various transport processes. Here, we describe the mechanisms responsible for the enrichment of sphingolipids in the plasma membrane and endocytotic organelles.

3. Topology of sphingolipid synthesis and mechanism of transport

The phosphosphingolipid sphingomyelin (SM), which accounts for some 10% of the cellular lipids, is synthesized by the transfer of phosphocholine from the DAG in PC onto ceramide. The bulk of the sphingomyelin synthase activity has been assigned to the lumen of the *cis*-Golgi [25,26] and no translocation of newly synthesized SM to the cytosolic surface was observed [27–29]. Transport of SM to the plasma-membrane surface must, therefore, occur by the exocytotic vesicular pathway (Figs. 1 and 2), and this is supported by the observation that newly synthesized native SM did not reach the cell surface in fibroblastic cells when vesicular transport from the Golgi to the plasma membrane was inhibited in the presence of brefeldin A [30] and during mitosis (van Helvoort, manuscript in preparation). No inhibition by brefeldin A was observed in liver [31].

It would be expected that if SM were able to reach the ER, it could translocate to the cytosolic leaflet [23] from where it

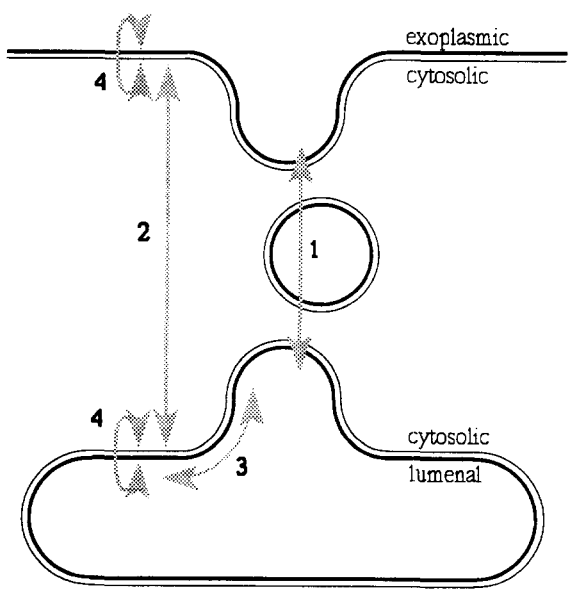

Fig. 2. Possible transport pathways for membrane lipids between an intracellular organelle and the plasma membrane. (1) Vesicular traffic. (2) Monomeric exchange. (3) Lateral diffusion. (4) Transbilayer movement.

would be able to reach the cytosolic surface of other membranes by exchange or by vesicular transport (Fig. 2). First of all, the SM content of the ER is low suggesting that SM is excluded from the extensive vesicular pathway from the *cis*-Golgi to the ER [20,32]. Second, exchange of SM through the cytosol does not seem a major process as the SM concentration in peroxisomes and mitochondria has been found to be extremely low [20]. However, we have observed that a short chain SM analog [33] reached the cell surface in the presence of brefeldin A and, in contrast with earlier work [34], in mitotic cells (van Helvoort, manuscript in preparation). Because vesicular traffic is inhibited under these conditions, SM seems to be translocated to the lumenal surface in a compartment late in the exocytotic pathway, which may reflect a mechanism to correct (minor) SM leakage to the cytosolic surface.

Part of the SM synthase activity has been localized to the cell surface [26,35]. Whether SM synthesis also occurs in endosomes must be shown by direct experiments [36]. The enzyme activity on the plasma membrane may be due to a different isoform from that in the Golgi and may serve a different purpose. We have suggested that this enzyme may regulate the concentration of the lipid second messengers DAG and ceramide in the plasma membrane during signal transduction processes [35]. Although DAG and ceramide have been claimed to be generated in the cytosolic leaflet, they rapidly equilibrate across the membrane due to their apolar character whereafter they would be substrate for the enzyme (unless DAG generated by phospholipase C would remain restricted to a signal transduction complex [37]). Also in the Golgi DAG may be involved in regulatory events as Golgi-specific protein kinase C isoforms have been found [38]. As SM synthase also clamps the levels of DAG and ceramide in the *cis*-Golgi, it may be part of a regulatory machinery in the Golgi as well. It has been suggested that PC [39], DAG [40] and ceramide [41] influence the rate of vesicle transport in the exocytotic pathway.

Glucosylceramide (GlcCer), the precursor for most higher GSL, is synthesized by the glucosyltransferase at the cytosolic site of the *cis*-Golgi, and some other Golgi-related compartment [29,42–44]. From its site of synthesis GlcCer can exchange through the cytosol. Indeed, GlcCer reached the plasma membrane in the presence of brefeldin A whereas SM did not [45]. Because spontaneous exchange of GlcCer is expected to be very slow, exchange may have been facilitated by a transfer protein [46]. It is unclear whether GlcCer can translocate from the inner to the outer leaflet of the plasma membrane. Short chain GlcCer reached the cell surface in the presence of BFA [33] and in mitotic cells (van Helvoort, manuscript in preparation), arguing for a translocase activity in a membrane late in the exocytotic pathway. On the other hand, GlcCer has been shown to translocate towards the lumenal leaflet of the Golgi where part of it is used for higher GSL synthesis [47], while the other part is transported to the cell surface by vesicular transport. Translocation of GlcCer from the outer to the inner leaflet of the plasma membrane has been reported to occur in some cells [48] but not in other cells [49]. Identification of translocator proteins and their localization in the cell may be expected to resolve these issues.

The galactosyltransferase synthesizing galactosylceramide, the major monohexosylceramide of myelin and of some epithelia, possesses a cytosolic ER-retrieval signal and its sequence suggests a lumenal orientation [50,51]. Although we recently confirmed its localization by gradient fractionation and protease digestion studies, the galactosylceramide product appeared to have access to the cytosolic surface of the ER. Indeed, direct translocation of both glucosyl- and galactosylceramide to the lumen of the Golgi could be demonstrated (Burger, van der Bijl, van Meer, manuscript in preparation). So the transport of the monohexosylceramides may be very similar.

The higher GSL are synthesized in the Golgi lumen and from what little evidence is available, they cannot translocate towards the cytosolic leaflet [47]. Their transport to the cell surface has been found to follow the kinetics of vesicular traffic [7,52].

4. Selectivity in sphingolipid transport

Although vesicular pathways connect the Golgi to both the ER and to the plasma membrane, SM and glycosphingolipids are enriched only in the latter. The same has been found for cholesterol [20]. The exocytotic pathway might be unidirectional and the high sphingolipid concentrations in the plasma membrane might be due to a relative high metabolic stability of sphingolipids as compared to phospholipids. This is unlikely in view of the retrograde transport through the Golgi to the ER [53]. As an alternative, based on our work on epithelial lipid sorting [18], we have suggested that sphingolipids and cholesterol aggregate in the lumenal leaflet of Golgi membranes and that these aggregates are preferentially included into anterograde vesicles [20]. Arguments have been provided to suggest that at the site of these domains the bilayer will be thicker (Fig. 1) and that the thickness selects for membrane proteins with longer transmembrane domains [54]. In turn, these proteins could be responsible for incorporation of the domains into the forward transport vesicles.

In the *trans*-Golgi network (TGN) of epithelial cells a further sorting of membrane components occurs into an apical and a basolateral complement of proteins and lipids. The enrichment of GSL in the apical domain of intestinal cells, which we also observed in our transport studies [33], led us to suggest that a different type of domains would assemble in the TGN of epithelial cells. The apical precursor domain would consist primarily of GSL whereas SM would be preferentially included in domains destined for the basolateral cell surface [18]. The apical enrichment of lumenal proteins that are attached to the membrane by a glycosylphosphatidylinositol (GPI) anchor is possibly due to preferential interaction of the GPI anchor with the GSL domains [55]. Interestingly, a common physico-chemical property of GSL and GPI-anchored proteins turns out to be a relative insolubility in detergent at low temperature [56], a feature previously described for an apical transmembrane protein [57]. Detergent-insolubility has been a valuable tool to identify proteins that may be involved in apical sorting, but it should not be overinterpreted to imply that the detergent-insoluble membrane remnants are the original apical precursor domains. First of all, SM is highly detergent-insoluble but is concentrated on the basolateral surface of intestinal cells [18]. Second, in a recent study not all GSL were preferentially transported to the apical cell surface (van der Bijl, Lopes-Cardozo, van Meer, manuscript in preparation). Third, it is difficult to see how a sphingolipid/cholesterol domain on the lumenal side of the membrane could persist when the phospholipids from the cytosolic leaflet are washed out by the detergent. Finally, espe-

cially in the case of lipids the addition of detergent will alter the equilibrium distribution of the native lipids between the apical and basolateral precursor domains. Similar arguments have been presented against using detergents for the isolation of two other lateral domains, the inner nuclear membrane [58] and caveolae [59].

Lateral segregation of sphingolipids and cholesterol is an attractive mechanism to explain their enrichment during transport to the cell surface. Also epithelial sorting is most easily understood in terms of lateral segregation of lipids and proteins in the lumenal leaflet of, in this case, the TGN. Epithelial cells of different origin display widely different sphingolipid compositions. It is a challenge to try and identify the physico-chemical rules that govern lipid domain formation in these complex environments. The next question is how these domains interact with the various membrane proteins. Lipid domains may also exist on the cytosolic surface of cellular membranes. It is not too difficult to come up with a myriad of potential biological functions. Let's sort them out.

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