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Lipid microdomains, lipid translocation and the organization of intracellular membrane transport (Review)

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Summary

Eukaryotic cells contain hundreds of different lipid species that are not uniformly distributed among their membranes. For example, sphingolipids and sterols form gradients along the secretory pathway with the highest levels in the plasma membrane and the lowest in the endoplasmic reticulum. Moreover, lipids in late secretory organelles display asymmetric transbilayer arrangements with the aminophospholipids concentrated in the cytoplasmic leaflet. This lipid heterogeneity can be viewed as a manifestation of the fact that cells exploit the structural diversity of lipids in organizing intracellular membrane transport. Lipid immiscibility and the generation of phase-separated lipid domains provide a molecular basis for sorting membrane proteins into specific vesicular pathways. At the same time, energy-driven aminophospholipid transporters participate in membrane deformation during vesicle biogenesis. This review will focus on how selective membrane transport relies on a dynamic interplay between membrane lipids and proteins.

Keywords: Sorting, lipid raft, sphingolipid, flippase, vesicle budding.

Introduction

Metabolic processes in eukaryotic cells are compartmentalized into distinct membrane-bound compartments or organelles. In general, these sub-cellular structures are not created *de novo*, but inherited by the daughter cell upon cell division (Warren and Wickner 1996). Hence, organelles are putative carriers of inheritable information that is not provided by the genome. Given that the various organelles display striking differences in the composition, texture and sidedness of their membrane lipids, part of this organelle-specific information might be encoded in the membrane lipid arrangement. For example, the plasma membrane (PM) has a strongly asymmetric transbilayer lipid distribution and contains high levels of sterols and sphingolipids (Figure 1). This is in sharp contrast to the situation in the endoplasmic reticulum (ER). These differences in lipid organization cannot be explained by local metabolism alone and are maintained in spite of extensive membrane trafficking between the two organelles. Without mechanisms imposing directionality and selectivity on lipid movement, lipids would readily equilibrate between the different organelles as well as between the two leaflets of each organellar membrane.

This review will describe one's current understanding of the transport mechanisms responsible for maintaining the non-random lipid distribution over and within organelles connected by vesicular traffic. Like membrane proteins, lipids travelling along the secretory and endocytic pathways must be preferentially incorporated into one type of transport vesicles or excluded from others to reach their proper destination. However, lipids lack the type of address labels by which proteins are sorted into vesicles. So, how then is lipid sorting accomplished? Studies in artificial bilayers reveal that lipids have a strong self-organizing capacity; lipid immiscibility can drive phase separation and give rise to domains with unique lipid compositions and biophysical properties. Current evidence supports the existence of such phase-separated lipid domains in cellular membranes, even though their composition, size and lifespan remain uncertain (Anderson and Jacobson 2002). The main attraction of a spontaneous lateral segregation of membrane lipids is that it would allow cells to sort membrane components independently of molecular interactions across the bilayer. One will discuss how cells may exploit lipid-based sorting mechanisms to safeguard the boundaries of their organelles.

Apart from the need for lipids to laterally segregate during vesicular traffic, they must adopt a correct distribution over the two membrane halves. In biogenic membranes like the ER, phospholipids produced in the cytoplasmic leaflet have to flip to the lumenal leaflet to allow balanced growth of the bilayer. Glucosylceramide synthesized on the surface of the Golgi has to cross the membrane to allow complex glycolipid production. In each case, transport of the lipid across the bilayer requires proteins. Protein-assisted flip-flop in the ER occurs independently of metabolic energy, is bi-directional and has little or no phospholipid specificity (Menon 1995). Hence, most phospholipid classes are thought to adopt a symmetrical distribution across the ER bilayer (Figure 1). The situation is different in the plasma membrane where aminophospholipids are concentrated in the cytoplasmic leaflet by an ATP-dependent aminophospholipid translocase (Devaux 1991).

A similar activity has been detected in Golgi-derived secretory vesicles. The action of the aminophospholipid translocase is counteracted by an outward-directed ATP-dependent lipid pump of broader substrate specificity. Hence, the various organelles along the secretory and endocytic pathways are equipped with different kinds of lipid flippases to control their transbilayer lipid arrangements. It appears that these activities serve a fundamental role in vesicular traffic, either by facilitating membrane curvature during vesicle biogenesis or by creating a membrane environment permissive for this process to occur.

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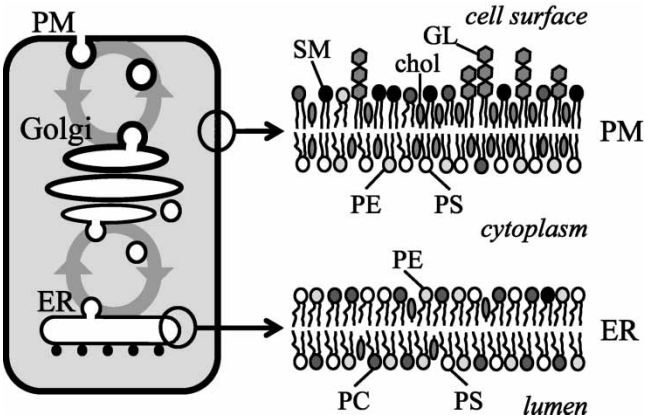

Figure 1. Cellular organelles display different membrane lipid compositions and transbilayer lipid distributions. The plasma membrane (PM) displays transbilayer lipid asymmetry and is rich in sphingolipids, sterols and saturated glycerolipids that promote the thickness and impermeability of the membrane. In contrast, the endoplasmic reticulum (ER) shows a symmetric lipid distribution and primarily contains unsaturated glycerolipids that make the membrane more flexible, hence facilitating incorporation of newly synthesized protein. Membrane traffic between the ER and the PM passes the Golgi, a multi-cisternal organelle where lipid sorting must occur to prevent randomization of the sub-cellular lipid distribution. PC; phosphatidylcholine, PE; phosphatidylethanolamine, PS; phosphatidylserine, SM; sphingomyelin, GL; glycosphingolipids, chol; cholesterol.

Lipid sorting during vesicular transport

Lipid trafficking rules

The various organelles of a cell require distinct protein and lipid compositions to perform their specialized tasks. In principle, each organelle acquires the correct mixture of these molecules through a three-step process: (1) protein and lipid biosynthesis, (2) selective transport of proteins and lipids across organellar membranes, and (3) selective transport of proteins and lipids between different organellar membranes. Over the last 20 years, tremendous progress has been made in uncovering the mechanisms responsible for inter- and intra-organellar protein transport. In comparison, knowledge of how cells move and sort lipids is still rather limited. This is likely due to the fact that the trafficking rules that apply to lipids are less absolute than those for proteins and, therefore, harder to translate into molecular terms.

Membrane lipids are much smaller than proteins and span only half the membrane. Consequently, lipids can be subject to transport mechanisms that are irrelevant for membrane proteins. To begin with, lipids can move between the two leaflets of the membrane bilayer, a process termed flip-flop. In model membranes, flip-flop is slow for lipids carrying a polar headgroup, but more rapid for lipids without one. Half times range from seconds/minutes for diacylglycerol, ceramide and cholesterol, to hours/days for phosphatidylcholine (PC), sphingomyelin and glycosphingolipids (Phillips *et al*. 1987, Trotter and Voelker 1994, Bai and Pagano 1997). Lipid transport across cellular membranes can be facilitated or driven by proteins termed flippases or translocases. Another mode of transport almost exclusively used by membrane lipids involves their monomeric exchange between the cytosolic membrane surfaces of different organelles. This process is especially relevant for organelles not connected by vesicular pathways like mitochondria and peroxisomes. Monomeric exchange requires desorption of the lipid from the membrane, passage through the aqueous phase and subsequent insertion into a different membrane. Spontaneous diffusion into the aqueous phase is extremely slow for lipids carrying two long fatty acid chains, but occurs at a significant rate for single chain lipids. Half times range from days/weeks for PC and glycosphingolipids to hours for cholesterol and seconds/minutes for lyso-PC and sphingoid bases (Phillips *et al*. 1987, Steck *et al*. 1988, Bai and Pagano 1997). Cytosolic proteins with specific lipid binding sites have been identified that are capable of accelerating lipid exchange rates *in vitro*. While some of these proteins may function as bona fide lipid carriers, others are thought to act primarily as membrane-bound lipid sensors with a regulatory role in lipid metabolism and membrane trafficking (Li *et al*. 2000). Monomeric exchange can also be facilitated by specialized contact sites between different organelles, like the ones existing between ER and mitochondria (Daum and Vance 1997). Ceramide, the precursor of all sphingolipids, can be transported from the ER to the Golgi by means of a non-vesicular mechanism that depends on ER-Golgi membrane contact sites and a cytosolic factor (Funato and Riezman 2001).

As a transport mechanism, monomeric exchange is irrelevant for sphingomyelin and complex glycosphingolipids because these lipids are produced in the lumenal leaflet of the Golgi and do not have access to the cytoplasmic leaflet (Burger *et al*. 1996). In fact, this may explain why sphingolipids are hardly found in peroxisomes or mitochondria. Although their synthesis occurs primarily in the Golgi, sphingolipids are enriched in the plasma membrane (Patton and Lester 1991, van Meer 1998). The only way they are able to get there is by vesicular transport. The Golgi is a polarized organelle situated right at the intersection of two major circuits of vesicular trafficking (Figure 1). One circuit interconnects the *cis*-side of the Golgi with the ER and the other one the *trans*-side with the plasma membrane. The Golgi consists of multiple compartments, called cisternae, which are stacked and communicate by shuttling vesicles. Sphingolipids are more abundant in the plasma membrane compared with the Golgi, and in the Golgi compared with the ER (van Meer 1998). Hence, a mechanism must exist by which the Golgi prevents the backflow of sphingolipids to the ER and promotes their concentration toward its *trans*-side, where membrane carriers depart for the cell surface. This can only be achieved if the surface density of sphingolipids is increased at sites of anterograde membrane transport and/or reduced at sites of retrograde vesicle budding. Consequently, sphingolipids produced in the lumenal leaflet of Golgi cisternae must be laterally segregated from other membrane lipids, in particular from unsaturated glycerolipids that are concentrated in the ER (Keenan and Morré 1970). The precise mechanism by which this lipid sorting is accomplished remains to be established. However, as discussed below, attractive lipid–lipid interactions seem to play a fundamental role.

Rafts, caveolae and other lipid domains

Biophysical studies on model membranes firmly established that mixtures of sphingolipids, unsaturated glycerolipids and cholesterol can segregate spontaneously into two fluid phases, where the sphingolipids and part of the cholesterol coalesce into a 'liquid-ordered' domain and break away from the unsaturated glycerolipids in a 'liquid-disordered' phase (Brown and London 1998). This phase behaviour has a clear physico-chemical basis. Due to their long and saturated fatty acyl chains, sphingolipids contact their neighbours along a greater and flatter surface than unsaturated glycerolipids. This results in a dramatic increase in the van der Waals attraction between neighbouring sphingolipid molecules. Van der Waals interactions are also held responsible for the preferential interaction between sphingolipids and cholesterol, which is a rigid and flat-cylindrical molecule (Boggs 1987). Moreover, the interfacial region that connects the polar headgroup with the non-polar hydrogen carbon moiety in sphingolipids contains both amide and hydroxyl groups that can function as hydrogen bond donors and acceptors. Glycerolipids have only hydrogen bond-accepting properties in this part of the molecule. This would allow sphingolipids, but not glycerolipids, to self-associate into a flexible hydrogen-bonded network in the plane of the membrane (Pascher 1976).

Phase behaviour of membrane lipids might be of considerable biological importance. Indeed, the structural differences between sphingolipids and glycerolipids that promote their immiscibility have been well preserved from vertebrates to flies, worms and fungi (Holthuis *et al*. 2001). When these differences are undermined by mutation, organisms exploit lipid-remodelling mechanisms to recreate structural diversity (Schneiter *et al*. 1999, Guillas *et al*. 2001). Most strikingly, the sphingolipid requirement in yeast can be bypassed by a suppressor mutation that enables the organism to synthesize a novel set of glycerolipids whose structural appearance and predicted biophysical properties closely mimic those of the sphingolipids (Lester *et al*. 1993).

Liquid-ordered domains can be isolated from model membranes as detergent-insoluble remnants that float in sucrose gradients (Brown and London 1998). The isolation of detergent-resistant membranes (DRMs) from a wide variety of cell types has been interpreted as evidence for the existence of phase-separated lipid domains in cellular membranes (Brown and London 1998). It was found that lipids associate with DRMs based largely on their degree of acyl chain order and that isolated DRMs are enriched in sphingolipids and sterols. The term 'rafts' refers to sphingolipid/sterol-rich domains that are characterized by their insolubility in non-ionic detergents at 4°C and a light buoyant density on sucrose gradients (Simons and Ikonen 1997). It has been suggested that DRMs exist in intact cells as rafts floating in a detergent soluble, liquid-disordered sea. However, the finding that model membranes present in a single uniform phase between the liquid-disordered and liquid-ordered state exhibit partial insolubility indicates that the latter characteristic by itself does not prove that membranes contain rafts (Brown and London 1998). Moreover, the detergent extraction method is not suitable to assess the size of the original raft, because separate rafts within the same membrane may coalesce upon removal of the surrounding glycerolipids.

Further insight into whether rafts exist in cellular membranes has come from morphological studies. Many proteins found in DRMs are linked to saturated acyl chains, which is likely to make them prefer an ordered lipid environment. Consistent with this notion, only glycosylphosphatidylinositol-anchored proteins (GPI-APs) and acylated proteins, but not proteins anchored by the branched isoprenyl groups can be recovered from DRMs (Melkonian *et al*. 1999). Given their apparent affinity for an ordered environment, GPI-APs have been used as morphological markers to probe the cell surface for the presence of rafts. Direct immunofluorescence and immunoelectron microscopy showed that GPI-APs display a uniform distribution on the cell surface (Maxfield and Mayor 1997). These proteins, however, become clustered when cells are exposed to cross-linking antibodies. Simultaneous addition of two antibodies recognizing different GPI-APs causes co-clustering of the two proteins, even though each antibody by itself clusters only the protein it was directed against (Maxfield and Mayor 1997). The affinity for the independently clustered proteins for each other suggests that both are present in rafts. Hence, rafts could be very small and might coalesce into larger rafts upon clustering of individual components. An alternative possibility is that stable rafts exist, but that individual GPI-APs have a relative low affinity for them. The affinity would then be increased by clustering. In any case, it seems that binding to a multi-valent ligand of molecules having an affinity for ordered domains reduces their lateral mobility and stimulates raft association.

Antibody-induced GPI-AP clusters co-localize with caveolae (Rothberg *et al*. 1990). These flask-shaped invaginations of the plasma membrane have a cytoplasmic coat consisting of the cholesterol-binding protein caveolin and serve a role in endocytosis and signal transduction (Anderson 1998). Caveolae are detergent insoluble and rich in cholesterol and sphingolipids, although their precise lipid composition remains to be established. So far they form the only example where the structure of a putative lipid domain has been well established. Some cell types do not express caveolin and lack caveolae, yet DRMs isolated from these cells are enriched in many of the same molecules found concentrated in caveolae of caveolin-expressing cells (Fra *et al*. 1994, Wu *et al*. 1997). The recent application of several high-resolution optical techniques on living cells has led to the identification of areas on the cell surface with enhanced resistance to lateral diffusion that are preferred by some, but not by other membrane molecules. Depending on the technique used, the estimated size of these areas ranges from 50 nm (3000 molecules; Praelle *et al*. 2000) to 700 nm (600 000 molecules; Schutz *et al*. 2000). A special case may be the apical surface of epithelial cells of kidney and intestine, which is believed to be completely covered by sphingolipids. The absence of GPI-AP clustering on the apical surface of MDCK cells has led to the idea that the apical membrane of these cells is one big raft (Kenworthy and Edidin 1998). Hence, a considerable body of evidence suggests that rafts exist on the surface of cells, even though a general consensus about their size, shape and dynamics is lacking.

The finding that Src-family kinases are present in DRMs, although their acyl chains have only access to the cytoplasmic leaflet, suggests that rafts might exist there as well. The plasma membrane is enriched in saturated species of phosphatidylserine (PS) and phosphatidylethanolamine (PE; Keenan and Morré 1970, Schneiter *et al*. 1999) that are confined to the cytoplasmic leaflet by the aminophospholipid translocase. These lipids could form the basis for a liquid-ordered phase. The observation that antibody-induced clustering of a GPI-AP results in co-clustering of the doubly acylated Src-like kinase *fyn* indicates that lipid domains situated in opposite sides of the plasma membrane may interact (Harder *et al*. 1998). Coupling of cytosolic and lumenal rafts could involve transmembrane proteins, but may also be achieved solely on the basis of their constituent lipids (Dietrich *et al*. 2001).

Perhaps the best evidence for raft organization in the plasma membrane is functional. Numerous studies have implicated rafts in the compartmentalization, modulation and integration of signalling events by providing platforms for the assembly of cell surface receptors and their down stream effectors (Simons and Toomre 2000). Moreover, SNAREs are concentrated in cholesterol-dependent microdomains that define docking and fusion sites for exocytosis (Lang *et al*. 2001). Caveolae have been implicated in clathrin-independent endocytosis of GPI-APs and glycosphingolipid-binding toxins (Puri *et al*. 1999, Nichols *et al*. 2001). It appears that the different endocytic routes followed by GPI-APs in different cell types depend, at least in part, on the residence time of GPI-APs in rafts (Fivaz *et al*. 2002).

Coupled lipid and protein sorting in the Golgi

Sphingolipid synthesis primarily occurs in the Golgi, hence spatially separated from glycerolipid and sterol production in the ER. This arrangement is a conserved feature of eukaryotic cells (van Meer 1998, Levine *et al*. 2000). The assembly of sphingolipids in Golgi cisternae likely has an impact on the lateral organization of other membrane molecules and may provide a physical basis for sorting events that help establish the compositional and functional differences between the ER, the plasma membrane and the Golgi itself.

After synthesis in the Golgi, sphingolipids accumulate in the plasma membrane. In spite of extensive bi-directional membrane trafficking at the Golgi-ER interface, the ER contains much lower sphingolipid levels than the plasma membrane. Cholesterol forms a remarkably similar gradient along the secretory pathway (Liscum and Munn 1999). Cholesterol is synthesized in the ER and its intracellular distribution is likely affected by its high affinity for sphingolipids. Indeed, altering the sphingolipid distribution also affects cholesterol distribution (Puri *et al*. 1999). Hence, anterograde sorting of newly synthesized sphingolipids in the Golgi may serve as a mechanism to deplete cholesterol from the ER and promote its concentration in the plasma membrane (van Meer 1998). This brings one back to the question of how anterograde sorting of sphingolipids in the Golgi is accomplished.

There is now general agreement that anterograde transport through the Golgi is mediated, at least in part, by the cisternae (Pelham 2001). Briefly, it is believed that Golgi cisternae arise by the fusion of vesicular intermediates from the ER and then mature from a *cis*- to a *trans*-form by the exchange of Golgi-associated enzymes in small COPI-coated vesicles that move in a retrograde fashion. Therefore, anterograde sphingolipid sorting could be achieved if sphingolipids were prevented from leaving the cisternae in which they are made. In support of this scenario, COPI vesicles contain significantly reduced levels of sphingomyelin and cholesterol compared with their parental Golgi membranes (Brugger *et al*. 2000). In principle, there are two possibilities of how a segregation of sphingomyelin and cholesterol from COPI vesicles could be accomplished. First, segregation may occur because the coat machinery is recruited to sphingolipid/sterol-poor regions in the membrane (Figure 2(*a*)). Thus, sphingolipids produced in Golgi cisternae may attract ER-synthesized sterols and trigger a phase separation, creating domains enriched in unsaturated glycerolipids that function as donor sites for COPI vesicle biogenesis versus sphingolipid/sterol-rich domains that do not. There is evidence that sphingolipid/sterol-rich domains exist in the

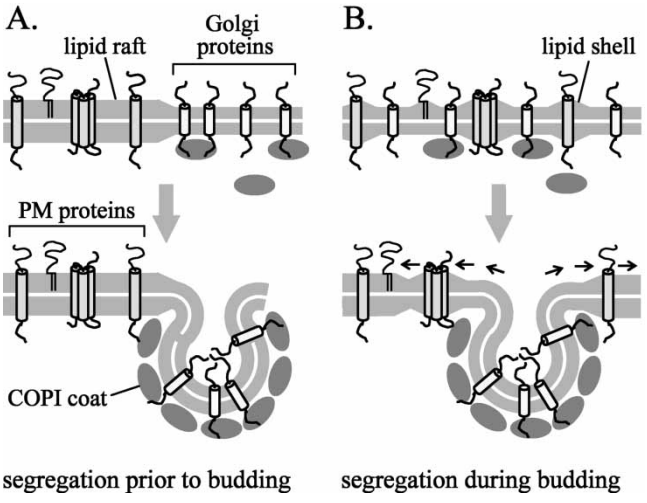

Figure 2. Models for coupled lipid and protein sorting in the Golgi. COPI vesicles involved in recycling Golgi proteins contain lower levels of sphingolipids and sterols than the cisternal membranes from which they bud. According to the model in A, this lipid segregation occurs because ongoing sphingolipid synthesis triggers a phase separation and generates sphingolipid/sterol-poor domains that serve as donor sites for COPI mediated vesicle budding versus sphingolipid/sterol-rich domains (rafts) that do not. The model in B shows an alternative possibility, namely that the coat machinery influences the lipid composition at the bud site. The high curvature imposed onto the vesicular membrane results in selective exclusion of sphingolipids, sterols and other lipids that tend to form flat and rigid bilayers. Due to their short membrane spans, Golgi proteins segregate from the thick sphingolipid/sterol-rich membrane regions and preferentially partition into the glycerolipid rich COPI vesicles. PM proteins, on the other hand, do not have access to COPI vesicles, either because their membrane anchors prefer an ordered sphingolipid/sterol-rich environment (rafts/shells) or because of the preferential partitioning of Golgi proteins at sites of vesicle budding. Retention of PM proteins in Golgi cisternae would result in their unidirectional transport toward the cell surface by cisternal maturation.

early Golgi (Gkantiragas *et al*. 2001). Secondly, segregation may occur because the coat machinery influences the lipid composition at the bud site (Figure 2(*b*)). For example, the high curvature imposed onto the vesicular membrane may cause a specific exclusion of lipid species that tend to form flat and rigid bilayers (sphingolipids, sterols and saturated glycerolipids). Consistent with this notion, formation of COPI-coated buds on giant liposomes is negatively influenced by fatty acyl chain saturation (Spang *et al*. 1998).

In any case, exclusion of sphingolipids and sterols from COPI vesicles will cause their progressive accumulation in maturing cisternae and this process is likely coupled to a gradual depletion of unsaturated glycerolipids along the secretory pathway. This remodelling of the cisternal membrane will alter its biophysical properties and promote a more compact, thicker and less permeable bilayer. Sphingomyelin with a C18:0 acyl chain forms up to 30% thicker membranes than monounsaturated PC (C16:0/C18:1; Nezil and Bloom 1992, Maulik and Shipley 1996). The enrichment in sphingolipids and sterols may explain the observed increase in bilayer thickness of *trans*-Golgi and plasma membranes (Grove *et al*. 1968). Bretscher and Munro (1993) proposed a model for protein sorting in the Golgi that is based on preferential interactions between lipids and transmembrane proteins with the best matching hydrophobic length. Golgi enzymes generally have shorter membrane spans than plasma membrane proteins and this property would prevent them from being included in the thick sphingolipid/sterol-rich membrane regions that depart for the cell surface. Consistent with this idea, lengthening the membrane spans of Golgi enzymes results in their movement to the plasma membrane (Munro 1995), while shortening the membrane spans of plasma membrane proteins causes their retention in the Golgi (Cole *et al*. 1998). Model membrane studies indicate that insertion of an hydrophobic peptide into a bilayer requires a proper relationship between membrane width and peptide length (Webb *et al*. 1998). Hence, during cisternal maturation, ongoing sphingolipid synthesis would cause a thickening of the cisternal bilayer and eventually drive all Golgi enzymes into recycling COPI vesicles (Figure 2). This would allow Golgi enzymes to keep their proper location. Plasma membrane proteins, on the other hand, would achieve the desired directionality in transport if their membrane anchors would partition preferentially in the more organized, sphingolipid/sterol-rich regions from which COPI vesicles do not bud. As discussed above, GPI-APs have a high affinity for such regions, and this characteristic appears critical for their efficient delivery to the cell surface (Mays *et al*. 1995, Skrzypek *et al*. 1997).

However, the first model for coupled protein and lipid sorting was put forward to explain the sorting of apical and basolateral surface components in polarized epithelial cells (van Meer *et al*. 1987, Simons and van Meer 1988). The basolateral surface of these cells has a lipid composition very similar to that of non-polarized cells, but the apical surface is highly enriched in glycosphingolipids. To explain this distribution, glycosphingolipid-rich microdomains containing apically directed GPI-APs were proposed to form in the *trans*-Golgi lumen and to be packaged into apically-directed membrane carriers (Lisanti *et al*. 1988, Brown *et al*. 1989). A similar mechanism is believed to mediate sorting of axonal and dendritic membrane proteins in neurons (Ledesma *et al*. 1998). In model membranes, glycosphingolipids display a stronger tendency than sphingomyelin to segregate from glycerolipids into a liquid-ordered phase (Silvius 1992). Hence, ongoing sphingolipid synthesis in the Golgi may initially cause a phase separation between unsaturated glycerolipids and sphingolipids, but after COPI-mediated removal of unsaturated glycerolipids eventually culminate in a phase separation between apical and basolateral sphingolipids. Basolateral protein sorting typically relies on discrete targeting motives in the cytoplasmic tails of membrane proteins and involves adaptor protein (AP) coats (Folsch *et al*. 1999). Apical sorting, on the other hand, seems more dependent on a cooperation of weak interactions between glycolipids and glycoproteins in the *trans*-Golgi membrane and lumen (Ikonen and Simons 1998). A role for vesicular coats in the formation of apical transport carriers remains elusive. It is feasible that apical transport intermediates are formed by a progressive maturation of *trans*-Golgi cisternae driven by AP-dependent removal of basolateral and endosomal/lysosomal material (Holthuis *et al*. 2001). Hence, sorting of apical cargo may occur by means of a 'sorting by retention' principle, based on the co-aggregative properties of the apical proteins and membrane lipids. Such a mechanism would bypass the requirement of an apical coat complex, as it would be the terminal step in the cisternal maturation process.

Lipid translocation during vesicular transport

Lipid flippases in early secretory organelles

The bulk of glycerophospholipid synthesis in eukaryotic cells occurs on the cytoplasmic leaflet of the ER (Bell *et al*. 1981). Growth of a stable ER bilayer requires that half of the newly synthesized lipids are flipped to the exoplasmic leaflet at a rate similar to that of their production. Indeed, flip-flop of phospholipids and their analogues in the ER is a fast process, with a half-time ($t_{1/2}$) of seconds to minutes (Buton *et al*. 1996). The observation that spontaneous movement of phospholipids across protein-free liposomes is very slow ($t_{1/2}$: hours to days) has led to the idea that flip-flop in the ER is protein mediated, involving one or more phospholipid flippases (Bretscher 1973). Consistent with this notion, transbilayer lipid movement in the ER is sensitive to proteases and protein-modifying reagents (Bishop and Bell 1985, Buton *et al*. 1996). ER flippases catalyze a transverse diffusion of most, if not all phospholipid classes in both directions and function independently of metabolic energy (Menon 1995). As facilitators of non-vectorial lipid transport, these activities would promote a symmetric phospholipid distribution across the bilayer.

Even though consensus has been reached that flip-flop in the ER is protein mediated, it is unclear whether there is a dedicated lipid flippase, a group of flippases or whether flip-flop is accomplished by the mere presence of proteins in the ER bilayer. Genetic work in yeast has identified a candidate flippase, Rft1p, with an essential role in the translocation of

dolichol-pyrophosphoryl oligosaccharides from the cytoplasmic to the lumenal leaflet of the ER, an obligatory step in *N*-linked protein glycosylation (Helenius *et al*. 2002). A possible functional counterpart of Rft1p is the *E. coli* Wzx protein, a polytopic membrane protein implicated in the translocation of O-antigen-pyrophosphorylbactoprenol complex across the bacterial cytoplasmic membrane (Feldman *et al*. 1999). In search for ER proteins mediating fast phospholipid movement, Menon *et al*. (2000) fractionated detergent-solubilized rat liver ER and reconstituted different protein fractions in proteoliposomes. This approach yielded specific protein pools with increased flip-flop activities, suggesting that distinct proteins are capable of catalyzing transbilayer lipid transport in the ER. Remarkably, peptides mimicking the transmembrane α-helices of proteins have been reported to induce phospholipid translocation in liposomes (Kol *et al*. 2001). The latter finding has reinforced the idea that the ability to catalyze flip-flop in the ER is not restricted to a single dedicated protein. However, the hypothesis that the mere presence of transmembrane helices would be sufficient to mediate flip-flop only holds for specific organellar membranes. Flip-flop in the plasma membrane, for example, is a slow process, even though this organelle contains a large variety of transmembrane proteins (Seigneuret and Devaux 1984, Buton *et al*. 2002). Interestingly, helix-mediated flip-flop in liposomes is strongly inhibited by cholesterol (Kol *et al*. 2003), an abundant component of the plasma membrane. Cholesterol may exert this inhibitory effect by causing an increased packing of the acyl chains through which the polar head group has to travel. Hence, the gradual increase in cholesterol levels along the secretory pathway may serve as a regulatory device to switch from constitutive flip-flop in the ER to a more tightly regulated lipid translocation in the plasma membrane (Kol *et al*. 2003).

Glucosylceramide (GlcCer) produced on the surface of *cis/medial*-Golgi cisternae is converted to complex glycosphingolipids in the Golgi lumen. GlcCer can flip across the Golgi bilayer by means of an energy-independent mechanism resembling lipid flip-flop in the ER (Burger *et al*. 1996). Transbilayer movement of spin-labelled analogues of GlcCer in Golgi and ER membranes occurs at a 30-fold higher rate than in plasma and model membranes (Buton *et al*. 2002). Kinetic data as well as chemical modification and proteolysis experiments suggest that this transbilayer movement is protein mediated and occurs in both directions with the same efficiency. This implies that monohexosylsphingolipids synthesized in the Golgi would adopt a symmetric distribution over the two leaflets. LacCer, which is formed by galactosylation of GlcCer in the Golgi lumen, is unable to cross the Golgi bilayer and remains in the lumenal leaflet where it undergoes further glycosylation (Burger *et al*. 1996, Buton *et al*. 2002). Hence, a monohexosylsphingolipid flippase may play a role in the regulation of complex glycosphingolipid synthesis. A protein(s) responsible for GlcCer flip-flop across the Golgi bilayer has not yet been identified.

Lipid flippases in late secretory organelles

Lipid asymmetry is a well-known feature of eukaryotic plasma membranes. In all cells where reliable measurements have been made, the majority of the aminophospholipids PS and PE are located in the cytoplasmic leaflet while sphingomyelin and glycosphingolipids are concentrated in the exoplasmic leaflet (Devaux 1991, Zachowski 1993). This asymmetry is made possible by lipid translocation machinery that uses hydrolysis of ATP to translocate lipids against a concentration gradient. The use of short-chain lipid analogues has led to the discovery of an aminophospholipid translocase that catalyzes a fast ($t_{1/2}$ of minutes), inwardly directed transport of PS and PE across the plasma membrane (Seigneuret and Devaux 1984, Daleke and Lyles 2000). A distinct energy-dependent flippase mediates a non-selective outward movement of lipids to compensate for the active maintenance of aminophospolipids in the inner leaflet (Connor *et al*. 1992). Conditions of cellular activation that are accompanied with increased levels of intracellular Ca^{2+} may cause a collapse of lipid asymmetry by switching on an energy-independent scramblase (Sims and Wiedmer 2001). This activity accelerates a bidirectional movement of all phospholipid classes across the plasma membrane. As a result, PS is exposed on the cell surface where it functions as a recognition signal for the engulfment of apoptotic cells by macrophages (Fadok *et al*. 2000). PS exposure on the surface of activated platelets is important for initiating blood coagulation (Rosing *et al*. 1980).

Although the various activities mediating lipid transport across the plasma membrane have been characterized in great detail, most of the corresponding lipid transporters still await unambiguous identification. A prime candidate for the inward aminophospholipid translocase is ATPase II, a vanadate-sensitive Mg^{2+}-ATPase of 110–120 kD (Zachowski *et al*. 1989). ATPase II has been purified from human erythrocytes, but a similar activity is also present in chromaffin granules, synaptic vesicles and clathrin-coated vesicles (Daleke and Lyles 2000). Cloning of the gene encoding ATPase II from bovine chromaffin granules revealed that the protein belongs to an ancient sub-family of P-type ATPases (Tang *et al*. 1996). A defect in low temperature internalization of NBD-labelled PS in yeast cells in which the homologous gene, *DRS2*, was deleted has been interpreted as evidence for the biochemical function of the chromaffin granule P-type ATPase as aminophospholipid translocase (Tang *et al*. 1996, Gomès *et al*. 2000). This conclusion was called into question when the dependence of NBD-PS uptake on the expression of *DRS2* could not be confirmed in two independently constructed null strains (Siegmund *et al*. 1998, Marx *et al*. 1999). Moreover, Drs2p does not reside in the PM, but in the *trans*-Golgi (Chen *et al*. 1999). However, recent work identified two Drs2p-related ATPases, Dnf1p and Dnf2p, that are essential for the inward translocation of NBD-labelled PE, PS and PC across the yeast PM (Pomorski *et al*. 2003). Although the noted lipid preference disqualifies these proteins as specific aminophospholipid translocases, NBD-labelled sphingolipids, phosphatidic acid and phosphatidylglycerol are not recognized. In the Δdnf1Δdnf2 deletion mutant, endogenous PE accumulates at the cell surface, in particular when Drs2p is also removed (Pomorski *et al*. 2003). Collectively, these findings provide new evidence for a role of ATPase II, Drs2p and related ATPases in aminophospholipid transport. Moreover,

it appears that these proteins help regulate the transbilayer lipid arrangement in multiple organelles of the late secretory and endocytic pathways.

A recent genetic screen for yeast mutants hypersensitive to a cytolytic PE-binding peptide identified Ros3p, a polytopic transmembrane protein whose removal severely affects the inward translocation of NBD-PE and -PC across the PM (Kato *et al*. 2002). Ros3p belongs to a conserved protein family whose members are unrelated to P-type ATPases or other known transporters. Conceivably, Ros3p and related proteins represent essential components of the P-type ATPase-dependent translocation machinery. Alternatively, they may be required for the proper functioning or localization of Drs2p-related ATPases. It will be necessary to reconstitute these proteins into proteoliposomes to establish their precise role in transbilayer lipid transport.

Studies originally related to multi-drug resistance in cancer cells and in yeast identified several ATP-binding cassette (ABC) transporters that, in addition to amphiphilic drugs, catalyze the movement of lipids from the inner to the outer leaflet of the PM. Mice having null-alleles for the liver-specific ABC transporter Mdr2 (ABCB4) are unable to secrete PC into the bile (Smit *et al*. 1993). Further evidence for PC translocase activity of Mdr2 was obtained upon its heterologous expression in yeast (Ruetz and Gros 1994). The closely-related MDR1 P-glycoprotein (ABCB1) translocates a broad variety of short-chain lipid analogues as well as natural short-chain PC platelet activated factor (van Helvoort e*t al*. 1996, Raggers *et al*. 2000). The impaired efflux of cholesterol from the PM to exogenous HDL in Tangier disease is due to mutations in ABC1 (ABCA1). It has been suggested that ABC1 indirectly affects the cholesterol distribution across the PM by translocating PS toward the outer leaflet in a Ca^{2+}-dependent fashion (Hamon *et al*. 2000). The yeast ABC transporters Pdr5p and Yor1p exhibit outward-directed lipid translocase activity (Decottignies *et al*. 1998) and their over-expression causes an increased aminophospholipid exposure at the cell surface (Pomorski *et al*. 2003).

A protein called phospholipid scramblase (PLSCR1) has been identified that exhibits Ca^{2+}-activated phospholipid scrambling *in vitro* (Sims and Wiedmer 2001). However, this protein is not required for PS exposure during apoptosis. This would suggest that cells are equipped with a second, PLSCR1-independent mechanism for PS scrambling that is responsive to the apoptotic cascade.

Role of lipid flippases in vesicle biogenesis

In multicellular organisms, maintenance of PM lipid asymmetry is critical to cell survival. This becomes evident when it is dissipated by the action of the phospholipid scramblase. As a result, PS appears on the cell surface where it acts as a signal for phagocytic clearance by macrophages (Fadok *et al*. 2000). What significance lipid asymmetry has for the functioning of individual cells is less clear. That it is important can be inferred from the fact that cells, including those living in solitude, invest energy to maintain non-random lipid distributions across their PMs. During cell division, PE is transiently exposed on the cell surface of the cleavage furrow. Immobilization of cell surface PE by a PE-binding peptide inhibits disassembly of the contractile ring, thereby preventing the final separation of daughter cells (Emoto and Umeda 2000). These findings suggest that local redistributions of PE across the PM are essential for progression of cytokinesis.

An increasing body of evidence indicates that a dynamic regulation of the transbilayer lipid arrangement in the PM, and most likely also in late Golgi and endosomal compartments, serves a critical role in yet another fundamental cellular process, namely in the biogenesis of transport vesicles. Vesicle budding imposes locally a high curvature on the membrane that must be associated with a difference in the surface area of the inner and outer monolayers. At the level of the head groups, the outer leaflet of a 60-nm diameter vesicle contains 1.5 times the number of lipid molecules of the inner leaflet. In addition, formation of a vesicle requires the bilayer to adopt a positive curvature at the site of the emerging bud, as well as an extreme negative curvature at the site of membrane fission. Only lipids with a small headgroup, like PE, would be suitable to fit in areas of high constriction. These areas arise primarily in the inner leaflet at the level of the bud neck. Adopting a transbilayer lipid arrangement permissive for vesicle formation might not pose a problem to ER or *cis*-Golgi membranes where phospholipids can freely cross the bilayer in both directions (Figure 3). In these flexible membranes, the assembly of a COP coat may exert a force sufficient to deform the bilayer into a bud. The situation might be different in the PM, the *trans*-Golgi and endosomes where the free 'flip-flop' of phospholipids across the bilayer is constrained (Seigneuret and Devaux 1984, Buton *et al*. 2002). These membranes lack the energy-independent lipid flippase described for the ER and contain high levels of sterols and sphingolipids that reduce lipid diffusion rates (Kol *et al*. 2003). It has been postulated that the ATP-consuming aminophospholipid translocase, responsible for inward transport of PS and PE across the PM, drives the budding of endocytic vesicles by expanding the surface area of the cytoplasmic leaflet at the expense of the exoplasmic leaflet (Devaux 1991). According to this scenario, coat assembly would help localize the process, rather than providing the driving force. Aminophospholipid asymmetry would then merely be a consequence of the fact that, without any lipid selectivity, the translocase would be trapped in a continuous pumping of the whole PM.

A direct participation of the aminophospholipid translocase in vesicle budding is supported by the observation that stimulating its activity provokes endocytic-like vesicles in erythrocytes (Birchmeier *et al*. 1979, Muller *et al*. 1994) and accelerates endocytosis in erythroleukemia K562 cells (Farge *et al*. 1999). In both cases, endocytosis activity was enhanced by external addition of PS or PE, but inhibited by PC, lyso-PS and other lipids not transported by the aminophospholipid translocase. Intruigingly, the yeast Δdnf1Δdnf2 mutant, but most prominently Δdnf1Δdnf2Δdrs2 cells, are defective in the internalization step of endocytosis (Pomorski *et al*. 2003). Moreover, deletion of the *DRS2* gene causes synthetic lethality with mutations in the genes for ADP-ribosylation factor 1 (*ARF1*) and clathrin heavy chain (*CHC1*; Chen *et al*. 1999) and perturbs the formation of a specific class of clathrin-coated vesicles carrying invertase and acid

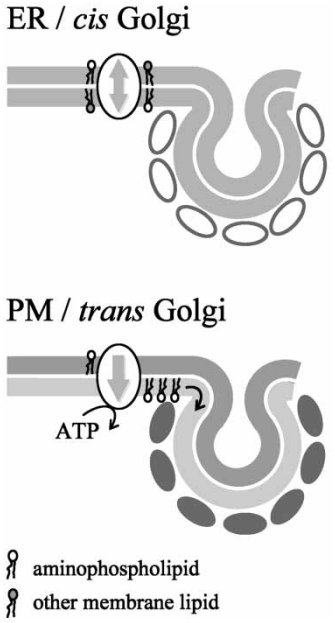

Figure 3. Role of uni- and bi-directional lipid flippases in vesicle budding. Membrane curvature during vesicle budding requires a selective increase in surface area of the inner monolayer. In ER and *cis*-Golgi membranes, phospholipids can rapidly cross the bilayer in both directions due to the presence of energy-independent, bi-directional flippases. Here, assembly of a protein coat may be sufficient to deform the bilayer into a bud. In *trans*-Golgi and PMs, however, free lipid flip-flop across the bilayer is constrained due to high sphingolipid and sterol levels and/or the absence of a constitutive bi-directional flippase. In these organelles, the transbilayer lipid imbalance required for vesicle budding must be generated by inward-directed ATP-driven lipid transporters. Here, coat assembly would help localize the process, rather than providing the driving force. Selectivity of the inward translocases for aminophospholipids will prevent destabilization of the bilayer, but may also help create a lipid environment favourable for coat recruitment.

phosphatase to the yeast PM (Gall *et al*. 2002). Considering the translocase activity of Dnf1p and Dnf2p (Pomorski *et al*. 2003), these data strongly implicate a functional link between P-type ATPase-controlled lipid asymmetry and biogenesis of transport vesicles. Electron microscopy on ATPase-deficient cells and reconstitution of the ATPases in giant liposomes may help establish whether (amino)phospholipid transport is required at the level of coat recruitment, membrane deformation (budding) or vesicle fission.

Yeast contains five P-type ATPases related to ATPase II. Two of these are associated with the PM (Dnf1p, Dnf2p), two with the *trans*-Golgi (Drs2p, Dnf3p) and one with endosomes (Neo1p; Hua *et al*. 2002, Pomorski *et al*. 2003). Hence, P-type ATPase-dependent lipid transport may well serve a general function in vesicular pathways operating at the Golgi-PM interface. The involvement of ATPase II homologues in lipid translocation and membrane trafficking in yeast may provide a paradigm that could help elucidate the molecular basis of some human disorders. Mutations in the *ATP8B1/FIC1* gene, which encodes one of the 12 known ATPase II homologues in man, causes familial intrahepatic cholestasis, a defect in bile secretion (Bull *et al*. 1998). How ATP8B1 is involved in bile secretion remains to be established. However, rather than being directly responsible for bile transport, it seems more likely that ATP8B1 is required for the correct delivery of a bile salts export pump to the canalicular membrane. ATP10C, another human ATPase II homologue, has been linked to the neurological disorders Angelman syndrome and autism (Meguro *et al*. 2001) and may serve a role in membrane trafficking steps that are critical for cell signalling in the central nervous system. Although phospholipid translocation is the most likely biochemical activity of ATPase II and its homologues, it should be noted that definitive evidence for this remains elusive. The establishment of whether these ubiquitous proteins are directly responsible for transbilayer lipid transport forms an important target for future research in this field.

Future perspectives

Understanding the mechanisms that maintain the non-random lipid distributions among and across cellular membranes provides a major challenge in current cell biology. The concept of phase-separated lipid domains has dramatically altered one's view on membrane organization. Lipid domains or rafts represent versatile devices for compartmentalizing membrane-bound processes, ranging from vesicular trafficking to signal transduction. However, in spite of the increasing numbers of papers on raft function, the dynamics of raft assembly in cellular membranes and the precise nature of the relevant protein-lipid interactions remain poorly understood. Future advances in this field will largely depend on the development of novel techniques and on the insightful integration of information gained from genetic, cell biological and biophysical approaches. The identification of new candidate lipid flippases has boosted investigations on the origin and significance of transbilayer lipid asymmetry. Reconstitution into proteoliposomes will be an obligatory step to proof that these proteins are directly responsible for transbilayer lipid transport and may help uncover the functional link between lipid pumping and vesicle biogenesis. This methodology is technically demanding and will require major efforts. The good news is that lipids are invading the minds and experiments of an increasing number of researchers, and it seems they will stay there for a considerable time to come.

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