Regulating membrane curvature

Joost C. M. Holthuis

Abstract

Membranes of living cells are subject to a variety of shape changes. Membrane deformation is essential for cell division, locomotion, organelle biogenesis, and creation of the high-curvature vesicles and tubules that mediate cargo transport along the endo- and exocytic pathways. Yet membrane bilayers are stabilized against deformations by a powerful hydrophobic effect. Moreover, the various membrane-bound organelles of a cell are composed of different mixtures of lipids, with each lipid species contributing differently to membrane elasticity and shape. So how do cells adopt the desired radii of curvature in their bilayers? A long-standing concept is that membrane curvature can be induced by assembly of a protein coat onto one side of the bilayer. Recent studies indicate that lipids too can serve as the molecular workers in accomplishing the structural changes needed to deform cellular membranes.

1 Introduction

Membrane deformation is a universal feature of living cells. It is crucial for cell division, organelle biogenesis, sustaining endo- and exocytosis, and for the release of enveloped viruses from infected cells. In many cases, (e.g. cell division, vesicle formation), but not always (e.g. formation of plasma membrane protrusions), membrane budding culminates in the physical separation, or fission, of the bud from the donor membrane. Fission requires strong membrane bending and the formation of a highly constricted neck where the opposing membranes eventually make contact and fuse. However, curling up a planar membrane, or flattening a curved one, is energetically unfavourable and does not occur spontaneously. So how does a cell induce the desired curvature in its bilayers?

It appears that cytoskeletal elements, apart from providing the network over which membrane traffic flows, are capable of driving membrane tubulation by pushing a developing bud into the extracellular environment or pulling it into the cytoplasm (Lippincott-Schwartz et al. 2000). Another classical paradigm for the acquisition of membrane curvature is the polymerisation of cytosolic proteins into a coat scaffold on the membrane surface (Kirchhausen 2000). Here, budding would be a consequence of the intrinsic curvature in the structure of the assembled coat. Consistent with this notion, *in vitro* studies with protein-free liposomes show that coat assembly is fully sufficient to deform lipid bilayers into buds and tubules,

Topics in Current Genetics
S. Keränen, J. Jäntti (Eds.): Regulatory Mechanisms of Intracellular Membrane Transport
DOI 10.1007/b98566 / Published online: 25 May 2004

and, in some cases, even to pinch off bilayer vesicles (Matsuoka et al. 1998; Takei et al. 1998; Bremser et al. 1999). Whether coat assembly or cytoskeletal mechanisms are also sufficient to pay the energy costs for membrane remodelling *in vivo* has remained an open issue. The matter is complicated by the fact that cellular membranes contain mixtures of 100 or more different lipids that are often asymmetrically distributed across the bilayer (Zachowski 1993; Holthuis et al. 2003), a situation that is difficult, if not impossible, to reproduce with model membranes. According to a recent estimate, the rigidity of the clathrin coat does not supersede the mechanical bending resistance of biological membranes (Nossal 2001). This would indicate that clathrin polymerisation by itself may stabilize budding vesicles, but unlikely provides a force sufficient to drive vesicle biogenesis *in vivo*.

Indeed, a growing body of evidence indicates that for remodelling cellular membranes, mechanisms applying external forces on the membrane (coat assembly, cytoskeletal mechanisms) often operate in conjunction with mechanisms exerting intrinsic ones, i.e. that cause a physical perturbation of the lipid bilayer. Several proteins have now been identified that function in concert with classical coat proteins and likely promote vesicle budding by inserting an amphipathic helix into one monolayer of the membrane, causing an intrinsic curvature by pushing the lipid head groups apart. Moreover, cells contain ATP-driven lipid translocases that facilitate or induce membrane curvature by controlling the lipid distribution across the bilayer. Enzymatically-induced changes in the geometry of the lipid constituents has emerged as yet another mechanism to modify membrane curvature directly. Finally, model membrane studies show that lipid phase separation and microdomain formation can cause membrane budding and fission events, raising the possibility that cells exploit lipid immiscibility to drive membrane vesiculation.

This review focuses on recent insights into the mechanisms by which cells control the radii of curvature in their bilayers. Particular attention is paid on how lipid composition affects membrane folding, how different membrane-deforming mechanisms may be combined to accomplish the dynamic shape transformations that are necessary to sustain membrane traffic in live cells, and how lipids and proteins cooperate to lower the energy costs of these transformations.

2 Topological considerations

Cellular membranes bud into two principal topological spaces, the cytoplasm and the extracytoplasmic space, with the latter comprising both the lumen of intracellular organelles and the extracellular environment (Fig. 1). Budding into the cytoplasm, or inward budding, is required for generating the transport vesicles and tubules that carry cargo between the different subcellular organelles (Lippincott-Schwartz et al. 2000), but also for creating the transverse tubular invaginations of the plasma membrane in striated muscle cells (Ishikawa 1968). Budding into the extracytoplasmic space, or outward budding, occurs during cell division, during apoptosis when microvesicles and blebs are shed from the plasma membrane

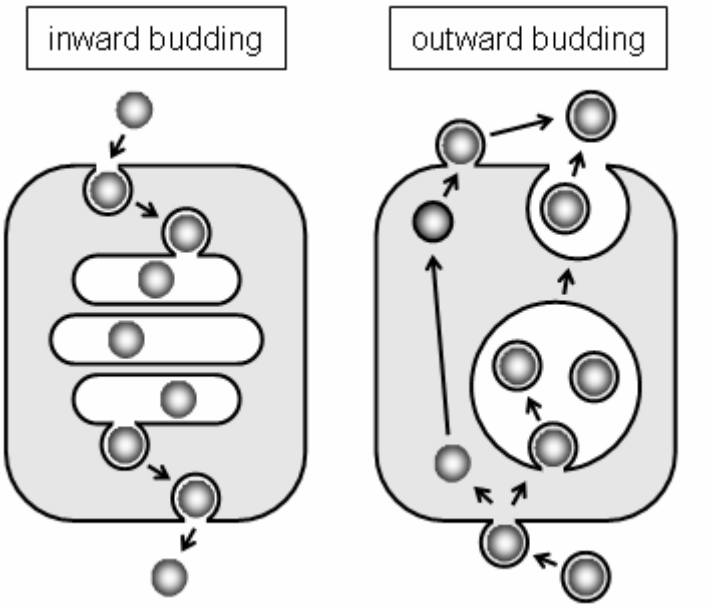

Fig. 1. Orientation of membrane budding events. Inward budding allows vesicular transport from the plasma membrane to intracellular compartments (endocytosis) and from intracellular compartments to the plasma membrane (exocytosis), with transport vesicles containing material taken up from the extracellular environment or the lumen of intracellular compartments, respectively. Outward budding allows the release of vesicles into the extracellular environment or into the lumen of intracellular compartments. These vesicles contain material taken up from the cytosol. Outward budding occurs during the shedding of milk-fat droplets or enveloped viruses form the cell surface, or during formation of the internal vesicles of multivesicular endosomes. Note that budding not necessarily culminates in fission and formation of a vesicle; budding also mediates formation of filopodia, microvilli and other cell surface projections as well as the biogenesis of T-tubules in muscle cells.

(Coleman et al. 2001), during the release of milk-fat globules from mammary gland epithelial cells (Deyrup-Olsen and Luchtel 1998), and during formation of filopodia, microvilli and other cell surface projections. Outward budding also concerns the assembly of enveloped viruses in infected cells (Pornillos et al. 2002) as well as the generation of the internal vesicles in multivesicular endosomes that, when released into the extracellular environment, are called exosomes (Katzmann et al. 2002). Exosomes contain MHC class II molecules and T cell co-stimulatory factors, and as such are potent modulators of the immune system (Thery et al. 2002). The variation in shape changes imposed on cellular membranes provides a strong indication that cells are equipped with more than one type of budding machinery.

3 Extrinsic forces affecting membrane curvature

3.1 Cytoskeleton

Cytoskeletal elements seem to play a prominent role in the regulation of membrane curvature. A direct participation of the microtubule network in the formation and movement of tubular transport intermediates as well as in the tubular dynamics of the endoplasmic reticulum (ER) has been well established (Dabora and Sheetz 1988; Vale and Hotani 1988; Lippincott-Schwartz et al. 2000). The interactions of membranes with microtubules are mediated by several classes of proteins, notably the motor proteins of the dynein and kinesin families (Hirokawa 1998). While moving along a preformed microtubule track, motor proteins would exert forces on the membrane to which they are attached, resulting in the formation of membrane tubes and tubular networks (Fig. 2 A). Consistent with this view, depletion of kinesin heavy chain or depolymerization of microtubules causes a retraction of the ER toward the cell centre and abolishes formation of new tubes (Lee et al. 1989; Feiguin et al. 1994). Importantly, recent work has demonstrated that synthetic giant liposomes, kinesin-coated beads, immobilized microtubules and ATP provide a minimal system for generating tubular structures that resemble tubes formed from cellular membranes (Roux et al. 2002; Koster et al. 2003). The forces required for pulling a tube are higher than can be generated by individual motor proteins, indicating that multiple motor proteins must work together. Membrane tube formation is sensitive to lipid composition; when the bending rigidity of the liposome is increased by the addition of cholesterol, the threshold concentration of motor proteins for tubulation is doubled (Koster et al. 2003).

Polymerisation forces generated by the cytoskeleton itself provide an alternative to motor proteins in affecting membrane curvature (Fig. 2 B). Animal cells change shape and move by polymerising actin at the leading edge of lamelipodia and filipodia (Lauffenburger and Horwitz 1996; Mitchison and Cramer 1996). Model systems using synthetic liposomes encapsulating actin or tubulin indicate that self-assembly of these proteins in principle can generate sufficient mechanical force to deform a lipid bilayer. Hence, actin polymerisation causes spherical liposomes to transform into stable disk or dumbbell-shapes (Miyata and Hotani 1992; Palmer et al. 2003), whereas, tubulin polymerisation results in the development of a bipolar shape (Hotani and Miyamoto 1990). Maintenance of the shape changes induced by polymerised tubulin requires microtubule-associated proteins (Kaneko et al. 1998).

The force required for pulling or pushing a tubule from a giant vesicle is determined not only by the bending rigidity, but also the tension of the membrane. When more and longer tubes are being pulled, the membrane tension will rise in part because of the fixed area-to-volume ratio of the giant liposome. This tension can be released by the introduction of pores in the bilayer (Koster et al. 2003). Hence, regulation of lumenal volume may also contribute to tubule shape, and an obvious way of doing this would be with an ion pump. Whether cells exploit ion pumps in regulating membrane curvature is unclear. However, note that membrane tubulation can occur independently of the cytoskeleton. In *Xenopus* egg

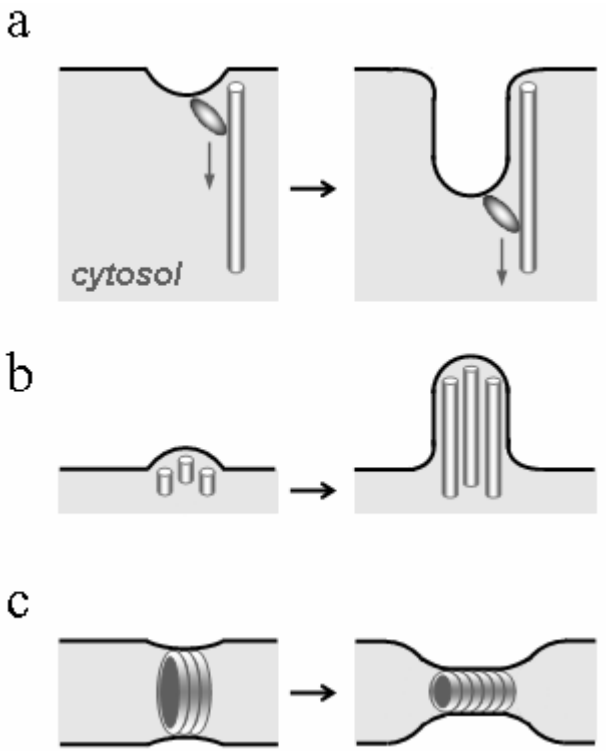

Fig. 2. Cytoskeleton-induced membrane curvature. (a) Membrane tubule pulled by a cytoskeletal motor protein. (b) Membrane tubule pushed by the polymerisation of actin or tubulin. (c) Membrane shaft constricted by a contractile ring composed of actin and myotubulin.

extracts, ER networks form *de novo* in the absence of microtubules or an actin scaffold (Dreier and Rapoport 2000). The exact mechanism by which this happens remains to be established. Moreover, cytosolic proteins with potent tubulogenic activities have been identified that do not form part of the cytoskeleton (see sections 4.1 and 4.2).

Finally, contractive forces generated by the cytoskeleton may also influence membrane curvature. A well-known example is the contractile ring of overlapping actin and bipolar myosin II filaments that forms during cytokinesis and exerts a force on the plasma membrane to generate a cleavage furrow at the site where the two daughter cells will be separated (Fig. 2 C). A redistribution of phosphatidyl ethanolamine (PE) from the inner to the outer leaflet of the plasma membrane appears to be involved in regulating the disassembly of the contractive ring and is essential for a proper progression of cytokinesis (Emoto et al. 1996; Emoto and Umeda 2000). Since PE has an effective cone shape (section 4.2), its redistribution to the outer leaflet may serve to facilitate a further inward bending of the plasma membrane.

3.2 Adhesion to curved particles

The acquisition of membrane curvature can also result from enwrapping a cytoplasmic or exoplasmic particle (Fig. 3 A). Here, the driving force would derive from adhesion of the membrane to an already curved particle. Examples include cells eating beads (Desjardins and Griffiths 2003), the outward budding of milk-fat droplets from the plasma membrane (Deyrup-Olsen and Luchtel 1998) and the biogenesis of dense-core secretory granules at the trans-Golgi network (TGN; Burgoyne and Morgan 2003). In the latter case, membrane deformation is thought to result from a pH and calcium-dependent aggregation of secretory proteins in the TGN lumen. Chromogranin A (CgA) appears to be a key mediator of this process, since CgA depletion in neuroendocrine cells reduces granule number while CgA expression in fibroblasts induces dense-core granule formation (Kim et al. 2001). Hence, the aggregative properties of CgA, coupled to its propensity to interact with membranes (Huttner et al. 1991) would allow wrapping of the TGN membrane around a forming CgA aggregate. Although CgA alone seems capable of triggering secretory granule formation, its restricted expression in neuroendocrine tissues implies that other proteins may fulfil this role in other cell types. Indeed, heterologous expression of pro-von Willebrand factor in neuroendocrine or epithelial cell lines causes the induction of rod shaped structures resembling Weibel-Palade bodies, the organelles in which von Willebrand factor (vWF) is normally stored within endothelial cells (Wagner et al. 1991; Voorberg et al. 1993). Formation of these bodies requires the ability of vWF proteins to condensate into multimers. How nascent granule buds are subsequently pinched off to form secretory granules is unclear. Cholesterol is essential for this process as cholesterol depletion blocks granule biogenesis at a late stage with dense-core buds observable at the TGN (Wang et al. 2000). The role of cholesterol in vesicle fission may be direct, by facilitating the strong membrane curvature at the bud neck, or indirect, as a key component of lipid microdomains in the recruitment of proteins that drive vesicle fission (e.g. dynamin).

3.3 Coat assembly

The idea that cytosolic coat proteins bind membranes and then deform them to initiate vesicle budding is a long-standing one (Fig. 3 B). This concept, first developed for the clathrin coat, and then extended to other vesicle coats like COPI and COPII, is supported by studies revealing an intrinsic curvature in the structure of assembled coat complexes (Musacchio et al. 1999; Bi et al. 2002). Indeed, coat assembly, membrane budding and even fission can occur on protein-free liposomes, indicating that interfaces between lipids and cytosolic coat proteins are sufficient for pinching off bilayer vesicles (Matsuoka et al. 1998; Spang et al. 1998; Takei et al. 1998; Bremser et al. 1999). However, recent progress in the field has led to a partial revision of this view.

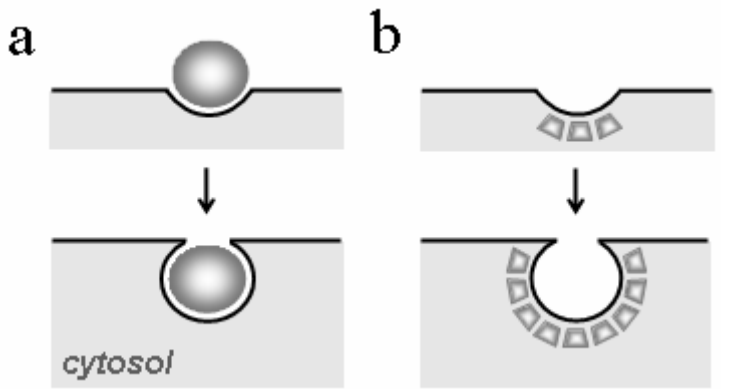

Fig. 3. Membrane curvature induced by adhesion to a body with intrinsic curvature. (a) Membrane budding driven by adhesion to a curved particle, for example during phagocytosis of bacteria or the biogenesis of secretory granules. (b) Membrane budding driven by coat proteins polymerising with an intrinsic curvature, for example during COPI-, COPII-, or clathrin-coated vesicle formation.

First, real-time imaging of green fluorescent protein (GFP)-cargo proteins in mammalian cells revealed the existence of clathrin- or COPI-containing membrane carriers that are larger and more pleiomorphic than the conventional, 60-100 nm-diameter coated vesicles produced *in vitro*. These carriers include vesicles up to 1 μm in diameter, tubules several μm in length and vesicular-tubular structures of various sizes and shapes (Lippincott-Schwartz et al. 2000). Second, electron microscopy studies demonstrated that coat complexes adopt a larger variety of shapes than previously anticipated. For example, early endosomes are partially decorated with flat 'bilayered' clathrin coats. Rather than giving rise to clathrin-coated vesicles, these structures seem to function in retaining lysosomally targeted cargo proteins when recycling proteins are being removed by vesicles or tubules that bud from early endosomes (Sachse et al. 2002). Third, fluorescence recovery after photobleaching (FRAP) experiments on GFP-tagged clathrin and COPI indicate that protein coats are much more dynamic than previously envisioned. Instead of providing a rigid assembly that is put on and taken off only once in a single round of coated-vesicle formation and fusion, protein coats undergo a continuous exchange between membranes and the cytosol by a process that can be uncoupled from vesicle formation (Presley et al. 2002). Fourth, in order to drive membrane curvature efficiently, the rigidity of an assembled coat polymer has to supersede the mechanical bending resistance of the membrane. This notion has been challenged for clathrin, because of estimations that the rigidity of the clathrin triskelia is at best similar to the mechanical bending resistance of the plasma membrane (Nossal 2001). Finally, at least two clathrin-binding proteins, epsin and amphiphysin, induce membrane curvature in the absence of clathrin (Farsad et al. 2001; Ford et al. 2002). Remarkably, the curvature-stimulating properties of these pro-

teins do not depend on oligomerization, but rather on a unique and intimate interaction with the membrane bilayer (section 4.1).

Coat proteins are multifunctional, and likely stabilize nascent transport vesicles and tubules, select and concentrate their contents, prevent their fusion prior to completion of the budding process, and carry out post-budding reactions through the recruitment of accessory proteins that mediate interactions with the cytoskeleton. However, the precise function of coat assembly in driving the formation of the highly curved membrane carriers in cells is incompletely understood. There is growing evidence that mechanisms in addition to coat-protein lattice formation play a role in the biogenesis of coated transport intermediates.

4 Intrinsic forces affecting membrane curvature

4.1 Transbilayer area asymmetry

4.1.1 Physical considerations

Due to the high-energy price associated with exposing their carbon chains to water, the lipid molecules in a bilayer are tightly packed. In this condensed state, lipid bilayers display solid- or liquid-like material behaviour with the common feature of limited surface compressibility, i.e. great resistance to change in surface density (Tanford 1978; Rawicz et al. 2000). Yet budding a vesicle requires membrane bending that must be associated with an imbalance in surface area between the two membrane leaflets. At the level of the head groups, the outer leaflet of a 60-nm diameter synaptic vesicle accommodates 1.5 times the number of lipids of the inner leaflet. If lipids were free to cross the membrane, a change in the transbilayer lipid distribution would be easily accomplished and vesicle budding would happen as a manifestation of the thermal fluctuations exerted by the membrane. But in a lipid bilayer, it generally costs a lot of energy for a phospholipid to traverse the membrane. In model membranes, half times of spontaneous flip-flop of phosphatidylcholine range from several hours to days at physiological temperatures (Kornberg and McConnell 1971; Wimley and Thompson 1991), a time scale incompatible with the rate of vesicle budding in living cells.

Hence, vesicle budding requires transbilayer area asymmetry, and one way of achieving this is by the insertion of additional lipid in one of the two membrane halves. An artificial method for selectively increasing the lipid content of one leaflet involves the addition of lyso-phosphatidylcholine (lyso-PC) to a giant liposome or erythrocyte. Lyso-PC molecules will penetrate and selectively expand the outer leaflet since the spontaneous flip-flop rate of lyso-PC is extremely slow. Because the two leaflets of a lipid bilayer are coupled by a powerful hydrophobic effect, a progressive modification of the ratio between the inner and outer areas will force the membrane to bend (Sheetz and Singer 1974). In fact, insertion of a small amount of lyso-PC (equivalent to 0.1-1% of total lipid) already creates a mismatch in lateral pressure between the two leaflets sufficient to drive formation of a

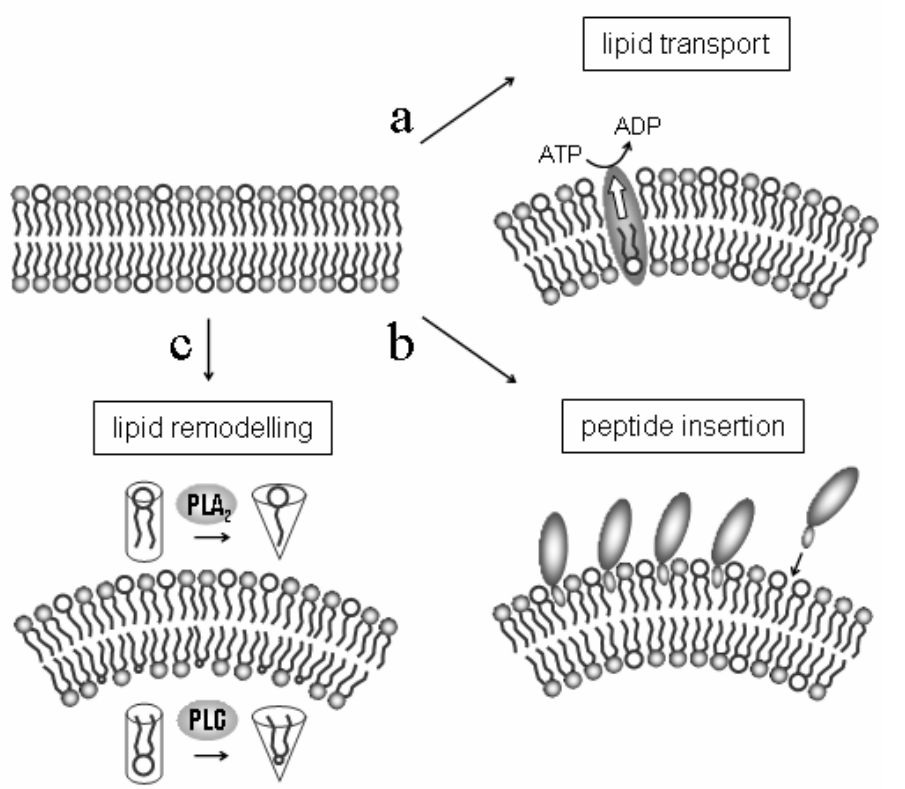

Fig. 4. Membrane curvature induced by a physical perturbation of the lipid-bilayer. (a) Transbilayer area asymmetry generated by a lipid translocase. (b) Transbilayer area asymmetry generated by proteins capable of physically penetrating into one face of the bilayer. (c) Transbilayer curvature asymmetry generated by lipid remodelling enzymes. PLA_2: phospholipase A_2; PLC: phospholipase C or sphingomyelinase.

microvesicle at the surface of a 50 μm-diameter giant liposome (Farge and Devaux 1992; Devaux 2000). Giant liposomes containing a small fraction (1 mol%) of phosphatidic acid or phosphatidylglycerol undergo similar shape changes when a transmembrane pH gradient is applied (Mathivet et al. 1996). In a living cell, net translocation of phospholipids can be achieved by lipid translocases that catalyse a transbilayer movement of phospholipids at the expense of ATP hydrolysis. Hence, lipid translocases may induce transbilayer area asymmetry and thus regulate membrane curvature *in vivo* (Fig. 4 A). As discussed below, a strong correlation between the activity of lipid translocases and the rate of endo- and exocytosis is consistent with this view.

4.1.2 Inward-directed lipid translocases

Eukaryotic plasma membranes generally display an asymmetric lipid arrangement across the bilayer. Whereas phosphatidylcholine (PC), sphingomyelin (SM), and glycosphingolipids are largely localized to the outer leaflet, the aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) reside predominantly in the inner leaflet (Zachowski 1993). Maintenance of this lipid asymmetry is a dynamic process involving two types of ATP-driven lipid transporters, namely an inward-directed transporter specific for PS and PE, known as the aminophospholipid translocase, and a less specific transporter responsible for a compensatory outward movement of lipids (Daleke 2003). By controlling the cytosolic levels of ATP, which is normally in the range of 2-3 mM, it is possible to modify the steady state distribution of the aminophospholipids and in turn to trigger shape changes in erythrocytes by shifting a fraction of the lipids from the inner to the outer leaflet and vice versa. When ATP levels are raised to 5-6 mM, erythrocytes form endocytic vesicles (Birchmeier et al. 1979). This process can be quantified by monitoring the acetylcholine esterase activity exposed on the cell surface, the reduction of which is an indication of the amount of plasma membrane internalised. Strikingly, membrane invagination and endovesiculation are enhanced by external addition and subsequent translocation of PS or PE, but inhibited when PC or SM are added to the outer leaflet (Seigneuret and Devaux 1984; Muller et al. 1994). On the basis of these findings, it has been postulated that active lipid transport catalysed by lipid translocases can act as a driving force in vesicle biogenesis (Devaux 1991; Devaux 2000). As discussed below, recent studies lend further support to this idea.

Erythrocytes do not represent the model of choice for studying endocytosis since they lack the ability to do so under physiological conditions. In fact, the aminophospholipid translocase activity in erythrocytes is rather weak in comparison to cells with natural endocytic activity; initial PS translocation rates in nucleated cells are nearly two orders of magnitude higher than in erythrocytes (Cribier et al. 1993; Pomorski et al. 1996). Addition of PS to the external surface of erythroleukemia K562 cells accelerates the endocytic uptake of fluoresceinated membrane proteins, whereas addition of lyso-PS, which is not transported by the aminophospholipid translocase, inhibits endocytosis (Farge et al. 1999). Complementary evidence for a role of lipid translocases in membrane vesiculation came from recent work in yeast. Disruption of the *DNF1* and *DNF2* genes abolishes inward phospholipid transport across the yeast plasma membrane and causes an accumulation of PE in the outer leaflet, especially when a related gene, *DRS2,* is also removed (Pomorski et al. 2003). Concomitantly, *dnf1dnf2* mutant cells, but in particular *dnf1dnf2drs2* cells exhibit a defect in the internalisation step of bulk phase and receptor-mediated endocytosis (Pomorski et al. 2003). Together with two additional yeast proteins (Neo1p and Dnf3p), Dnf1p, Dnf2p and Drs2p belong to a subfamily of P-type ATPases that also includes ATPase II, a putative aminophospholipid translocase purified from bovine chromaffin granules (Tang et al. 1996). Whereas Dnf1p and Dnf2p are primarily associated with the yeast plasma membrane, Drs2p resides in a late compartment of the yeast Golgi complex (Chen et al. 1999; Hua et al. 2002; Pomorski et al. 2003). Deletion of the *DRS2* gene is lethal

when combined with mutations in the genes for clathrin heavy chain or ADP-ribosylation factor 1, and perturbs formation of a specific class of clathrin-coated vesicles carrying invertase and acid phosphatase to the plasma membrane (Chen et al. 1999; Gall et al. 2002). Neo1p is localised to an early Golgi compartment and required for efficient COPI-dependent protein transport from the Golgi to the ER (Hua and Graham 2003).

All five members of the yeast Drs2/Neo1 P-type ATPase family have now been implicated in membrane trafficking at different steps along the endocytic and secretory pathways. Since these proteins most likely function as inward-directed lipid pumps (Tang et al. 1996; Gomes et al. 2000; Pomorski et al. 2003), it appears that a dynamic regulation of the transbilayer lipid arrangement plays a fundamental role in the formation of coated transport vesicles. Exactly how P-type ATPase-dependent lipid pumping participates in vesicle biogenesis remains to be established. A high concentration of aminophospholipids in the cytoplasmic leaflet may be necessary for an efficient recruitment of peripheral proteins such as ARFs, clathrin, amphiphysin, and endophilins (e.g. Takei et al. 1998). Yet this scenario is somewhat difficult to reconcile with the observation that cells lacking multiple Drs2/Neo1 family members down regulate the aminophospholipid content of their membranes, a response that would be counter productive for the rate of vesicle budding (Pomorski et al. 2003). Moreover, stimulation of inward lipid transport appears sufficient to trigger endocytic vesicle formation in erythrocytes, a cell type lacking the machinery for generating coated vesicles. This would imply that P-type ATPase-dependent lipid pumping primarily serves to facilitate inward vesicle budding by expanding the cytosolic leaflet of the membrane at the expense of the exoplasmic leaflet. According to this scenario, coat assembly would help localize the process, rather than providing the primary driving force. Aminophospholipid asymmetry would then merely be a consequence of the fact that, if there had been no lipid selectivity, the translocase would be trapped in a continuous pumping of the entire bilayer.

4.1.3 Outward-directed lipid translocases

Whereas Neo1/Drs2-related P-type ATPases appear to function as inward-directed lipid pumps, prime candidates for outward-directed lipid pumps are members of the ABC family of transport ATPases (Smit et al. 1993; Ruetz and Gros 1994; van Helvoort et al. 1996; Decottignies et al. 1998). Overexpression of ABC transporters with outward lipid translocase activity slows down endocytosis (Kean et al. 1997; Decottignies et al. 1998). Conversely, loss of ABCA1, a putative phospholipid transporter affected in Tangier disease, stimulates endocytosis (Zha et al. 2001). By catalysing an efflux rather than an influx of lipids across the bilayer, ABC transporters would promote an outward bending of cellular membranes. ABCA1 is required for the engulfment of apoptotic cells, a process characterized by an elaborate protrusion of the plasma membrane (Marguet et al. 1999).

Note that the action of a unidirectional lipid transporter would lead to a global, rather than a local difference in lateral pressure between the two membrane leaflets. However, a global difference in lateral pressure maintained by lipid pumps

could drive local membrane bending, with protein coats, membrane inserting peptides (below), or lipid microdomains (section 4.3) acting as the nucleation sites.

4.1.4 Bidirectional lipid flippases

Contrary to the situation in the plasma membrane and Golgi complex, flip-flop of phospholipids in the ER is a fast process with half times in the order of seconds or minutes (Seigneuret and Devaux 1984; Buton et al. 1996, 2002; Marx et al. 2000). This flip-flop is believed to be protein-mediated, involving one or more lipid flippases (Bretscher 1973; Bishop and Bell 1985; Menon et al. 2000). ER flippases operate independently of metabolic energy and facilitate a non-vectorial movement of most, if not all phospholipid classes in both directions. Consequently, they would promote a symmetric phospholipid distribution across the bilayer. Whether the rapid flip-flop in the ER is mediated by a single dedicated flippase, a group of flippases, or accomplished by the mere presence of integral membrane proteins in a particular lipid environment remains to be established. Peptides mimicking the transmembrane α-helices of proteins are capable of inducing phospholipid flip-flop in model membranes (Kol et al. 2003). This helix-mediated flip-flop is strongly inhibited by cholesterol, an abundant component of the plasma membrane. Cholesterol may exert this inhibitory effect by increasing the packing density of the acyl chains through which the polar head group has to travel. It has been postulated that the gradual increase in cholesterol levels along the secretory pathway serves as a regulatory device to switch from constitutive flip-flop in the ER to a more tightly controlled translocation of lipids in the Golgi and plasma membrane (Kol et al. 2002). An important implication of the fact that phospholipids can freely cross the ER bilayer in both directions is that a transbilayer lipid arrangement permissive for vesicle budding should be easily accomplished. Hence, unlike the situation at the plasma membrane where flip-flop is constrained, assembly of a coat on the surface of the ER may well be sufficient to drive membrane vesiculation.

Note that the plasma membrane of animal cells contains a Ca^{2+}-dependent scramblase that, when induced, mediates a complete intermixing of lipids between the bilayer leaflets, irrespective of head group specificity (Williamson et al. 1992). This activity is turned on during apoptosis, and perhaps facilitates the outward blebbing of the plasma membrane observed in apoptotic cells.

4.1.5 Amphipathic peptides

Recent work has led to the identification of proteins capable of mediating transbilayer area asymmetry through burying an amphipathic helix into one face of the membrane (Fig. 4 B), hence bypassing the requirement for a lipid rearrangement across the bilayer. A prominent example is epsin 1, an accessory protein that collaborates with clathrin and AP2 adopter complexes in the budding of clathrin-coated vesicles from the plasma membrane (Chen et al. 1998). Epsin 1 contains an epsin amino terminal homology (ENTH) domain that comprises a phosphoinositide-binding site. Upon binding phosphatidylinositol 4,5-bisphosphate (PIP_2), the

ENTH domain forms an amphipathic α-helix, H_0, in which the hydrophobic residues are facing the exterior (Ford et al. 2002). When added to PIP_2-containing giant liposomes, the ENTH domain causes a strong degree of membrane curvature, leading to the formation of 20 nm-diameter tubules. Mutation of hydrophobic residues in H_0 abolishes the tubulogenic behaviour of ENTH, suggesting that a physical penetration of H_0 in the outer membrane leaflet is responsible for inducing curvature (Ford et al. 2002). A striking example of the membrane deforming potential of an amphipathic helix can be found in the ability of a *de novo* designed, 18-mer amphipathic peptide to transform spherical liposomes of varying lipid composition into nanotubules up to several μm in length (Lee et al. 2001). By virtue of its ENTH domain and clathrin/adaptor-binding motifs, epsin 1 would provide both a driving force and a molecular link between membrane invagination, clathrin polymerisation and AP2 complex recruitment. By influencing local phosphoinositide metabolism, transduction events at the plasma membrane may create sites where epsin 1 would bind. This would allow cells to specify locations of membrane vesiculation.

The proposed working mechanism of epsin 1 may apply to other proteins implicated in clathrin-coated vesicle formation. EpsinR binds clathrin, the γ-appendage of the AP1 adapter complex, and contains an ENTH domain with a phosphatidylinositol-4 phosphate-binding site. Several lines of evidence indicate that epsinR is functionally equivalent to epsin 1, but in clathrin-coated vesicle budding from Golgi/endosomes rather than from the plasma membrane (Mills et al. 2003). Amphiphysin and endophilin, two major interactors of the GTPase dynamin, are capable of reshaping liposomes into narrow membrane tubules. Strikingly, the amino termini of these tubulogenic proteins contain a so-called N-BAR domain with an amino acid stretch predicted to form an amphipathic helix necessary for lipid bilayer tubulation (Farsad et al. 2001). Co-incubation of endophilin, clathrin and liposomes results in the formation of tubules capped with clathrin-coated buds in spite of the fact that endophilin has no known clathrin-binding properties. This suggests that proteins capable of driving membrane curvature on their own might facilitate clathrin-mediated bud formation by altering bilayer structure to favour this process (Farsad and De Camilli 2003). Membrane deforming proteins involved in cellular processes other than clathrin-coated vesicle formation have also been reported. A member of the amphiphysin protein family, amphiphysin 2, localises to the T-tubule system in striated muscle and induces tubular plasma membrane invaginations when expressed in non-muscle cells (Lee et al. 2002). Induction of the nanotubular network from the parasitophorous vacuole of *Toxoplasma gondi* requires a parasite secretory protein, Gra2, that has two amphipathic α-helical regions the integrity of which is essential for correct formation of the network (Mercier et al. 2002).

4.1.6 Lipid remodelling enzymes

Finally, transbilayer area asymmetry can also be achieved through chemical modifications affecting the molecular area or biophysical properties of the lipids in one leaflet. ATP depletion turns erythrocytes into echinocytes whose spiky appearance

has been ascribed to the breakdown of phosphatidylinositol-4,5-biphosphate and subsequent shrinkage of the inner membrane leaflet by 0.6% (Ferrell and Huestis 1984). Microinjection of sphingomyelinase into sphingomyelin-containing giant liposomes results in the appearance of microvesicles on the liposomal surface (Holopainen et al. 2000) and treatment of macrophages with externally added sphingomyelinase triggers ATP-independent endocytosis through the formation of large endocytic vesicles without discernable coats (Zha et al. 1998). Sphingomyelin constitutes nearly 10% of all phospholipids in mammalian cells and occurs primarily in the outer leaflet of the plasma membrane (Holthuis et al. 2001). While removal of its head group by sphingomyelinase would reduce crowding in the outer leaflet (Fig. 4 C), the breakdown product ceramide could flip across the bilayer due to its hydrophobicity (Pagano 1990) and hence induce a relative increase in lateral pressure in the inner leaflet. Both of these processes would promote inward curvature. Sphingomylinase-induced endovesiculation provides a mechanism potentially used by pathogenic bacteria like *Staphylococcus aureus* and *Neisseria gonorrhoeae* to invade human cells (Walev et al. 1996; Grassme et al. 1997).

4.2 Transbilayer curvature asymmetry

4.2.1 Lipid polymorphism and membrane shape

If there are no forces acting on its surface, a lipid monolayer adopts a curvature called the spontaneous or intrinsic curvature. Conventionally, the curvature of a monolayer is defined as positive if the monolayer bends in the direction of the polar heads, and negative for the opposite direction of bending. Unlike spontaneous bilayer curvature, which depends also on lipid number (see above), spontaneous monolayer curvature is primarily determined by the effective shapes of the lipids (Cullis and de Kruijff 1979; Israelachvili et al. 1980). Hence, cone-shaped lipids whose head groups are larger than the cross-sectional areas of their acyl chains (e.g. lyso-PC) will promote a positive spontaneous curvature. Inverted cone- or wedge-shaped lipids whose head groups are smaller than the cross-sectional areas of their acyl chains (e.g. unsaturated PE) will promote a negative spontaneous curvature. Cylindrically-shaped lipids with equally sized cross-sectional areas of the head group and acyl chains (e.g. di-monounsaturated PC, sphingomyelin) will assemble into monolayers with a close to zero spontaneous curvature. The curvature preference of lipids is not exclusively determined by their molecular structure, but also influenced by additional factors such as temperature, pH and ion concentrations.

Lipids promoting a pronounced positive or negative spontaneous curvature are also called non-bilayer lipids, although an equimolar mixture of a cone- and wedge-shaped lipid, for example lyso-PC and di-unsaturated PE, will form a stable bilayer (Madden and Cullis 1982). Cellular membranes consist of mixtures of bilayer and non-bilayer (primarily inverted cone-shaped) lipids. The latter are forced to remain in the lipid-bilayer, a situation generally referred to as ‘frustration’. The ratio of bilayer to non-bilayer lipids is strictly regulated, and likely

helps determine the propensity of cellular membranes to undergo fusion (Burger 2000). A planar membrane may be composed of either two monolayers having negative (or positive) spontaneous curvatures, or two monolayers having zero spontaneous curvatures. Because of symmetry, the net bilayer spontaneous curvature will be zero for both membranes, even though the membrane composed of lipids with non-cylindrical effective shapes will be stressed. But a difference in spontaneous monolayer curvature between the two membrane halves, or transbilayer curvature asymmetry, will result in membrane bending. This principle is perhaps best illustrated by the shape changes that occur in erythrocytes when natural, mono-unsaturated PC present in the outer leaflet is exchanged for synthetic di-saturated or di-unsaturated PC using a PC-specific transfer protein. While hardly affecting the surface area of the outer leaflet, the PC-exchange reaction causes dramatic shape changes with di-saturated PC triggering an outward membrane bending and echinocyte formation, and di-unsaturated PC producing the opposite effect (Kuypers et al. 1984; Christiansson et al. 1985).

By virtue of their ability to alter the lipid composition in the membrane, lipid transfer proteins, lipid translocases and lipid remodellases may all influence the spontaneous curvature of the membrane leaflets, and hence regulate membrane bending *in vivo* (Burger 2000).

4.2.2 Lipid hydrolases and lipid acyltransferases

The idea that local lipid metabolism and the generation of transbilayer curvature asymmetry are used *in vivo* to regulate membrane bending is supported by the recent discovery that phospholipid hydrolases and acyltransferases co-ordinately regulate physiological shape changes of the Golgi complex. Thus, inhibition of a cytoplasmic Ca^{2+}-independent phospholipase A_2 (PLA_2) prevents the formation of Golgi membrane tubules that form both constitutively and in response to brefeldin A (BFA) treatment (de Figueiredo et al. 1998, 1999). PLA_2 antagonists also inhibit retrograde protein transport from the Golgi to the ER (de Figueiredo et al. 2000) and the membrane tubule-mediated reassembly of a nocodazole-fragmented Golgi complex (Drecktrah et al. 2003). Peptide-induced stimulation of a cytoplasmic PLA_2 has the opposite effect, that of inducing Golgi membrane tubulation (Polizotto et al. 1999). Hence, by catalysing the conversion of cylindrical (or cone-shaped) phospholipids into inverted cone-shaped lyso-phospholipids in the cytosolic membrane leaflet, PLA_2 would promote an outward bending of the bilayer that at its most extreme would trigger membrane tubulation (Fig. 4 C). Consistent with this model, inhibition of a Golgi-associated lyso-phospholipid acyltransferase (LPAT), which reacylates lyso-phospholipids back to phospholipids, increases the levels of lyso-phosphatidylcholine in Golgi membranes and stimulates membrane tubulation and retrograde trafficking (Drecktrah et al. 2003). Importantly, preincubation of cells with PLA_2 antagonists prevents LPAT antagonists from inducing Golgi tubulation, providing further evidence that this process is directly controlled by the phospholipid/lyso-phospholipid ratio in the cytosolic leaflet of the Golgi. A similar mechanism seems to regulate tubulation of endosomes

and the recycling of transferrin receptors from endocytic compartments to the cell surface (de Figueiredo et al. 2001).

Other work indicates that LPATs are part of the fission machineries involved in Golgi tubule fragmentation and endocytic vesicle formation. Thus, a lysophosphatidic acid (lyso-PA) specific LPAT, CtBP/BARS, induces fission and vesicle formation from Golgi membrane tubules (Weigert et al. 1999). Likewise, inhibition of the intrinsic lyso-PA specific LPAT activity of endophilin reduces its ability to trigger endocytic vesicle formation (Schmidt et al. 1999), even though its tubulogenic properties do not require LPAT activity (Farsad et al. 2001). For both proteins, it has been postulated that condensation of membrane-embedded lyso-PA and cytosolic fatty acyl-CoA to phosphatidic acid (PA) would promote a negative spontaneous curvature in the cytosolic leaflet, in particular when unsaturated fatty acyl-CoA such as arachidonoyl-CoA is used. This, in turn, would drive inward bending of the membrane at the neck of a budding vesicle, thus, aiding in its fission (Burger 2000; Kooijman et al. 2003). In the Golgi, CtBP/BARS-derived PA is likely metabolised rapidly into diacylglycerol (DAG), a lipid with an even larger negative spontaneous curvature. Due to its hydrophobicity, DAG would readily equilibrate between the two membrane halves. A recent theoretical analysis suggests that the lipid transformation sequence lyso-PA→PA→DAG and subsequent partitioning of DAG may drive the progressive constriction of Golgi membrane tubules and explain the CtBP/BARS-induced 'pearling' of these tubules (Shemesh et al. 2003). While PA and DAG may serve as the direct mechanical workers in deforming the membrane, alternative scenarios cannot be excluded. For example, PA or DAG may recruit and activate downstream effectors involved in membrane bending or fission. Protein kinase D binds DAG and is required for secretory vesicle formation at the TGN (Baron and Malhotra 2002). The GTPase dynamin regulates membrane constriction and fission during receptor-mediated endocytosis (Hinshaw 2000). Dynamin is a membrane active protein whose ability to penetrate in between lipid head groups is greatly stimulated by PA (Burger et al. 2000). Hence, the PA generated by dynamin-bound endophilin may induce deep penetration of dynamin into the neck region of a budding vesicle or tubule, and hence promote fission indirectly. Note that cells may exploit the above principles simultaneously to accomplish membrane fission.

4.2.3 Lysobisphosphatidic acid in multivescicular endosome formation

Lysobisphosphatidic acid (LBPA) is an abundant component of internal vesicles in multivesicular endosomes, accounting for nearly 15 mole percent of total organelle phospholipids (Kobayashi et al. 1998). Recent work revealed that this unconventional cone-shaped lipid possesses the capacity to drive vesicle budding and fission within acidified liposomes. A cytosolic LBPA-binding protein, called Alix, controls this invagination process *in vitro* and is involved in the biogenesis of multivesicular endosomes *in vivo* (Matsuo et al. 2004). Precisely how LBPA and Alix participate in multivesicular endosome formation remains to be established, but the intrinsic capacity of LBPA to stimulate internal vesicle formation

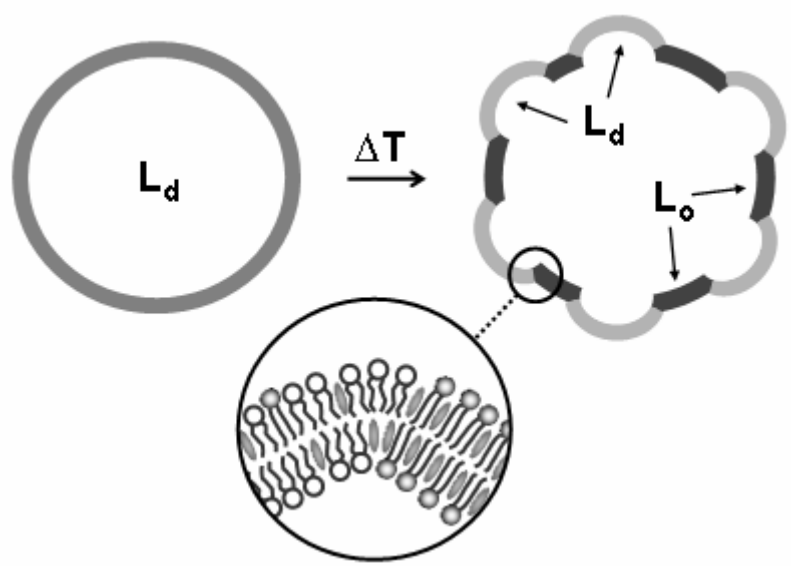

Fig. 5. Lipid domain-induced membrane curvature. Giant liposomes prepared from a ternary mixture of glycerolipids, sphingolipids and cholesterol are subjected to a temperature shift, ΔT, causing a lipid phase separation. As a result, two different liquid phases, or domains, are formed: a liquid ordered (L_o) domain enriched in sphingolipids and sterols, and a liquid disordered (L_d) domain containing the bulk of glycerolipids. A line tension builds up, which is proportional to the length of the boundaries between the two domains. This tension can be partially relieved by increasing the membrane curvature in one of the two domains. Due to a higher packing density of the lipids, the bending resistance of L_o domains is higher than that of L_d domains. Budding from L_d domains would, therefore, provide the most efficient way of reducing the line tension.

within acidic liposomes provides an important clue. At neutral pH, LBPA has a negatively charged head group that would impair its spontaneous movement across the bilayer. Lowering the pH would cause a reduction in charge and increase the flip-flop rate of LBPA. Acidification of the lumen of LBPA-containingliposomes would then result in a preferential accumulation of this cone-shaped lipid in the outer leaflet, thus promoting a negative spontaneous curvature of the outer leaflet at the expense of the inner leaflet. This, in turn, may drive membrane invagination and formation of internal vesicles. Consistent with an active role for LBPA in multivesicular endosome biogenesis, some patients afflicted with antiphospholipid syndrome produce auto-antibodies against LBPA that, when applied to cells, accumulate in late endosomes and disrupt their multivesicular morphology (Kobayashi et al. 1998).

4.3 Lipid domain-induced curvature

Studies on model membranes established that lipids have a strong self-organizing capacity; lipid immiscibility can trigger phase separation and create domains with unique lipid compositions and physical properties (Brown 1998). Thus, mixtures of sphingolipids, unsaturated glycerolipids, and cholesterol can segregate spontaneously into two fluid domains where the sphingolipids and part of the cholesterol coalesce into a liquid ordered (L_o) domain and break away from the unsaturated glycerolipids in a liquid disordered (L_d) phase. 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, resulting in a dramatic increase in the van der Waals attraction between neighbouring sphingolipid molecules (McIntosh et al. 1992). Intriguingly, high-resolution fluorescence imaging of giant liposomes composed of a ternary mixture of sphingomyelin, dioleoyl-PC and cholesterol has demonstrated that domain formation can drive vesicle budding and fission (Fig. 5; Baumgart et al. 2003). Here, the driving force is generated by the line tension, an energy associated with the edge of the domain that is proportional to the length of the edge. Domain-induced budding is a spontaneous process, governed by a competition between a decrease in line tension and an increase in bending energy (Julicher and Lipowsky 1993).

As there is evidence for the existence of phase-separated domains in cellular membranes (Simons and Ikonen 1997; Brown and London 1998; Anderson and Jacobson 2002), domain-induced budding may also occur *in vivo*. This concept could be particularly relevant for the Golgi complex, which is a major site of lipid sorting. Sphingolipids are synthesised in the Golgi, cholesterol in the ER (van Meer 1998; Huitema et al. 2004). In spite of extensive membrane trafficking at the ER-Golgi interface, both lipids accumulate in the plasma membrane. Cholesterol has a high affinity for sphingolipids, and anterograde sorting of newly synthesized sphingolipids in the Golgi has been put forward as a mechanism to deplete cholesterol from the ER and to promote its concentration in the plasma membrane (van Meer 1998). Anterograde sphingolipid sorting could be achieved if sphingolipids were prevented from leaving the Golgi cisternae in which they are made; cisternal maturation would then ensure their unidirectional transport toward the plasma membrane (Holthuis et al. 2001; Pelham 2001). By triggering a phase separation in Golgi membranes, newly synthesized sphingolipids may stimulate budding of retrograde COPI vesicles or tubules from which they themselves are excluded; note that this budding would preferentially occur from domains enriched in unsaturated glycerolipids, since it is negatively influenced by fatty acyl chain saturation and cholesterol (Spang et al. 1998; Brugger et al. 2000; Koster et al. 2003). A domain-induced budding mechanism has the particular appeal of being inherently self-organizing. Consequently, it may represent one of the cell's most primordial mechanisms for membrane vesiculation.

5 Concluding remarks

Cells clearly evolved more than one type of strategy to overcome the energy barrier of membrane deformation. So far, attention has been focused primarily on the oligomerization-driven mechanical deformation of lipid bilayers by 'classical' coat proteins, even though several of the intracellular budding events might not require a supramolecular coat complex. Indeed, two of the five endocytic pathways described in mammalian cells involve buds and vesicles that lack a discernable coat (Pelkmans and Helenius 2003). The same holds true for the internal vesicles of multivesicular endosomes, the secretory granules formed in neuroendocrine cells and the milk-fat droplets shed from mammary epithelial cells. At the same time, emerging data emphasize that lipids serve a more dynamic and active role in membrane deformation than previously envisioned. In fact, model membrane studies reveal that budding and fission can occur in the absence of added protein. In live cells, lipid remodelling and unidirectional lipid transport across the bilayer each seem to affect shape changes that support membrane tube formation and vesiculation. Current challenges include the unambiguous identification of the lipid remodellases and translocases involved. Reconstitution of these activities in giant liposomes will be important to further elucidate the functional link between lipid remodelling, lipid translocation and membrane folding.

One may wonder why cells invented so many different kinds of membrane-deforming mechanisms. Where and when do these mechanisms operate? Do cellular budding machineries apply multiple types of membrane bending simultaneously? If so, how are the actions of the proteins and lipids involved coordinated? At present, these issues are incompletely understood. The physical constraints associated with membrane folding are dependent on lipid composition. Our comprehension of this process in cells is hampered by a relative ignorance of the steady-state lipid compositions of the various organelles. The fact that lipids can freely cross the ER bilayer in both directions implies that this organelle would readily adopt a transbilayer lipid arrangement permissive for vesicle formation. This is in contrast to the situation at the plasma membrane where the lipid flip-flop rate is constrained due to high levels of sterols and sphingolipids. Hence, it is unlikely that the same membrane-deforming mechanism can be used for all organelles. This may help explain the multiplicity of budding machineries that are currently uncovered in cells.

Acknowledgements

I am grateful to Gerrit van Meer and Hein Sprong for critically reading the manuscript. Support was from a grant of the Dutch NWO-CW VIDI programme and from SENTER, the Dutch Ministry of Economic Affairs.

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Holthuis, Joost C. M.
Department of Membrane Enzymology, Faculty of Chemistry, Padualaan 8, 3584 CH Utrecht, The Netherlands
j.c.holthuis@chem.uu.nl