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Lipid distribution and transport across cellular membranes

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In eukaryotic cells, the membranes of different intracellular organelles have different lipid composition, and various biomembranes show an asymmetric distribution of lipid types across the membrane bilayer. Membrane lipid organization reflects a dynamic equilibrium of lipids moving across the bilayer in both directions. In this review, we summarize data supporting the role of specific membrane proteins in catalyzing transbilayer lipid movement, thereby controlling and regulating the distribution of lipids over the leaflets of biomembranes.

Key words: flip–flop / lipid asymmetry / translocase / flippases / ABC transporter

Introduction

The basic structure of biological membranes is the lipid bilayer. This structure is mainly formed from three different classes of lipids (glycerolipids, sterols and sphingolipids). In eukaryotic cells, the membranes of different intracellular organelles have different lipid compositions. For example, plasma membranes are typically enriched in sphingolipids, phosphatidylserine (PS) and cholesterol, while the endoplasmic reticulum (ER) is depleted in these lipids. In addition to the heterogeneous distribution of lipids between membranes, there are also striking differences in the distribution of lipids across the membrane bilayer. For instance, while the ER is assumed to have a symmetric lipid distribution, lipids in the plasma membrane of eukaryotic cells show a clear asymmetric arrangement, with the majority of the glycosphingolipids and phosphatidylcholine (PC) in the exoplasmic leaflet and the aminophospholipids, PS and phosphatidylethanolamine (PE), on the cytoplasmic face (reviewed in Reference 1).

The lipid distribution across membranes results from a continuous inward and outward movement of lipids between the two monolayers (Figure 1). Although neutral lipids like cholesterol and charged lipids in a protonated form, such as free fatty acid, phosphatidic acid or phosphatidylglycerol, can move fast between leaflets, spontaneous transbilayer movement of most lipids with a polar (or charged) lipid headgroup is a very slow process ($t_{1/2}$ = hours to days in model membranes; see Reference 1). However, the assembly of certain cellular membranes relies on a rapid transbilayer movement of polar lipids. Examples are the ER of eukaryotes or in the cytoplasmic membrane of prokaryotes, where lipid biosynthesis occurs predominately in the cytoplasmic leaflet. Furthermore, the compositional asymmetry of the plasma membrane does not correspond to the asymmetry of lipid synthesis or hydrolysis. Hence, lipid asymmetry must be formed and afterwards maintained by specific mechanisms that control lipid movement across the bilayer and counterbalance the lipid randomization caused by spontaneous transbilayer movement.

Techniques to assess transbilayer lipid movement

The asymmetric arrangement of phospholipids in plasma membranes was originally established for natural lipids in erythrocytes using chemical labeling,[2] hydrolysis by phospholipases,[3] and exchange by lipid transfer proteins,[4,5] and by the same techniques

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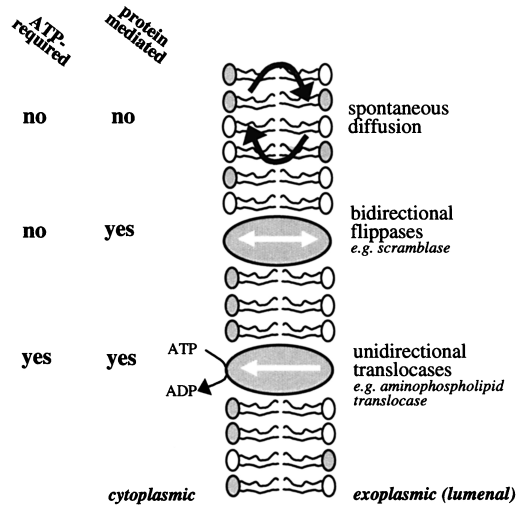

Figure 1. Lipids can cross biological membranes by various mechanisms. Spontaneous diffusion refers to the non-specific lipid movement occurring between the membrane leaflets; its rate is determined by the biophysical properties of both the lipid and the membrane (note that transfer of a lipid from one monolayer to the other is not necessarily coupled with movement of a lipid towards the opposite direction). Flippases facilitate an ATP-independent, bi-directional movement of lipids but are unable to accumulate a given lipid in one leaflet. Translocases directly use the energy released by ATP-hydrolysis to drive unidirectional lipid movement against a gradient in the membrane.

in plasma membrane-derived viruses.[6–8] Whereas initially lipid translocation across biomembranes was also measured by these techniques, evidence for the involvement of proteins is based on techniques using lipid analogs, where in general one fatty acid chain has been replaced by a short chain carrying a radiolabel, a spin-label or a fluorescent moiety[9] (Figure 2). Clearly, in any case where a protein has been identified as being involved in translocating lipid analogs across a biomembrane, the activity of the protein towards naturally occurring lipids will have to be established. Also the kinetics of transbilayer movement of natural lipids can only be determined by studying the lipids themselves, but due to technical complications this has been achieved in only a few cases. It is, therefore, a challenge to the field to develop methods to establish whether the putative lipid translocators use natural lipids as substrates, and by which the transbilayer movement of natural lipids can be measured in living systems. The use of fluorescent annexin V is a new method that has proved extremely sensitive for the detection of small amounts of endogeneous PS on the surface of apoptotic and aged cells.[10]

Lipid movement in the ER and bacterial membranes: evidence for flippases

The ER is the principal site of membrane assembly in eukaryotes and in that aspect similar to the cytoplasmic membrane in prokaryotes. Phospholipid biosynthesis in these membranes is an asymmetric process, resulting in the insertion of a newly synthesized lipid in the cytoplasmic leaflet. In order to create a bilayer, a fraction of the phospholipids has to be translocated to the other leaflet (Figure 3). Indeed, rapid phospholipid movement has been reported in microsomal membranes ($t_{1/2}$ = seconds to minutes).[11–17] Although the measured rate of transport varied between assays, probably due to differences in kinetic resolution, all observations to date indicate that phospholipid movement in the ER is bi-directional, ATP-independent and non-specific towards the phospholipid headgroup. Protein-modifying reagents partially inhibited phospholipid movement suggesting that proteins are directly or indirectly involved.[13,15–17] This notion was further substantiated when membrane proteins from rat liver microsomes were reconstituted into proteoliposomes and found to facilitate lipid translocation, while protein-free liposomes or proteoliposomes containing proteins from human erythrocyte membrane were inactive.[14] Recently, the transbilayer movement of short-chain (diC_4) water-soluble PC was assayed in proteoliposomes reconstituted from Triton X-100 soluble fractions of rat liver microsomes.[17] The transport activity was recovered in liposomes containing a protein fraction of a low sedimentation rate and was sensitive to proteolysis, whereas similarly prepared liposomes, containing solely the ER lipids, were inactive. Fractionation of the detergent extract resulted in proteoliposomes with different specific activity, indicating that specific microsomal membrane proteins were responsible for the transport.

Besides the phospholipids, the glycolipids mannosyl-phosphodolichol, glucosyl-phosphodolichol and an oligosaccharide-diphosphodolichol have to undergo transbilayer movement in the ER for the synthesis of the full length lipid-linked oligosaccharide that is subsequently transferred to a lumenal domain of proteins. Rapid flip–flop of water-soluble analogs of these glycolipids has been reported.[18]

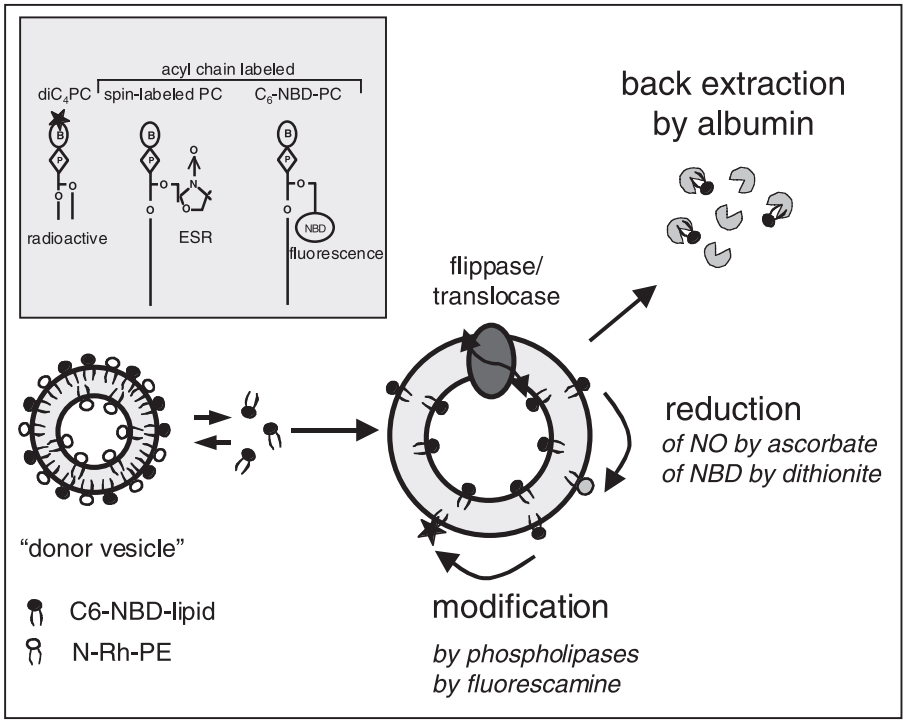

Figure 2. Schematic representation of the techniques used for monitoring transbilayer movement of lipid. Most techniques are based on the use of lipid analogs with either one or two short fatty acid chains. Dibutyroylphosphatidylcholine (diC_4PC) is rather water-soluble and will be released into the lumen when it redistributes from the outer to the inner leaflet of the vesicle. Thus, rapid separation of the vesicles from the incubation medium by filtration allows determination of lipid translocation by measuring the amount of radioactivity associated with the vesicles. Acyl-chain-labeled lipid analogs contain a radiolabel, a spin-label or fluorescent group on a short fatty acyl chain at the C2 position of the glycerolipid or on the amine of the sphingosine. These lipids can be incorporated into synthetic donor vesicles. Upon addition to cells, the lipid analogs (but not the normal long-chain lipids) transfer spontaneously and very rapidly into the outer leaflet of the biological membrane. Their transbilayer distribution can be monitored as a function of time by either back extraction of the probes still present in the exposed leaflet onto albumin, or by chemical modification of the non-translocated analogs with membrane impermeant reagents such as ascorbate (for spin-label) or dithionite (for the fluorescent NBD group). When using fluorescent analogs, vesicles can be prepared containing the non-exchangeable, headgroup labeled N-rhodamine PE and the NBD lipid of interest. During exchange and translocation of the NBD-lipid, a decrease of the energy transfer between the NBD lipid and the N-Rh-PE occurs which results in an increase in the NBD fluorescence and allows continuous measurement of lipid translocation. Modification of lipids present in the outer leaflet by chemical reagents or action of phospholipases can be applied to endogenous, long-chain lipids but has a limited temporal resolution.

Similarly, the synthesis of the glycosylphosphatidylinositol anchor of GPI-proteins is initiated on the cytosolic surface of the ER and, at some stage of its biosynthesis, must translocate to the ER lumen for addition to proteins.

As in the ER, fast transbilayer movement of phospholipids is needed to propagate the bilayer of the bacterial cytoplasmic membrane, at a rate sufficient for a rapidly growing bacterium. Phospholipid flip–flop in bacterial cytoplasmic membranes has accordingly been found to be fast.[19–23] Interestingly, phospholipid flip–flop in bacteria has similar characteristics as in the ER and is bi-directional, energy independent and non-specific towards the phospholipid headgroup. In support for the involvement of membrane proteins in the accelerated flip–flop, phospholipid movement in *Baccillus* membrane vesicles was found to be protease-sensitive.[22] However, protein modification of *E. coli* inner membrane proteins had no detectable effect on phospholipid transport,[21,23] probably because the assay used would not have allowed detection of partial inhibition.

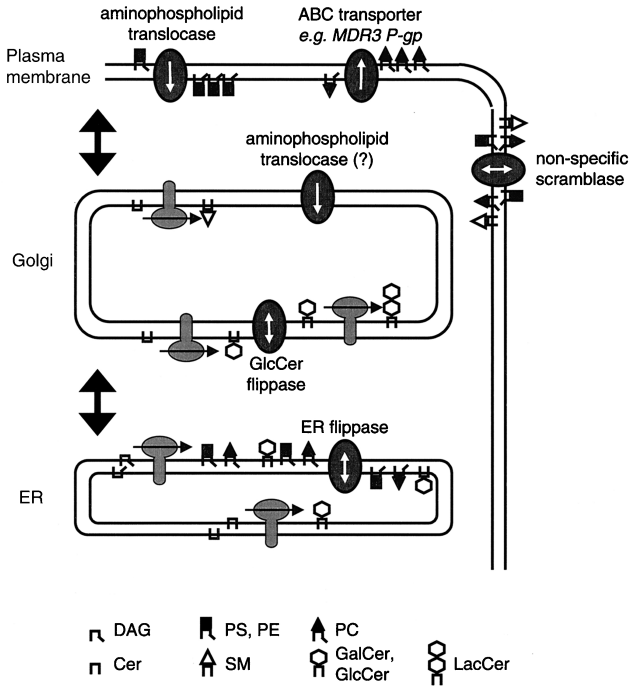

Figure 3. Various proteins control lipid sidedness across cellular membranes. Synthesis of phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylethanolamine (PE) occurs on the cytoplasmic leaflet of the endoplasmic reticulum (ER). PC and PE are synthesized by addition of the appropriate phosphoryl base to diacylglycerol (DAG), while PS is derived by base exchange of serine for the ethanolamine moiety of PE. Ceramide can be converted to galactosylceramide (GalCer) on the lumenal side of the ER. A non-specific flippase allows the rapid redistribution of the newly synthesized phospholipids[11–17] and GalCer.[74] Glucosylceramide (GlcCer), the precursor of higher glycosphingolipids, is synthesized at the cytosolic face of Golgi membranes. It can be translocated across the Golgi membrane and used in the biosynthesis of lactosylceramide (LacCer) and other glycosphingolipids, but it is unclear whether translocation is bi-directional. The complex glycolipids and sphingomyelin (SM), which are produced at the lumenal face, do not translocate towards the cytosolic face.[73,74] A candidate aminophospholipid translocase has been localized in the late Golgi.[35,39] In the plasma membrane, an ATP-dependent aminophospholipid translocase transports PS and PE towards the cytoplasmic leaflet and maintains a permanent lipid asymmetry.[9,25,27] Members of the ABC transporter family can translocate specific lipids from the cytoplasmic to the exoplasmic leaflet of the plasma membrane, e.g. MDR3 P-pg mediates PC translocation across the canalicular domain of the hepatocyte membrane.[43,45–47] Scramblase action, which depends on activation, results in rapid transbilayer movement of all phospholipids and loss of lipid asymmetry in the plasma membrane.[65–67]

Reconstitution of flippase activity from bacteria has been reported.[24] The transport of a short-chain phospholipid analog and a long-chain phospholipid was associated with the detergent extract of the membrane but not the lipid extract. Transport was protease-sensitive and was enriched in a fraction sedimenting at ~4S on a glycerol gradient. Recovery of activity in other gradient fractions was low despite the presence of a complex mixture of membrane proteins. These data suggest that bacteria contain specific proteins capable of facilitating phospholipid flip–flop. Whether the bacterial and the ER flippases turn out to be related proteins remains to be seen.

Lipid movement across the plasma membrane of eukaryotic cells

Maintenance and regulation of the asymmetric lipid distribution across the plasma membrane is governed by the concerted action of specific membrane proteins controlling lipid movement across the bilayer. The inward movement of PC and sphingomyelin (SM) from the exoplasmic to the inner plasma membrane is, under normal conditions for most cells, a slow, non-mediated process. The presence of cholesterol in the plasma membrane contributes largely to the stability of SM transbilayer distribution.

In contrast, the aminophospholipids, PS and PE, are rapidly transported from the outer to the inner leaflet by an active ATP dependent and protein-mediated process, thus maintaining lipid asymmetry. The involvement of proteins and ATP dependence has also been reported for the outward movement of lipids. The asymmetrical lipid distribution of the plasma membrane can be scrambled rapidly. This ATP-independent process triggered by cytosolic calcium, would involve the action of a scramblase. The maintenance of the nonrandom lipid distribution is important for cellular functions. Any change in this distribution generally triggers a physiological event. Exposure of PS at the surface of activated or injured blood cells or endothelium serves to promote blood coagulation (reviewed in Reference 25). Surface exposure of plasma membrane PS and PE signals the removal of injured apoptotic cells by the reticuloendothelia system.[26] Besides these functions of an asymmetric lipid distribution in specific cells, the transfer of lipids from one leaflet to the other in cellular membranes may be of general significance for the functioning of a single cell. For example, it could be involved in the regulation of membrane curvature, e.g. during endocytosis, or in the modulation of the activity of membrane proteins.[27,28]

Active inward translocation: the aminophospholipid translocase

The inward movement of aminophospholipids has been shown to depend upon an ATP-driven aminophospholipid translocase which transports PS and PE selectively from the exoplasmic to the cytosolic leaflet of mammalian plasma membranes ($t_{1/2}$ = minutes for PS). First described in human erythrocyte membrane,[9] this aminophospholipid translocating activity has now been demonstrated in various plasma membranes and in chromaffin granules (reviewed in References 1,25,27). A fast ATP-dependent inward movement of aminophospholipids analogs was also found for the yeast plasma membrane.[29,30] Here, aminophospholipid translocation was accompanied by rapid internalization of PC analogs suggesting the presence of either an additional PC-specific translocase or a new type of translocase, translocating aminophospholipids as well as PC towards the cytoplasmic leaflet. Such a rapid inward movement of PC was also found for the basolateral plasma membrane of hepatocytes[31] and in both the basolateral and apical membrane of kidney epithelial cells.[32]

The aminophospholipid translocase has not yet been unequivocally identified. Tentative purifications from erythrocytes[33,34] suggested a Mg^{2+} ATPase with a molecular mass of 115–120 kDa. However purification and cloning of the gene encoding the ATPase II from bovine chromaffin granules, another candidate protein, revealed a slightly bigger protein belonging to a novel subfamily of P-type ATPases.[35] Disruption of the homologous gene in yeast, the DRS2 gene, abolished the internalization of a fluorescent PS analog (C6-NBD-PS) at low temperature.[35] This observation was interpreted as evidence that the DRS2p and the ATPase from bovine chromaffin granules are aminophospholipid translocases. Interestingly, Axelsen and Palmgren[36] have claimed to have found an analog of the chromaffin ATPase II in plant cells that is responsible for PS translocation. In later studies, but under somewhat different experimental conditions, deletion of the DRS2 gene did neither specifically abolish the translocation of fluorescent PS and PE analogs, nor affect the preferential orientation of endogenous aminophospholipids towards the cytoplasmic leaflet of yeasts.[37,38] These observations and the recent localization of the DRS2p in the Golgi complex[39] argue against the idea that this protein acts as an exclusive or major aminophospholipid translocase in the plasma membrane of yeast.

Active outward translocation: involvement of ABC transporters

Evidence for an ATP-dependent and protein-mediated outward movement of lipids towards the cell surface were found first for erythrocyte membrane.[40,41] This transport activity has been observed for analogs of the aminophospholipids as well as of PC with rather slow rate ($t_{1/2} \approx 1.5$ hours). However, a role in the transport of endogenous lipids has not been well established.

A different outward-directed translocase activity located in the plasma membrane was identified in studies originally related to multidrug resistance (MDR) in cancer cells. One form of MDR results from overexpression of the MDR1 P-glycoprotein (P-gp). This membrane protein belongs to ATP-binding cassette (ABC) transporter family and extrudes a wide variety of amphipathic drugs from cells. The closely related ABC transporter MDR3 P-gp is highly expressed in the bile canalicular membrane of hepatocytes. Mice with a disruption of the mdr2 gene (the mouse homolog of MDR3), were found unable to transport PC into bile.[42] Studies on secretory vesicles

from yeast transfected with mdr2 P-gp showed that this protein transports the PC analog C6-NBD-PC from the cytoplasmic to the exoplasmic leaflet of the membrane bilayer[43] (however, *cf.* Angeletti and Nichols[44] for a methodological note). Nies *et al.*[45] confirmed this ATP-dependent PC translocation in isolated canalicular plasma membrane vesicles. Specificity for PC was demonstrated on polarized epithelial cells transfected with human MDR3 P-gp. Newly synthesized C6-NBD-PC could reach the apical plasma membrane in the absence of vesicular transport, while NBD analogs of PE, SM and glucosylceramide (GlcCer) were not translocated.[46] Evidence for translocation of endogenous PC was provided in fibroblasts from mdr2 knock out mice expressing MDR3 Pgp.[47] Besides MDR3 Pgp, evidence has been presented for an ATP-independent PC flippase in canalicular plasma membrane.[48]

Unexpectedly, the drug transporter MDR1 P-gp was found to be responsible for transporting a variety of short-chain lipid analogs from the inner to the outer leaflet of the plasma membrane.[46,49,50] Amongst these were short-chain analogs of PC and of GlcCer, two lipids synthesized on the cytosolic surface of ER and Golgi. MDR1 P-gp was found unable to rescue PC transport into the bile in the mdr2 knockout mice,[42] suggesting that natural long-chain PC is not a natural substrate. However, evidence has been obtained that secretion of the short-chain PC platelet activating factor (PAF) is greatly enhanced in cells transfected with MDR1 P-gp and is sensitive to inhibitors and substrates of MDR1 P-gp (Reference 51; R. Raggers, I. Vogels and G. van Meer, submitted). In addition, expression of MDR1 P-gp has been correlated with an increase in cellular GlcCer and higher glycolipids.[52,53] Using an enzymatic assay for GlcCer appearance on the cell surface, it has now been found that MDR1 P-gp rescues GlcCer from hydrolysis by a non-lysosomal enzyme through removal from the cytosolic leaflet of the plasma membrane and translocation towards the outer leaflet (R. Raggers *et al.*, manuscript in preparation). All cells express MDR1 P-gp, but expression is particularly high in the apical plasma membrane domain of epithelial cells. The observation that an MDR1 P-gp knockout mouse does not display lipid-related physiological defects suggests that its activity as a lipid translocator is physiologically irrelevant or that an alternative system(s) can compensate for the defect.[54]

The multidrug resistance protein MRP1, another drug-pumping ABC-transporter, was also found to translocate C6-NBD-analogs. In contrast to MDR1 P-gp, it did not translocate sphingolipids without the NBD moiety nor C6-NBD-PC.[55] In studies with MRP1 knockout mice, an MRP1-mediated transport of C6-NBD-PS (and C6-NBD-PC) was reported in erythrocytes. However, no changes in the distribution of endogenous PS were detected.[56,57] Therefore, it is still unclear whether MRP1 is involved in the translocation of natural lipids.

Finally, mutations of the ABC transporter ABC1 gene have been shown to be the cause of familial high-density lipoprotein (HDL) deficiency and Tangier disease, an autosomal recessive disorder of lipid metabolism, resulting in very low plasma HDL levels and increased amounts of cholesterol-ester storage in cells.[58–60] Tangier fibroblasts manifest a decrease in the HDL- or apolipoprotein A1-induced efflux of both cholesterol and phospholipids (radiolabeled PC and SM)[61,62] suggesting a possible role as cholesterol efflux transporter. ABC1 was reported to be involved in the engulfment of apoptotic cells by increasing the transbilayer movement of PS.[63] Recent studies with ABC1 knockout mice revealed that calcium triggered spin-labeled PS redistrubution is indeed accelerated by ABC1.[64]

Bi-directional movement: the scramblase

Calcium-influx accompanying cellular activation causes a loss of phospholipid asymmetry in the plasma membrane. The scrambling process is bidirectional and involves all major phospholipid classes, moving at comparable rates ($t_{1/2} \approx 10$ to 20 minutes), with slower mobility for SM.[65–67] One report has suggested that, at least in platelets, PS and PE are preferentially externalized.[68] Inhibition of the aminophospholipid translocase alone does not directly lead to the exposure of PS. Since phospholipid scrambling is inhibited by protein-modifying reagents[67] and is ATP-independent, the activation of a specific calcium-dependent flippase, called scramblase, has been suggested. A rare and very severe bleeding disorder called Scott syndrome was thought to correspond to the lack of the scramblase; at least it is associated with an impairment of calcium triggered lipid redistribution in platelets.[69] A 37 kDa membrane protein from erythrocytes was capable of mediating calcium-dependent transbilayer movement of phospholipids in reconstituted liposomes.[70,71] However, the apparent rate of phospholipid scrambling was rather low ($t_{1/2} \approx 2$ hours)

and this protein is present in blood cells of the Scott patients.[72] Hence, the identity of the scramblase is still obscure.

Lipid movement across other intracellular membranes

In the Golgi, GlcCer, the precursor for the higher glycosphingolipids, is synthesized on the cytoplasmic leaflet. All higher glycosphingolipids are synthesized on the lumenal leaflet, resulting in the presence of numerous different glycosphingolipids on the cell surface. Therefore, GlcCer has to translocate across the Golgi membrane for synthesis of higher glycolipids. This has been demonstrated with a short-chain analog (C6-NBD-GlcCer) on isolated Golgi membranes,[73,74] suggesting that a GlcCer flippase is present in the Golgi membrane. The flippase did not require exogenous energy, allowed passage of galactosylceramide as well as GlcCer and did not allow the metabolic products lactosylceramide, $(galactosyl)_2$ceramide or sulfatide to translocate back to the cytosolic surface. The localization of a putative aminophospholipid translocator in the late Golgi,[39] raises the possibility that lipid asymmetry might be established in the Golgi complex.

Evidence for a novel ATP-dependent phospholipid translocase has been recently reported for gastric vesicles which translocates PS, PE and PC from the cytosolic to the lumenal leaflet.[75] Transport was dependent on ATP on the cytosolic side and abolished by an inhibitor of the gastric H^+, K^+-ATPase but not by an inhibitor or substrates of the multidrug resistance proteins. A similar translocase activity was reported for synaptic vesicles but assumed to depend on lumenal ATP.[76]

Summarizing remarks

Although the last decades have brought us first insights into the transversal orientation of membrane lipids and the mechanisms responsible for generating and maintaining this organization, many questions remain. With respect to the transbilayer distribution of the lipids, little is known concerning the orientation of cholesterol, one of the major lipids in the plasma membrane, and other more minor lipids like phosphatidylinositol and its phosphorylated derivatives. An additional complexity is the fact that the lateral distribution of the various lipids in the plane of the membrane is probably not homogeneous. Apart from difficulties in defining the relevant parameters of lipid asymmetry, this predicts that not all molecules of a certain lipid class present in one membrane leaflet will translocate with the same kinetics. The flippases or translocases may be preferentially localized in a specific lipid environment.

A number of proteins have now been identified as being involved in the translocation of lipids. In essentially all cases it remains to be established whether these proteins can translocate a certain lipid across a lipid bilayer on their own, or whether they are part of an oligomeric complex. To resolve this issue, reconstitution of purified proteins into liposomes would seem the only suitable method. Only then will we be ready to start addressing the problem of how the activities of the various lipid transporters are regulated and coordinated to satisfy the physiological demands of a dynamic cell.

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