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Dynamic Transbilayer Lipid Asymmetry

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Cells have thousands of different lipids. In the plasma membrane, and in membranes of the late secretory and endocytotic pathways, these lipids are not evenly distributed over the two leaflets of the lipid bilayer. The basis for this transmembrane lipid asymmetry lies in the fact that glycerolipids are primarily synthesized on the cytosolic and sphingolipids on the non-cytosolic surface of cellular membranes, that cholesterol has a higher affinity for sphingolipids than for glycerolipids. In addition, P4-ATPases, "flippases," actively translocate the aminophospholipids phosphatidylserine and phosphatidylethanolamine to the cytosolic surface. ABC transporters translocate lipids in the opposite direction but they generally act as exporters rather than "floppases." The steady state asymmetry of the lipids can be disrupted within seconds by the activation of phospholipases and scramblases. The asymmetric lipid distribution has multiple implications for physiological events at the membrane surface. Moreover, the active translocation also contributes to the generation of curvature in the budding of transport vesicles.

A lipid bilayer consisting of phosphatidylcholine (PC) with one saturated and one unsaturated acyl chain is stable, flexible, and semipermeable. It is the simplest model of a biomembrane. In such membranes, PC with a spin label on its choline headgroup diffused rapidly in the plane of the membrane with a diffusion coefficient of 1.8 μm^2/sec (Devaux and McConnell 1972). In contrast, PC movement between leaflets, "flip-flop," was slow with a half-time of >6 h at 30°C (Kornberg and McConnell 1971). Similar half-times for PC flip-flop were measured in erythrocyte membranes, a mammalian plasma membrane with a complex lipid composition (Rousselet et al. 1976; Renooij and Van Golde 1977; van Meer et al. 1980). Interestingly, the erythrocyte membrane maintains an asymmetric lipid distribution across the lipid bilayer with all of its phosphatidylserine (PS) and most of its phosphatidylethanolamine (PE) in the cytosolic leaflet (Bretscher 1972; Verkleij et al. 1973). A critical discussion of these early data and the techniques used can be found in (Op den Kamp 1979).

It was then observed that the enrichment of aminophospholipids in the cytosolic leaflet is maintained by an ATP-consuming translocator that flips these lipids from the outer leaflet across the lipid bilayer (Seigneuret and Devaux 1984). The flippase was later identified as a P4-ATPase (Tang et al. 1996; Soupene and Kuypers 2006). Around the same time it was found that an ABC transporter, ABCB4, was

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involved in transporting PC into the bile (Smit et al. 1993), and studies on the closely related ABCB1 proved that these transporters can translocate lipids across the plasma membrane onto acceptors in the extracellular space (van Helvoort et al. 1996). Finally, evidence was provided for passive, bidirectional movement of lipids across the ER membrane and under some conditions across the plasma membrane, in which cases the responsible proteins have not yet been unequivocally identified (Sanyal and Menon 2009; Bevers and Williamson 2010). Thus, we now have a general picture of how lipid asymmetry is generated, maintained, and disrupted. However, there are still important gaps in our knowledge. For example, the transbilayer orientation of the sterols that make up one-third of the lipids in eukaryotic plasma membranes has still not been resolved satisfactorily. Moreover, we do not understand mechanistically how translocators and exporters work and how their activity is regulated.

TRANSBILAYER LIPID ASYMMETRY

Model Membrane Lipid Asymmetry

Gentle hydration of mixtures of membrane lipids generally results in multilamellar liposomes with a symmetrical distribution of the various lipids across the bilayer. However, when the curvature of the membranes is increased by sonication phospholipids with a small headgroup tend to be enriched in the more highly curved inner leaflet at the cost of the more cylindrical PC (Berden et al. 1975). Asymmetric model membranes can be prepared in several ways, the simplest being the adjoining two lipid monolayers of different chemical composition into an "asymmetric black lipid membrane" (Montal and Mueller 1972). Asymmetric vesicles have been formed by inserting a specific lipid to preformed liposomes, spontaneously (van Meer and Simons 1986) or via methyl-beta-cyclodextrin (Cheng et al. 2009), or by the exchange of short-chain lipids between liposome populations (Pagano et al. 1981). Alternatively, phospholipid asymmetry was induced by a transmembrane pH gradient (Hope et al. 1989). Asymmetric planar bilayers have also been prepared on solid supports (Kiessling et al. 2006).

Natural Membrane Lipid Asymmetry

Erythrocytes

An asymmetric distribution of phospholipids was first established for erythrocytes. Erythrocytes are a convenient experimental model for eukaryotic plasma membranes: because they lack internal membranes, their lipids exist in only two pools, that in the outer leaflet and that in the inner leaflet. Quantitative experiments are not complicated by a pool of lipids in intracellular membranes, which may contain some 85% of all cellular lipids (Griffiths et al. 1989). Initially, PE was found to be less accessible for an amino-reagent in intact erythrocytes than in opened cells (Bretscher 1972). It was then observed that most of the erythrocyte sphingomyelin (SM) and PC were accessible to exogenous phospholipases, whereas most of the PE and essentially all PS were protected (Verkleij et al. 1973). Whereas cholesterol has been shown by many biophysical approaches to have a preferential interaction with SM, indirect evidence assigned most of it to the cytosolic leaflet (see below) (Schroeder et al. 1991).

Viral Membranes

A number of membrane-enveloped viruses obtains its membranes by a budding event whereby the nucleocapsid has enveloped itself in a part of the plasma membrane. Although they contain virus specific membrane proteins, their lipid comoposition and organization may reflect that of the plasma membrane of origin. It turns out that the transbilayer distribution of the phospholipids is remarkably similar to that found in the erythrocyte membrane with most of the PS and PE inside (for a summary, see van Meer et al. 1981). These studies have taught some additional lessons. (a) Most SM was found to be accessible to exogenous phospholipase C (Tsai and Lenard 1975) or sphingomyelinase (Allan and Quinn 1989), and it was concluded that all SM resides in the

outer leaflet. However, only 40% of the SM was calculated to be on the outside of the same Semliki Forest virus when the rate of hydrolysis was kinetically analyzed (van Meer et al. 1981), and even less in influenza virus when analyzed by a phospholipid transfer protein or phospholipase C (Rothman et al. 1976). It has been reported for liposomes that phospholipase C (Sundler et al. 1978) and sphingomyelinase (Contreras et al. 2003) can induce transbilayer translocation of inner leaflet lipids, unless the substrate lipid is only a minor fraction of the lipids in the outer leaflet. This may have been the case in the Rothman study (see under b). Thus, the orientation of SM remains to be settled. (b) From the fact that Rothman and colleagues found only 30% of the phospholipids in the virus outer leaflet, they concluded that the outer surface of influenza virus that budded from kidney cells must have been enriched in glycolipids (Rothman et al. 1976). Indeed, it was found later that influenza virus buds from the apical surface of polarized epithelial cells, and that the apical surface of such cells is enriched in glycosphingolipids (reviewed in Simons and van Meer 1988). Experimentally, the sialic acid-containing glycolipids (gangliosides) were found exclusively on the outer viral surface (Stoffel et al. 1975; Stoffel and Sorgo 1976) in line with their presumed presence in the outer leaflet of the cellular plasma membrane.

Nucleated Cells

The studies on viral membranes suggested that the plasma membrane of nucleated cells displays an asymmetric distribution of lipids similar to that of erythrocytes, albeit less outspoken. This was confirmed by studies on isolated chromaffin granules (Buckland et al. 1978) and phagosomes (Sandra and Pagano 1978). In these membranes, which have their cytosolic surface exposed to the medium, 70% of the PE and only a minor proportion of the SM (<20%) was found on the cytosolic surface. Also, introducing the SM-specific equinatoxin II into the cytosol caused labeling of the Golgi, showing the presence of SM in the cytosolic leaflet, but not of the plasma membrane (Bakrac et al. 2010). The application of phospholipases and amino-reagents on intact erythroleukemic cells followed by plasma membrane isolation led to the conclusion that 80%–85% of SM and 10%–20% of PS was present in the outer leaflet of these plasma membranes, with a roughly equal distribution of PC, PE and phosphatidylinositol (PI) (Rawyler et al. 1985). Although PI is phosphorylated by cytosolic kinases, significant fractions of various phosphoinositides have been found on the outer surface of plasma membranes (Gascard et al. 1991; Kale et al. 2010).

Independent but indirect evidence was provided by studies on the lipid organization in the apical and basolateral plasma membrane domains of epithelial cells. It was found that the tight junction that separates the two domains, acts as a barrier to lipid diffusion in the outer but not the cytosolic leaflet of the plasma membrane bilayer (Dragsten et al. 1981; van Meer and Simons 1986). As a consequence, if the free diffusion of lipid molecules in the cytosolic leaflet of the plasma membrane leads to an identical lipid composition of the cytosolic leaflets of both domains, the compositional differences between the two domains must have been because of different compositions of the outer leaflets of those domains. If the exoplasmic leaflet of the apical domain were predominantly occupied by glycosphingolipids, as is probably the case in intestinal cells (reviewed in Simons and van Meer 1988), the phospholipids of the apical domain would be mainly situated in its cytosolic leaflet. The phospholipid composition of the cytosolic leaflet of the basolateral membrane would be identical with that of the apical domain, and the distribution of the individual phospholipid classes across the basolateral membrane bilayer could be predicted from the total phospholipid composition of the basolateral membrane. For three independent studies on the apical and basolateral lipid composition (Kawai et al. 1974; van Meer and Simons 1982, 1986) the calculation for the two major phospholipid classes leads to the following numbers: 65%–90% of the PE and only 10%–25% of the PC would be localized in the cytoplasmic leaflet.

Just like the findings in erythrocytes, also in nucleated cells all (indirect) evidence points to an enrichment of cholesterol in the cytosolic leaflet of the plasma membrane (Mondal et al. 2009). This is counterintuitive because the sphingolipids in the noncytosolic leaflet should enrich the sterols there. In addition, at the typical plasma membrane content of 40 mol% cholesterol (and with a cholesterol surface area half that of PC), restriction of the cholesterol to one leaflet would yield a ratio cholesterol/phospholipid in that leaflet of 2, which is the limiting solubility of cholesterol in PC but (far) above that of PE containing membranes (Huang et al. 1999). Interestingly, sphingolipid-sterol domains in one leaflet of the bilayer can apparently be recognized by glycerophospholipid-sterol domains on the opposite side (Collins 2008; Collins and Keller 2008; Wan et al. 2008; Kiessling et al. 2009). The biophysical details of the relevant interactions remain to be resolved.

TRANSBILAYER LIPID TRANSLOCATION

Model Membrane Lipid Translocation

Lipid asymmetry in model membranes is stabilized by the low tendency of the regular membrane phospholipids to flip-flop across the bilayer. However, the rate of spontaneous transmembrane translocation is very different for various lipids. Generally, lipids with a large or charged polar group, like the phospholipids and glycolipids do not move across a PC bilayer for hours, whereas lipids with a small uncharged headgroup like cholesterol, diacylglycerol (DG) or ceramide flip-flop on a (sub)-second timescale (Lange et al. 1981; Bai and Pagano 1997; López-Montero et al. 2005; Krasilnikov and Yuldasheva 2009). Some charged lipids can translocate when their charge is neutralized, for example by pH. Free fatty acids readily move across membranes at neutral pH (Hamilton 2003; Simard et al. 2008).

The low rate of translocation of PC across a PC membrane (Kornberg and McConnell 1971) is enhanced when defects are introduced in the membrane. Such a defect can be the boundary between liquid and solid phases at the phase transition temperature (John et al. 2002), or the presence of nonbilayer phases induced by, for example, the addition of Ca^{2+} to a cardiolipin-containing bilayer (Gerritsen et al. 1980) or by the generation of ceramide in membranes (Contreras et al. 2003, 2005). In addition, it has been observed that transmembrane peptides can stimulate lipid flip-flop and that this process strongly depended on the lipid composition of the liposomal bilayer (Kol et al. 2003).

Natural Membrane Lipid Translocation

Erythrocytes

The spontaneous rate of PC translocation across the erythrocyte membrane was found to be slow with a half-time of hours (Renooij et al. 1976), which was confirmed by phospholipid exchange studies (Crain and Zilversmit 1980; van Meer et al. 1980; van Meer and Op den Kamp 1982). Subsequently, Seigneuret and Devaux were able to show ATP-dependent translocation of spin-labeled PE and PS to the inner leaflet of the erythrocyte membrane (Seigneuret Devaux 1984). The responsible protein, the "flippase," was then identified as a member of the P4 subfamily of P-type transporting ATPases: ATPase II now known as ATP8A1 (Tang et al. 1996; Soupene and Kuypers 2006; Paulusma and Oude Elferink 2010). In the meantime, evidence was found to suggest that PC synthesized on the inner surface of the erythrocyte membrane by acylation of lysoPC was actively translocated outward (Andrick et al. 1991). This "floppase" activity may be because of an ABC-transporter (Kälin et al. 2004).

Finally, it had been observed early on that blood platelets display the same lipid asymmetry across their plasma membrane as that of erythrocytes (Schick et al. 1976; Chap et al. 1977). Disruption of this asymmetry during platelet activation (Bevers et al. 1983) exposes PS, which turns out to be crucial for blood coagulation (Zwaal et al. 1977). The sudden loss of lipid asymmetry is mediated by a "scramblase." Various scramblase candidates have been proposed but none has been validated (Bevers and Williamson 2010).

Viral Membranes

The transbilayer translocation of PC in viral membranes was found to be very slow: 7 h – several days (Rothman et al. 1976; Shaw et al. 1979; van Meer et al. 1981). There is presently no evidence that viruses actively translocate lipids.

Nucleated Cells

Aminophospholipid Flippases. The erythrocyte aminophospholipid translocator ATP8A1 (Soupene and Kuypers 2006) was originally purified from chromaffin granules (Tang et al. 1996), and 14 family members have been identified in mammals at present (Paulusma and Oude Elferink 2010). Yeast expresses five P4 ATPases (Catty et al. 1997) and they are located in the sterol- and sphingolipid rich membranes of the late secretory and endocytotic pathways (Pomorski et al. 2003). The P4-ATPases require additional subunits for their proper intracellular localization and presumably for their activity, notably the CDC50 proteins (Kato et al. 2002; Saito et al. 2004; Lenoir et al. 2009).

The P4 ATPases clear PS from the surface of blood cells: PS stimulates blood coagulation by the activation of factor X and the subsequent proteolytic production of thrombin (Bevers and Williamson 2010). An unexpected function is their involvement in vesicle budding. By moving lipid mass from the noncytosolic into the cytosolic leaflet they increase the lateral pressure in the cytosolic as compared to the noncytosolic leaflet, which results in curving and vesicle budding. Evidence has been provided for a role in endocytosis (Farge et al. 1999; Pomorski et al. 2003), and in vesicle transport from the Golgi (Chen et al. 1999; Hua et al. 2002; Hua and Graham 2003). As is to be expected of a physiologically relevant system, the aminophospholipid translocases are regulated by a network of kinases (Nakano et al. 2008; Roelants et al. 2010), and an asymmetry sensing system in yeast has been reported (Ikeda et al. 2008).

ABC Transporters. Searching for the function of ABCB4, a close relative of the multidrug transporter ABCB1 (P-glycoprotein, MDR1), Smit and colleagues generated Abcb4$^{-/-}$ mice and observed that these mice were unable to secrete PC into the bile (Smit et al. 1993). Subsequent studies (van Helvoort et al. 1996) showed that both ABCB1 and ABCB4 were capable of utilizing ATP to translocate a number of short-chain analogs of membrane lipids like PC, PE, SM, and the glycosphingolipid glucosylceramide (GlcCer) across the plasma membrane. It later turned out that ABCB1 only translocates short-chain lipids, like platelet activating factor (PAF) (Ernest and Bello-Reuss 1999; Raggers et al. 2001), whereas ABCB4 is a real PC exporter (Morita et al. 2007). Also, a number of other members of the 50 human ABC transporters have now been characterized as being lipid exporters (van Meer et al. 2006; Nagao et al. 2010). The most likely general working mechanism of the mammalian ABC transporters seems to be that they enclose hydrophobic molecules into a binding site that is open to the cytosolic leaflet. A conformational change opens this binding site to the extracellular space (or the lumen of an intracellular organelle). Molecules with a rather high water solubility will diffuse out of the binding pocket into the extracellular medium. This would be the case for many drugs (ABCB1; ABCC1) or lipids with high water solubility like PAF (ABCB1; ABCB4) and sphingosine-1-phosphate (ABCC1; ABCG2) (Takabe et al. 2010). More hydrophobic molecules will not leave the binding pocket even after it opens up to the extracellular side of the membrane unless an extracellular (or lumenal) acceptor is present. This acceptor can be a lipoprotein (cholesterol, ABCA1) (Boadu et al. 2008); phospholipids (Linsel-Nitschke et al. 2005), a bile salt micelle (PC, ABCB4) (Morita et al. 2007); plant sterol (ABCG5/G8) (Levy et al. 2007), a lumenal membrane structure (PC, ABCA3; glucosylceramide, ABCA12) Mitsutake et al. 2010), or a soluble enzyme complex (very long-chain fatty acyl-SCoA, ABCD1-4) (Wanders et al. 2007). It may be the case that from the binding pocket on the outside of the plasma membrane the lipid is able to move into the noncytoplasmic leaflet when an acceptor is not present. In that case, the ABC transporter would function as a floppase, as was found in

a study on isolated erythrocytes in the absence of any natural acceptor (Andrick et al. 1991; Kälin et al. 2004). It is very unlikely that the ABC transporters generally act as floppases translocating their substrates into the outer layer of the plasma membrane, after which this substrate would move out of the membrane onto the acceptor. In the latter model it is difficult to see what would be the driving force to release the substrate from the membrane, which is so nicely explained in the "exporter" or "projection" model (van Meer et al. 2006; Nagao et al. 2010).

Scramblases. In mammalian cells, one main function of the P4 ATPases is to prevent PS from appearing on the outer surface of the cell (Leventis and Grinstein 2010). Indeed, after platelet activation PS appears on the surface within seconds. In addition, PS appears on the surface of apoptotic cells where it is recognized by a PS receptor on macrophages. This is followed by ingestion of the cell corpse by the macrophage (Fadok et al. 1992). The nonspecific and bidirectional scrambling of the membrane lipids is induced by an elusive scramblase, which can be activated in various ways (Bevers and Williamson 2010). Two independent mechanisms may be involved (Schoenwaelder et al. 2009). Unexpectedly, a P4-ATPase in *Caenorhabditis elegans*, which should translocate PS to the cytosolic surface, appears to be required for the apoptotic appearance of PS on the cell surface (Züllig et al. 2007). Evidence has been provided that the activation of SM hydrolysis during cell signaling is because of lipid scrambling which brings outer leaflet SM to the neutral sphingomyelinase on the cytosolic surface (Tepper et al. 2000). However, the story of SM hydrolysis during signal transduction is more complex because hydrolysis can be limited to a special SM pool on a cytosolic surface (Andrieu et al. 1996), and on the other side some stimuli activate the acidic sphingomyelinase on the outside of the cell (Lin et al. 2000). Some 20%–30% of the SM and PC were found to be converted to ceramide and DG (Kolesnick 1989; Okazaki et al. 1989). This high concentration of ceramide might induce scrambling by its nonbilayer propensity (Contreras et al. 2003).

Other Translocators. In Farber's disease, mutations in acid ceramidase result in lysosomal storage of ceramides. Similarly, cholesterol accumulates in lysosomes in Niemann-Pick type C disease. It is unlikely that storage is because of the absence of a translocator in the lysosomal membrane because ceramide and cholesterol readily flip across membranes spontaneously. Indeed, it is now concluded that these lipids are present in vesicular structures in the lysosomal lumen and are unable to enter the lumenal leaflet of the lysosomal limiting membrane. Ceramide needs to be degraded to sphingosine and fatty acid. These can reach the limiting membrane spontaneously, followed by transmembrane translocation and release into the cytosol. In contrast, to enter the lysosomal membrane cholesterol needs the soluble NPC2 protein and the membrane protein NPC1 (Kolter and Sandhoff 2010). It has been argued that NPC1 is also involved in translocating sphingosine out of the lysosome (Lloyd-Evans et al. 2008): At the low lysosomal pH, sphingosine is positively charged and does not move spontaneously across the lysosomal membrane. NPC1L1 is closely related to NPC1 and is involved in moving cholesterol across the apical membrane of intestinal cells. Probably, both proteins are needed to transport cholesterol across the glycocalyx that covers both the apical epithelial surface and the lumenal side of the lysosomal membrane.

Unlike plasma membranes, the ER membrane displays high rates of transbilayer translocation for all lipids tested (Herrmann et al. 1990; Buton et al. 1996, 2002). Evidence has been provided that distinct proteins allow passive translocation of glycerophospholipids and oligosaccharide diphosphate dolichols across the ER (Sanyal et al. 2008) and photoreceptor membranes (Menon et al. 2011). The conclusion that the yeast protein RFT1 is required for the translocation of these glycophosphodolichols (Helenius et al. 2002) was not supported by other data (Rush et al. 2009), suggesting there may be more than one mechanism. One other class of lipids that translocates across the ER membrane is the class of simple glycosphingolipids (Buton et al. 2002). The simple glycosphingolipid

GlcCer is synthesized on the cytosolic side of the Golgi but is converted to complex glycosphingolipids in the Golgi lumen. Evidence has been presented to suggest that GlcCer is transported back to the ER to cross the membrane. Interestingly, galactosylceramide is synthesized in the ER lumen, and appeared to mix with the GlcCer pool in the cell according to their similar kinetics in various intracellular transport steps (Halter et al. 2007).

Bacterial and Mitochondrial Membranes

The cytoplasmic membrane of bacteria is a biogenic membrane like the ER. Lipids rapidly flip across it (Rothman and Kennedy 1977). This property is maintained in proteoliposomes prepared from these membranes, and not in protein-free membranes (Kubelt et al. 2002; Watkins and Menon 2002), but the responsible protein has so far escaped identification. Interestingly, two distinct but interchangeable mechanisms were identified that are required for flipping lipid-linked oligosaccharides to the outside of the cytoplasmic membrane (Alaimo et al. 2006). Surprisingly, one was a passive system and the other an ABC transporter. A different ABC transporter, MsbA, translocates nascent LPS and phospholipids to the exoplasmic surface (Doerrler et al. 2004) as was recently shown in a reconstituted system (Eckford and Sharom 2010). An independent bacterial protein translocates phospholipids and lysophospholipids (Harvat et al. 2005; Tefsen et al. 2005). As could be expected a rapid (minutes) bidirectional and energy-independent phospholipid translocation was observed across mitochondrial inner membrane (Gallet et al. 1999).

PERSPECTIVES

Even after nearly 40 years of intense research on the transbilayer organization of lipids, there are still dramatic gaps in our knowledge. It is for example unclear how cholesterol is distributed, and as argued above the methodology by which SM has been assigned to the noncytosolic leaflet is potentially flawed. Also the molecular mechanism by which the P4-ATPases and the ABC transporters move lipids across membranes is unknown, although the tremendous progress in membrane protein structure determination provides hope that such data will also explain the specificity of these systems. Lipid asymmetry and transmembrane translocation are not an isolated phenomenon, but a central aspect of the lipid economy of the cell. The process feeds into vesicle transport, protein recruitment and function, signal transduction, and physiological issues like cell death and blood clotting. The field needs input from biochemists, biophysicists, structural and cell biologists, physiologists, and clinicians: It is a great challenge to study on the one hand the molecular details of a lipid translocator and on the other hand extend this to finding a cure for hearing disorders (Stapelbroek et al. 2009). It is time to apply chemical biology and systems approaches to the unsolved questions. The field deserves it. After all, isn't it dynamic asymmetry that characterizes life?

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