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Review

Proteins involved in lipid translocation in eukaryotic cells

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Abstract

Since the first discovery of ATP-dependent translocation of lipids in the human erythrocyte membrane in 1984, there has been much evidence of the existence of various ATPases translocating lipids in eukaryotic cell membranes. They include P-type ATPases involved in inwards lipid transport from the exoplasmic leaflet to the cytosolic leaflet and ABC proteins involved in outwards transport. There are also ATP-independent proteins that catalyze the passage of lipids in both directions. Five P-type ATPase involved in lipid transport have been genetically characterized in yeast cells, suggesting a pool of several proteins with partially redundant activities responsible for the regulation of lipid asymmetry. However, expression and purification of individual yeast proteins is still insufficient to allow reconstitution experiments in liposomes. In this review, we want to give an overview over current investigation efforts about the identification and purification of proteins that may be involved in lipid translocation.

Keywords: Lipid asymmetry; Aminophospholipid translocase; Flippase; P-glycoprotein; Drs2p; Lipid scramblase

Contents

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1. Introduction: the quest of lipid translocases

In spite of experimental uncertainties concerning in particular the distribution of lipids in organelle membranes and a lack of data on the lipid asymmetry in plant cells, it is widely accepted that eukaryotic cell membranes have an asymmetrical lipid distribution (Zachowski, 1993; Devaux, 1991; Pomorski et al., 2001). The plasma membrane (PM) outer leaflet contains essentially sphingomyelin (SM), phosphatidylcholine (PC) and glycosphingolipids. The inner leaflet has practically all phosphatidylserine (PS), the majority of phosphatidyletholamine (PE) and the phosphatides. This asymmetrical topology is possible because of the slow spontaneous transmembrane diffusion of phospholipids as demonstrated in 1971 by Kornberg and McConnell in artificial lipid vesicles (Kornberg and McConnell, 1971), and because several membrane proteins are believed to transport selective lipids from one side of the membrane to the other at the expense of ATP hydrolysis. Such proteins, named "phospholipid flippases", were postulated by Bretscher (1974). Their existence in human erythrocytes were shown in 1984 using spin-labeled lipids (Seigneuret and Devaux, 1984) and confirmed later with many different techniques in erythrocytes as well as in other mammalian cells (Daleke and Huestis, 1985; Martin and Pagano, 1987; Tilley et al., 1987; Connor and Schroit, 1987; Tannert et al., 2003; Zachowski et al., 1987a). In fact, in all eukaryotic cells where a flippase activity was assayed, the ATP-dependent translocation of aminophospholipids (PS and PE) from the outer to the inner monolayer was demonstrated, which shows the ubiquitous character of this lipid pumping system. An ATP-dependent flippase activity was found also in chromaffin granules (Zachowski et al., 1989), indicating the occurrence of an ATP-dependent transport of lipid in intracellular organelles as well. On the other hand, the rapid lipid flip–flop in the endoplasmic reticulum (ER) suggests the existence of an ATP-independent lipid flippase that allows newly synthesized lipids in the ER to be randomly distributed between the two leaflets of this membrane (Bishops and Bell, 1985; Herrmann et al., 1990; Buton et al., 1996, 2002; Menon et al., 2000; Marx et al., 2000).

Rapid transmembrane diffusion of lipids may not always require a specific protein. Lipids that do not possess an important polar head group such as cholesterol, ceramide, diacylglycerol or free fatty acids and fatty esters can flip in a lipid bilayer in a time scale of the order of a minute or less (Broring et al., 1989; Müller and Herrmann, 2002; Hamilton, 1998, 2003; López-Montero et al., 2005a). Other lipids with a polar head group that can be protonated such as phosphatidic acids and many drugs can traverse a membrane in a minute and accumulate on one side simply because of a pH difference between the two sides of a membrane (Eastman et al., 1991). Additionally, in eukaryotic cells, lipids are transported via vesicle traffic from one membrane to another. The spontaneous transverse diffusion may be slow in the PM, which contains a high proportion of

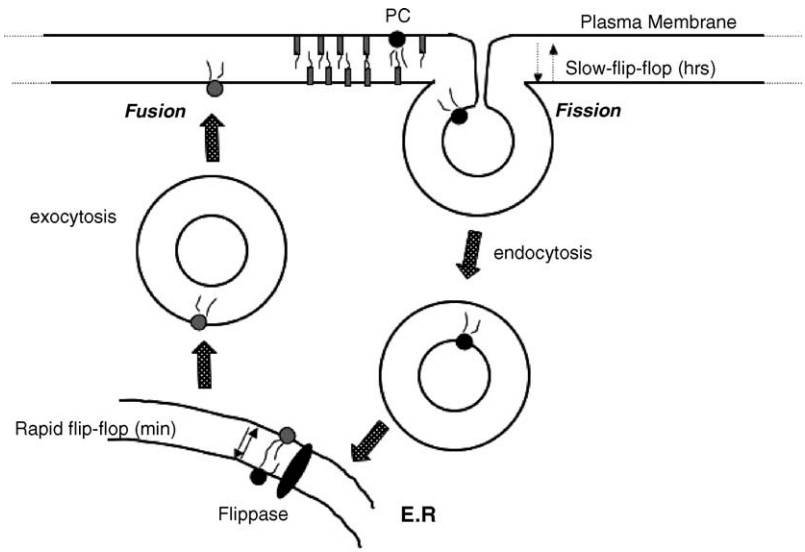

Fig. 1. Rapid flip–flop of phospholipids in the plasma membrane of eukaryotic cells may in fact be associated with rapid lipid traffic between the PM and organelles, where the flip–flop can be much more rapid than in the PM, which is enriched in cholesterol. This schematic drawing suggests that the apparent flip–flop in the PM can be the consequence of rapid lipid traffic.

cholesterol, and be much faster in the membrane of an intracellular organelle such as the ER. Thus, as suggested schematically in Fig. 1, the transmembrane diffusion of a phospholipid depends on the membranes visited within the cell by each specific lipid.

The purpose of this review is neither to summarize the latest data on transmembrane lipid asymmetry in biological membranes, nor to review the numerous biological functions that are linked to the topological organization of lipids in eukaryotic cells. Our intention is to give an objective view on the present research concerning the identification and purification of the protein, or rather the proteins that are involved in lipid translocation. In many ways, this subject has been rather disappointing during the last 20 years, and the conclusions may seem confusing for outsiders. Indeed, 20 years after the clear demonstration of a ubiquitous phenomenon in eukaryotic cells, there are still controversies concerning the identity and actual biological function of proteins able to translocate lipids in biological membranes. However, in spite of the very slow emergence of a clear situation, there are yet many reproducible experiments that indicate that progress is being made. Although some historical aspects will be provided here that tentatively explain some contradiction in the literature, emphasize will be put to the most recent publications devoted to the assignment of flippase activity to specific proteins. The reader is referred to former reviews for more details on particular aspects (Devaux, 1991; Schroit and Zwaal, 1991; Daleke and Lyles, 2000; Menon et al., 2000; Daleke, 2003; Sprong et al., 2001; Graham, 2004; Pomorski et al., 2004; Holthuis and Levine, 2005). Table 1 contains the most relevant references.

2. Inward ATP-dependent lipid translocation (flippases)

2.1. Human erythrocytes

As indicated out above, erythrocytes seem to be the most convenient cell system to use for a successful aminophospholipid translocase (APLT) purification. Due to the absence of intracellular organelles, the plasma membrane can be easily purified. Furthermore, human erythrocyte or erythrocytes from animal origin are not difficult to obtain in relatively large quantities. However, the drawback of erythrocytes is that direct genetic approach is almost impossible. In practice, erythrocytes have been very useful to investigate translocase lipid specificity as well as to investigate the inhibitory role of ions such as calcium or vanadate or the influence of protein reagents such as *N*-ethyl-maleimide. The observed response of aminophospholipid translocation to ATPase inhibitors strongly suggest that a P-type ATPase, part of which displays a Mg^{2+}-ATPase activity, is involved in aminophospholipid translocation (Beleznay et al., 1997).

Involvement of Rh blood group polypeptides in the maintenance of aminophospholipid asymmetry was proposed in the early 1990s (Schroit et al., 1990) but was contested later (Smith and Daleke, 1990; Geldwerth et al., 1997) and do not seem to be considered anymore as a likely candidate.

Attempts to purify the APLT by classical biochemical methods used for protein purification were carried out by several groups using, for example, photoaffinity labeling with modified PS derivatives (Schroit et al., 1987; Zachowski et al., 1987b). However, these probes labeled numerous proteins in the red cell membrane and did not give a clear answer. An erythrocyte ATPase with a molecular weight of 110 kDa was purified by the group of Roufogalis in Australia by gel chromatography and its reconstitution into proteoliposomes gave promising results (Auland et al., 1994). But later it was found impossible to obtain this ATPase without severe proteolytic degradation (Dolis et al., 1997). In fact, the major problem encountered by the groups who attempted to purify the translocase from human erythrocytes was that the red cells contain proteases, which are difficult to inhibit and which very rapidly degrade the purified or partially purified protein (Table 1).

2.2. From chromaffin granules to yeasts

In 1989, we showed that spin-labeled PS and PE are transported from the lumenal side of purified bovine chromaffin granules to the external side of theses organelles (which corresponds to the cyosolic leaflet) by an ATP-dependent mechanism (Zachowski et al., 1989). We simultaneously suggested that the granules ATPase II, now named ATP8A1, which had been purified from bovine chromaffin granules in Nelson's laboratory (Moriyama and Nelson, 1988) but had no known function, is the APLT. Following this track, the laboratories of Williamson and of Schlegel cloned ATPase II and discovered a strong sequence analogy with a yeast *Saccharomyces cerevisiae* ATPase, named Drs2p. Importantly, they demonstrated that a yeasts strain Δ*drs2*, which lacks this ATPase gene was unable to internalize fluorescent PS, while the wild type cell line did (Tang et al., 1996).

2.3. From yeast to plant cells

The experiments performed by Tang et al. (1996) with yeast cells were challenged afterwards by two lab-

Table 1
Eukaryotic proteins involved in phospholipids translocation between the two leaflets of a biological membrane or of liposomes containing a purified protein

Name	Specificity	ATP	References	Systems used
ATP-independent flippase	No	No	Bishops and Bell (1985), Herrmann and Devaux (1990), Gummadi and Menon (2002)	Rat liver ER/liposomes with ER proteins
Oligosaccharide flippase	$Man_5GlcNac_2$-PPdol	No	Helenius et al. (2002)	Yeast
Aminophospholipid translocase (APLT)	PS, PE	Yes	Seigneuret and Devaux (1984), Daleke and Huestis (1985), Tilley et al. (1986), Connor and Schroit (1987)	Human erythrocytes -idem -idem
			Martin and Pagano (1987)	Hamster fibroblasts
			Suné et al. (1987)	Platelets
			Zachowski et al. (1987b)	Lymphocytes
			Julien et al. (1993)	Endothelial cells
			Cribier et al. (1993)	Erythroblasts (K562)
			Kean et al. (1993)	Yeast
			Libera et al. (1999)	Human osteoblasts
			Pomorski et al. (1999)	Epithelial cells
			Fellmann et al. (2000)	Human fibroblasts
			Araujo-Santos et al. (2003)	Leishmania infantum
			Tannert et al. (2003)	Canalicum membrane
ALA1	PS (PE)		Gomes et al. (2000)	Arabidopsis/yeast
ATPase II (ATP8A1)	PS, PE	Yes	Zachowski et al. (1989)	Chromaffin granules
Drs2p	PS, PE, (PC)	Yes	Tang et al. (1996), Chen-Ying et al. (1999), Pomorski et al. (2003)	Yeast (*Saccharomyces cerevisiae*)
Dnf1p, Dnf2p, Dnf3p	PS, PE, (PC)	Yes	Hua et al. (2002), Pomorski et al. (2003)	-idem
Neo1p		Yes	Hua and Graham (2003)	-idem
ABCA1	PS, chol.	Yes	Hamon et al. (2000)	Mice erythrocytes
ABCB1 P-glycoprotein (MDR1)	Low specificity	Yes	van Helvoort et al. (1996)	Epithelial cells
	PS, PE, PC	Yes	Romsicki and Sharom (2001)	Proteoliposomes
	PS		Pohl et al. (2002)	Carcinoma cell line
	GluCer, GalCer	Yes	Eckford and Sharom (2005)	Proteoliposomes
Pdr5p, Yor1p	PE		Decottignies et al. (1998)	Yeast
ABCB4 (mdr2/MDR3)	PC	Yes	Ruetz and Gros (1994)	Yeast secretory vesicles
	PC		Smith et al. (1994)	Fibrobl. (transgenic mice)
	PC		van Helvoort et al. (1996)	Epithelial cells
ABCC1 (MRP1)	PS	Yes	Kamp and Haest (1998)	Human erythrocytes
	PS, PC		Dekkers et al. (1998)	Human erythrocytes
Scramblase	No specificity	No	Schroit and Zwaal (1991)	Human platelets
			Williamson et al. (1992)	Human erythrocytes
			Bassé et al. (1992), Zhou et al. (1997)	Human erythrocytes

Table of putative or purified proteins for which assays have indicated their role in lipid translocation between two leaflets of a lipid bilayer. When several laboratories have tested the same biological systems, only references to the first published experiments are indicated. This table does not include proteins of the human subfamily of P-type ATPases that correspond to yeast ATPases family because there is no direct experimental evidence so far on flippase activity of the former proteins. The lipid specificity is quoted without restriction for fluorescent or spin-labeled analogues.

oratories that had difficulties to reproduce their data (Siegmund et al., 1998; Marx et al., 1999) but the laboratory of Palmgren in Denmark working with plant cells discovered a novel P-type ATPase in Arabidopsis that belongs to the gene family ALA1 to ALA12. The amino acid sequence of ALA1 is homologous with those of yeast Drs2p and bovine ATPase II (the putative APLT). Yeast strain carrying a deletion of DRS2 are viable but cannot grow at 20 °C or below ALA1 reconstituted the deficiency in PS internalization into intact

cells that is exhibited by the Δ*drs2* yeast mutant, and expression of ALA1 resulted in increased translocation of aminophospholipids in reconstituted yeast membrane vesicles (Gomes et al., 2000). Therefore, the importance of the discovery by Tang and collaborators concerning the yeast ATPase was confirmed.

What seemed to be a serious objection to Drs2p putative role came from Graham's laboratory where the localization of Drs2p to the yeast Golgi network rather than in the PM, was demonstrated in 1999 (Chen-Ying et al., 1999) and confirmed later by Pomorski et al. (2003). The localization of Drs2p into the yeast Golgi membranes had not been suspected by previous investigators, and could appear as a major objection to the hypothesis put forward by Zachowski et al. (1989) or by Tang et al. (1996) who identified, respectively, ATPase II of bovine chromaffin granules and Drs2p in yeast as the eukaryotic APLT. Indeed, an implicit assumption in the article by Zachowski et al. (1989) was that the PM had a protein analogous (or identical) to the ATPase II of chromaffin granules.

Eventually, four other ATPases with a high percentage of sequence identity with DRS2, forming a subfamily of Drs2p-related P-type ATPases, were discovered in yeast and named Dnf1p, Dnf2p and Dnf3p (Hua et al., 2002; Pomorski et al., 2003) and Neo1p. The yeast PM contains the majority of Dnf1p and Dnf2p while Dnf3p and Drs2p are situated in the late Golgi membranes (Hua et al., 2002; Pomorski et al., 2003; Natarajan et al., 2004). The DNF genes can be deleted without consequence to growth, however the Δ*drs2*Δ*dnf1*Δ*dnf2* Δ*dnf3* combination is lethal (Hua et al., 2002). The influx of aminolipids was tested with NBD-labeled lipids and also by the binding of trinitrobenzene sulfonic acid. Using various knockout mutants. Pomorski et al. (2003) showed that simultaneous depletion of dnf1p and dnf2p abolished the ATP-dependent influx of NBD-labeled PE and PS. Surprisingly, an ATP-dependent influx of NBD-PC was also observed and was abolished in Δ*dnf1*Δ*dnf2*. Loss of Drs2p in Δ*dnf1*Δ*dnf2* cells enhanced cell surface exposure of aminophospholipids. Thus, in spite of its localization in the Golgi, Drs2p influences the aminophospholipid transmembrane distribution in the plasma membrane. This may occur if Drs2p participates in the formation of vesicles from the Golgi by PS translocation. These vesicles may then circulate between the Golgi and the plasma membrane and fuse with the PM, thus providing a supply of new PS with the correct transmembrane orientation.

It can be explained by the participation of Drs2p in the formation of vesicles from the Golgi by PS translocation, thereby indirectly influencing the lipid distribution in other membranes. The role of P-type ATPases in the formation of vesicles is an hypothesis coherent with shape change observations in pure lipid vesicles where it has been shown that a very small excess of phospholipids in one leaflet triggers vesicle budding (Farge and Devaux, 1992; López-Montero et al., 2005a). Actually the stimulation of APLT activity is now believed to be a mechanism used in vivo during the first step of endocytosis (Farge et al., 1999; Devaux, 2000). Consistent with this model, in the triple mutant depleted of dnf1p, dnf2p, and Drs2p, endocytosis was totally blocked (Pomorski et al., 2003). This confirms that P-type ATPases involved in lipid translocation could be the molecular motor of the first stage of endocytosis.

Other membrane proteins are reported to be involved in the formation of asymmetrical membranes in yeasts. In particular, Neo1p seems to be necessary. It is an ATPase which is localized to the endosomes (Wicky et al., 2004) and may maintain lipid asymmetry in these organelles. However, Hua and Graham (2003) believe that it localizes to the ER. Two additional proteins are required for phospholipids translocation across the plasma membrane: Ros3p (also named Lem3p) and Cdc50p. They are not ATPases but could be chaperons and could be necessary for the proper localization of the P-type ATPases family involved in lipid translocation (Graham, 2004).

To date there are 5 P-type ATPases in yeast that are potential APLTs. Fig. 2 attempts to summarize in a schematic way the localization of the various P-type ATPases involved in lipid movement in yeast. In humans, the number of ATPase of the family comprised 14 members as based on phylogenic analyses (Halleck et al., 1999). Their precise localization is not known and can only be extrapolated from data in yeast. However, it should be kept in mind that one of the main features of a living eukaryotic cell is its dynamic character associated with an intense vesicle traffic that results in the transport of lipid and proteins from one membrane to the another, obviously not randomly, but enabling nevertheless each protein synthesized to visit several membranes. Apparent contradictions in the literature may reflect experimental uncertainties, but they may be also associated with the fact that proteins can be found in more than one organelle membrane.

Experimental data, up to now, do not show a clear specificity of the lipid translocation associated with specific members of the ATPase subfamily. In fact experiments with KO yeast cells depleted of one, two or three P-type ATPases, which show that cells can survive and grow even when any one of the DNF proteins are lack-

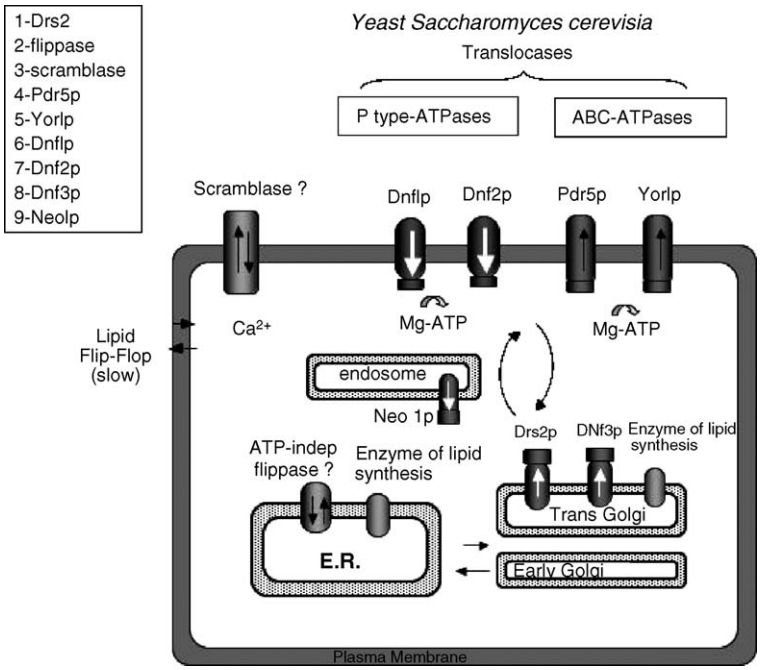

Fig. 2. Tentative localization of the main lipid flippases that are believed to be involved in lipid transmembrane distribution in yeast as indicated from the literature. More proteins should be involved in the case of mammals (see text for relevant references).

ing, suggest a certain redundancy. Only the quadruple mutant deprived of the three DNF proteins and of Drs2p is not viable. Similarly, as pointed out above, only in the triple mutant depleted of dnf1p, dnf2p and drs2p was endocytosis totally blocked (Pomorski et al., 2003). The only essential protein is Neo1p (Hua and Graham, 2003).

3. Outward ATP-dependent lipid translocation (floppase)

Another important question related to the establishment of lipid asymmetry in eukaryotic cells is the following: what creates the accumulation of PC and SM in the outer monolayer of the PM? In normal eukaryotic cells (i.e. cells, which unlike erythrocytes, have an internal vesicular traffic), the synthesis of PC in the ER excludes the possibility to attribute the polarization of lipid organization in the PM to the enzymes involved in lipid synthesis. Indeed the ER contains an ATP-independent flippase that randomizes the lipid distribution in this membrane. In the case of SM, the question is more debatable since SM is synthesized in the luminal face of trans-Golgi membrane (Pomorski et al., 2001) and the spontaneous transmembrane diffusion of SM is slow in the Golgi, which is a cholesterol containing membrane. Thus, the external exposure of SM in the PM could be a direct consequence of its synthesis and its orientation in the trans Golgi membrane coupled with a very slow flip–flop.

Already in 1988, we reported that an ATP-dependent system seemed to be responsible for the outwards transport of phospholipids in erythrocyte membranes (Bitbol and Devaux, 1988). Connor et al. (1992) suggested that the 32 kDa Rh protein could be involved in the outward transport of lipids. However the function of lipid translocases able to catalyze the outwards movement of PC and SM in eukaryotes has been generally assigned to ATPases from the ABC superfamily of proteins such as ABCB1 commonly named P-glycoprotein. The suggestion that the P-glycoprotein responsible multidrug resistance in cancer therapy would transport various phospholipids from the cytosolic leaflet to the exoplasmic leaflet and not only amphiphilic drugs was proposed by Higgins and Gottesman (1992). Since, several papers reported experimental evidence of NBD-labeled phospholipid outward translocation in cells expressing the P-glycoprotein or in proteoliposomes containing a purified ABC protein. See the review by Pohl et al. (2005). The articles by Ruetz and Gros (1994) and by Smith et al. (1994) were the first to show a PC floppase activity carried out by an ABC protein. van Helvoort et al. (1996), using various short chain NBD-lipids, found a floppase activity in epithelial cells expressing MDR1or MDR3 (now named, respectively, ABCB1 and ABCB4) with a difference in

lipid specificity. The outward transport of PC in the bile was associated selectively with MDR3 in humans (and mdr2 in mice), while MDR1 had a low lipid specificity. Later, Sharom and collaborators reported successful reconstitution experiments with purified P-glycoprotein into PC large unilamellar vesicles, which showed also the capacity of ABC proteins to transport many different fluorescent analogues: PC, PS, SM, GluCer (Romsicki and Sharom, 2001; Eckford and Sharom, 2005). Other groups suggested that MRP1 (or ABCC1) was directly responsible for the accumulation of endogenous SM and PC in the outer leaflet in erythrocytes (Dekkers et al., 1998, 2000; Kamp and Haest, 1998).

If the results obtained with ABC proteins are applicable to natural long chain lipids, it raises the problem of a futile systems in biological organization since the PM of eukaryotes, in particular erythrocytes, contains an active APLT which pumps very efficiently PS and PE from the outer to the inner leaflet. Possibly apart from driving SM and PC towards the outer leaflet, ABC proteins can regulate the activity of the P-type ATPases involved in aminolipids inward movements. Kamp and Haest (1998) reported a 20% decrease of NBD-PS exposure in the inner leaflet of erythrocyte membranes containing the multidrug resistance protein MRP1. Pomorski et al. (2003) have reported a decrease cell surface exposure of endogenous PE caused by a loss of ABC transporter in yeasts. Recently, we have found in K562 cells expressing P-glycoprotein (ABCB1), a reduced rate of internalization of spin-labeled PS of about 10% (Encinar et al., manuscript in preparation). The above-mentioned data obtained with NBD or spin-labeled analogues possessing a short β chain suggest a competition between the two lipid pumps. Although it is hard to compare at this stage the number of copies of the two type of lipid pumps present in these cells, in particular because of the unknown APLT identity, in all examples cited the data show that the P-type inward pump (APLT) is more efficient than the ABC outward pump.

A paradox is that the presence of the P-glycoprotein in cancer cells should favour the elimination of such cells. Indeed, if they contain more exposed PS, they should be considered as apoptotic by macrophages since PS exposure on the outer surface is a characteristic of apoptotic cells. Another paradox is that there are cells that do not express ABC proteins in their PM and yet there are no reports of cells in which exist an accumulation of PC and SM in the inner leaflet of the PM. In conclusion, the role of ABC proteins as the unique translocators of choline containing phospholipids is not firmly established.

Is there another credible explanation of SM and PC accumulation in the outer monolayer that does not need to postulate the existence of new selective "floppases"? Brumen and collaborators proposed an interesting model that could explain lipid asymmetry in red blood cells on the ground of a single type of lipid pump, namely an APLT (Brumen et al., 1999). Briefly, the model postulates that if a fraction of lipids are transported towards the inner monolayer, the remaining population of lipids, which are not substrates of the transporter (SM and PC), should experience an accelerated transmembrane diffusion towards the outer monolayer. This is what one should deduce from the second Fick'law of diffusion, if it can be applied. The problem in the case of lipid is that the spontaneous transmembrane diffusion is extremely slow. A bud in a liposome obtained by an excess of phospholipids on one side (Farge and Devaux, 1992) or the insertion of chlorpromazine or lyso-PC in erythrocytes generates stable membrane bending (Sheetz and Singer, 1974) and suggests that there is no significant acceleration of outwards diffusion. In fact, intuitively, the increased surface pressure may even reduce the spontaneous diffusion. Similarly the crowding of molecules in a three-dimensional environment reduces the local viscosity. However, Raphael and collaborators pointed out that in a fluid system like a biological membrane, significant fluctuations both in local curvature and in thickness, i.e. local defects, are the points of lipid flip–flop (Raphael and Waugh, 1996; Raphael et al., 2001). These defects create pores probably with a short lifetime but their number could be enhanced by a mismatch between the tension in two monolayers or by a defect such as a mere transmembrane helice (Gerritsen et al., 1980; Kol et al., 2004). Thus, instead of the surface tension growing until the membrane breaks, small pores can form temporarily that allow the membrane to relax the mismatch by a local flow of lipids from the more constrained surface to the less constrained surface of the membrane. Raphael and collaborators showed that an asymmetrical bilayer not only can form defects but also should lead to more rapid exchange between the two leaflets than if pores are formed randomly in a symmetrical membrane (see Fig. 3).

Additionally, erythrocytes are not typical eukaryotic cells in particular because they do not experience membrane traffic by endocytosis–exocytosis. Obviously, giant vesicles do not either resemble a real cell membrane precisely because they do not have a continuous renewal of their own membrane by fusion–fission processes. We therefore postulate that the perturbation caused in normal eukaryotic cells by the vesicular traffic suffices to stimulate the formation of flip–flop sites. We have observed with erythrocytes that if pores are formed artificially by

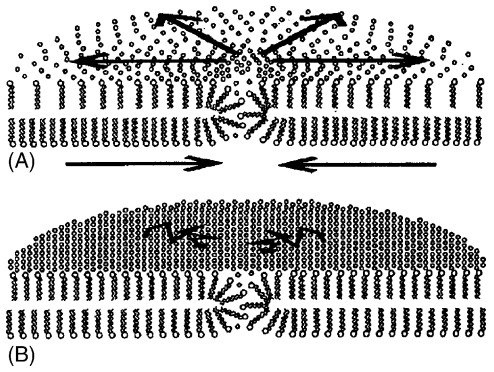

Fig. 3. Models assuming that the translocation of lipids depends on the formation of defects forming transient pores and on the rate at which a lipid diffuses to the defect site. The net flux is faster in case A, which corresponds to an asymmetrical membrane (Raphael et al., 2001).

an osmotic shock, phospholipids scrambling takes place (Schreir et al., 1992).

4. Calcium-dependent phospholipid scramblase

The so called "lipid scramblase" was postulated to be a transmembrane protein responsible for the calcium triggered lipid randomization in the PM of cells in the blood circulation. Elevation of cytosolic calcium concentration not only inhibits APLT but also, and by a different process, rapidly triggers the exposure of PS on the outer surface of platelets and erythrocytes. The inhibition of the inward pump is not sufficient to explain the outward lipid flux since the spontaneous diffusion would take several hours even at physiological temperatures to randomize the two leaflets of the bilayer. PS exposure on the outer leaflet is a crucial stage for the sequence of events leading to platelet aggregation and the formation of a clot. PS exposure, which can be revealed by the binding of fluorescent annexin V (Tait and Gibson, 1994), is an indication of cell aging and apoptosis. Cells exposing PS are recognized by macrophages and are eliminated from the blood circulation. Attempts to purify the scramblase were carried out by several laboratories. The group of Sims in the USA claimed that they purified from erythrocytes a 37 kDa protein responsible for lipid scrambling (Bassé et al., 1996; Zhou et al., 1997). However, cells of patients with a Scott syndrome characterized by the absence of effect of calcium entry (Stout et al., 1997) possess this 37 kDa protein (Fadeel et al., 1999).

We made recently an alternative suggestion to explain the calcium scrambling of lipids in erythrocytes, which can be triggered artificially with calcium ionophores (López-Montero et al., 2005b). Endogenous sphingomyelinase (SMase) has been reported to be present in the cytosol of erythrocytes and to play a role in scramblase activation (Lang et al., 2005). Our suggestion is that an endogenous (SMase) triggered by calcium could generate the formation of ceramides in the inner monolayer of erythrocytes in a sufficient amount for the mismatch between the two leaflets of erythrocytes or platelets to create an invagination. Eventually, an instable situation can be relaxed by the temporary formation of pores. During the lifetime of pores, lipids have free access to both sides of the cell membrane and should randomize the composition of the two leaflets (Schrier et al., 1991). The idea that the scramblase could be in fact a SMase was inspired by experiments carried out in Goñi's laboratory where they added SMase to vesicles containing a high proportion of SM (Contreras et al., 2003). They observed that when the percentage of SM was of the order of 5%, the lipids of the two leaflets scrambled. However, we do not think that it is an intrinsic property of ceramides as suggested by Goñi and collaborators but rather an artifact generated by the mismatch induced between the two leaflets. In model systems we have observed pores triggered by SMase in vesicles containing 5% of SM, but no acceleration of scrambling in vesicles containing initially 5% of ceramide (López-Montero et al., 2005a).

5. ATP-independent flippases

Functionally, this protein, or this family of proteins, has been recognized a long time ago. Purified ER membranes allowed Bishops and Bell (1985) to suggest the existence of ATP-independent enzymes that would accelerate the transmembrane diffusion of phospholipids in the ER where lipid synthesis takes mainly place. A search for such a protein is very difficult because any transmembrane segment of a protein or a simple hydrophobic helix may in fact perturb the lipid bilayer and induce a slight increase in the rate of lipid flip–flop. It was even suggested from experimental data that the transmembrane segment of glycophorin, or band 3 or any hydrophobic peptide might behave like a non-specific flippase capable of accelerating the spontaneous transmembrane diffusion of a phospholipid (Gerritsen et al., 1980; Vondenhof et al., 1994; Kol et al., 2004). The role of proteins was also clearly established by comparing lipid diffusion rates in proteoliposomes, with the diffusion in liposomes made with lipid extract either from the ER of mammals or from *E. coli* membranes (Kubelt et al., 2002).

One of the difficulties is the lack of an easy test for assaying lipid flip–flop caused by specific proteins extracted from biological membrane. Because there is no need of ATP and very little specificity, control exper-

iments are difficult to perform in the case of ATP-independent flippases.

6. Conclusions

In conclusion, we end this review by a few remarks:

(i) First, and this may seems a trivial comment but has been often neglected, it is dangerous to infer general conclusions about eukaryotic cells by the mere observation of human erythrocytes. Erythrocytes are very specialized eukaryotic cells depleted of all intracellular organelles and without vesicular traffic. It is possible that mature red cells have lost some of the proteins that are found in intracellular organelles of nucleated cells, such as yeasts. For example, the absence of a PC translocator from the outer to the inner monolayer of erythrocyte PM may be a late evolution of these cells. After all, erythrocytes only have to preserve their lipid asymmetry in particular to rigorously exclude PS from the cell surface during their lifetime and not to establish this lipid asymmetry. The absence of endocytosis–exocytosis and of vesicular traffic preserves these cells from accidental lipid scrambling associated probably with fusion and fission processes.

Moreover, although human disease can be associated with a mutation of erythrocyte proteins involving lipid–protein interactions as in the case of the Scott syndrome (Stout et al., 1997) and knockouts can be achieved with mice (Hamon et al., 2000), it is nevertheless rather difficult to carry a systematic genetic investigation with erythrocytes.

Yeast, on the other hand, are primitive cells and the number of ATPases required in yeasts for the establishment and maintenance of subtle equilibration of lipid distribution may not be sufficient to describe human cells. The fact that the yeast P-type ATPases subfamily involved in the regulation of lipid asymmetry comprises 5 proteins while the equivalent family in humans has 14 members (Graham, 2004) is certainly an indication of the degree of complexity one should expect with human cells.

(ii) To fully demonstrate the role of specific proteins in the translocation of phospholipids in biological membranes, it will be necessary to express and purify sufficient amounts of the candidate proteins and to reinsert them into lipid vesicles. Each P-type ATPase from yeast, which are only considered yet as *potential* lipid translocators, need to be purified and their transport activity must be assayed in proteoliposomes containing a single ATPase. A serious difficulty may be the required presence of accompanying proteins such as Ros3P (Lem3p) or Cdc50p. The use of labeled lipids has been criticized, sometimes in an unjustified fashion, sometimes with real arguments associated with the polarity of spin-labeled probes or the steric hindrance of fluorescent lipid analogues (Devaux et al., 2002). Up to now, most reconstitution experiments with potential lipid flippases have been performed in lipid vesicles with a typical size of about 100 nm obtained by dialysis or with biobeads (Auland et al., 1994; Romsicki and Sharom, 2001; Rothnie et al., 2001; Eckford and Sharom, 2005). We have pointed out several times that the unidirectional translocation of lipids in such vesicles with a limited radius raises an intrinsic problem in particular when the major lipid composition comprises a lipid, which is a flippase substrate, for example phosphatidylcholine (Rothnie et al., 2001; Traikia et al., 2002). Indeed, the accumulation of the host lipids in a single leaflet of a bilayer vesicle is obviously impossible and in fact the transfer of even a small fraction of lipids from one leaflet to the other without compensation should lead to an increased surface tension, which very likely blocks the lipid translocase activity. This phenomenon can explain the modest effects observed in most case with reconstituted systems. There is actually a scaling effect that has been discussed theoretically, which predicts that in small vesicles the surface tension will be generated more rapidly than in giant vesicles if the lipid asymmetry increases (Farge and Devaux, 1993).

Future reconstitution experiments should be performed preferably with unlabeled lipids and with naturally occurring long chains in giant vesicles. One approach that we suggest is based on the observation with an optical microscope of the shape change triggered by a small mismatch between the two leaflets of a giant unilamellar vesicle. This type of experiment which requires neither labeled lipids nor short chain lipids, is illustrated in Fig. 4. In this experiment, there were no attempts to purify the proteins except for the removal of cytoskeleton proteins (spectrin and actin). Fig. 4 shows the sequence of shape changes undergone by a giant unilamellar vesicle containing proteins from human erythrocytes. The formation of a bud and its evolution with time was triggered by the addition of Mg-ATP in the vicinity of a giant prolate vesicle containing solely the endogenous lipids of a red

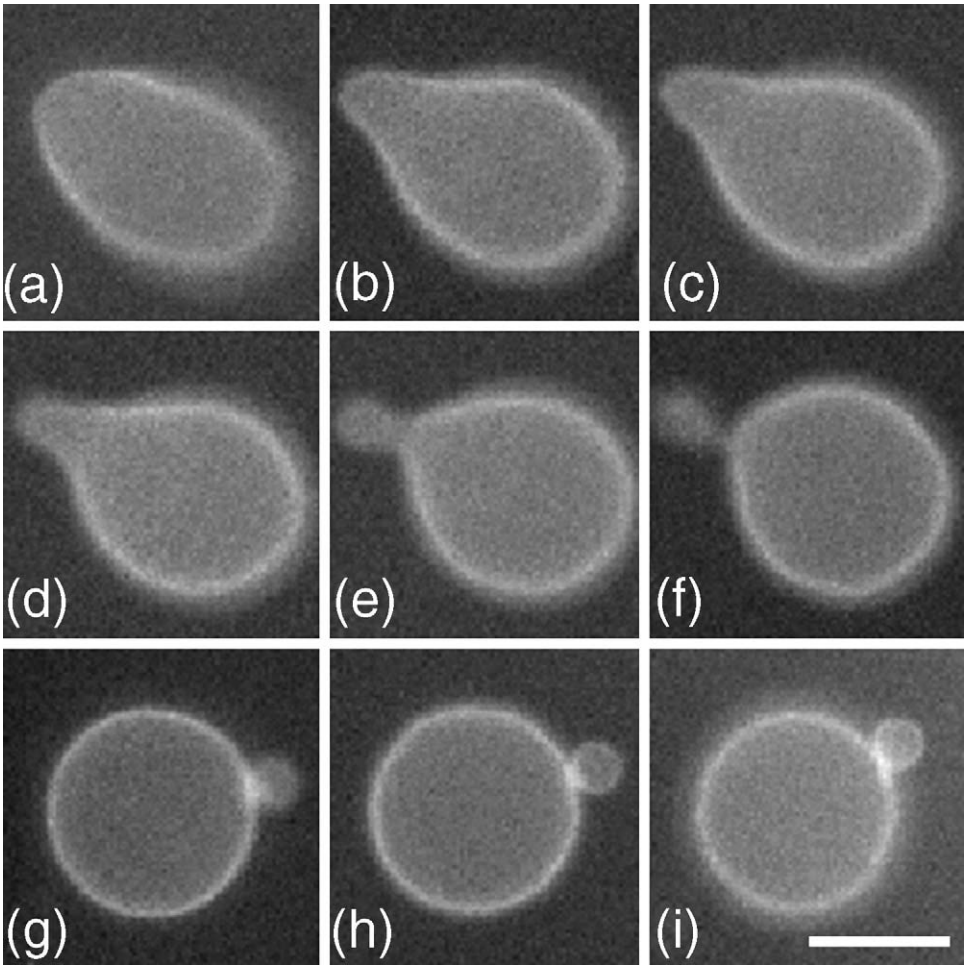

Fig. 4. Shape change of a giant unilamellar vesicle obtained from human erythrocyte inside out vesicles (depleted of spectrin and actin) to which ~0.5% of rhodamine-PE was added. Vesicles were partially dehydrated and submitted to an oscillating electric field, following the technique used to generate giant unilamellar vesicles (Angelova and Dimitrov, 1986). Shape changes were triggered by injection of 1 mM Mg-ATP in the medium at time 0. (a) $t = 0$ s; (b) $t = 45$ s; (c) $t = 46$ s; (d) $t = 47$ s; (e) $t = 48$ s; (f) $t = 52$ s; (g) $t = 1$ min; (h) $t = 1$ min and 1 s; (i) $t = 1$ min and 2 s. Scale bar: 10 μm. Temperature: 20 °C.

cell membrane. The shape change indicates in a spectacular way the translocation of a small fraction of lipids. Similar experiments have been carried out with DOPC giant unilamellar vesicles to show the rapid and spontaneous transmembrane diffusion of ceramides (López-Montero et al., 2005a). Control experiments with giant unilamellar vesicles depleted of protein and without an excess of lipids on one side did not show such shape transformation even after ATP injection.

(iii) Needless to say the ultimate goal, after protein assignment and purification, is to obtain the three-dimensional structure of a phospholipid flippase. Clearly, the mechanism by which a hydrophobic protein is able to flip an amphiphilic molecule within a lipid bilayer and accumulate against a gradient of concentration and against the surface pressure can only be understood through the knowledge of the structure at high resolution of at least one of these ATP-dependent phospholipid flippases. Up to now, the only relevant structure that has been determined by X-ray crystallography (at a resolution of 4.5 Å) is that of the bacterial ABC protein MsbA from *E. coli*, which is an homolog of the multidrug resistance proteins ABC transporters in eukaryotes (Chang and Roth, 2001). The three-dimensional structures of the mammalian multidrug resistance P-glycoprotein was solved at a resolution of approximately 2 nm by electron crystallography of negatively stained crystals and demonstrated major conformational changes in the transmembrane domains upon nucleotide binding (Rosenberg

et al., 2003). The structure of yeast Pdr5 was also determined at a resolution of 2.5 nm by electron microscopy after purification and reinsertion into liposomes (Ferreiras-Pereira et al., 2003). However, only better resolution will allow one to describe the complete transport mechanisms involved.

(iv) There has been some ambiguity concerning what is really a lipid translocation within a membrane and this concerns particularly the "transport" of molecules like cholesterol and fatty acid, which spontaneously can flip rapidly within a lipid bilayer and do not need the help of a protein (Hamilton, 2003). For example, ABCA1 was reported to be a cholesterol translocators, however the present interpretation is that it is *adaptor* for the connection between a membrane and lipoproteins (Hamon et al., 2000; Chambenoit et al., 2001). Similarly, fatty acid *translocators* may in fact be adaptors. Multidrug resistance proteins were discovered because of their aptitude to eject amphiphilic drug out of a cell, after their spontaneous insertion. Assays for flippase activity were carried out on ABC proteins with labeled lipids possessing at least one short chain, which provided them with non-negligible water solubility. Therefore, the process that was tested was not exactly a phospholipids translocation. Future work on flippase activity of ABC proteins, in particular, should concentrate on the translocation of long chain natural lipids.

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