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Aminophospholipid translocase and proteins involved in transmembrane phospholipid traffic

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Abstract

The transmembrane distribution of phospholipids in the membranes of eukaryotic cells depends on specific proteins (called flippases). The aminophospholipid translocase is responsible for the sequestration of phosphatidylserine and phosphatidylethanolamine in the cytosolic leaflet of plasma membranes. Several laboratories are presently working on the identification, purification and cloning of this Mg-ATPase, first recognized in the human red cell membrane. In accordance with the 1992 hypothesis of Higgins and Gottesman, proteins of the *MDR*1 family appear to be able to translocate certain phospholipids from the inner to the outer monolayer of the plasma membrane. It has been reported in particular that expression of the human *MDR*3 and mouse *mdr*2 genes promote translocation of long chain phosphatidylcholine, while expression of the *MDR*1 gene stimulates the outward motion of phospholipids possessing at least one short chain. ATP-independent flippases activities were recognized not only in microsomes but also in Golgi membranes. © 1997 Elsevier Science B.V.

Keywords: Phospholipid asymmetry; Aminophospholipid translocase; Flippase; P-glycoprotein; Scramblase

1. Introduction: transmembrane lipid traffic

The transverse diffusion of phospholipids in liposomes, also called phospholipid flip-flop, is a slow process with a characteristic time of several hours as measured originally by Kornberg and McConnell [1] in sonicated vesicles. On the other hand, neutral lipids such as diacylgycerol, ceramides or fatty esters and very likely cholesterol can diffuse from one leaflet to the other with characteristic times of seconds or minutes. In addition, some lipids including fatty acids and phospholipids, such as phosphatidic acid and phosphatidylglycerol, are in equilibrium between a charged and a neutral form allowing their rapid exchange between both sides of a membrane. After the discovery in 1984 of the active inward transport of phosphatidylserine and phosphatidylethanolamine in the human red cell membrane [2,3], investigations on biological membranes by many laboratories have demonstrated that the transmembrane lipid distribution is largely governed by specific proteins capable of catalyzing lipid transverse diffusion. The same proteins also accumulate

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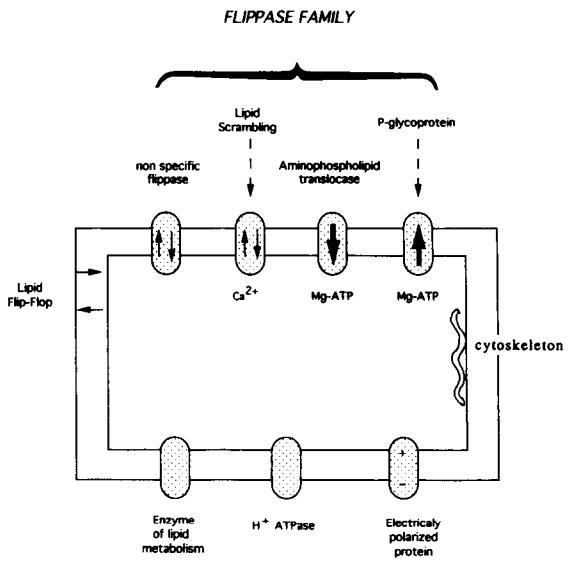

Fig. 1. Schematic representation of various proteins involved in the control of phospholipid transmembrane distribution.

specific lipids on one side of a membrane. As a consequence of this protein-dependent lipid traffic, the lipid distribution in biological membranes is asymmetrical [4,5]. Proteins may also be responsible for the sudden redistribution or 'scrambling' of phospholipid that takes place in specific cells under stimulation.

Fig. 1 represents schematically the different classes of proteins that are involved in transmembrane lipid traffic. They are normally distributed within the different membranes of an eukaryotic cell. In particular, the enzymes of lipid metabolism are mostly present in the ER which also contains the nonspecific phospholipid flippase. Whereas, plasma membranes contain the lipid pumps such as the aminophospholipid translocase and the protein responsible for lipid scrambling. ATPases that create a pH gradient and transmembrane proteins forming a stable electrical dipole as well as cytoskeletal proteins may participate in the stabilization of lipid asymmetry. It should be noted, however, that a transmembrane electrical field or a pH gradient is not sufficient to pull a charged lipid from one side of a membrane to the other.

Several preceeding reviews have summarized our rapidly evolving knowledge on lipid transverse diffusion, the formation of asymmetrical membranes and the putative biological functions associated with transmembrane lipid asymmetry [6–13]. Significant progress was made recently on the identification of phospholipid flippases and on the concerted actions of flippases and scramblases. Here, we shall give an overview of the most recent discoveries concerning the various proteins controlling transmembrane lipid distribution in eukaryotic cells, with particular emphasis on the aminophospholipid translocase. Biological functions associated with lipid asymmetry will be only briefly discussed.

2. The aminophospholipid translocase

2.1. Asymmetrical lipid distribution in the plasma membrane of eukaryotic cells

It is now well established that in the human erythrocyte membrane, the inner and outer leaflets have a different phospholipid composition. Phosphatidylcholine (PC), sphingomyelin (SM) and glycolipids are located predominantly in the outer monolayer, while phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphoinositides are located predominantly, if not exclusively, in the inner monolayer. Although the lipid topology was established only in the plasma membrane of a few animal cells, it is generally held that the asymmetrical character of the lipid topology, as it exists in the red cell membrane, is an ubiquitous property of eukaryotic cells. Experimental protocols and quantitative results compiled from the literature can be found in former reviews [6–13]. The actual data reveal small differences according to the type of cells. Possibly, some variations reported can be attributed to experimental uncertainties. Overall, the segregation of PS from the outer monolayer seems to be a general property.

It may be inferred that the asymmetrical distribution of phospholipids originates from the asymmetrical topology of the enzymes involved in lipid synthesis. This is certainly true in part. However, PC, PS and PE are synthesized mainly in the ER which is reported to contain a symmetrical lipid distribution. Newly synthesized lipids are exported to the plasma membrane where they acquire an asymmetrical dis-

tribution, thus proteins other than those directly involved in lipid synthesis must be responsible for the transmembrane orientation. Furthermore, human erythrocytes have an average lifetime of 120 days which far exceeds the time of spontaneous lipid randomization. The stable asymmetrical lipid distributions observed in red cells and in other plasma membranes must be associated with a mechanism which counteracts the effect of spontaneous transverse diffusion. It was first believed that interactions with cytoskeleton proteins could stabilize lipid asymmetry. However, in 1984, it was shown that, in human erythrocyte membranes, aminophospholipids are selectively transported from the outer to the inner leaflet by a process that requires hydrolysis of cytosolic ATP [2]. This was the first evidence of the role of phospholipid transport proteins in the establishment and/or stabilization of membrane asymmetry. The existence of such proteins had been postulated by Bretscher [5] in 1974 who named the proteins as 'flippases'. Phospholipid flippase activity was later demonstrated in the plasma membrane of many other eukaryotic cells.

2.2. Experimental evidence of an active transport of amino-phospholipids

To demonstrate the existence of a selective lipid transport, fluorescent or spin-labelled phospholipids with a short β chain (Fig. 2) can be introduced in the external surface of red cells and their translocation to the inner membrane leaflet determined from the percentage of probes that can be extracted with bovine serum albumin. After incubation for approximately 5 min in the red cell membrane at 37°C, 95% of the spin-labelled PS is inaccessible to BSA. The rapid disappearance of PS from the outer surface requires hydrolyzable Mg^{2+}-ATP in the cytosol and is inhibited by protein reagents such as *N*-ethyl maleimide and by cytosolic calcium ions. PE is transported also, though at a slower rate, with a characteristic time of about 45 min at 37°C. Exogenous SM or PC diffuses very slowly. SM, in particular, remains in the external leaflet with practically no flip-flop in a day. These results led us to postulate the existence of a specific protein, aminophospholipid translocase, which would be responsible for the accumulation of PS and PE in the cytosolic leaflet of human red cells [14,15].

Investigation of the lipid specificity indicated that not only the head group is recognized by the aminophospholipid translocase but also the glycerol backbone and the ester bond on the *sn*-2 chain [15]. Plasmalogen-PE is transported like the diacyl-PE [16], in accordance with the asymmetrical distribution of endogenous plasmalogen-PE. Recent results from this laboratory showed that aminophospholipids with di-ether bonds instead of di-ester bonds are also transported (Fig. 2) (Fellmann et al., unpublished). Because the chain length and degree of unsaturation are not crucial, chemical modifications of the chains do not impede the rapid translocation of PS analogs. For example, a nitroxide radical on the 4th or 5th carbon position of the *sn*-2 chain does not seem to influence the rapid transport, however, a NBD moiety at the 6th carbon position effectively slows down the transport of the PS analog and blocks the transport of NBD-PE in red cells [17].

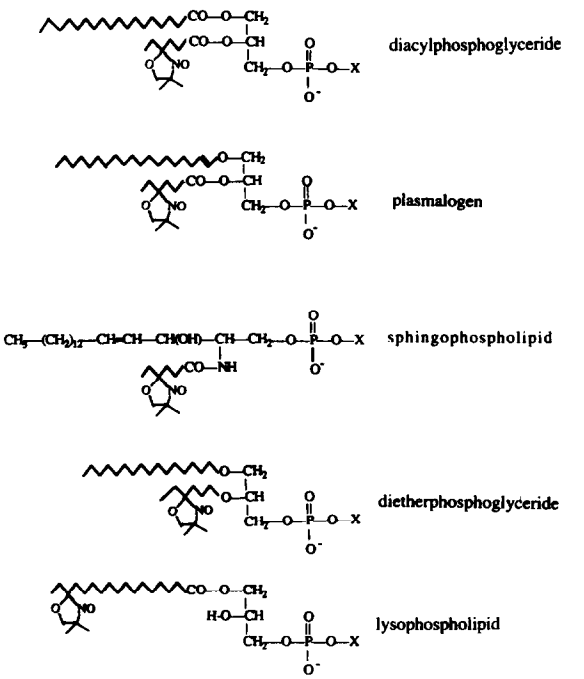

Fig. 2. Spin-labelled phospholipids used to measure lipid flip-flop in biological membranes. Because of the short β chain, these molecules are slightly water soluble (CMC around 1 μM). This feature allows a rapid incorporation in the outer monolayer of any cell membrane and permits the monitoring of their transmembrane distribution by back exchange with bovine serum albumin. *X* stands for choline, serine or ethanolamine.

The K_m for Mg^{2+}-ATP is approximately 1mM [2]. Nonhydrolyzable analogs of ATP cannot substitute for ATP. The exact stoichiometry was determined in DMPC-induced red cell vesicles which contain ATP but no regenerating system. We found that one molecule of ATP was hydrolyzed each time one aminophospholipid was transported [18]. Mg-ATPase activity and PS translocation in red cell ghosts loaded with ATP were studied in the presence of vanadate, suramin, eosin y or elaiophylin, all inhibitors of P-type ATPase. A parallel between the inhibition of both activities indicates that the ATPase involved in the translocation of PS is indeed a P-type ATPase [19].

In summary, it is well established that a Mg^{2+}-ATPase is involved in the translocation of aminophospholipids from the outer to the inner leaflet of red cells. Experiments were also carried out by several groups with other eukaryotic cells: platelets, lymphocytes, reticulocytes, K562 cells (which are transformed cells derived from human erythroblasts), endothelial cells, human fibroblasts, sperm cells, chromaffin cells, yeasts. Fluorescent probes are used most frequently. Internalization by endocytosis as well as hydrolysis of the short β chain by endogenous phospholipases A_2 makes the experiments more difficult at a quantitative level than in the case of erythrocytes. Nevertheless, the ATP-dependent rapid internalization of PS and PE in these cells indicates the ubiquitous character of the protein-dependent translocation of aminophospholipids in the plasma membrane of animal cells [6–13].

2.3. Identification of the aminophospholipid translocase: ups and downs

Based on labelling experiments with an iodinated photoaffinity PS analog, Schroit et al. [20] have identified the aminophospholipid transporter with the Rh(D) protein which has a molecular weight of 32 kDa. However, Smith and Daleke [21] found that Rh_{null} cells which are deficient in the expression of Rh proteins transport PS normally. Recent experiments carried out in France, on a series of 7 patients with no Rh protein have confirmed Smith and Daleke's former conclusion (Geldwerth et al., submitted). Furthermore, the aminoacid sequence of the Rh(D) protein possesses no apparent ATP binding sites [22], implying that if an Rh-like protein is involved in flippase activity, it must be associated with a separate ATPase [7]. Purification of a red cell Mg-ATPase by solubilization in detergent and fractionation by column chromatography of erythrocyte ghosts was carried out simultaneously in Paris [23] and in the United States [24]. A partially purified ATPase, vanadate-sensitive and requiring addition of PS for reactivation was isolated in both laboratories. ATP-γS labelled a band in the region 110–120 kDa. Simultaneously, Auland and Roufogalis [25], in Australia, claimed to have purified to homogeneity a Mg-ATPase from red cells, having a molecular weight also in the region 110 kDa. The latter ATPase was reconstituted in egg PC liposomes in our laboratory, and a partial transport of spin-labelled PS from the inside to the outside could be demonstrated when Mg-ATP was added externally [25]. No transport of spin-labelled PC was observed. The transport of spin-labelled PE required the presence of an anionic phospholipid on the side of the membrane where the ATP was added. In practice, a partial reorientation of spin-labelled PE was achieved in the presence of 1% unlabelled phosphatidylinositol. In all these experiments, we could not achieve 100% translocation of the spin-label initially inside the vesicles. This may be attributed to a fraction of vesicles that did not contain the protein or contained a denatured protein or contained an improperly oriented protein. In conclusion, it appears that a single protein of apparent molecular weight 110 kDa would suffice to provoke an active translocation of aminophospholipids. However, optimum translocation may require other components.

Recent experiments in our laboratory based on Auland and Roufogalis's purification procedure led systematically to proteolysed fractions. Various protease inhibitors were tested but could not be used because they also induce an irreversible inhibition of the ATPase activity. A modified protocol was set up in order to minimize the proteolysis and obtain more reproducible results. The first step consists of a DEAE-Sepharose column which allows the exclusion of at least 80% of the multicatalytic protease (or MCP), the latter being in the flow-through while ATPases stay bound to the anion exchanger and are eluted using a KCl gradient [25,26]. Fractions with a significant Mg^{2+}-ATPase activity are pooled and

dialyzed to remove the salt. The second step of purification involves Calmodulin-Agarose and Heparin-Agarose columns. The Calmodulin column was used to separate the Ca-ATPase from the other ATPases. The Ca-ATPase stays coupled to the Calmodulin column while the other ATPases are found in the flow-through which is directly loaded on Heparin-Agarose column. The ATPases bind to the beads and are eluted by a linear KCl gradient. After labelling by [γ^{32}P]-ATP proteins of different fractions are separated on SDS-PolyAcrylamide Gel Electrophoresis and visualized by silver staining (Fig. 3). In the fractions of high Mg^{2+}-ATPase activities, three bands are present in the range of molecular weight 100–130 kDa. Only the middle band, which corresponds to a molecular weight of 115 kDa is labelled by [γ^{32}P]-ATP (Fig. 4). This result suggests that the band around 115 kDa could be the aminophospholipid translocase of human erythrocytes. Until the amino acid sequence of the phosphorylated band is known, the possibility that this protein corresponds to the ATP citrate lyase cannot be excluded. Indeed we, as well as Lyles and Daleke [27], have found that a citrate lyase can contaminate the purified Mg-ATPase fraction. However, the necessity of citrate being present as substrate for the ATPase activity of the latter protein, and the overlapping of the ATPase activity profile and [^{32}P] phosphorylation are arguments in favor of the human erythrocyte aminophospholipid translocase. Note that two lower molecular weight bands around 95 kDa and 85 kDa are also labelled by [γ^{32}P]-ATP (Fig. 4). These may correspond to proteolytic fractions of the ATPase. Lyles and Daleke [27] also reported the existence of a protein around 83 kDa in a partially purified fraction of the red cell Mg-ATPase. The same laboratory recently succeeded in obtaining a polyclonal antibody made against the partially purified Mg-ATPase which was able to inhibit the aminophospholipid translocase activity in erythrocyte ghosts when added prior to resealing [28].

The lower molecular weight proteins could correspond to proteolytic fractions of the 115 kDa protein. In favor of this hypothesis is the result obtained by Tang et al. [29] in the United States. These investigators have purified to homogeneity a Mg-ATPase from bovine chromaffin granules and succeeded in cloning the gene coding for this ATPase. Sequence

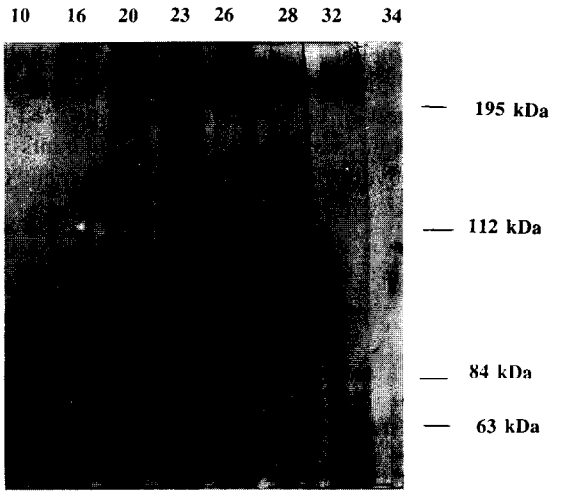

Fig. 3. Polyacrylamide (6% acrylamide) gel electrophoresis under denaturation conditions of purified protein fractions from human erythrocyte membranes. Gels stained with silver nitrate. *Experimental protocol*: Eryhrocyte ghosts were solubilized at 4°C by agitation in buffer A (Triton×100 0.5%, $CaCl_2$ 2mM, $MgCl_2$ 5mM, Hepes 20mM, glycerol 10%, pH 7.4). After 30 min, the mixture was centrifuged, filtered (0.45 μm pore diameter) and the filtrate loaded on a DEAE-Sepharose 6B Fast-Flow column (Pharmacia) equilibrated at 4°C in buffer B (Triton×100 0.2%, $MgCl_2$ 5mM, Hepes 20mM, glycerol 10%, pH 7.4). The flow rate was 80 ml h^{-1}. After washing, the column was eluted with 50 ml of a gradient from 0 to 200mM KCl into buffer B. Vanadate-sensitive ATPase activity was measured. Active fractions were pooled and dialyzed overnight against 30 volumes of buffer B to which 2mM $CaCl_2$ had been added. The dialyzate was loaded on a Calmodulin-Agarose column (Sigma) and the flow through directly loaded onto a Heparin-Agarose HS column (Sigma). The two columns were equilibrated in buffer B. After washing in buffer B containing 2mM $CaCl_2$ and 1mM DTE, the Calmodulin column was disconnected and the Heparin column washed with 50 ml of the same buffer. The ATPase is eluted with 100 ml of a gradient from 0 to 250mM KCl into buffer B containing 1mM DTE. The gel shown in this figure was obtained after elution from Heparin-Agarose. The numbers refer to the fractions collected from the column.

analysis indicates that it is a member of a novel subfamily of P-type ATPases that includes the product of the DRS2 gene in yeast. A DRS2 null mutant was found to be defective in transport of fluorescent PS, suggesting that the Mg-ATPase isolated from chromaffin granules is an ATP-dependent lipid transporter. The molecular weight calculated from the derived sequence is 130 kDa while the molecular weight estimated from SDS PAGE is 120 kDa. A

cDNA coding for a human ATPase with 94.3% homologies with the bovine MgATPase potentially implicated in lipid translocation has been recently cloned [30]. The two clones have to be expressed in aminophospholipid translocase defective mutants in order to definitively demonstrate the assignment of this Mg-ATPase with the lipid translocator.

3. Flippase activity of MDR proteins

In 1992, Higgins and Gottesman [31] realized that the activity of the multidrug transporter corresponds to the reorientation of hydrophobic molecules from one side of a lipid membrane to the other and thus has certain similarities to the activity of a phospho-

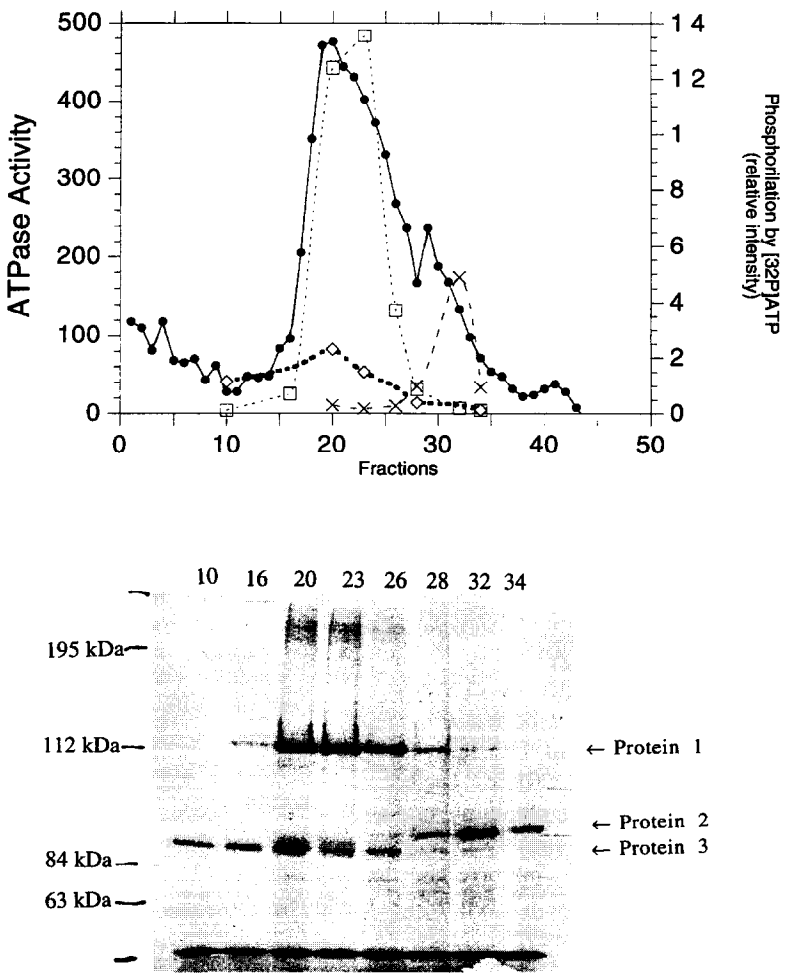

Fig. 4. Upper panel. Vanadate- and PS-sensitive ATPase activity in the fractions eluted from the Heparin-Agarose column, superimposed with the extent of [^{32}P]-labelling corresponding respectively to: (□) protein 1 (112–115 kDa); (×) protein 2 (95 kDa); (◇) protein 3 (85 kDa), defined in lower panel. Fractions supplemented with 0.3 mg ml^{-1} brain PS and 2.5mM EGTA were incubated with [γ^{32}P] ATP (5 mCi from Amersham) for 1 min. The reaction was stopped by addition of 10% TCA and 50mM KH_2PO_4. After centrifugation, the protein pellet was dissolved and gel electrophoresis was run (see Fig. 2). The amount of labelling was quantified with Phosphor Imager and the relative intensities plotted (upper panel).

lipid flippase. The P-glycoprotein encoded by the *MDR*1 gene confers drug resistance to drug sensitive cells. This Mg-ATPase translocates apparently very different compounds from the inner monolayer to the outer monolayer of the cells where it is expressed. Although the substrate specificity of the multidrug resistance is very poorly defined, it appears that a common feature is the amphiphilic character of the drugs transported. The flippase model contrasts with the classical pump model for transporters in which substrates move between the aqueous phases on either side of the membrane without ever coming into contact with membrane lipids. Can the P-glycoprotein translocate phospholipids and, if it does, are all phospholipids translocated from the inner to the outer membrane surface? A priori this cannot be because it is hard to imagine a membrane accumulating all of its lipids in the same leaflet. It seems that this would result in a complete destruction of the bilayer. Yet in 1996, van Helvoort et al. [32], in the Netherlands, demonstrated that epithelial cells, in which the *MDR*1 gene had been overexpressed, were capable of translocating various fluorescent phospholipid analogs possessing a short β chain when the control cells were not. This phenomenon was inhibited by *MDR*1 inhibitors such as verapamil and also by energy depletion of the cells. The authors conclude, however, that it was unlikely that natural long chain lipids would be translocated. If it did happen, aminophospholipids would be translocated from inside to outside cell surface by the Pgp and from outside to inside surface by the aminophospholipid translocase leading to a futile cycle of utilizing ATP to pump such lipids back and forth. It should be pointed out that most experiments to demonstrate the existence of aminophospholipid translocase activity were carried out with short chain analogs, either spin-labelled or fluorescent lipids. The exception is the experiments carried out on red cells in Utrecht in 1986 by Tilley et al. [33], who used radioactive long-chain phospholipids introduced in the red cell membrane with the help of a phospholipid exchange protein. van Helvoort et al. speculated that the biological function of the translocation of phospholipids with a short chain by the P-glycoprotein could be to excrete peroxidized lipids or lyso-derivatives out of a cell [32]. A direct assay of the translocation of long chain phospholipids remains needed, e.g., in reconstituted proteoliposomes containing the purified P-glycoprotein.

Human *MDR*3 and mouse *mdr*2 Pgp are proteins with a high homology with the human *MDR*1 Pgp, but the latter proteins do not confer multidrug resistance. However, in the Netherlands again, it was found that transgenic mice with an homozygous *mdr*2 gene disruption had severe liver disease with a total inability to secrete phosphatidylcholine in the bile [34]. Ruetz and Gros [35] later carried out an experiment with secretory vesicles from yeast in which the *mdr*2 or the human *MDR*3 equivalent genes had been expressed. They found that *mdr*2 expression caused a slight enhancement of PC translocation. Maximal NBD-PC translocation resulted only in the transfer of about 3.5–4% of the total NBD-PC molecules incorporated initially in the vesicles. Nevertheless, the authors took this as an indication of a possible physiological role of the *mdr*2 gene. They later found enhancement of *mdr*2 mediated PC translocation by the bile salt taurocholate and implicated the role of *mdr*2 protein in the process of bile formation and secretion [36].

According to Ruetz and Gros [35], expression of the rodent *MDR*3 P-glycoprotein which is responsible for drug resistance was without effect on NBD-PC translocation in secretory vesicles derived from yeast. Smith et al. [37], on the other hand, showed that expression in fibroblasts from transgenic mice of human *MDR*3 gene identical to the mouse *mdr*2 gene promotes translocation of [^{14}C] labelled long chain phosphatidylcholine through the plasma membrane. Finally, van Helvoort et al. [32] found that a short chain NBD-PC was specifically transported from the inner to the outer leaflet of the plasma membrane of epithelial cells transfected with *MDR*3 but not other phospholipid analogs. Note that the latter result contradicts the finding of Ruetz and Gros [35], which was mentioned earlier.

In conclusion, it appears that proteins of the MDR family which are members of the ABC (ATP Binding Cassette) superfamily of active transporters are able, in specific cases, to translocate phospholipids from the inner leaflet to the outer leaflet of plasma membranes and particularly phosphatidylcholine. Thus, one may wonder if such proteins could be involved in the formation of the transmembrane lipid asymmetry existing in the plasma membrane of eu-

karyotic cells. However, there is no evidence of an active outward transport of PC (or SM) in normal cells, i.e., in cells which do not express *mdr*2 or *MDR*3 genes. For example, in erythrocytes at normal steady state equilibrium, there are some PC in the inner monolayer and some PE in the outer monolayer; increasing the cytosolic concentration of ATP from the normal level which is about 1mM–5mM results in the activation of the pumps with a transfer of more PE to the inner monolayer (shift of equilibrium), and is accompanied by the formation of stomatocytes but it does not appear to modify the distribution of PC.

4. ATP-independent flippases

In 1985, Bishop and Bell [38] showed the existence of a rapid ATP-independent translocation of the water soluble dibutyroyl-PC in microsomal membranes which they attributed to the action of a specific ER protein, named again phospholipid flippase. Clearly, this is a different protein from the protein whose existence had been found a year before in red cell membrane and which translocates specifically aminophospholipid at the expense of the hydrolysis of ATP. While Bishop and Bell claimed that the ER flippase is specific for PC, we have found using spin-labelled phospholipids with one long chain that many different phospholipids traverse the ER membrane with a $t_{1/2} < 20$ mn [39]. More recent results from this laboratory indicate that the half time for the translocation of glycerophospholpids could be of the order of a few seconds [40] and about one order of magnitude slower for sphingomyelin. This rapid translocation is bidirectional, saturable and displays a competitive behavior. It does not involve ATP. It is partially inhibited by *N*-ethyl maleimide (20mM) and by trypsin treatment, again indicating the role of a protein. The relationship between phospholipid flip-flop and nonbilayer structures occurring in the ER was also investigated by ^{31}P-NMR. Several conditions were found under which the ^{31}P isotropic signal previously attributed to nonbilayer structures is decreased or abolished whereas transbilayer diffusion was unaffected, suggesting that the flip-flop process is independent of such structures.

Rapid transbilayer movement of glycerophospholipids was also found in purified Golgi fractions from rat liver. Furthermore, protein mediated transbilayer movement of glucosylceramide was evidenced in rat liver ER and Golgi apparatus. In contrast no transbilayer motion of spin-labelled lactosylceramide could be detected. Thus, glucosylceramide has access to the lumenal leaflet of the Golgi membrane where it is converted to lactosylceramide and then may undergo subsequent glycosylation before export to the plasma membrane by vesicule traffic [41]. Finally, protein-mediated transmembrane movement of a water soluble analogue of mannosylphosphoryldolichol in rat liver ER has been reported, indicating that this very particular membrane possesses a whole series of proteins allowing the rapid equilibration of newly synthesized lipids or lipid precursors [42].

5. Phospholipid scramblase

The entry of calcium in red cells with an ionophore provokes a very rapid PS exposure on the outer monolayer; it also triggers the transmembrane movement of other lipids, e.g., a fraction of SM and PC flips inside the cell with a time scale of the order of 10–20 mn. These experiments were carried out with probed lipids, either fluorescent or spin-labelled [43], and also with long chain endogenous lipid. PS exposure in particular can be detected by very sensitive methods such as the stimulation of prothombinase activity or the binding of radioactive annexine V [44,45]. This rapid lipid reorientation or 'lipid scrambling' cannot be caused solely by the calcium inhibition of the aminophospholipid translocase because the time scale of spontaneous diffusion is of the order of several hours. Similar results were obtained in platelets where half times of lipid scrambling are even shorter, typically on the order of a few minutes. In the latter case, it corresponds to a physiological event. Indeed, increase of cytosolic calcium in platelets is the result of their stimulation by natural agonists. The exposure of PS, in turn, promotes blood coagulation as well as cell–cell recognition and phagocytosis.

Zwaal and collaborators have postulated the existence of a 'scramblase' which would be a calcium-dependent enzyme responsible for the rapid flip-flop of phospholipids. Two laboratories recently pub-

lished data reporting a scramblase activity in proteoliposomes containing proteins purified either from platelet membranes [46] or from erythrocytes [47]. In the experiment of Bassé et al. [47], however, the scrambling activity triggered by calcium has a characteristic time of about 4 h which is very different from what is observed in red cells where the reorientation time is on the order of 10–15 min. A rare syndrome was found in a person whose platelets do not respond by PS exposure to a stimulation. By analyzing the protein pattern of this patient's platelets, it will perhaps be possible to tell which protein is lacking and causes the Scott syndrome.

An alternative mechanism proposed recently to explain phospholipid scrambling involves the synergistic action of PIP_2 and calcium [48]. Using spin-labelled lipids to monitor the extent of phospholipid reorientation, we have found that the extent of the Ca^{2+} induced lipid scrambling was dependent upon the level of PIP_2 contained in the external leaflet of inside-out erythrocyte vesicles. The level of PIP_2 in this leaflet, which normally accounts for 80% of the total membrane PIP_2, was manipulated either by ATP depletion of the original erythrocytes or by incorporation of exogenous PIP_2. Similarly, loading the outer monolayer of the membrane of intact erythrocytes with exogenous PIP_2 caused, in a dose dependent way, the scrambling of spin-labelled PE, PS, PC and SM. These results were challenged by a group of laboratories who reported that in Scott erythrocytes which do not respond to calcium stimulation, the modulation of the level of PIP_2 did not impair lipid scrambling even in the presence of Ca^{2+} ionophore [49]. Their conclusion is that scrambling requires the existence a specific protein (or scramblase) that would be absent in pathological cells. Yet, at least partial lipid scrambling can be induced by calcium ions in liposomes depleted of proteins providing they contain PIP_2 [50]. No other di-anionic phospholipid (PI or cardiolipin) can substitute for PIP_2. In this artificial system where PIP_2 is obviously the only target of Ca^{2+}, scrambling is inhibited by spermine, a polyamine which also inhibits the scrambling phenomenon in red cell ghosts [51]. In conclusion, the scramblase may be a complex containing a protein and PIP_2. The absence of lipid redistribution in Scott cells could be due to the presence of an inhibitory protein acting like spermine.

6. Concluding remarks: physiological functions of lipid asymmetry

The existence of protein involvement in transmembrane traffic of lipids has not yet permitted one to fully explain how the equilibrium transmembrane lipid distribution is set up nor for what purposes the asymmetry is engendered. Indeed, although the establishment of a complete asymmetrical membrane with only active transport of aminophospholipids was considered as possible in a theoretical work carried out in Herrmann's laboratory, their model implied an accelerated passive diffusion of choline containing lipids in the presence of a gradient for which there is no experimental evidence [52]. Bitbol and Devaux [53] as well as Connor et al. [54] have reported a protein dependent accelerated outward diffusion of PC, however, in both instances the time scale of the PC outward movement was considerably longer than that of the PS inward movement. Therefore, a hypothetic erythrocyte membrane with a random lipid distribution would undergo very drastic shape deformation with possible formation of endocytic vesicles because of the mismatch between the inward and outward lipid traffic. The correct answer to the problem of the in vivo formation of asymmetrical membranes is probably that membranes are formed progressively by means of growing, fusion and fission of already asymmetrical membranes. Indeed, it is much easier to understand how a membrane can adjust continuously its lipid distribution with the help of flippases molecules than it is to understand how a totally random lipid distribution can become asymmetrical. It should be reminded that the erythrocyte membrane is formed in precursor cells containing organelles as all eukaryotic cells and that the lipid asymmetry is installed progressively during internal membrane traffic between organelles and the plasma membrane.

As for the physiological function of lipid asymmetry, the topic has been discussed at length in former reviews [6–11,13]. There are generally two ways of thinking. The first is that of biologists who see particular lipid exposure as a way to modulate

protein activity. The paradigm is that the recognition between two molecules triggers a new event, typically receptor-ligand recognition or enzyme-cofactor recognition. Good examples are the triggering of blood clotting following the stimulation of prothrombinase activity by PS exposure on platelets, or the disposal of apoptotic lymphocytes by macrophages due to PS recognition on the surface of apoptotic cells. The second way of thinking is that of physicists who analyze the physical consequences of lipid redistribution in terms of shape deformation, generation of surface tension, possibility to modulate adhesion and fusion of vesicles by changing the lipid distribution. These ideas have not always proven to be applicable but they are stimulating and may become more important than previously believed by biologists.

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