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Topical Review

Role of Translocases in the Generation of Phosphatidylserine Asymmetry

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Introduction

The distribution of phospholipids across the plasma membrane of eukaryotic cells is not random, and certain phospholipids are distributed asymmetrically across the lipid bilayer. This is particularly true both for the choline-containing phospholipids, phosphatidylcholine (PC) and sphingomyelin, which are primarily located in the cell's outer leaflet, and for the aminophospholipids, phosphatidylserine (PS) and phosphatidylethanolamine (PE) which are found at the membrane's cytoplasmic face.

Although it has been known for more than two decades that membranes are asymmetric (Bretscher, 1972) the biological significance of this likely ubiquitous phenomenon has only recently been addressed. While our understanding is still limited, it is clear that transmembrane orientation of lipids influences membrane structure and the function of various membrane-bound enzymatic systems. There is also evidence to suggest that certain lipids localized at specific locations within the cell membrane participate in processes as diverse as cell-cell recognition and blood coagulation.

Because spontaneous transbilayer movement of charged phospholipids is very slow in artificial phospholipid bilayers (Kornberg & McConnell, 1971; Pagano & Sleight, 1985), the generation of lipid asymmetry in cells must be controlled and regulated by specific lipid transport processes. Indeed, the movement of aminophospholipids from the cells outer-to-inner leaflet has been shown to be dependent upon an ATP-driven aminophospholipid translocase/flipase which transports PS and PE across the cell membrane (Seigneuret & Devaux, 1984).

In this review, we summarize data supporting the concept that specific membrane proteins constitute the machinery that generates transbilayer aminophospholipid movement, thereby controlling and regulating the equilibrium distribution of lipids between both membrane leaflets. Important recent results concerning the identification of the aminophospholipid flipase from red blood cells will be discussed. Its possible cooperativity with other lipid flopases/scramblases that ultimately regulate the membrane sidedness of PS and determine the equilibrium distribution of lipids across the cell's plasma membrane will also be discussed. This review focuses on data obtained with red cells and platelets. The reader is also referred to other recent reviews on lipid flipases and related topics (Devaux, 1992; Schroit, 1994; Devaux & Zachowski, 1994; Menon, 1995).

Membrane Lipid Asymmetry

The steady-state phospholipid composition of cell membranes is known to be heterogeneous and asymmetric. Because red blood cells are dynamic structures that cannot synthesize new lipids, changes in membrane composition that are dictated by changes in function can only be accomplished by membrane remodeling through constant leaflet-directed alterations in transbilayer lipid distributions.

The transbilayer distribution of the four major phospholipids in human erythrocytes — PC, sphingomyelin, PS and PE — was established more than 20 years ago through the use of side-specific phospholipases (Verkleij

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et al., 1973; Zwaal et al., 1975) and nonpermeable amine-reactive probes (Bretscher, 1972; Gordesky et al., 1975; Rawyler et al., 1984). These studies showed that about 80% of the sphingomyelin and 75% of the PC is located in the outer leaflet (Verkleij et al., 1973), and 80% of the PE and essentially all of the PS is in the cell's inner leaflet (Verkleij et al., 1973; Gordesky et al., 1975; Zwaal et al., 1975; Marinetti & Crain, 1978). Although these studies proved that membrane lipid asymmetry does exist, the participation of energy-dependent, ATP-utilizing lipid flipases in its generation was not recognized until the mid 1980s (Seigneuret & Devaux, 1984; Daleke & Huestis, 1985). New approaches that introduced exogenous lipids and probe-labeled lipids into the outer leaflet of cells, together with methods for monitoring the movement and appearance of lipids in opposing membrane leaflets, were essential to the developing concept of aminophospholipid movement.

Transbilayer Lipid Movement

Aminophospholipid Flip Flop

Direct evidence for the involvement of specific proteins in lipid movement has been provided by the development of synthetic lipid analogues containing spin, fluorescent, and radioactive labels for monitoring lipids within the cell membrane. The first report describing protein-mediated aminophospholipid translocation across the red cell membrane by Seigneuret and Devaux (1984) utilized spin-labeled lipids. By examining the fraction of spin-labeled probe accessible to reduction by ascorbate, it was shown that exogenously-supplied analogues of PS and PE were transported from the site of insertion at the cell's outer ascorbate-sensitive leaflet, to the cell's inner ascorbate-resistant leaflet. Approximately 95% of the PS, 80% of the PE, and 20% of the PC were transported to the red cell's inner membrane leaflet and adopted the distribution of endogenous lipids.

Similar observations were made when the movement of isotopically- (Tilley et al., 1986; Schroit et al., 1987; Connor et al., 1992*a*) and fluorescent-labeled (Connor & Schroit, 1987) lipids was monitored. Lipid movement with these probes was monitored by either removing the fraction of residual lipid not transported to the cells' inner leaflet by the so-called ''back-exchange'' procedure (Struck & Pagano, 1980) or, similar to the chemical reduction of spin-labeled lipids, by destroying the fraction of fluorescent lipid (in the cells' outer leaflet) that was accessible to a reductant added to the buffer (Mcintyre & Sleight, 1991).

These and other experiments showed that PS transport was ATP-dependent, because transport did not occur in ATP-depleted cells, was inhibited by vanadate, and could be reconstituted in erythrocyte ghosts resealed in the presence of Mg^{2+}-ATP but not ADP (Seigneuret & Devaux, 1984). Additional evidence that the transport of aminophospholipids depended on lipid-specific protein transporters came from experiments which showed that transport was inhibited by agents that react with membrane cysteines (Seigneuret & Devaux, 1984; Daleke & Huestis, 1985; Tilley et al., 1986; Connor & Schroit 1988, 1990) and histidines (Conner et al., 1992*a*).

Although these observations implicated the involvement of a specific protein, conclusive evidence of the transporters' specificity was shown by the cell's inability to transport D-isomers of PS and PE (Martin & Pagano, 1987). Substrate specificity is also dictated by the glyceride backbone and esterification of the sn-2 position. Translocation was abolished when ceramide was substituted for glyceride even when the polar head group contained the phosphoserine moiety (Morrot et al., 1989) and translocation of lysophosphatidylserine across the bilayer membrane occurred only at very slow rates (Bergmann et al., 1984). Although transport is influenced by the length of the fatty acid side chains and the presence of a reporter group (e.g., fluorescent, iodinated or spin-labeled moieties), translocation rates are determined primarily by the lipid's polar head group. This was shown in a series of elegant experiments in which progressive methylation of PE to its monomethyl-, dimethyl- and trimethyl (pc) - adducts resulted in a methylation-dependent decrease of transport (Morrot et al., 1989).

Together with rapid flipase-mediated aminophospholipid inward movement, an ATP-dependent flopase seems to be involved in phospholipid outward movement. Using spin-labeled analogues, Bitbol and Devaux (1988) showed that outward movement of aminophospholipids was faster than that of PC. Although these studies revealed some degree of lipid specificity, other experiments using NBD-labeled lipids suggested that the process was lipid species independent (Connor et al., 1992*a*). These studies showed that while outward movement was much slower than translocase-mediated inward movement, it had similar biochemical properties. Outward movement was Mg^{2+}- and temperature-dependent and was inhibited by vanadate and several sulfhydryl oxidants. Conceivably, these opposing activities — the slow transport of phospholipids to the cell's outer leaflet, concurrent with the transport of aminophospholipids to the cell's inner leaflet — could create conditions that lead to lipid asymmetry.

Lipid Scramblase

Inhibition of transmembrane lipid movement with reagents that abrogate the activity of both the flipase and flopase does not result in loss of membrane asymmetry.

This suggests that active translocases are not required to maintain it (Tilley et al., 1986; Comfurius et al., 1990; Connor & Schroit, 1990; Henseleit et al., 1990; Schroit & Zwaal, 1991, Basse et al., 1993). If, on the other hand, transport is inhibited by increasing the cytosolic Ca^{2+} concentration, rapid nonspecific redistribution of all phospholipids between bilayer leaflets occurs with a concomitant rise in procoagulant activity (Bevers et al., 1990; Williamson et al., 1992; Smeets et al., 1994). In red cells and platelets, elevation of intracellular Ca^{2+} is also accompanied by changes in cell shape, membrane blebbing, and the release of cytoskeleton-free microvesicles (Allan & Mitchell, 1975; Sims et al., 1989); events which closely parallel the loss in phospholipid asymmetry.

The mechanism by which Ca^{2+} affects membrane phospholipid asymmetry remains unclear. Although elevated cytoplasmic Ca^{2+}-induced formation of microvesicles closely parallels the loss of phospholipid asymmetry that occurs in the cell and in the shed vesicles (Sims et al., 1989; Comfurius et al., 1990; Zwaal et al., 1992; Chang et al., 1993), it is also associated with many other phenomena, especially elevated calpain activity (Fox et al., 1991; Yano et al., 1993) and the formation of phosphatidylinositol 4,5-bisphosphate (PIP2)-Ca^{2+} complexes (Sulpice et al., 1994). Data suggest, however, that neither direct Ca^{2+} effects, protein degradation (Basse, 1993), PIP2 (Bevers et al., 1995) nor inhibition of the translocase (Bitbol et al., 1987; Bevers et al., 1989; Tilley et al., 1990) can singularly accommodate the membrane rearrangements that occur upon the elevation of intracellular Ca^{2+}. These findings raise the possibility that another component is activated upon Ca^{2+} elevation which is responsible for the scrambling of membrane lipids. Indeed, recent studies indicate that the purported scramblase activity is sensitive to sulfhydryl oxidants (Williamsson et al., 1995) and requires ATP (Martin & Jesty, 1995). Consistent with the concept that distinct mechanisms are responsible for translocase and scramblase activity, removal of Ca^{2+} restores flipase (and possibly flopase) activity and results in transport of the scrambled aminophospholipids back to cell's inner leaflet (Comfurius et al., 1990). Under normal conditions, however, activity is not restored in the shed vesicles probably because of insufficient ATP to sustain it (Zwaal et al., 1993).

The strong association between Ca^{2+}-dependent loss of membrane phospholipid asymmetry and the subsequent membrane blebbing and release of microvesicles from the cell membrane requires fusion of membrane segments at the point where the budding microvesicle is released from the parent cell (Sims et al., 1989). Because red cells do not exhibit spontaneous cell fusion reactions, it would seem likely that the fusion site is a consequence of a local event that results in loss of membrane lipid asymmetry. While vesicle release is coincidental to lipid scrambling, the budding membrane must also be released from cytoskeletal proteins (Chang et al., 1993; Basse et al., 1993, 1994; Dachary-Prigent et al., 1995) which requires proteolysis by (Ca^{2+}-activated) calpain (Phillips & Jakabova, 1977; Fox et al., 1991). This implies that vesiculation is at least a two-step process and that membrane scrambling must precede microvesiculation. Indeed, both events are separable and membrane scrambling can occur in the absence of microvesiculation (Dachary-Prigent et al., 1995). It is unlikely, however, that microvesiculation can occur in the absence of scrambling.

Studies using red cells and platelets from a patient with a moderately severe bleeding disorder known as Scott syndrome (Sims et al., 1989; Bevers et al., 1992), suggest that these cells may be deficient in a specific membrane protein upon which lipid scrambling is dependent (Zwaal et al., 1993; Williamson et al., 1995). Despite normal calpain activation following stimulation, these erythrocytes and their resealed ghosts do not undergo normal discocyte to echiocyte shape changes, expose outer leaflet PS, or spiculate and release microvesicles in response to Ca^{2+} (Bevers et al., 1992).

The results of Zwaal et al. (1993) provide additional evidence in support of the concept that a specific membrane protein is responsible for lipid scrambling. Cytoskeleton-free vesicles released from RBC through expansion of the cells outer monolayer by insertion of dimyristoyl-phosphatidylcholine (DMPC) from sonicated liposomes, contained scramblase activity that was preferentially sorted from the parent cell. This preferential sorting is similar to the selective sorting of other membrane proteins which occurs upon vesiculation (Butikofer et al., 1989; Hagelberg & Allan, 1990).

Identification of the Aminophospholipid Transport Protein

A significant amount of data suggests that at least two different red blood cell proteins play a role in aminophospholipid transport. These are the 110–120 kDa Mg^{2+}-ATPase proposed by Zachowski and Devaux (Zachowski, Henry & Devaux, 1989; Morrot et al., 1990; Auland et al., 1994), and the 32-kDa Rh-associated proteins suggested by Connor & Schroit (Schroit et al., 1990).

Based on the transport activity of spin-labeled lipids in ATP-depleted and vanadate-treated RBC, Devaux and collaborators suggested that Mg^{2+}-ATPase, the function of which was previously unknown, is likely to function as an aminophospholipid translocase (Seigneuret & Devaux, 1984). This was inferred from observations that the activity of partially purified Mg^{2+}-ATPase was stimulated by PS and was sensitive to the known inhibi-

tors of aminophospholipid translocation, vanadate, *N*-ethylmaleimide (NEM) and Ca^{2+}. Because its inhibition also prevents echinocyte to biconcave disc transitions (Xu et al., 1991; Morris et al., 1992, 1993), this Mg^{2+}-ATPase is probably involved in the regulation of erythrocyte shape as well.

Some direct support for the participation of a Mg^{2+}-ATPase in lipid movement has recently been obtained by Auland and coworkers (1994). They showed that a partially purified 110-kDa ATPase reconstituted into proteoliposomes supported a moderate degree of ATP- and lipid composition-dependent spin-labeled PS movement. Because the inside/outside orientation of the reconstituted protein could not be controlled however, the direction of PS movement was determined by adding ATP to only the external medium. The addition of hydrolyzable ATP activated enzyme in the outside face of the liposomes and facilitated spin-labeled PS movement from the inner-to-outer leaflet of the vesicles, a finding consistent with PS transport toward the lumenal (ATP side) side of red cell membranes. These results directly demonstrate reconstitution of transporter activity and indeed suggest the participation of this Mg^{2+}-ATPase in lipid movement. However, the reorientation of PS and PE was incomplete and raises the possibility that other components might be required for full transport activity.

Interestingly, protocols used to isolate the 110–120 kDa Mg^{2+}-ATPase also result in the purification of 32 kDa and 50 kDa proteins (Zimmerman & Daleke, 1993). Studies using ^{125}I-labeled lipid substrate analogues and transport inhibitors prompted Schroit and colleagues to suggest that 32 kDa, Rh-associated polypeptides are involved in aminophospholipid translocation. They showed that photoactivation of RBC incubated with ^{125}I-azido-lipids resulted in two types of labeling: Specific labeling of a 32 kDa polypeptide by the PS analogue and random labeling of proteins by the PC analogue (Schroit et al., 1987). When the same cells were treated with known inhibitors of aminophospholipid translocation, the distribution of the proteins labeled by the photoactivated PS became random and resembled that of the PC-treated cells (Connor & Schroit, 1991). Similarly, treatment of RBC with ^{125}I-labeled pyridyldithiolethylamine (PDA), a potent inhibitor of lipid transport, also labeled 32 kDa polypeptides (Connor & Schroit, 1988). Other experiments showed that the polypeptide(s) labeled by both reagents were the same because inhibition of transport activity abrogated labeling with the photolabeled substrate (Connor & Schroit 1991).

Observations of the similarities between several key features of the putative 32 kDa aminophospholipid transport protein and Rh polypeptides led to the suggestion that the two may be analogous (Saboori, Smith & Agre, 1988; Connor & Schroit, 1989). The proteins that compose the Rh membrane cluster possess the typical membrane-spanning architecture common to other membrane channels and transporters (Avent et al., 1990; Cherif-Zahar et al., 1990). In addition, both have similar molecular weights, are sensitive to sulfhydryl reagents, are nonglycosylated, and are functionally dependent upon membrane lipids (Agre & Cartron, 1991). Evidence directly supporting their association came from immunoprecipitation experiments which showed that monoclonal Rh antibodies immunoprecipitated 32 kDa proteins labeled with both the transportable substrate ^{125}I-azido-PS and with the iodinated transport inhibitor ^{125}I-PDA (Schroit et al., 1990). Furthermore, incubation of red blood cells with dilaurylphosphatidylcholine results in the release of plasma membrane vesicles that contain no cytoskeletal components but are enriched in 32 kDa proteins and Rh polypeptides (Bruckheimer et al., 1995). When these vesicles are produced under conditions that maintain high levels of ATP (Beleznay et al., 1993), the vesicles transport PS in a manner indistinguishable from that of normal cells.

Other data suggest, however, that transport activity and Rh are distinct. Data showing that Rh_{null} cells, which do not express Rh antigen, have normal PS transport activity and that Rh antibodies do not inhibit lipid movement, suggest that transport activity and Rh are independent (Schroit et al., 1990). These data might also indicate that the putative 32 kDa transporter is distinct albeit tightly associated with the Rh complex and inseparable by immunoprecipitation from Triton-X100 solubilized membrane complexes (Connor et al., 1992*b*). Indeed, hydrodynamic analysis of Triton X-100 solubilized membranes which showed that Rh proteins are present in a large oligomeric complex of ~170 kDa cannot be explained by the presence of a single 32 kDa polypeptide chain (Hartel-Schenk & Agre, 1992). This issue will be resolved upon molecular identification of the PS transporter.

It should be emphasized that the designation of transport activity to the 110 kDa Mg^{2+}-ATPase or to 32 kDa polypeptides is not necessarily mutually exclusive. In addition to ATPase activity, transbilayer lipid transport is likely to require a structure that forms a protective environment for the lipid's polar head group to cross through the hydrophobic bilayer. Although a single ATPase could fulfill this requirement, the functional ''transporter'' could be a complex of an ATPase and a distinct multispanning membrane polypeptide that forms a transmembrane channel (Schroit & Zwaal, 1991; Schroit, 1994). This hypothesis is supported by data which show that the movement of fluorescent PS analogues required the participation of a 32 kDa polypeptide and a distinct protein located at the cell's endofacial surface (Connor & Schroit, 1990) and by unrelated studies which showed that Rh protein forms a complex or

cluster with several membrane components (Bloy et al., 1988).

Function of the Aminophospholipid Transporter

The maintenance and regulation of a particular transmembrane distribution of lipids between both leaflets of the cell's plasma membrane is tightly regulated during the lifespan of the cell. Because the cell expends energy to keep its asymmetry through the consumption of ATP, it is logical to conclude that reorientation of the phospholipids would have serious consequences. Indeed, PS in the cell's outer leaflet does result in the expression of altered surface properties that influence and regulate the cell's interaction with its environment.

Because phospholipid transport is apparent only when the lipids do not occupy the correct side of the membrane bilayer, the actual role of the translocase may be to regenerate phospholipid asymmetry after its perturbation. Although the translocase would correct for any backflow or leak of PS to the cell's outer monolayer, its major function could be to restore phospholipid asymmetry when major structural rearrangements of the membrane occur. Experiments with activated platelets and Ca^{2+}/ionophore-treated red cells have shown that lipid scrambling that occurs during vesiculation and possibly fusion events can only be corrected by an active aminophospholipid translocase (Comfurius et al., 1990; Tilly et al., 1990). This finding suggested that the translocase may play a principal role in preserving membrane asymmetry in cells undergoing endocytosis or exocytosis, cell division, and other membrane fusion events and predicts a corrective role for the translocase in reestablishing membrane asymmetry following its deterioration (Schroit & Zwaal, 1991).

Physiology of Altered Membrane Lipid Asymmetry

Membranes containing PS provide a catalytic surface that serves as a point of assembly for the coagulation factors Va and Xa into the prothrombinase complex (Rosing et al., 1985), enhance Ca^{2+}-mediated membrane fusion events (Schewe et al., 1992; Lacy, 1993) and trigger PS-dependent cell recognition and subsequent engulfment by phagocytic cells (Schroit et al., 1984; McEvoy, Williamson & Schlegel, 1986; Fadok et al., 1992*a*).

Studies in platelets and red cells have indicated that these events are the result of elevated intracellular Ca^{2+} which destroys membrane asymmetry. In certain cases, Ca^{2+} influx is accompanied by membrane fusion events that result in the release of highly procoagulant microvesicles from the cell surface.

In contrast to the rapid redistribution of membrane lipids that occurs upon platelet activation and Ca^{2+} influx into red cells, the intrinsic appearance of outer leaflet PS seems to be progressive and cumulative. Determination of external PS by prothrombinase assay on RBC isolated according to age on self-forming Percoll gradients[1] revealed an age/density-dependent accumulation of endogenous PS on the cell's outer leaflet (Connor et al., 1994). In addition, analysis of the transport rates and equilibrium distribution of exogenously-supplied fluorescent lipid analogues showed decreased aminophospholipid transport activity and increasingly symmetric equilibrium distributions with increasing red cell age (Connor et al., 1994). Using the PS/Ca^{2+}-dependent binding assay of isotopically-labeled annexin V, Tait and Gibson (1994) showed that endogenous PS redistributed to the outer leaflet of red cells in a manner that was proportional to the duration of storage. Aminophospholipid transport activity is also decreased after storage in vitro (Herrmann & Devaux, 1990; Geldwerth et al., 1993). Studies on the topography of spin-labeled lipids in platelets also indicated that stored cells had lost their ability to transport PS and were less asymmetric than young cells (Gaffet, Basse & Bienvenue, 1994). Interestingly, decreased aminophospholipid translocase activity and lipid scrambling can be modeled by the oxidation of red cells with peroxide or malonyldialdehyde (Jain, 1984, Herrmann & Devaux, 1990) and by amphipath-induced vesiculation (Diaz. Morkowski. & Schroit, 1996), respectively.

Cells undergoing programmed cell death/apoptosis also accumulate increasing amounts of cell surface PS. This has been shown by the PS-dependent Russel viper venom assay, by amine-specific PS labeling (Fadok et al., 1992*a*,*b*), and, more recently, by direct measurement of fluorescein-conjugated annexin V binding (Dachary-Prigent et al., 1993; Koopman et al., 1994). Similar to platelet activation, one of the first manifestations of apoptosis is reorientation of PS to the cells outer leaflet (Martin et al., 1995) a process that also seems to involve activation of scramblase and inactivation of the translocase (Verhoven, Schlegel & Williamson, 1995). PS is also detectable on the surface of certain tumor cells (Utsugi et al., 1991). In contrast to its appearance upon aging and programmed cell death however, differentiation to a normal nontumorigenic phenotype (Connor et al., 1989), results in its reorientation to the cell's inner leaflet.

Other studies have suggested that the exposure of PS in the outer membrane leaflet is a prerequisite for making a cell fusion competent. According to this theory, bilayer imbalance caused by the preferential movement of

[1] Separation of RBC on self-forming Percoll gradients isolates distinct cell populations based on their densities (Lutz et al., 1992) which are assumed to be directly correlated with their ages (Piomelli & Seaman, 1993).

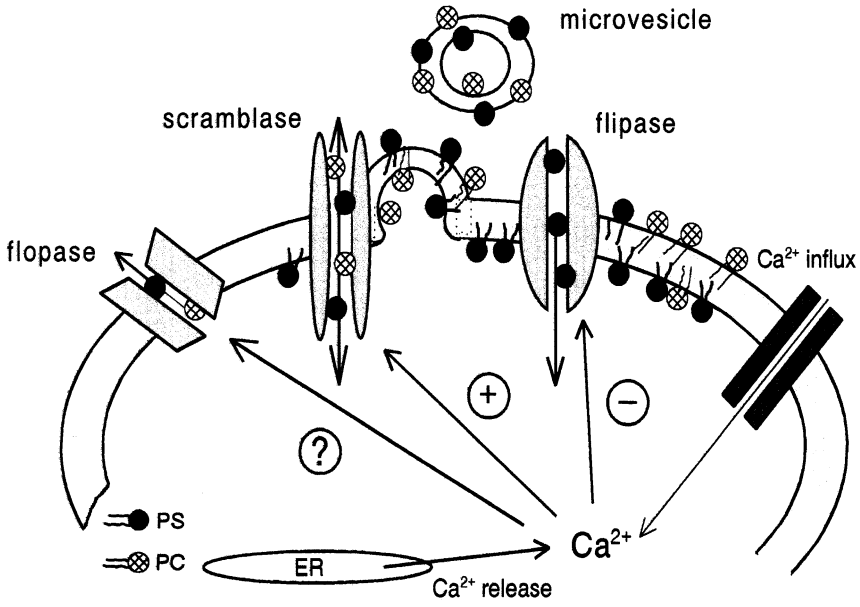

Fig. 1. Possible targets for Ca^{2+} action in the generation of PS asymmetry. This model predicts that elevated intracellular Ca^{2+} induces PS randomization across the cell's plasma membrane and the formation of microvesicles by providing a stimulus that positively and negatively regulates scramblase and flipase activities, respectively. At physiological Ca^{2+} concentrations, PS asymmetry is promoted because of an active flipase but inactive scramblase. Depending upon the type of cell, elevated intracellular Ca^{2+} levels can be achieved by the influx and accumulation of extracellular Ca^{2+} or by its release from intracellular stores after activation with agonists. This can also be achieved experimentally with extracellular Ca^{2+} and ionophores or by the accumulation of cytosolic Ca^{2+} from intracellular stores by inhibition of Ca^{2+}-ATPases with thapsigargin.

lipid to one leaflet would induce membrane invaginations which, together with the fusion competence of PS, create conditions conducive to endocytosis and exocytosis (Devaux, 1990; 1991).

Control of Membrane Lipid Asymmetry

In view of the dramatic physiologic consequences that occur upon the exposure of PS in activated platelets, aging red cells and apoptotic cells, lipid sidedness must be precisely controlled and highly regulated during the cell's lifespan. A considerable amount of evidence indicates that increases in the concentration of free cytosolic Ca^{2+} is involved in all major platelet functional responses, in particular the expression of PS-dependent procoagulant activity and the formation of microvesicles. In platelets and red cells treated with Ca^{2+}/ionophore, scramblase activity can be clearly distinguished from the translocase by its energy independence, opposing response to intracellular Ca^{2+} and bidirectionality (Comfurius et al., 1990; Basse, Gaffet & Bienvenue, 1993; Smeets et al., 1994). The induction of procoagulant activity and microvesiculation can also be brought about by inhibiting the Ca^{2+} pump (Williamson et al., 1995, Dachary-Prigent et al., 1995). For example, the Ca^{2+} pool released in platelets treated with thapsigargin and thrombin (which by itself results in granule release without PS externalization) results in lipid scrambling (Smeets et al., 1993). These events may be synonymous with those that occur in RBC, where an age-dependent increase in cytosolic free Ca^{2+}, (Aiken, Satterlee & Galey, 1992) and a decrease in Ca^{2+}-ATPase activity (Vincenzi & Hinds, 1988; Samaja et al., 1989), is associated with the expression of cell surface PS (Connor et al., 1994). This suggests that the mechanism responsible for scrambling induced by Ca^{2+}-ATPase inhibitors shares a common pathway with that induced by Ca^{2+}/ionophore and physiologic agonists.

Similar Ca^{2+}-regulated mechanisms also seem to be important in the induction of apoptosis (McConkey & Orrenius, 1994). Apoptosis and the characteristic blebbing/vesiculation of the cell's membrane can be induced directly with Ca^{2+}, either by introducing exogenous Ca^{2+} with ionophores or by the release of Ca^{2+} from intracellular stores with thapsigargin (McConkey et al., 1989*a*; Jiang et al., 1994). Apoptosis can also be initiated by stimulation of the T-cell receptor in thymocytes (Smith et al., 1989; McConkey et al., 1989*b*) and by stimulation of glutamate (NMDA) receptors in neurons (Choi, 1992), both of which trigger sustained Ca^{2+} increases. Consistent with the ability of EGTA to stop scrambling and activate translocase in platelets and red cells (Comfurius et al., 1990; Williamson et al., 1995), Ca^{2+} chelators and Ca^{2+} channel blockers delay or abolish apoptosis in several model systems (McConkey & Orrenius, 1994).

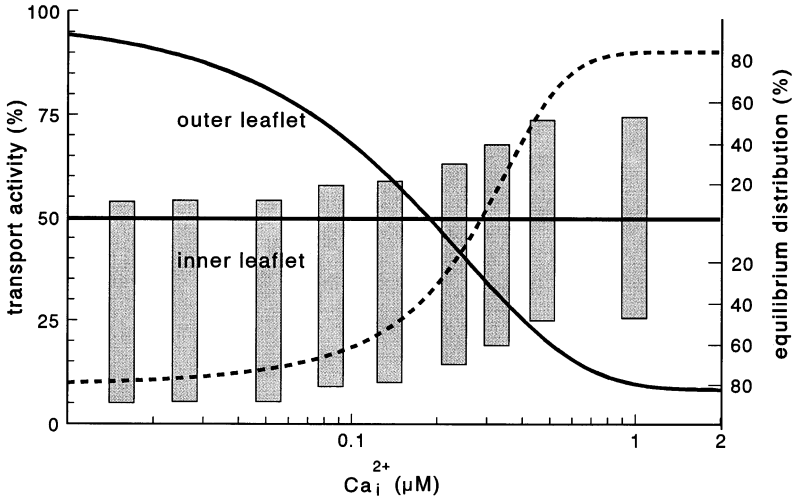

FIG. 2. Transbilayer PS distribution in response to intracellular Ca^{2+}. This model integrates data on the transport rates and equilibrium distribution of spin-labeled PS in red cells from Bitbol et al. (1987) and the distribution of endogenous PS in platelets from Dachary-Prigent et al. (1995). The continuous and broken lines show the inverse dependence of flipase and scramblase activity on the concentration of intracellular Ca^{2+}, respectively. The hypothetical curve for scramblase activity reflects the response of red cells and platelets to physiological (<0.05 μM) and elevated (>0.2 μM) intracellular Ca^{2+} and assumes the response to be sigmoidal. The bars show that the equilibrium distribution of spin-labeled PS favors the inner leaflet at physiological concentrations and becomes more symmetric upon Ca^{2+} elevation (data from Bitbol et al., 1987).

One can postulate that separate Ca^{2+}-regulated transport activities are required for translocating PS across the membrane bilayer; one induced by the scramblase, and the other by the translocase. These opposing activities generate, respectively, a random symmetric PS distribution and a highly ordered asymmetric PS distribution. This conceptual model (Fig. 1) argues that PS distribution is controlled by the Ca^{2+}-dependent synchronous and cooperative activation of the PS-specific aminophospholipid translocase, the nonspecific flopase, and the lipid scramblase. The model predicts that PS asymmetry is inversely proportional to the concentration of free cytosolic Ca^{2+}. At physiologic (low) Ca^{2+} concentrations, the aminophospholipid translocase and nonspecific flopase are active and the lipid scramblase is inactive. Conversely, at high Ca^{2+} concentrations the lipid scramblase is active and the aminophospholipid translocase is inactive (Fig. 2). Thus, under normal conditions the exposure of PS by energy-dependent flop, spontaneous lipid leak, membrane fusion, or exocytotic or endocytotic events would be corrected by the ATP-dependent translocase. If intracellular Ca^{2+} concentrations begin to increase, however, a concomitant decrease in the activity of the aminophospholipid translocase and an increase in the activity of the lipid scramblase would ensue, resulting in increased PS at the cell's outer leaflet. Conceivably, such a mechanism can accommodate a wide range of transbilayer PS distributions.

Conclusions

Phosphatidylserine is not only a simple structural component of the plasma membrane, but it also plays an important role in blood coagulation, aging, apoptosis, membrane fusion, and cell-cell recognition. Because the transport of lipids is a complex process that cannot be attributed to a single dominant mechanism, a unifying model for the establishment and maintenance of PS sidedness cannot account for all the existing data. Although certain pathways of transbilayer PS movement have been determined and several candidate proteins have been identified, more studies are clearly needed. The recent selection of a mutant cell line which has a defect in its ability to transport PS (Hanada & Pagano, 1995) should promote the ultimate identification and characterization of all the components and mechanisms responsible for the control and establishment of leaflet-specific lipid composition.

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References

Agre, P., Cartron, J.P. 1991. *Blood* **78:**551–563

Aiken, N.R., Satterlee, J.D., Galey, W.R. 1992. *Biochim. Biophys. Acta* **1136:**155–160

Allan, D., Mitchell, R.H. 1975. *Nature* **258:**348–349

Auland, M.E., Roufogalis, B.D., Devaux, P.F., Zachowski, A. 1994. *Proc. Natl. Acad. Sci. USA* **91:**10938–10942

Avent, N.D., Ridgwell, K., Tanner, M.J.A., Anstee, D.J. 1990. *Biochem. J.* **271:**821–825

Basse, F., Gaffet, P., Bienvenue, A. 1994. *Biochim. Biophys. Acta* **1190:**217–224

Basse, F., Gaffet, P., Rendu, F., Bienvenue, A. 1993. *Biochemistry* **32:**2337–2344

Beleznay, Z., Zachowski, A., Devaux, P.F., Navazo, M.P., Ott, P. 1993. *Bichemistry* **32:**3146–3152

Bergmann, W.L., Dressler, V., Haest, C.W.M., Deuticke, B. 1984. *Biochim. Biophys. Acta* **772:**328–336

Bevers, E.M., Tilly, R.H.J., Senden, J.M.G., Comfurius, P., Zwaal, R.F.A. 1989. *Biochemistry* **28:**2382–2387

Bevers, E.M., Verhallen, P.F.J., Visser, A.J.W.G., Comfurius, P., Zwaal, R.F.A. 1990. *Biochemistry* **29:**5132–5137

Bevers, E.M., Wiedmer, T., Comfurius, P., Shattil, S.J., Weiss, H.J., Zwaal, R.F.A., Sims, P.J. 1992. *Blood* **79:**380–388

Bevers, E.M., Wiedmer, T., Comfurius, P., Zhao, J., Smeets, E.F., Schlegel, R.A., Schroit, A.J., Weiss, H.J., Williamson, P., Zwaal, R.F.A., Sims, P.J. 1995. *Blood* **86:**1983–1991

Bitbol, M., Devaux, P.F. 1988. *Proc. Natl. Acad. Sci. USA* **85:**6783–6787

Bitbol, M., Fellmann, P., Zachowski, A., Devaux, P.F. 1987. *Biochim. Biophys. Acta* **904:**268–282

Bloy, C., Blanchard, D., Dahr, W., Beyreuther, K., Salmon, C., Cartron, J.P. 1988. *Blood* **72:**661–666

Bretscher, M.S. 1972. *Nature New Biol.* **236:**11–12

Bruckheimer, E.M., Gillum, K.D., Schroit, A.J. 1995. *Biochim. Biophys. Acta* **1235:**147–154

Butikofer, P., Kuypers, F.A., Xu, C.M., Chiu, D.T.-Y., Lubin, B. 1989. *Blood* **74:**1481–1485

Chang, C.-P., Zhao, J., Wiedmer, T., Sims, P.J. 1993. *J. Biol. Chem.* **268:**7171–7178

Cherif-Zahar, B., Bloy, C., Le Van Kim, C., Blanchard, D., Bailly, P., Hermand, P., Salmon, C., Cartron, J.P., Colin, V. 1990. *Proc. Natl. Acad. Sci. USA* **87:**6243–6247

Choi, D.W. 1992. *Science* **258:**241–243

Comfurius, P., Senden, J.M.G., Tilly, R.H.J., Schroit, A.J., Bevers, E.M., Zwaal, R.F.A. 1990. *Biochim. Biophys. Acta* **1026:**153–160

Connor, J., Bucana, C., Fidler, I.J., Schroit, A.J. 1989. *Proc. Natl. Acad. Sci. USA* **86:**3184–3188

Connor, J., Schroit, A.J. 1987. *Biochemistry* **26:**5099–5105

Connor, J., Schroit, A.J. 1988. *Biochemistry* **27:**848–851

Connor, J., Schroit, A.J. 1989. *Biochemistry* **28:**9680–9685

Connor, J., Schroit, A.J. 1990. *Biochemistry* **29:**37–43

Connor, J., Schroit, A.J. 1991. *Biochim. Biophys. Acta* **1066:**37–42

Connor, J., Bar-Eli, M., Gillum, K.D., Schroit, A.J. 1992*b. J. Biol. Chem.* **267:**26050–26055

Connor, J., Pak, C.C., Schroit, A.J. 1994. *J. Biol. Chem.* **269:**2399–2404

Connor, J., Pak, C.H., Zwaal, R.F.A., Schroit, A.J. 1992*a. J. Biol. Chem.* **267:**19412–19417

Dachary-Prigent, J., Freyssinet, J.M., Pasquet, J.M., Carron, J.C., Nurden, A.T. 1993. *Blood* **81:**2554–2565

Dachary-Prigent, J., Pasquet, J.M., Freyssinet, J.M., Nurden, A.T. 1995. *Biochemistry* **34:**11625–11634

Daleke, D.L., Huestis, W.H. 1985. *Biochemistry* **24:**5406–5416

Devaux, P.F. 1990. *NIPS* **5:**53–58

Devaux, P.F. 1991. *Biochemistry* **30:**1163–1173

Devaux, P.F. 1992. *Annu. Rev. Biophys. Biomol. Struct.* **21:**417–439

Devaux, P.F., Zachowski, A. 1994. *Chem. Phys. Lipids* **73:**107–120

Diaz, C., Morkowski, J., Schroit, A.J. 1996. *Blood* **87:**2956–2961

Fadok, V.A., Savill, J.S., Haslett, C., Bratton, D.L., Doherty, D.E., Campbell, P.A., Henson, P.M. 1992*b. J. Immunol.* **149:**4029–4035

Fadok, V.A., Voelker, D.R., Campbell, P.A., Cohen, J.J., Bratton, D.L. 1992*a. J. Immunol.* **148:**2207–2216

Fox, J.E.B., Austin, C.D., Reynolds, C., Steffen, P.K. 1991. *J. Biol. Chem.* **266:**13289–13295

Gaffet, P., Basse, F., Bienvenue, A. 1994. *Eur. J. Biochem.* **222:**1033–1040

Geldwerth, D., Kuypers, F.A., Butikofer, P., Allary, M., Lubin, B.H., Devaux, P.F. 1993. *J. Clin. Invest.* **92:**308–314

Gordesky, S.E., Marinetti, G.V., Love, R. 1975. *J. Membrane Biol.* **20:**111–132

Hagelberg, C., Allan, D. 1990. *Biochem. J.* **271:**831–834

Hanada, K., Pagano, R.E. 1995. *J. Cell Biol.* **128:**793–804

Hartel-Schenk, S., Agre, P. 1992. *J. Biol. Chem.* **267:**5569–5574

Henseleit, U., Plasa, G., Haest, C.W.M. 1990. *Biochim. Biophys. Acta* **1029:**127–135

Herrmann, A., Devaux, P.F. 1990. *Biochim. Biophys. Acta* **1027:**41–46

Jain, S.K. 1984. *J. Biol. Chem.* **259:**3391–3394

Jiang, S., Chow, S.C., Nicotera, P., Orrenius, S. 1994. *Exp. Cell Res.* **212:**84–92

Koopman, G., Reutelingsperger, C.P.M., Kuijten, G.A.M., Keehnen, R.M.J., Pals, S.T. van Oers, M.H.J. 1994. *Blood* **84:**1415–1420

Kornberg, R.D., McConnell, H.M. 1971. *Biochemistry* **10:**1111–1120

Lacy, J.A. 1993. *Biochem. Soc. Trans.* **21:**51–54

Lutz, H.U., Stammler, P., Fasler, S., Ingold, M., Fehr, J. 1992. *Biochim. Biophys. Acta* **1116:**1–10

Marinetti, G.V., Crain, R.C. 1978. *J. Supramolecular Structure* **8:**191–213

Martin, D.W., Jesty, J. 1995. *J. Biol. Chem.* **270:**10468–10474

Martin, O.C., Pagano, R.E. 1987. *J. Biol. Chem.* **262:**5890–5898

Martin, S.J., Reutelingsperger, C.P.M., McGahon, A.J., Rader, J.A., van Schie, R.C.A.A., LaFace, D.M., Green, D.R. 1995. *J. Exp. Med.* **182:**1545–1556

McConkey, D.J., Hartzell, P., Nicotera, P., Orrenius, S. 1989*a. FASEB J.* **3:**1843–1849

McConkey, D.J., Hartzell, P., Perez, J.F., Orrenius, S., Jondal, M. 1989*b. J. Immunol.* **143:**1801–1806

McConkey, D.J., Orrenius, S. 1994. *Trans Cell. Biol.* **4:**370–374

McEvoy, L., Williamson, P., Schlegel, R.A. 1986. *Proc. Natl. Acad. Sci. USA* **83:**3311–3315

Mcintyre, J.C., Sleight, R.G. 1991. *Biochemistry* **30:**11819–11827

Menon, A.K. 1995. *Trends Cell Biol.* **5:**355–360

Morris, M.B., Auland, M.E., Xu, Y.-H., Roufogalis, B.D. 1993. *Biochem. Mol. Biol. Internat.* **31:**823–832

Morris, M.B., Monteith, G., Roufogalis, B.D. 1992. *J. Cell. Biochem.* **48:**356–366

Morrot, G., Hervé, P., Zachowski, A., Fellmann, P., Devaux, P.F. 1989. *Biochemistry* **28:**3456–3462

Morrot, G., Zachowski, A., Devaux, P.F. 1990. *FEBS Lett.* **266:**29–32

Pagano, R.E., Sleight, R.G. 1985. *Science* **229:**1051–1057

Phillips, D.R., Jakabova, M. 1977. *J. Biol. Chem.* **252:**5602–5605

Piomelli, S., Seaman, C. 1993. *Am. J. Hematol.* **42:**46–52

Rawyler, A., Roelofsen, B., Op den Kamp, J.A.F. 1984. *Biochim. Biophys. Acta* **769:**330–336

Rosing, J., van Rijn, J.L.M.L., Bevers, E.M., van Dieijen, G., Comfurius, P., Zwaal, R.F.A. 1985. *Blood* **65:**319–322

Saboori, A.M., Smith, B.L., Agre, P. 1988. *Proc. Natl. Acad. Sci. USA* **85:**4042–4045

Samaja, M., Rubinacci, A., DePonti, A., Portinaro, N. 1989. *Biochem. Biophys. Res. Comm.* **159:**432–438

Schewe, M., Muller, P., Korte, T., Hermann, A. 1992. *J. Biol. Chem.* **267:**5910–5915

Schroit, A.J. 1994. Cell Lipids. D. Hoekstra, editor. pp. 47–74. Academic Press, New York

Schroit, A.J., Bloy, C., Connor, J., Cartron, J.-P. 1990. *Biochemistry* **29:**10303–10306

Schroit, A.J., Madsen, J., Ruoho, A.E. 1987. *Biochemistry* **26:**1812–1819

Schroit, A.J., Tanaka, Y., Madsen, J., Fidler, I.J. 1984. *Biol. Cell* **51:**227–238

Schroit, A.J., Zwaal, R.F.A. 1991. *Biochim. Biophys. Acta* **1071:**313–329

Seigneuret, M., Devaux, P.F. 1984. *Proc. Natl. Acad. Sci. USA* **81:**3751–3755

Sims, P.J., Wiedmer, T., Esmon, C.T., Weiss, H.J., Shattil, S.J. 1989. *J. Biol. Chem.* **264:**17049–17057

Smeets, E.F., Comfurius, P., Bevers, E.M., Zwaal, R.F.A. 1994. *Biochim. Biophys. Acta* **1195:**281–286

Smeets, E.F., Heemskerk, J.W.M., Comfurius, P., Bevers, E.M., Zwaal, R.F.A. 1993. *Thromb. Haemost.* **70:**1024–1029

Smith, C.A., Williams, G.T., Kingston, R., Jenkinson, E.J., Owen, J.J.T. 1989. *Nature* **337:**181–183

Struck, D.K., Pagano, R.E. 1980. *J. Biol. Chem.* **255:**5404–5419

Sulpice, J.-C., Zachowski, A., Devaux, P.F., Giraud, F. 1994. *J. Biol. Chem.* **269:**6347–6354

Tait, J.F., Gibson, D. 1994. *J. Lab. Clin. Invest.* **123:**741–748

Tilley, L., Cribier, S., Roelofsen, B., Op den Kamp, J.A.F., van Deenen, L.L.M. 1986. *FEBS Lett.* **194:**21–27

Tilly, R.H.J., Senden, J.M.G., Comfurius, P., Bevers, E.M., Zwaal, R.F.A. 1990. *Biochim. Biophys. Acta* **1029:**188–190

Utsugi, T., Schroit, A.J., Connor, J., Bucana, C.D., Fidler, I.J. 1991. *Cancer Res.* **51:**3062–3066

Verhoven, B., Schlegel, R.A., Williamson, P. 1995. *J. Exp. Med.* **182:**1597–1601

Verkleij, A.J., Zwaal, R.F.A., Roelofsen, B., Comfurius, P., Kastelijn, D., van deenen, L.L.M. 1973. *Biochim. Biophys. Acta* **323:**178–193

Vincenzi, F.F., Hinds, T.R. 1988. *Blood Cells* **14:**139–148

Williamson, P., Bevers, E.M., Smeets, E.F., Comfurius, P., Schlegel, R.A., Zwaal, R.F.A. 1995. *Biochemistry* **34:**10448–10455

Williamson, P., Kulick, A., Zachowski, A., Schlegel, R.A., Devaux, P.F. 1992. *Biochemistry* **31:**6355–6360

Xu, Y.H., Lu, Z.Y., Conigrave, A.D., Auland, M.E., Roufogalis, B.D. 1991. *J. Cell. Biochem.* **46:**284–290

Yano, Y., Shiba, E., Kambayashi, J., Sakon, M., Kawasaki, T., Fujitani, K., Kang, J., Mori, T. 1993. *Thromb. Res.* **71:**385–396

Zachowski, A., Henry, J.-P., Devaux, P.F. 1989. *Nature* **340:**75–76

Zimmerman, M.L., Daleke, D.L. 1993. *Biochemistry* **32:**12257–12263

Zwaal, R.F.A., Comfurius, P., Bevers, E.M. 1992. *Biochim. Biophys. Acta* **1180:**1–8

Zwaal, R.F.A., Comfurius, P., Bevers, E.M. 1993. *Biochem. Soc. Trans.* **21:**248–253

Zwaal, R.F.A., Roelofsen, B., Comfurius, P., van Deenen, L.L.M. 1975. *Biochim. Biophys. Acta* **406:**83–96