Regulation of Transbilayer Plasma Membrane Phospholipid Asymmetry

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Abstract

Lipids in biological membranes are asymmetrically distributed across the bilayer; the amine-containing phospholipids are enriched on the cytoplasmic surface of the plasma membrane, while the choline-containing and sphingolipids are enriched on the outer surface. The maintenance of transbilayer lipid asymmetry is essential for normal membrane function and disruption of this asymmetry is associated with cell activation or pathologic conditions. Lipid asymmetry is generated primarily by selective synthesis of lipids on one side of the membrane. Because passive lipid transbilayer diffusion is slow, a number of proteins have evolved to either dissipate or maintain this lipid gradient. These proteins fall into three classes: 1] cytofacially-directed, ATP-dependent transporters (“flippases”), 2] exofacially-directed, ATP-dependent transporters (“floppases”), and 3] bidirectional, ATP-independent transporters (“scramblases”). The flippase is highly selective for phosphatidylserine and functions to keep this lipid sequestered from the cell surface. Floppase activity has been associated with the ABC class of transmembrane transporters. Although they are primarily non-specific, at least two members of this class display selectivity for their substrate lipid. Scramblases are inherently non-specific and function to randomize the distribution of newly synthesized lipids in the endoplasmic reticulum or plasma membrane lipids in activated cells. It is the combined action of these proteins and the physical properties of the membrane bilayer that generate and maintain transbilayer lipid asymmetry.
The transbilayer distribution of lipids across biological membranes is asymmetric (1). The choline-containing lipids, phosphatidylcholine (PC) and sphingomyelin (SM) are enriched primarily on the external leaflet of the plasma membrane or the topologically equivalent lumenal leaflet of internal organelles. In contrast, the amine-containing glycerophospholipids, phosphatidylethanolamine (PE) and phosphatidylserine (PS), are located preferentially on the cytoplasmic leaflet. Other minor phospholipids, such as phosphatidic acid (PA), phosphatidylinositol (PI), phosphatidylinositol-4-monophosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP$_2$) are also enriched on the cytofacial side of the membrane. This lipid asymmetry has been most well characterized in the erythrocyte membrane, the outer monolayer of which contains 75 –80 % of the PC and SM, 20% of the PE, PA, PI, and PIP$_2$, and no detectable PS or PIP (2-6). The distribution of glycosylsphingolipids, another significant membrane component, favors the external leaflet of the plasma membrane (7).

Loss of transmembrane phospholipid asymmetry, with consequent exposure of PS in the external monolayer, occurs in both normal and pathologic conditions. PS externalization is induced early in apoptotic cells (8) and during platelet activation (9). This perturbation results in a change in cell surface properties, including conversion to a procoagulant state (10), increased adhesion (11), increased aggregation (12) and recognition by phagocytic cells (13, 14). While these processes are essential for normal cell development and hemostasis, unregulated loss of PS asymmetry may contribute significantly to heart disease and stroke and has been associated with diseases that have high cardiovascular risk, such as diabetes (15, 16).
A number of recent reviews contain excellent discussions of lipid asymmetry (17), the role of transporters in the maintenance of lipid asymmetry (18-20) and the consequences of a loss of asymmetry (21, 22). This review will describe the role of transbilayer lipid transporters, with emphasis on their substrate specificity, or lack thereof, in the maintenance of lipid asymmetry across the bilayer of the plasma membrane.

**Generation of Transmembrane Lipid Asymmetry**

Lipid biosynthesis is inherently asymmetric. The enzymes responsible for lipid synthesis are localized typically only on one side of the membrane in which biosynthesis occurs. For the major glycerophospholipids (PS, PE, PC and phosphatidylinositol), *de novo* synthesis occurs on the cytosolic side of the endoplasmic reticulum (23). With the exception of PC, this places the newly synthesized lipids on the side of the membrane in which they are ultimately enriched in the plasma membrane. Because of the thermodynamic barrier to spontaneous transbilayer movements, these lipids should remain enriched on the cytoplasmic side of the membrane, provided that there is no perturbation to the membrane. However, the asymmetric addition of newly synthesized phospholipids to one leaflet of the bilayer generates an unstable membrane. Accumulation of lipid on one side of the membrane can induce membrane bending and consequent membrane shape changes (24-26). In addition, evidence indicates that ER and Golgi membranes may be less asymmetric than the plasma membrane (27). These problems are addressed by the presence of a lipid transporter that redistributes ER phospholipids across the membrane (28-31). Although *de novo* glycerophospholipid
synthesis is asymmetric, the action of this transporter defeats vectoral biosynthesis and results in a more random distribution of lipids across the bilayer.

Sphingolipids are localized predominately on the external leaflet of the plasma membrane. Unlike PC synthesis, sphingolipid synthesis occurs predominantly on the side of the membrane in which these lipids ultimately reside. With the exception of glucosylceramide (Glc-Cer), which is synthesized on the cytofacial side of the Golgi, all of the sphingolipids are synthesized on the lumenal surface of the ER or Golgi, including sphingomyelin, galactosylceramide and complex sugar-linked sphingolipids (7, 32, 33). Because Glc-Cer is a precursor of many glycosylsphingolipids, a mechanism must exist to transport this lipid to the lumenal surface of the ER or Golgi. A transporter that catalyzes the transbilayer movement of short chain analogs of Glc-Cer has been discovered (34, 35) that may server this function.

The selective accumulation of glycerophospholipids on one side of the plasma membrane requires that during, or as a result of, membrane trafficking from the ER to the plasma membrane that the transbilayer randomizing process be inhibited or that an asymmetry-generating process be activated. Thermodynamic considerations require an input of energy to generate, or to maintain, a transbilayer lipid gradient. Consistent with these needs, both inward- and outward ATP-dependent lipid transport activities have been discovered that selectively move lipids, across the plasma membrane. The asymmetric distribution of phospholipids in the plasma membrane may be the result of the selective trafficking or regulation of lipid transporting proteins. The retention of ATP-independent non-selective lipid transporters in the ER, combined with the trafficking of substrate-specific ATP-dependent transporters to the plasma membrane
may account for the creation of a highly asymmetric plasma membrane from the more symmetric ER and Golgi membranes. Alternatively, lipid randomizing and asymmetry generating lipid transporters may coexist in multiple membranes, but be differentially regulated. Discrimination between these models awaits the positive identification, verification of intracellular location, and characterization of the biochemical regulation of these transporters.

**Maintenance of Plasma Membrane Lipid Asymmetry by Lipid Transporters**

Once lipid asymmetry has been established it is maintained by a combination of slow transbilayer diffusion, protein-lipid interactions and protein-mediated transport. The presence of binding sites for acidic lipids, including PS, on the cytoskeletal proteins spectrin and band 4.1(36-38) and soluble membrane-binding proteins such as annexins (39) suggest that cytofacial protein-membrane interactions may play a role in sequestering PS in the cytofacial monolayer. Indeed, lipid-symmetric membranes bind cytoskeletal proteins more poorly than lipid-asymmetric membranes at low ionic strength and have lower mechanical stability (40). However, the number and magnitude of the available binding sites is not sufficient to trap PS (41-44). In addition, spectrin-depleted membranes (45) and pathologic cells with defective or deficient cytoskeletal proteins (46, 47) are capable of generating and maintaining a PS gradient. These data indicate that, although the plastic properties of the erythrocyte membrane require close association with cytofacial lipids, this interaction does not play a major role in the maintenance of lipid asymmetry.
The thermodynamic barrier to passive lipid flip-flop prevents rapid spontaneous transbilayer diffusion of phospholipids. The half time for phospholipid flip-flop is approximately several hours to days (48) and depends on the nature of the lipid and the membrane. In the human erythrocyte, flip-flop rates are dependent on phospholipid acyl chain length and degree of unsaturation (49-51). Considering that the half time of flip is much shorter than the average lifespan of most cell types, it is unlikely that this phenomenon could account for the maintenance of phospholipid asymmetry. Other perturbations to membrane structure may induce a rapid reorientation of lipids. For example, chronic in vitro hyperglycemia (16) or diabetes (52) induces the exposure of inner monolayer lipids on the surface of the plasma membrane of erythrocytes and may contribute to the vascular damage associated with this disease (53).

Perhaps the most significant contributors to the maintenance and dissipation of transbilayer lipid asymmetry are proteins that catalyze the movement of lipids across the membrane. These activities are classified according to substrate specificity, requirements for energy, and direction of transport (Figure 1). Two classes of transport activities have been described that are responsible for the ATP-dependent transport of lipids. The most well-characterized activity is the aminophospholipid translocase or “flippase” which transports PS from the outer monolayer to the cytoplasmic surface of the plasma membrane. A second activity, catalyzed by “floppases,” transport lipids in the opposite direction. The most well-characterized floppase activities have been shown to catalyze the inner-to-outer monolayer transport of short chain fluorescent lipids and the selective efflux of PC or cholesterol. Three ATP-independent and relatively non-specific scramblase activities have been reported; a plasma membrane Ca\(^{2+}\)-activated transporter,
an ER glycerophospholipid-specific transporter and an ER monohexosyl-lipid transporter.

The ultimate transbilayer distribution of lipids is determined by the specificity of the lipid transporters located in the membrane. Each of the transport activities described above displays a unique specificity or non-specificity that defines its function in the determination of lipid organization. A number of excellent reviews have surveyed this subject recently (17, 18, 20, 54). The following summarizes the current state of knowledge regarding the specificity of these transport activities and, where evidence is available, the protein(s) involved.

Flippases

Aminophospholipid flippase activity was first reported by Devaux and coworkers who measured the ATP-dependent uptake of spin labeled lipid analogs in human erythrocytes (55). Phospholipids labeled with fluorescent fatty acids, particularly 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD), have also been used extensively to study this transporter (56-58). The addition of this polar, bulky substituent to the fatty acid component of lipids may alter transporter-lipid interactions, thus questioning whether movements measured reflect the behavior of endogenous lipids. The use of these probes requires careful interpretation and has been critically evaluated recently (59, 60).

Although the transport of these reporter molecules differs somewhat from endogenous lipids, for the most part their movements reflect those of endogenous lipids, at least for aminophospholipid flippase activity. In addition to spin-labeled and fluorescent lipids, native and radiolabeled short (24, 61, 62) and long (63) chain fatty acid-containing
species have been used to measure flippase activity. These lipids may more accurately reflect the behavior of endogenous lipids.

Flippase-catalyzed transport requires ATP (24, 55) and Mg$^{2+}$ (61, 64) and is inhibited by vanadate (55). The stoichiometry of transport is approximately 1 ATP consumed per lipid transported (65). Flippase activity is sensitive to temperature and to a number of non-specific reagents, including sulfhydryl oxidizing and alkylating agents (24, 66), and histidine reagents (67). Flippase activity is also inhibited by Ca$^{2+}$ (24, 64), indicating that the activity of this enzyme may be regulated in activated cells.

The flippase is widely distributed and is present in most plasma membranes. Aminophospholipid flippase activity is ubiquitously expressed in erythrocytes (68) and has been detected in a wide variety of cell types and membranes, including platelets (24, 69), lymphocytes (70), aortic endothelial cells (71), fibroblasts (57, 72, 73), pheochromacytoma cells (74), hepatocytes (75), spermatozoa (76), synaptosomes (77), and chromaffin granules (78). Activity is present at both the apical and basolateral surfaces of polarized epithelial cells (79).

The aminophospholipid flippase is perhaps the most selective of the lipid transporters. It prefers aminophospholipids over other lipids (24, 55) and the specificity for PS is defined by each of the functional groups of the lipid (Figure 2). The amine group is absolutely required; phosphatidylhydroxypropionate, a PS analog without an amino group, is not a substrate for transport (80). The enzyme can tolerate mono-methylation of PS (80) and, to a limited extent, PE (81). However, progressive methylation of PE reduces transport significantly (81). The carboxyl group is not essential (PE is also a transport substrate), but its absence lowers the rate of transport approximately ten-fold
(82) and methyl esterification of the carboxyl group reduces transport activity significantly (80, 81). In contrast to other PS-specific proteins, such as protein kinase C (83) and the macrophage PS receptor (84, 85), the stereochemistry of the L-serine headgroup is unimportant for recognition by the flippase; both the D- and L-serine isomers are transported equally well (80, 81, 86). The glycerol backbone is another important recognition element. Although diacylglycerophosphoserine is the preferred substrate, 1,3,4-butanetriol analogs are transported with similar rates (87). Sphingolipid (81) and diether analogs (88) of PS are also recognized as transport substrates, but transport rates are reduced compared to diacylglycerophosphoserine. This indicates that there is some flexibility in lipid backbone recognition by the enzyme. However, the enzyme displays an absolute requirement for the stereochemistry of the glycerol backbone; the \(sn\)-2,3-glycerol analog of the naturally-occurring \(sn\)-1,2-glycero-lipid is not a substrate for transport (72, 80). In contrast to the polar headgroup specificity, the flippase is capable of accepting PS molecules containing fatty acids of various lengths and composition, including spin, fluorescent, and photoactivatable groups (24, 55, 58, 59, 63, 89-91), but prefers reporter groups attached to longer acyl chains (58, 59).

The identity of the aminophospholipid flippase remains elusive. Attempts at direct purification of the protein from erythrocytes have yielded preparations with varying characteristics (for review see Daleke & Lyles (92)). However, at least one of these purified candidate erythrocyte flippases (an PS-stimulated \(Mg^{2+}\)-ATPase) has been reconstituted and demonstrated to transport PS (93).

Another candidate flippase (ATPase II) has been purified and cloned from bovine chromaffin granules (94-96). Close homologs of this protein have been identified from
bovine brain (97) and human (ATP8A1; (98)) sources. These proteins are members of a new class of P-type ATPases, the P₄-ATPases (96, 99, 100). Defects in genes of this family produce alterations in ribosomal assembly (Drs2; (101, 102)), cold sensitivity in plants (ALA1; (103)), and familial intrahepatic cholestasis (ATP8B1, FIC1; (104)). Although some controversy exists regarding whether Drs2 is a flippase (102, 105) it is widely believed that the P₄-ATPase family are lipid transporters. Like the erythrocyte Mg²⁺-ATPase, these enzymes are selectively activated by PS (97, 106). Recent evidence also indicates that another member of this family (ATP10C) may be associated with Angelman syndrome, a neurological disorder in humans (107), and fat metabolism in mice (108). Although no direct evidence for transbilayer phospholipid transport has been reported, it is likely that the P₄-ATPases are involved directly or indirectly in amphipath transport. Whether this activity is involved in the maintenance of transmembrane asymmetry remains to be resolved. The high degree of specificity that the flippase demonstrates for its substrate should enable the eventual positive identification of this protein.

Floppases

The second class of ATP-dependent lipid transporters are the exofacially-directed floppases. Early studies in red blood cells revealed a non-specific outward flux pathway for NBD- and spin-labeled lipids (67, 109). It was recognized subsequently that some members of the ABC transporter superfamily are capable of transporting lipids (for recent reviews see (18, 19)).

ABC transporters are a diverse group of transporters that, in general, are responsible for the ATP-dependent export of amphipathic compounds. These include the multidrug
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resistance proteins, which export cytotoxic xenobiotics and were first discovered in drug-resistant tumor cells. Multidrug resistance proteins are also present in yeast and some members of this sub-family (C. albicans CDR1, CDR2, CDR3) have been shown to be lipid transporters (110, 111). ABC transporters are also widely expressed in prokaryotes. One of these proteins, MsbA, is an inner membrane transporter involved in lipid A export to outer membrane (112). The ATPase activity of purified MsbA is selectively activated by hexacylated lipid A (113).

Consistent with their role in general xenobiotic amphipath export, floppase proteins are, for the most part, nonspecific. However, some members of this class demonstrate a unique specificity for their respective substrate. The most well-characterized lipid floppase activities are those catalyzed by ABCA1, ABCB1, ABCB4, and ABCC1.

The ABC transporter ABCA1 (ABC1) has been shown to transport cholesterol out of cells for collection by HDL. ABCA1 is defective in Tangier’s disease (114-116), an autosomal recessive disorder characterized by low HDL levels and peripheral accumulation of cholesterol. ABCA1 has also been linked to PS efflux (117, 118) and is required for macrophage engulfment of prey in C. elegans (119). This transporter may act as a floppase for both cholesterol and PS. Whether cholesterol and PS efflux are linked is unclear, but this protein is likely an efflux transporter and is not involved in the maintenance of lipid asymmetry.

ABCB1 (MDR1) is a ubiquitous multidrug resistance xenobiotic transporter and is a lipid transporter of broad specificity. In LLC-PK1 epithelial cells, it catalyzes the efflux of NBD or short chain PC, PE, SM, Glc-Cer, and Gal-Cer (120), but not NBD-PS (121), and may also function to facilitate the movement of platelet activating factor to the cell.
surface (122). The reconstituted enzyme is similarly non-specific but, in contrast to ABCB1 \textit{in situ}, also transports NBD-PS (123). The lack of specificity exhibited by ABCB1 makes it unlikely to be a regulator of transbilayer lipid asymmetry.

ABCB4 (MDR3, mMdr2) is a selective PC transporter (120). Mice homozygous for a disruption in the mMdr2 gene do not secrete phospholipid into the bile and, as a result, develop liver disease (124). Secretory vesicles isolated from yeast transfected with mMdr2 are capable of ATP-dependent, vanadate-sensitive uptake of short chain fluorescent PC (125). Further confirmation of the role of this enzyme as a PC transporter was the demonstration of ATP-dependent PC transport, but not PE transport, in bile canilicular membranes vesicles (126). In keeping with its role in bile formation, the tissue distribution of ABCB4 is restricted to the liver and bile canilicular membrane (124, 127). Like ABCA1 and ABCB1, ABCB4 may be involved in lipid efflux, rather than in the maintenance of plasma membrane lipid asymmetry.

ABCC1 (MRP1) was identified initially as a conjugated glutathione transporter (128, 129). This enzyme was also shown to catalyze the efflux of short chain NBD phospholipid analogs, but not endogenous lipids (such as PS), from the inner to the outer monolayer of the erythrocyte (67, 109, 130, 131) and may account for the previously described efflux activity of these lipids (57, 77). Cells from an ABCC1 -/- knockout mouse show no efflux of NBD-PC or –PS but possess functional flippase activity (132). Also located on the basolateral surface of LLC-PK1 cells, ABCC1 is capable of transporting the labeled sphingolipids, NBD-Glc-Cer and NBD-SM, to the exofacial side of the membrane (133). Extensive (24-48 h) treatment of erythrocytes with inhibitors of ABCC1 causes a disruption in the distribution of NBD-labeled and native choline
phospholipids (PC and SM), but has no effect on PS or PE distribution (131). These data indicate that ABCC1 is an outwardly directed PC and sphingolipid-selective transporter.

It is interesting to note that not all ABC lipid transporters are floppases. ABCR is another ABC protein with lipid transport activity although it is a flippase, rather than a floppase. ABCR is present in retinal rod cell outer segment disc membranes and transports \( N \)-retinylidene-PE from the disc lumen to the cytofacial side of the membrane (134). This protein may serve to transport all trans-retinal to the cytoplasm for subsequent export. A deficiency in this gene leads to retinal degeneration (135, 136).

**Scramblases**

Rather than assist in the maintenance of lipid asymmetry, scramblases function to degrade transbilayer phospholipid gradients by catalyzing energy-independent bidirectional transbilayer transport. Three scramblase activities have been reported; two are involved in dissipating lipid gradients in biogenic membranes and the third is activated by \( Ca^{2+} \) in stimulated cells.

The ER scramblase was first described as a bi-directional transporter of PC and its metabolites (28, 137) and has been subsequently shown to be relatively non-specific (29, 138). Transport activity has been reconstituted from crude (30, 137) and purified (31) ER membrane proteins. The previously described mono-hexosylsphingolipid transporter is also bidirectional and is selective for Glc-Cer or Gal-Cer (34, 35). Evidence has not been found for the activity of these transporters in the plasma membrane. Thus, they may server only to redistribute newly synthesized lipids or lipid precursors in ER and Golgi membranes.
The Ca$^{2+}$ activated scramblase plays an important role in plasma membrane reorganization in response to cell stimulation, such as that accompanying platelet activation and apoptosis. The dissipation of transbilayer asymmetry results in the exposure of PS on the surface of the cell, which activates blood clotting factors (9) and recognition of the cell by macrophages (8, 139, 140).

Putative scramblases have been purified from erythrocytes (141) and platelets (142). The erythrocyte protein (PLSCR1) has been cloned (143) and several additional isoforms have been discovered recently (144). However, blood cells from a mouse PLSCR1 knockout are not deficient in activation-induced lipid scrambling (145), suggesting that this protein may not be a scramblase. The presence of potential signaling motifs (146), protein phosphorylation sites (147, 148), and accumulation in plasma membrane lipid rafts (149), indicates that this protein may play a role in cellular signaling rather than, or in addition to, its role in lipid randomization.

The Ca$^{2+}$ activated scramblase randomizes the distribution of all of the major classes of endogenous (150) and NBD-labeled phospholipids (151). Recent evidence indicates that the scramblase prefers glycerophospholipids analogs and is sensitive to headgroup size (152). In general, this transporter is non-selective and serves primarily to express PS on the surface of activated cells.

Conclusion

The interplay between these non-selective and selective transporters results in the maintenance and, in some cases, the generation of transbilayer lipid asymmetry. Although the tissue distribution and the expression of some of these transporters is...
restricted, in general lipid asymmetry is maintained by selective inward flux of aminophospholipids and outward flux of choline- and sphingophospholipids. Non-selective scramblases in biogenic membranes equalize the distribution of newly synthesized lipids. Selective, ATP-dependent transporters maintain the asymmetric distribution of lipids in the plasma membrane. The concentration gradients generated by these transporters can be dissipated by a non-selective Ca^{2+}-activated scramblase in response to cell stimulation. Advances have been made in the identification of the proteins involved, but positive identification will require reconstitution and demonstration of lipid transport activity.
Acknowledgements

This work was supported by grants from the NIH (GM47230) and the American Heart Association.
References


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Footnotes

1 The methods for measuring transbilayer lipid asymmetry have been reviewed recently (54).

2 Using an endocytosis mutant, Nichols demonstrated a similar ATP-dependent transport activity in yeast, although NBD-PC was transported in addition to NBD-PE (153, 154). The relationship between this protein and the aminophospholipid flippase is not yet clear.
Figure Legends

Figure 1. Schematic representation of the action of transbilayer transporters in the eucharistic plasma membrane. Phosphatidylcholine (PC), sphingomyelin (SM), and sugar-linked sphingolipids (X-Sph) are enriched in the outer monolayer, while phosphatidylserine (PS) and phosphatidylethanolamine (PE) are sequestered on the cytoplasmic monolayer. This distribution is maintained by (left) an inwardly directed PS flippase. In some tissues an outwardly directed PC or cholesterol floppase (middle) is responsible for the efflux of these lipids. A non-specific, Ca\textsuperscript{2+}-stimulated scramblase (right) randomizes lipid distribution in activated cells.

Figure 2. Structural determinants of substrate specificity by the aminophospholipid flippase. \( R_1, R_2 = \) acyl preferred, but alkyl accepted; length and unsaturation independent; fluorescent, spin label, and photoaffinity groups also accepted; \( R_3 = H\) or methyl; \( n = 1-2 \).
# Tables

Table 1. Lipid Specificity of Transbilayer Lipid Transporters

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<th>Class</th>
<th>Protein</th>
<th>Specificity</th>
<th>Ref.</th>
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<tr>
<td>Flippases</td>
<td>$P_4$-ATPases</td>
<td>amphiphaths</td>
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<td></td>
<td>erythrocyte Mg$^{2+}$-ATPase</td>
<td>PS</td>
<td>(92)</td>
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<td></td>
<td>ABCR</td>
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<td>Scramblases</td>
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<tr>
<td></td>
<td>ER flippase</td>
<td>none</td>
<td>(28-31)</td>
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Figure 1
Figure 2
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ERRATA

In the article “Regulation of transbilayer plasma membrane phospholipid asymmetry” by David L. Daleke, published in the February 2003 issue of the *Journal of Lipid Research* (Volume 44, pages 233–242), in Fig. 2 the stereochemistry of the glycerol C2 position is not rendered correctly. The stereochemistry described in the text (sn-1,2-glycerol configuration) is shown in the corrected Fig. 2 below.