ATP-Dependent Transport of Phosphatidylserine Analogues in Human Erythrocytes†

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Received June 30, 2006; Revised Manuscript Received November 13, 2006

ABSTRACT: The plasma membrane of most cells contains a number of lipid transporters that catalyze the ATP-dependent movement of phospholipids across the membrane and assist in the maintenance of lipid asymmetry. The most well-characterized of these transporters is the erythrocyte aminophospholipid flippase, which selectively transports phosphatidylserine (PS) from the outer to the inner monolayer. Previous work has demonstrated that PS and to a lesser extent phosphatidylethanolamine (PE) are substrates for the flippase and that other phospholipids move across the membrane only by passive flip-flop. The present study re-evaluates these results. The incorporation and transbilayer movement of a number of short-chain (dilauroyl) phospholipid analogues in human erythrocytes was measured by observing lipid-induced changes in cell morphology, and the effect of an ATPase inhibitor (vanadate) and a sulfyhdryl reagent (*N*-ethylmaleimide) was determined. Incubation of cells with these lipids causes the rapid formation of echinocytes, because of the accumulation of the lipid in the outer monolayer. While dilauroylphosphatidylcholine-treated cells retained this shape, cells treated with *sn*-1,2-DLP-L-S, *sn*-1,2-DLP-D-S, or *N*-methyl-DLPS rapidly changed morphology to stomatocytes, which is consistent with the transport and accumulation of the lipid in the inner monolayer. A similar, although slower, stomatocytic shape change was induced by *sn*-2,3-DLP-L-S. Other lipids that were tested (dilauroylphosphatidylhydroxypropionate, dilauroylphosphatidylhomoserine, DLPS-methyl ester, or *sn*-2,3-DLP-D-S) reverted to discocytes only. In all cases, pretreatment with vanadate or *N*-ethylmaleimide inhibited the conversion of echinocytes to discocytes or stomatocytes. This is the first report of a protein- and energy-dependent pathway for the inwardly directed transbilayer movement of lipids other than PS and PE in the erythrocyte membrane and suggests that the flippase has broader specificity for substrates or that other lipid transporters are present.

The asymmetric distribution of phospholipids in biological membranes is sustained by the concerted action of transmembrane transporters of differing directionality and specificity (*1*). Perhaps the most well-characterized transporter is the aminophospholipid translocase or "flippase" of the erythrocyte membrane, which catalyzes the energy-dependent selective transport of aminophospholipids from the exofacial side to the luminal/cytoplasmic side of the membrane (*2*, *3*). Transport of aminophospholipids is an ATP-dependent process, and the flippase requires a hydrolyzable form of Mg^{2+}-ATP (*2*, *4*). Transport is inhibited by calcium (*4*, *5*) and sulfhydryl-oxidizing (*2*) and -alkylating reagents (*6*).

Phosphatidylserine (PS)[1] is the preferred substrate of the flippase (*2*, *3*, *7*, *8*), although phosphatidylethanolamine (PE) is also transported but at a rate 10-fold slower than PS (*3*, *9*). Substrate recognition by the flippase is highly selective and recognizes the glycerol and headgroup moieties of the lipid but not the fatty acid composition. PS molecules with long and short fatty acyl chains (*2*, *3*, *10*), spin or fluorescent-labeled fatty acyl chains (*9*, *11*, *12*), or ether linkages are transported, although the latter are transported at a slower rate (*13*). The structure of the glycerol backbone is a key recognition element in the transport of PS; replacing the diacylglycerol moiety with ceramide reduces the transport by 100-fold, but extending the length of the glycerol moiety by replacing it with 1,3,4-butanetriol does not affect transport (*8*, *14*). Removal of one fatty acyl chain (lyso PS) eliminates ATP-dependent transport of PS (*2*), but transport of PS containing a fatty acid as short as acetate is supported (*8*). Recognition of the glycerol group is stereospecific. While the naturally occurring *sn*-1,2-glycerol isomers of PS are substrates for transport, the *sn*-2,3-glyerol isomers of PS are not (*15*, *16*). The flippase recognizes only certain elements of the polar headgroup. The amine group of PS is essential, but monomethylation is tolerated (*15*). Progressive methylation of PE further reduces the transport of this lipid; PC (*N*,*N*,*N*-trimethyl-PE) is not transported (*8*, *15*). Esterification of the carboxyl group of PS also reduces transport of this lipid significantly (*8*, *15*).

The present studies were undertaken to re-evaluate the ability of PS structural and stereochemical analogues to move across the erythrocyte membrane. Using short-chain lipid

† This work was supported by grants from the American Heart Association, National Division, and the Walther Cancer Institute, Indianapolis, IN.

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[1] Abbreviations: PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin; DLPS, dilauroylphosphatidylserine; DLPC, dilauroylphosphatidylcholine; rac-DLPC, 1,2-didodecanoyl-rac-glycero-3-phosphocholine; DLPP, dilauroylphosphatidylhydroxypropionate; DLPhS, dilauroylphosphatidylhomoserine; DLPS-*N*-Me, *N*-methyl-dilauroylphosphatidylserine; DLPS-*O*-Me, dilauroylphosphatidylserine-methyl ester; BSA, bovine serum albumin; CM-52, carboxymethyl-52; TLC, thin-layer chromatography; NEM, *N*-ethylmaleimide.

10.1021/bi061333x CCC: $37.00
Published on Web 02/01/2007

analogues, we report that, with the exception of PC, these lipid analogues were capable of moving across the erythrocyte membrane by a vanadate- and *N*-ethylmaleimide (NEM)-sensitive mechanism, although most did not accumulate in the inner monolayer. These data indicate that a wider variety of lipids is translocated across the erythrocyte membrane by an ATP-dependent protein-mediated process than previously recognized. These data further indicate either that the PS flippase is more flexible in the recognition of its substrate than previously determined or that the erythrocyte possesses multiple flippases of differing specificities.

MATERIALS AND METHODS

Materials. Dilauroylphosphatidylcholine (DLPC) was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Bovine serum albumin (BSA, fatty acid free), 1,2-didodecanoyl-rac-glycero-3-phosphocholine (rac-DLPC), phospholipase D from *Streptomyces* species, phospholipase A_2 from *Naja naja*, L-serine, D-serine, L-homoserine, L-serine methyl ester, and fluorescamine were purchased from Sigma Chemical Co. (St. Louis, MO). *N*-Methyl-L-serine was purchased from Advanced Chemtech, Inc. (Louisville, KY). 3-Hydroxypropionic acid was purchased from TCI America (Portland, OR). Carboxymethylcellulose 52 was purchased from Whatman International Ltd. (Hillsboro, OR). Thin-layer chromatography (TLC) plates (silica HL) were purchased from Analtech, Inc. (Newark, DE). All other chemicals were of reagent grade.

Preparation of Erythrocytes. Human erythrocytes were obtained from healthy volunteers by venipuncture into citrate buffer (87.5 mM sodium citrate and 17.5 mM citric acid). Erythrocytes were isolated from plasma by centrifugation (200*g*, 20 min) and were washed 3 times with 4 volumes each of NaCl (0.15 M) by centrifugation (2000*g*, 10 min). Packed erythrocytes were obtained after washing with phosphate-buffered saline (PBS: 150 mM NaCl, 5 mM KCl, 6 mM Na_2HPO_4, 1.4 mM NaH_2PO_4, 1 mM $MgCl_2$, and 5 mM glucose at pH 7.4) at 16000*g* for 5 min and were kept on ice until use. Cells were used within 6 h of being collected.

Synthesis of sn-2,3-DLPC. sn-2,3-DLPC was synthesized by the selective cleavage of *sn*-1,2-DLPC with phospholipase A_2. Briefly, rac-DLPC (50 mg) was dissolved in diethyl ether (250 μL) and vortexed until clear. The lipid suspension was added to an aqueous solution of Tris at pH 8 (3 mL of 100 mM), $CaCl_2$ (250 μL of 1 M), and ethyl acetate (2 mL). The reaction was initiated by the addition of phospholipase A_2 (25 IU), and a similar amount of enzyme was added every 30 min. The reaction was stopped after 5 h by the addition of 300 μL of 100 mM ethylenediaminetetraacetic acid (EDTA). Progression of the reaction was monitored by one-dimensional silica TLC in an acidic solvent system (65:25:5 chloroform/methanol/formic acid). Approximately 40% of the rac-DLPC was cleaved. *sn*-2,3-DLPC was purified from *sn*-1,2-lyso-PC and fatty acid using CM-52 cation-exchange cellulose chromatography as described (*17*). Stereochemical purity was verified by circular dichroism.

Synthesis of PS Structural and Stereo Analogues. PS analogues were synthesized by a phospholipase D-catalyzed headgroup exchange or transphosphatidylation reaction with the appropriate alcohol (*18*) (Figure 1). *sn*-1,2-DLPC was used to synthesize the following structural and stereoisomers of PS: dilauroylphosphatidylhydroxypropionate (DLPP), dilauroylphosphatidylhomoserine (DLPhS), dilauroylphosphatidylserine methyl ester (DLPS-*O*-Me), dilauroylphosphatidyl-*N*-methyl-serine (DLPS-*N*-Me), *sn*-1,2-DLPC-L-serine, and *sn*-1,2-DLPC-D-serine. *sn*-2,3-DLPC was used to synthesize *sn*-2,3-DLPC-L-serine and *sn*-2,3-DLPC-D-serine. Synthetic lipids were purified using CM-52 column chromatography as described (*17*). All lipids were analyzed by one-dimensional silica TLC by elution with chloroform/methanol/formic acid (65:25:5) and quantified by inorganic phosphate analysis (*19*). The structures of lipids were verified by nuclear magnetic resonance (NMR) and mass spectrometry (*20*, *21*). Stereochemical purity was verified by circular dichroism. Lipids were stored in chloroform solution at −20 °C.

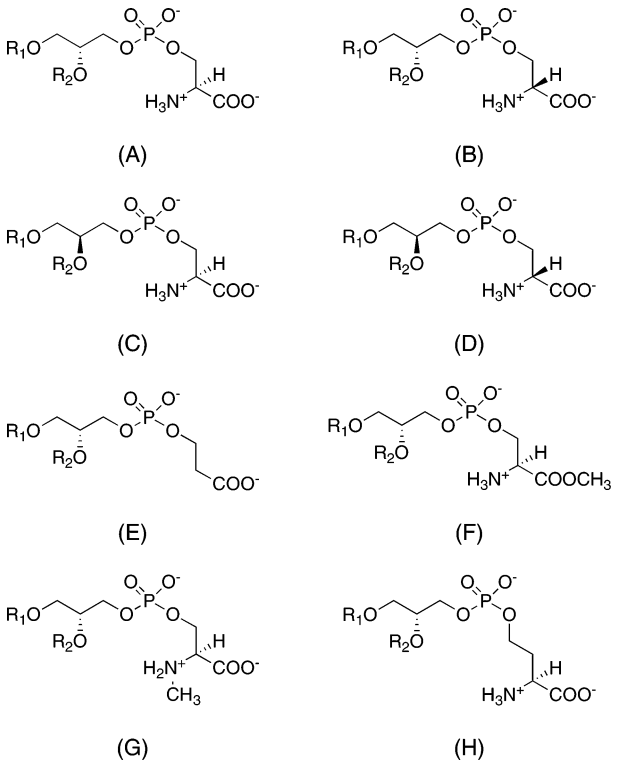

FIGURE 1: Structure of phosphatidylserine (PS) and the lipids synthesized for this study. (A) *sn*-1,2-DLPC-L-serine, (B) *sn*-1,2-DLPC-D-serine, (C) *sn*-2,3-DLPC-L-serine, (D) *sn*-2,3-DLPC-D-serine, (E) DLPP, (F) DLPS-*O*-Me, (G) DLPS-*N*-Me, and (H) DLPhS. R_1 and R_2 represent lauric acid.

Preparation of Vesicles. Small unilamellar phospholipid vesicles were prepared by first removing the solvent from aliquots of lipids in chloroform with a stream of nitrogen gas and then placing the dried lipids under vacuum for 30 min to remove traces of the solvent. PBS was then added to yield the indicated lipid concentrations. The suspensions were vortexed and sonicated in a bath sonicator for 1−3 min or until clear.

Cell-Vesicle Incubations and Fixation of Cells. Packed, washed erythrocytes were mixed with an equal volume of sonicated vesicles to yield a final hematocrit (HCT) of 50% and were incubated in a shaking water bath at 37 °C. At the indicated times, cells were fixed by mixing 5 μL of cell suspension with 45 μL of 0.5% glutaraldehyde prepared in 0.15 M NaCl.

Determination of the Morphological Index (MI) of Fixed Erythrocytes. Fixed erythrocytes were viewed by light microscopy under bright field illumination, and cell morphology was indexed as reported (*5*). Crenated cells or echinocytes were graded from +1 to +5; discocytes were graded 0; and invaginated cells or stomatocytes were graded from −1 to −4. The average score of a field of 100 erythrocytes was designated as the MI.

Vanadate and NEM Treatments. For vanadate pretreatments, packed erythrocytes were suspended at 50% HCT in PBS containing sodium orthovanadate (250 μM). The suspension was agitated at 37 °C for 15 min, and cells were pelleted by centrifugation (16000*g*, 5 min). The supernatant was removed, and the cells were resuspended at 50% HCT with sonicated lipid vesicles and vanadate (250 μM). In some experiments, untreated cells were incubated with lipid for 10 min at 37 °C before adding vanadate (250 μM). Samples were incubated at 37 °C with slight agitation. For NEM treatment, erythrocytes were suspended in PBS containing NEM (2 mM) at 20% HCT. The suspension was incubated at 37 °C for 1 h with shaking, and cells were then pelleted by centrifugation (16000*g*, 5 min). The supernatant was removed; cells were resuspended at 50% HCT with the indicated concentrations of sonicated lipid vesicles; and the suspension was incubated at 37 °C with slow shaking. At the indicated time intervals, aliquots of vanadate- and NEM-treated cell suspensions were fixed with 0.5% glutaraldehyde for morphological indexing.

Back Extraction of Lipids from the Erythrocyte Outer Monolayer with BSA. To determine the transbilayer distribution of incorporated lipid analogues, erythrocytes (20 μL of 50% HCT) were incubated twice (5 min each) with 180 μL of 1% BSA on ice, with one wash in between with PBS, to selectively remove outer monolayer synthetic short-chain lipids. Cells were washed 3−4 times with 1 mL of PBS to remove traces of BSA and fixed in 0.5% glutaraldehyde. Fixed cells were counted to determine the MI as described above.

Extraction of Lipids from Red Cells and Separation by TLC. Lipids were extracted from red cell membranes or unfractionated cells after treatment with synthetic lipids. To isolate membranes (ghosts), red blood cell suspensions (100 μL, 50% HCT) containing incorporated lipid analogues were lysed by the addition of 10 volumes of ice-cold Tris lysing buffer [10 mM Tris-HCl, 0.5 mM ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 3.8 mM $MgSO_4$, 200 μM PMSF, and 1 mM dithiothreitol (DTT) at pH 7.6] and incubated on ice for 5 min. Ghosts were centrifuged (16000*g*, 20 min) and washed 4 times with Tris lysing buffer at 4 °C. Lipids were extracted by the method of Comfurius and Zwaal (*17*). Briefly, 4.3 volumes of mixture (5:8 chloroform/methanol) was added to the ghosts and incubated at room temperature for 5 min with intermittent vortexing. Water (1 volume) and chloroform (3.7 volumes) were added, and the organic/aqueous phases were separated by centrifugation (3000*g*, 10 min). The lower organic layer was collected, and the solvent was removed with a stream of nitrogen gas.

To extract lipids from unfractionated red cells, a modification of the method of Rose and Oaklander was used (*22*). Briefly, after lipid treatment, cells were isolated by centrifugation (16000*g*, 10 min), placed in a glass tube with a Teflon-lined cap, and lysed by the addition of 1 volume of water. The suspension was vortexed and incubated on ice for 15 min, and 11 volumes of isopropanol were added slowly while mixing. The mixture was incubated on ice for 1 h, and 7 volumes of chloroform were added while mixing. After another 1 h of incubation on ice, the organic and aqueous layers were separated by centrifugation (500*g*, 30 min) and the organic layer was removed and washed with 0.2 volume of 50 mM KCl. The phases were separated by centrifugation (500*g*, 30 min), and the organic layer was collected. The solvent was removed with a stream of nitrogen gas.

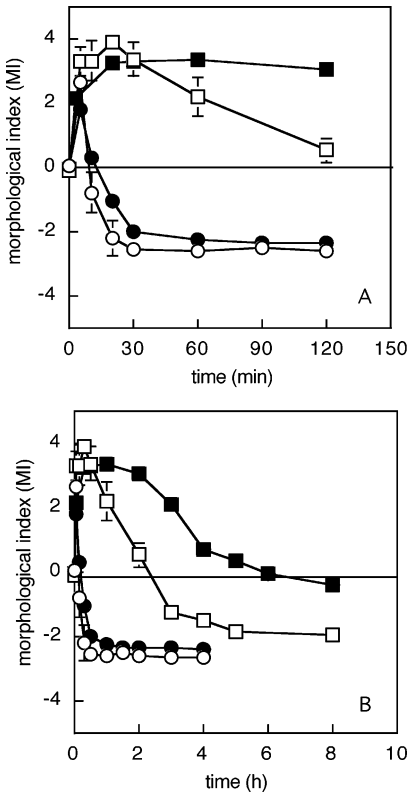

FIGURE 2: Time course of erythrocyte shape change (MI) during incubation with PS diastereomers (200 μM) at 37 °C for (A) 0−120 min and (B) 0−8 h. At the indicated times, aliquots were fixed and the MI was calculated as described in the Materials and Methods. *sn*-1,2-DLP-L-serine (○), *sn*-1,2-DLP-D-serine (●), *sn*-2,3-DLP-L-serine (□), and *sn*-2,3-DLP-D-serine (■).

After isolation, lipids were reconstituted in chloroform and separated by two-dimensional TLC, by elution with a basic solvent (65:25:2.5:2.5 chloroform/methanol/water/ammonia) in the first dimension and an acidic solvent (65:25:5 chloroform/methanol/formic acid) in the second dimension. Lipid spots were visualized with iodine vapor and scraped, and the phosphate in each lipid was determined by phosphate analysis (*19*).

RESULTS

Morphological Response of Red Blood Cells Incubated with DLPS Diastereomers. The shape changes of red blood cells incubated with sonicated vesicles of *sn*-1,2-DLP-L-serine and *sn*-1,2-DLP-D-serine at 37 °C for times up to 120 min have been described previously (*2*, *16*); cells treated with these lipids convert rapidly from discocytes to echinocytes but then revert within 30 min to stomatocytes (Figure 2). Similar to the *sn*-1,2-DLPS stereoisomers (L and D forms),

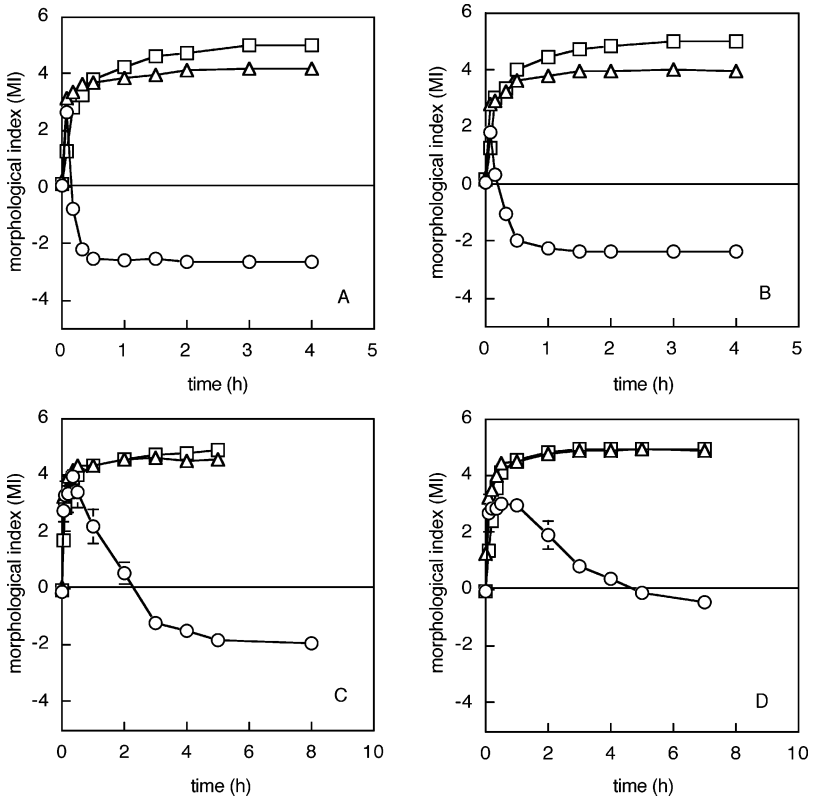

FIGURE 3: Effect of sodium orthovanadate and NEM on the morphology of erythrocytes incubated with PS diastereomers (200 μM) at 37 °C. Untreated cells (○) or cells pretreated with 250 μM vanadate (△) or 2 mM NEM (□) were incubated with (A) *sn*-1,2-DLP-L-serine, (B) *sn*-1,2-DLP-D-serine, (C) *sn*-2,3-DLP-L-serine, or (D) *sn*-2,3-DLP-D-serine. At the indicated times, cells were fixed and the MI was calculated as described in the Materials and Methods.

sn-2,3-DLP-L-serine and *sn*-2,3-DLP-D-serine liposomes rapidly induced echinocytes but no reversion to a less echinocytic morphology was observed for 30 min (*sn*-2,3-DLP-L-serine) or 120 min (*sn*-2,3-DLP-D-serine) (Figure 2A). Continued incubation induced the formation of stomatocytes in *sn*-2,3-DLP-L-serine-treated cells within 3 h (Figure 2B). In contrast, cells incubated with *sn*-2,3-DLP-D-serine reverted slowly from echinocytes to discocytes in approximately 6 h ($t_{1/2} \sim 3$ h, Figure 2B). Upon reversion of cells to stomatocytes (*sn*-1,2-DLP-L-serine, *sn*-1,2-DLP-D-serine, and *sn*-2,3-DLP-D-serine) or discocytes (*sn*-2,3-DLP-D-serine), shapes were retained for several hours (Figure 2). These results indicate that *sn*-2,3-DLPS stereoisomers migrate slowly from the outer to inner monolayer compared to *sn*-1,2-DLPS stereoisomers and that the L-serine headgroup increases the rate of movement of the *sn*-2,3 isomer.

Morphological Response of Vanadate- and NEM-Treated Red Blood Cells to sn-1,2-DLP-L-serine, sn-1,2-DLP-D-serine, sn-2,3-DLP-L-serine, and sn-2,3-DLP-D-serine. PS transport in human red blood cells is dependent upon ATP and is sensitive to vanadate and sulfhydryl reagents (*2*, *3*, *23*). Red blood cells pretreated with sodium orthovanadate (250 μM) and incubated with *sn*-1,2-DLP-L-serine vesicles changed rapidly (<5 min) from discocytes to echinocytes and attained a MI of +3; however, no echinocyte to stomatocyte shape changed was observed (Figure 3A). Instead, the cells retained an echinocytic shape for 4 h, indicating that the out-to-in movement of *sn*-1,2-DLP-L-serine is dependent upon ATP hydrolysis (Figure 3A). Vanadate inhibition was not dependent upon pretreatment with the reagent; cells treated with vanadate after a 10 min exposure to *sn*-1,2-DLP-L-serine stopped reverting from echinocytes to discocytes (data not shown). Cells pretreated with the sulfhydryl-alkylating agent NEM prior to incubation with *sn*-1,2-DLP-L-serine also changed morphology from discocytes to echinocytes within 5 min and retained this shape for several hours, indicating that the lipid remained in the outer monolayer. These data and similar experiments performed by others have established that PS transport in the red blood cell plasma membrane is both protein-mediated and ATP-dependent.

To determine whether the transbilayer movement of the PS diastereomers were also ATP-dependent and protein-mediated, cells were pretreated with vanadate or NEM and the morphological effects of the lipid analogues were assessed. Vanadate pretreatment of cells incubated subsequently with *sn*-1,2-DLP-D-serine demonstrated a change in morphology from discocytes to echinocytes analogous to the shape changes induced by *sn*-1,2-DLP-L-serine (Figure 3B). Pretreatment of cells with vanadate also blocked the reversion of echinocytes to discocytes or stomatocytes induced by *sn*-2,3-DLP-L-serine and *sn*-2,3-DLP-D-serine, suggesting that the transport of these PS stereoisomers from the outer to inner monolayer requires ATP hydrolysis (parts C and D of Figure 3). Treatment with vanadate subsequent to exposure to *sn*-2,3-DLP-L-serine or *sn*-2,3-DLP-D-serine for 10 min caused the cessation of the morphological shape change from echinocytes to stomatocytes or discocytes (data not shown). Cells pretreated with NEM and exposed to *sn*-2,3-DLPS serine isomers exhibited no reversion to discocytes or stomatocytes, suggesting that a cysteine-containing protein is involved in the transbilayer movement of *sn*-2,3-DLP-L-serine and *sn*-2,3-DLP-D-serine (parts C and D of Figure 3). Cells treated with vanadate or NEM, in the absence of lipids,

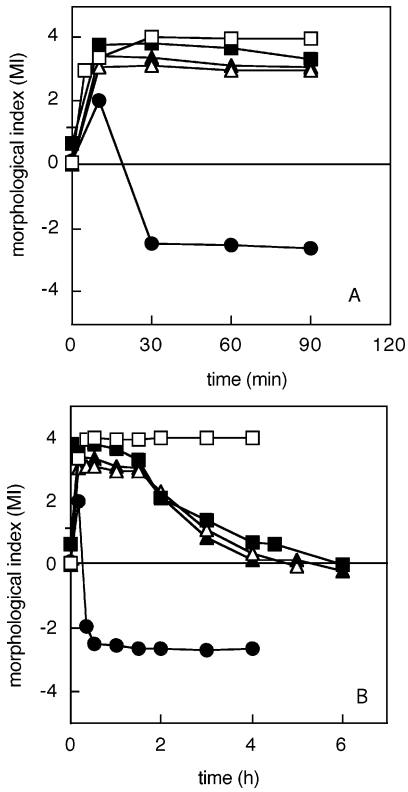

FIGURE 4: Time course of erythrocyte shape change (MI) induced by PS structural analogues (200 μM) at 37 °C for (A) 0−120 min and (B) 0−6 h. DLPC (□), DLPP (■), DLPhS (△), DLPS-*O*-Me (▲), and DLPS-*N*-Me (●). At the indicated times, cells were fixed and the MI was calculated as described in the Materials and Methods.

exhibited no change in morphology, thus ruling out an independent effect of these reagents on the cell shape (data not shown). Vanadate and NEM sensitivity implicate an ATPase in the transbilayer movement of *sn*-2,3-DLPS stereoisomers from the outer to inner monolayer of the red cell membrane.

Morphological Response of Red Blood Cells Incubated with Structural Analogues of PS. Cells exposed to DLPC, DLPP, DLPhS, DLPS-*N*-Me, and DLPS-*O*-Me vesicles became echinocytic within 10 min of exposure to the lipid (Figure 4A). Cells treated with DLPC remained echinocytic for 2 h and retained their morphological stage for 4 h, consistent with the retention of this lipid in the outer monolayer (*2*) (parts A and B of Figure 4). In contrast, cells incubated with DLPS-*N*-Me reverted from echinocytes to stomatocytes after 10 min and attained a stomatocytic MI (−2.4) within 30 min (parts A and B of Figure 4), similar to the time course and extent of the shape change induced by DLPS. However, cells incubated with DLPP, DLPhS, and DLPS-*O*-Me retained an echinocytic morphology for 90 min but, upon continued incubation, reverted to discocytes at similar rates by 4 h, indicating that there is movement of the lipids from the outer to inner monolayer of the cell membrane (Figure 4B). The morphology of these cells remained discoid (Figure 4).

Morphological Response of Vanadate- and NEM-Treated Cells to Structural Analogues of PS. Cells were treated with vanadate and NEM to test the possibility of energy-dependent and protein-mediated transport of these structure-based PS analogues in human erythrocyte membranes. Pretreatment of DLPP-incubated cells with vanadate and NEM had no effect on the conversion of discoid cells to echinocytes but prevented the reversion of DLPP-induced echinocytes to discocytes (Figure 5A), indicative of an outer monolayer location of the lipid and lack of transbilayer transport. In a separate experiment, incubation of untreated cells with DLPP for 1 h prior to exposure to vanadate (250 μM) resulted in the termination of the shape change from echinocytes to discocytes (data not shown). Other structural analogues (DLPhS, DLPS-*N*-Me, and DLPS-*O*-Me) responded similarly to pretreatment with vanadate or NEM; the cells became echinocytic but did not revert to discocytes (parts B−D of Figure 5), although NEM treatment resulted in echinocytes with a lower MI after exposure to DLPP- or DLPhS-incubated cells (parts A and B of Figure 5). In general, shape changes induced by lipids in NEM-treated cells were qualitatively different than those induced by lipids in untreated or vanadate-treated cells; the spikes formed were shorter, finer, and more numerous. This effect is characteristic of NEM treatment, but the echinocytic shapes were still reflective of the outer monolayer location of the lipid. These results indicate that transbilayer movement of PS structural analogues is mediated by a sulfhydryl-containing protein and is linked to ATP hydrolysis.

Transbilayer Distribution of DLPS Analogues. The echinocyte−discocyte shape changes induced by DLPS analogues may be due to equilibration across the membrane or the transbilayer movement of the incorporated lipid followed by removal from the inner monolayer (degradation or cytosolic partitioning). To address these hypotheses, cells exposed to DLPS analogues were treated with BSA to remove the residual synthetic lipids from the outer monolayer. If the reversion to discocytes was the result of equilibration of the lipid across the membrane, BSA back extraction of the outer monolayer lipid should result in an increased stomatocytic shape because of the resulting bilayer imbalance because of excess synthetic lipid in the inner monolayer. If the discocytic morphology was due to translocation followed by removal of the lipid from the inner monolayer, then BSA back extraction should result in little or no change in morphology. To test this strategy, cells were incubated with DLPS or DLPC and aliquots were removed, treated with BSA, fixed with glutaraldehyde, and examined by light microscopy. DLPC induces a discocyte−echinocyte shape change because of the incorporation of this lipid in the red cell outer monolayer. BSA treatment induced a reversion of these echinocytes to discoid morphology, consistent with the removal of DLPC from the outer monolayer (Figure 6A). For DLPS, BSA treatment of cells incubated with DLPS for a short time (<15 min) induced a conversion of echinocytic cells to discocytes, consistent with the removal of all of the DLPS from the outer monolayer and indicating that little DLPS had been transported to the inner monolayer during this time period (Figure 6A). As the incubation with DLPS progressed, BSA treatment resulted in progressive stomatocytosis, consistent with the transport of an increasing amount of DLPS from the outer to inner monolayer. After approximately 20 min of incubation with DLPS, BSA treatment had no additional effect on the stomatocytic shape of the cells, consistent with the near-complete accumulation of the incorporated lipid in the inner monolayer by this time point. Control cells treated with BSA

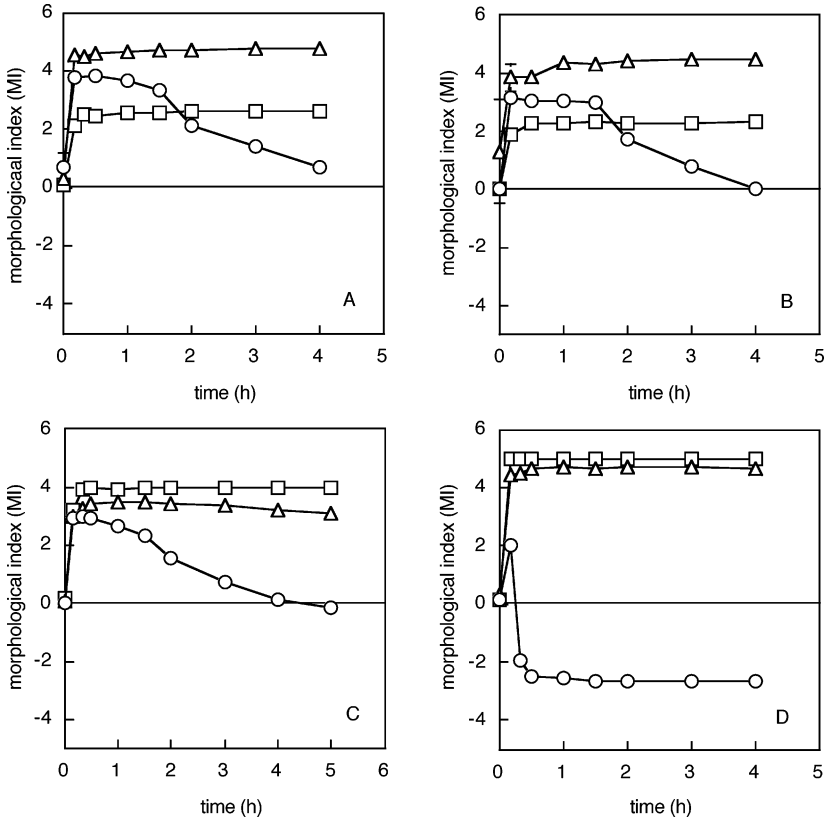

FIGURE 5: Effect of vanadate and NEM on the morphology of erythrocytes incubated with PS analogues. Untreated cells (○) or cells pretreated with 250 μM vanadate (△) or 2 mM NEM (□) were incubated with (A) DLPP (150 μM), (B) DLPhS (200 μM), (C) DLPS-*O*-Me (200 μM), or (D) DLPS-*N*-Me (200 μM). At the indicated times, cells were fixed and the MI was calculated as described in the Materials and Methods.

became only slightly stomatocytic (MI ~ -0.5), possibly because of the extraction of endogenous lipids, such as cholesterol, from the outer monolayer (data not shown).

The BSA back extraction method was used to determine the transbilayer distribution of lipids in cells incubated with DLPP, DLPhS, DLPS-*O*-Me, DLPS-*N*-Me, *sn*-2,3-DLP-D-serine, or *sn*-2,3-DLP-L-serine (Figure 6). BSA treatment of cells incubated with these analogues for 5 min resulted in a conversion from echinocytic morphology to discocytes or early-stage stomatocytes, consistent with an outer monolayer location of the lipid. At longer incubation times, the behavior of cells treated with these lipids fell into two categories, depending upon the equilibrium morphology induced by the lipid. The effect of BSA on shape changes induced by DLPS-*N*-Me, a lipid that induces extensive stomatocytosis, was identical to the effects of BSA on cells incubated with *sn*-1,2-DLP-L-serine; at early times (<15 min) of incubation with the lipid, BSA back extraction resulted in the conversion of echinocytes to stomatocytes, while at later times, the shape of DLPS-*N*-Me-induced stomatocytes was unaffected by BSA treatment (Figure 6A). Cells incubated with lipids that produce an appreciable degree of stomatocytes, although at a slower rate (*sn*-2,3-DLP-L-serine), responded similarly to BSA. Back extraction of *sn*-2,3-DLP-L-serine-treated cells with BSA induced progressive stomatocytosis (Figure 6B), consistent with the removal of residual outer monolayer *sn*-2,3-DLP-L-serine and increasing accumulation of *sn*-2,3-DLP-L-serine in the inner monolayer. After extended incubation (5 h) with this lipid, BSA treatment induced a small increase in stomatocytosis, indicating that, at this time point, most of the *sn*-2,3-DLP-L-serine had accumulated in the inner monolayer. The other PS analogues behaved differently. All of the remaining lipids (DLPP, DLPhS, DLPS-*O*-Me, and *sn*-2,3-DLP-D-serine) induced the formation of echinocytes, but the cells reverted to discoid and not stomatocytic morphology (parts B and C of Figure 6). BSA treatment of cells incubated with these lipids resulted in the conversion of the echinocytic shapes to discocytes, indicating that all of the lipid was present in the outer monolayer and accessible to back extraction. After extensive incubation (3−6 h), back extraction of cells incubated with these lipids had no effect or resulted in the conversion of the discoid cells to early-stage stomatocytes (parts B and C of Figure 6).

Effect of MRP1 Inhibition on Lipid-Induced Shape Changes. In addition to the inwardly directed PS flippase, red cells contain an ABC transporter, MRP1, that facilitates the movement of PC and perhaps PS from the inner to outer monolayer (*24*). To rule out the possibility that the shape changes induced by the synthetic PS analogues were influenced by outward transport to the outer monolayer by MRP1, cells exposed to PS analogues were subsequently treated with MK571, an inhibitor of MRP1 (*24*, *25*). Aliquots were drawn from suspensions of cells incubated with lipid vesicles for 10 min (*sn*-1,2-DLP-L-serine) or 1 h (DLPP, DLPhS, and DLPS-*O*-Me) to allow for full incorporation of the lipids in the outer monolayer and were treated subsequently with MK571 (50 μM). The time course of the shape change induced by these lipids was unaffected by MK571 treatment, indicating that MRP1 did not transport these analogues from the inner to outer monolayer (data not shown).

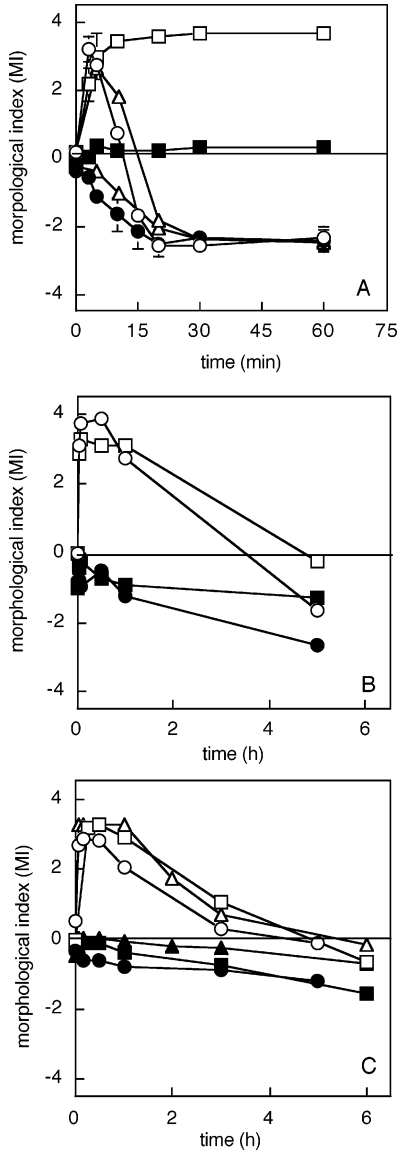

FIGURE 6: Effect of BSA back extraction on the morphology of cells incubated with dilauroyl phospholipids. Cells were incubated with sonicated vesicles of dilauroyl phospholipids for the indicated time, and aliquots were fixed with glutaraldehyde before (○, □, and △) or after (●, ■, and ▲) treatment with 1% BSA. The MI was calculated as described in the Materials and Methods. (A) *sn*-1,2-DLP-L-serine (200 μM, ○ and ●), DLPC (200 μM, □ and ■), and DLPS-*N*-Me (200 μM, △ and ▲); (B) *sn*-2,3-DLP-L-serine (200 μM, ○ and ●) and *sn*-2,3-DLP-D-serine (150 μM, □ and ■); and (C) DLPP (150 μM, ○ and ●), DLPhS (200 μM, □ and ■), and DLPS-*O*-Me (200 μM, △ and ▲).

Analysis of the Incorporated Short-Chain Lipids. The apparent lack of accumulation of DLPP, DLPhS, DLPS-*O*-Me, and *sn*-2,3-DLP-D-serine in the erythrocyte inner monolayer could be the result of the removal of the newly transported lipid from the inner monolayer (i.e., by partitioning into the cytosol) or the degradation of the lipid by intracellular phospholipases. To test the former possibility, lipids from cells treated with PS analogues were extracted from isolated membranes immediately after incorporation of the lipid (0 min) or after a 4 h incubation. Extracted lipids were separated by conventional TLC. Because of the difference in acyl chain composition and the increased resolution afforded by the silica HL plates, the dilauroyl

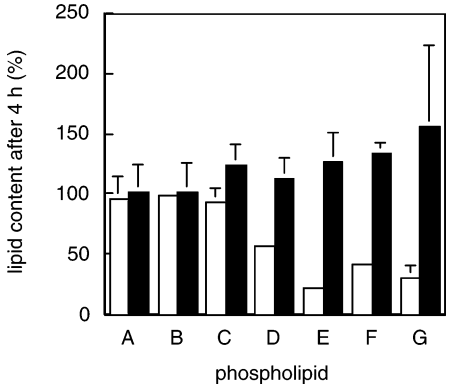

FIGURE 7: Amount of cell-associated short-chain lipids. Cells were pretreated with buffer (open bars) or vanadate (250 μM, closed bars) and incubated with (A) *sn*-1,2-DLP-L-serine (200 μM), (B) DLPS-*N*-Me (200 μM), (C) *sn*-2,3-DLP-L-serine (200 μM), (D) *sn*-2,3-DLP-D-serine (150 μM), (E) DLPP (150 μM), (F) DLPhS (200 μM), or (G) DLPS-*O*-Me (200 μM) for 4 h. Cells were lysed; membranes were isolated; and the lipids were extracted and separated by TLC. Lipids were identified by reference to standards separated on parallel TLC plates, and the lipid content was determined by phosphate analysis. Data are expressed as percentage of each short-chain lipid initially associated with the cell.

phospholipids were well-separated from endogenous lipids, allowing for the independent assessment of synthetic lipid content by phosphate analysis. The R_f values of the DLPS diastereomers, DLPhS, and DLPS-*N*-Me were less, and the R_f values of DLPP and DLPS-*O*-Me were greater than that of the corresponding native PS in a one-dimensional acidic elution system (65:25:5 chloroform/methanol/formic acid). However, to ensure good separation, a two-dimensional system was used to isolate the PS analogues. While the amount of *sn*-1,2-DLPS, DLPS-*N*-Me, and *sn*-2,3-DLP-L-serine was unchanged (parts A−C of Figure 7, respectively), the 4 h incubation resulted in a significant loss of *sn*-2,3-DLP-D-serine, DLPP, DLPhS, and DLPS-*O*-Me from the membrane (a reduction of 42.5, 76.9, 58.4, and 68.5%, respectively; parts D−G of Figure 7). However, if the cells were preincubated with vanadate before exposure to these lipids and analysis by TLC, no change in the amount of *sn*-1,2-DLPS, *sn*-2,3-DLP-L-serine, and DLPS-*N*-Me associated with the cells occurred within 4 h (parts A−C of Figure 7) but an increase in the amount of the *sn*-2,3-DLP-D-serine, DLPP, DLPhS, and DLPS-*O*-Me associated with the cells was observed (an increase of 13, 27.7, 33.6, and 56.4%, respectively).

To address the question of whether the apparent loss of lipid determined in the previous experiment was due to degradation or cytosolic extraction of the synthetic lipid, unfractionated red blood cells, rather than membranes, were subjected to extraction with isopropanol/chloroform (*22*). This procedure should capture all of the synthetic lipid associated with the cell (membrane or cytosol), regardless of location. Cells treated with lipids for 0 or 5 h were extracted with isopropanol/chloroform; the organic phase was isolated; and the lipids were analyzed by TLC as described above. Cells treated with DLPP, DLPhS, and DLPS-*O*-Me retained 70.5, 118.2, and 79.5%, respectively, of the lipids initially associated with the cells. These data are consistent with the partitioning of the transported lipids into the cytoplasm, rather than inner-monolayer-dependent degradation.

DISCUSSION

The mechanism by which transbilayer phospholipid asymmetry in biological membranes is maintained is through the combined action of a number of lipid transporters that vary in substrate specificity, energy requirements, and direction of transport (*1*). These include outwardly direct transporters or "floppases", such as the multidrug resistance proteins ABCB1, ABCB4, and ABCC1, and the inwardly directed "flippases", such as the plasma membrane PS flippase. Previous studies have shown that some floppases are relatively nonspecific for their lipid substrate, while others display a greater degree of selectivity (*26*, *27*). In contrast, erythrocyte PS flippase activity is exquisitely sensitive to the structure of its substrate and recognizes multiple structural elements of the lipid (*7*). The present study demonstrates that, although *sn*-1,2-PS is the optimal substrate for the inwardly directed flippase activity in erythrocytes, structural homologues and stereoisomers of PS are also capable of ATP- and protein-dependent transport across the membrane.

Erythrocytes and platelets are unique in that the transbilayer distribution of incorporated amphipathic compounds can be deduced from cell morphology. The effects of amphipathic compounds on red cell shape have been well-documented, and independent confirmation of the monolayer location of the incorporated lipids has shown that the induction of these shape changes are consistent with the bilayer couple hypothesis of Sheetz and Singer (*3*, *28*, *29*); amphipaths that partition into the outer monolayer induce an echinocytic shape, while those that partition into the inner monolayer induce a stomatocytic shape. These changes in cell shape can be used as a sensitive indicator of the movement of lipids across the membrane bilayer. Indeed, the first reports of PS flippase activity in erythrocytes used these characteristic shape changes to report the transbilayer distribution of exogenously incorporated synthetic PS (*2*, *3*). Subsequent studies have employed cell morphology as a measure of transbilayer lipid location (*5*) and have applied this approach to other cells, such as platelets (*2*, *30*, *31*), and to synthetic systems, such as giant unilamellar vesicles (*32*). Further, the lipids that can be studied with this technique do not require the presence of additional, potentially perturbing, reporter groups, such as fluorophores or spin labels, and may more accurately represent the behavior of native phospholipids. Amphipath-induced changes in cell morphology were exploited in the present study to address the specificity of PS homologue transport in erythrocyte membranes.

Previous studies have shown that PS is unique among the glycerophospholipids in that it is a substrate for an ATP- and protein-dependent inwardly directed flippase in the erythrocyte membrane. Using a variety of methods and lipid analogues, it has been well-established that other glycerophospholipids do not traverse the membrane at an appreciable rate compared to PS. These data have led to the proposition that a single, PS-selective ATP-dependent transporter is present in erythrocyte membranes. However, these studies did not address the energy requirement of the slowly moving glycerophospholipids (*8*, *15*). We have re-evaluated these experiments and found that a set of glycerophospholipid analogues representing select modifications of the PS structure flip across the erythrocyte membrane in a vanadate- and NEM-dependent manner.

Vanadate is a pentavalent phosphate analogue that inhibits a variety of ATP-dependent enzymes, including ATPases, and has been used as an indicator of energy-dependent lipid transport (*3*). Sensitivity to sulfhydryl reagents, such as NEM, has been used as a criterion for whether PS transport is protein-mediated (*2*, *23*, *33*). The present study is the first demonstration of an ATP-dependent transport activity in erythrocytes for lipids other than PS, PE, and *N*-methyl-PE.

The transport activity of lipids tested in this work fall into three kinetic categories: (1) rapidly transported lipids ($t_{1/2}$ < 15 min; *sn*-1,2-DLPS and DLPS-*N*-Me), (2) lipids that have intermediate transport rates ($t_{1/2} \sim 1-3$ h; *sn*-2,3-DLP-L-serine, DLPP, DLPhS, DLPS-*O*-Me, and *sn*-2,3-DLP-D-serine), and (3) lipids that are not transported significantly after several hours ($t_{1/2}$ > 6 h; DLPC; Table 1). A detailed study of the transport of spin-labeled PS and PE analogues has been reported previously (*8*, *15*) and is consistent with the present study. Although spin-labeled lipids generally have higher rates of inward movement, they can also be grouped in the classification described above. Spin-labeled PE, *N*-methyl-PE, and *N*,*N*-dimethyl-PE fall into the second class (intermediate rate of transbilayer movement). Methylation of the amine group of PE reduces the amount and increases the half-time of lipid transport. The rate of transport of the spin-labeled PS analogues with headgroup [phosphatidyl-(1-methyl-ethanolamine)] and acyl group [1-(16-doxyl-stearoyl)-2-acetyl-phosphatidylserine] modifications (*8*) places them in the slowly transported class. Similar rates of transport were also reported for the short-chain (this paper) and spin-labeled (*8*) analogues of PS-*O*-Me. The lipids belonging to this class represent the modification of a number of lipid structural elements, suggesting that rapid inwardly directed flipping activity is sensitive to almost all structural elements of the PS molecule.

With the exception of PC, all of the lipids tested accumulate on the cytosolic side (in the inner monolayer or cytosol) of the erythrocyte membrane. This is not readily apparent from the morphological data. Indeed, the shape changes induced by the lipids used in this study, except for DLPS, N−Me-DLPS, and *sn*-2,3-DLP-L-S, appear to indicate that at equilibrium an equal amount of lipid is present on each side of the membrane. If this were the case, BSA back extraction would result in the induction of a significant degree of stomatocytosis. However, removal of residual outer monolayer lipids induces a similar, discocytic morphology at all time points. It is also possible that the lipids are being transported to the inner monolayer but are then transported out of the cell by another mechanism, such as the multidrug-resistance-related protein, MRP1 (*24*, *34*). However, inhibition of MRP1 has no effect on the morphology of treated cells, indicating that lipid efflux by this mechanism is unlikely. Instead, these data are consistent with the transport of the lipid from the outer to inner monolayer followed by removal from the inner monolayer, either by metabolic conversion to a nonmembrane-perturbing lipid or by partitioning into the cytosol. The BSA lipid extraction data support this hypothesis. Cells treated with *sn*-2,3-DLP-D-serine, DLPP, DLPhS, or DLPS-*O*-Me show a substantial (40−70%) loss of the incorporated lipid after a 4 h incubation (Figure 7). Prior transport to the inner monolayer is required; inhibition of transport with vanadate prevents the loss of lipid

Table 1: Rate and Extent of Lipid Transport in Human Erythrocytes

lipid	abbreviation	$t_{1/2}$ at 37 °C	maximum internalization (%)	ref
1-palmitoyl-2-(4-doxylpentanoyl)-*sn*-glycero-3-phospho-L-serine	PS	4 min	93	*8*
1,2-octanoyl-*sn*-glycero-3-phospho-L-serine	DOPS	15 min	nd[a]	*50*
1,2-heptanoyl-*sn*-glycero-3-phospho-L-serine	DHPS	15 min	nd	*50*
1,2-decanoyl-*sn*-glycero-3-phospho-L-serine	DDPS	15 min	nd	*50*
1,2-dilauroyl-*sn*-glycero-3-phospho-L-serine	DLPS	15 min	80−90	*2, 5*
1,2-dilauroyl-*sn*-glycero-3-phospho-D-serine	*sn*-1,2-DLP-D-serine	15 min	80−90	*b*
1,2-dilauroyl-*sn*-glycero-3-phospho-*N*-methylserine	DLPS-*N*-Me	15 min	80−90	*b*
1-(16-doxylstearoyl)-2-acetyl-*sn*-glycero-3-phospho-L-serine	Ac-PS	36 min	65	*8*
1-palmitoyl-2-(4-doxylpentanoyl)-*sn*-glycero-3-phospho-*N*-methyl-ethanolamine	PME	52 min	56	*8*
1-palmitoyl-2-(4-doxylpentanoyl)-*sn*-glycero-3-phosphoethanolamine	PE	55 min	87	*8*
1-palmitoyl-2-(4-doxylpentanoyl)-*sn*-glycero-3-phosphoserine methyl ester	PS-MeE	1 h	79	*8*
1,2-dilauroyl-*sn*-glycero-3-phosphoethanolamie	DLPE	1.25 h	nd	
2,3-dilauroyl-*sn*-glycero-3-phospho-L-serine	*sn*-2,3-DLP-L-serine	2 h	>70	*b*
2,3-dilauroyl-*sn*-glycero-3-phospho-D-serine	*sn*-2,3-DLP-D-serine	3 h	>50	*b*
1,2-dilauroyl-*sn*-glycero-3-phospho-propionic acid	DLPP	3 h	>50	*b*
1,2-dilauroyl-*sn*-glycero-3-phospho-serine methyl ester	DLPS-*O*-Me	3 h	>50	*b*
1,2-dilauroyl-*sn*-glycero-3-phospho-homoserine	DLPhS	3 h	>50	*b*
1-(16-doxylstearoyl)-2-hydroxy-*sn*-glycero-3-phospho-L-serine	lyso PS	3 h	38	*8*
bovine brain lyso PS	lyso PS	3 h	nd	*2*
1-palmitoyl-2-(4-doxylpentanoyl)-*sn*-glycero-3-phospho-*N*,*N*-dimethyl-ethanolamine	PDE	3 h	42	*8*
1-palmitoyl-2-(4-doxylpentanoyl)-*sn*-glycero-3-phospho-L-alaninol	PE-β Me (L)	3 h	42	*8*
1-palmitoyl-2-(4-doxylpentanoyl)-*sn*-glycero-3-phospho-D-alaninol	PE-β Me (D)	3 h	42	*8*
1,2-dilauroyl-*sn*-glycero-3-phospho-choline	DLPC	>6 h	0	*2, 5*
1-palmitoyl-2-(4-doxylpentanoyl)-*sn*-glycero-3-phospho-L-choline	PC	9 h	9	*8*
1-palmitoyl-2-(4-doxylpentanoyl)-*sn*-glycero-3-phospho-3-amino propanol	PP	16 h	80	*8*
1-(16-doxylstearoyl)-2-hydroxy-*sn*-glycero-3-phosphocholine	lyso PC	14 h	4	*8*
1-(16-doxylstearoyl)-2-acetyl-*sn*-glycero-3-phospho-L-choline	Ac-PC	14 h	50	*8*

[a] n.d. = not determined. [b] This paper.

from the membrane fraction. In addition, restricting the lipid to the outer monolayer with vanadate also increases the amount that can accumulate in the cell. In contrast, lipids that induce a significant degree of stomatocytosis (DLPS, *N*-Me-DLPS, and *sn*-2,3-DLP-L-S) are retained in the membrane fraction. The disappearance from the membrane of the slowly transporting species is the result of partitioning into the cytosol rather than degradation or conversion to another lipid metabolite; extraction of lipid from unfractionated cells indicates that the incorporated synthetic lipids are retained by the cell. Similar cytosolic partitioning behavior induced by peroxide treatment has been reported for short-chain synthetic lipids incorporated into red blood cells (*35*). The significance of these results is unclear but may indicate that, once internalized, at least some of these lipids rapidly dissociate from the membrane and may reflect a previously uncharacterized role for hemoglobin in binding lipids and other amphipathic compounds (*36*).

Transbilayer Movement of PS Diastereomers. The changes in cell morphology upon incubation with PS diastereomers from discocytes to echinocytes to stomatocytes reflects their incorporation in the outer monolayer by monomer transfer, followed by transport to the inner monolayer (Figure 2B). Although the shape changes determined by the cells incubated with *sn*-1,2-DLP-L-serine, *sn*-1,2-DLP-D-serine, and the glycerol isomer *sn*-2,3-DLP-L-serine were similar, their $t_{1/2}$ of inward movement was different. *sn*-1,2-DLP-L-serine and *sn*-1,2-DLP-D-serine were transported into the inner monolayer within 15−30 min, whereas *sn*-2,3-DLP-L-serine required 3−5 h to reach equilibrium (parts A and B of Figure 2). In contrast, cells incubated with *sn*-2,3-DLP-D-serine began to revert to less echinocytic shapes only after 2 h and attained a slight stomatocytic morphology (MI < −1), indicating that, on this time scale, *sn*-2,3-DLP-D-serine was transferred to the inner monolayer. The transbilayer movement of each of the diastereomers of PS is inhibited by vanadate and NEM, indicating that transport is ATP- and protein-dependent.

These data have significant implications for the structure of the substrate recognition sites of the putative lipid transporter(s). Of the two chiral centers in the PS molecule, the primary determinant of rapid transport is the glycerol moiety, while the serine component is a secondary determinant; *sn*-1,2-PS is transported regardless of serine stereochemistry; however, *sn*-2,3-PS is transported, albeit more slowly, at a rate dependent upon the serine stereoconfiguration. This indicates that both chiral centers are recognized by the lipid transporter(s). The stereochemical centers may not bind to a spatially complementary structure on the protein but might instead form a pair of two-position contacts. This would allow for the combination of the *sn*-2,3-glycerol isomer and the L-serine group to create a conformer that more closely mimics *sn*-1,2-DLPS than *sn*-2,3-DLP-D-serine, considering that the distance between these structures provides for a significant degree of conformational flexibility.

Transbilayer Movement of PS Structural Analogues. A hallmark of rapid ATP-dependent transport across the erythrocyte membrane is the presence of a primary or secondary amine group in the lipid substrate. Previously, only PS and PE were considered to be substrates for this transport activity. Modification of the amine group of PE results in a decrease in the rate of transport; progressive methylation of PE results in a progressive decrease in transport activity (*8*); and acylation of the amine group of PE eliminates transport activity (*37*). In contrast, the present study demonstrates that methylation of the amine group of PS has no effect on the

ability of the lipid to be transported across the membrane. Indeed, DLPS-*N*-Me is the only lipid that is transported across the erythrocyte membrane at a rate comparable to DLPS. The present study also demonstrates that the presence of an amine group is not essential for ATP- and protein-mediated transport. A PS analogue that does not contain an amine group (DLPP) is transported across the erythrocyte membrane at a rate ($t_{1/2} \sim$ 10-fold slower than PS) similar to that of PE (*2*, *7*). Thus, the presence of both an amine and carboxyl group are necessary for maximal transport; however, the lack of either of these groups does not preclude transport, although more slowly, by an ATP- and protein-mediated process.

Other modifications to the structure of the PS molecule produce a similar reduction in the rate of transport and indicate that transport is sensitive to the headgroup size and charge. Increasing the size of the serine headgroup, through the addition of a methylene between the phosphate group and serine α carbon (DLPhS) is sufficient to reduce the rate of transport by 10-fold. A similar reduction is observed with the carboxyl methyl ester of PS (DLPS-*O*-Me), although both of these lipids retain an ATP- and protein-dependent mechanism of transport. These data are consisted with Morrot et al. (*8*), who reported that a spin-labeled analogue of PS-*O*-Me was transported across erythrocyte membranes at a rate similar ($t_{1/2} \sim$ 1 h) to that reported here.

When these data are taken together, they indicate that the ATP- and protein-mediated transport of glycerophospholipids across the erythrocyte membrane is much more flexible in regard to substrate recognition but that PS and *N*-methyl-PS are the optimal substrates. The lack of ATP-dependent transport of PC and lyso PS and the slow transbilayer movement of phosphoinositides [$t_{1/2} \sim$ 3 h (*38*)] indicates that there is a limit to substrate recognition and suggests that for optimal binding the lipid must be a diacyl glycerophospholipid with a headgroup possessing both a protonatable amine and a negatively charged (carboxylate) recognition element.

Potential Lipid Transporters. Several erythrocyte proteins have been suggested to transport lipids across the membrane, including the anion exchanger band 3 [AE1 (*39*)], the multidrug-resistance-related protein 1 [MRP1 (*24*, *34*)], a Ca^{2+}-dependent scramblase (*40*−*42*), and the aminophospholipid-specific flippase. It is unlikely that the transport activity described here is due to AE1 or the erythrocyte scramblase, because neither are ATP-dependent. MRP1 transports choline-containing lipids primarily from the inner to outer monolayer and is also unlikely to be responsible for this activity. The remaining possibilities are that these lipids are transported by the PS flippase or that some other, as yet unidentified, transporter is responsible for their transbilayer movement. Although the red blood cell plasma membrane flippase has not been identified, members of the P_4-type ATPase family have been proposed to participate in the regulation of transbilayer membrane asymmetry (*1*, *43*, *44*). Yeast P_4-ATPases have been shown to play a role in the uptake of fluorescent analogues of PS, PE, and PC across the plasma membrane, and similar low-specificity, multiple transporter systems may exist in other cells, including erythrocytes. A recent study from our group demonstrated that lipid specificity for enzyme activation of a P_4-family member (murine Atp8a1) is similar but not identical to the structural requirements for ATP-dependent transport reported here (*45*). Whether these protein are directly involved in transport activity awaits purification and functional reconstitution.

Attempts to purify the human erythrocyte flippase have resulted in various preparations of PS-stimulated Mg^{2+}-ATPases that are likely candidates (*33*, *46*−*49*). The ATPase activity of these ATPases demonstrates a lipid specificity that is similar to the requirements demonstrated here; they are selectively stimulated by *sn*-1,2-PS, insensitive to serine stereochemistry, and are partially activated by negatively charged lipids (Daleke, D. L., and Zimmerman, M. L., unpublished observations; *7*, *15*, *49*). The identity of this protein and whether it is a PS flippase are the subject of ongoing studies.

CONCLUSION

This work demonstrates that the inwardly directed transport of short-chain glycerophospholipid analogues of PS across the plasma membrane of the human erythrocyte is ATP-dependent and protein-mediated. P-L-S, P-D-S, and *N*-methyl-PS are the optimal substrates, but this transport mechanism(s) accepts modification of many structural features of the PS molecule. Almost all of these modifications increase the $t_{1/2}$ of the out-to-in movement by at least 10-fold, but the previously unrecognized sensitivity of the transbilayer movement of these lipids to vanadate and NEM suggests the involvement of an energy-dependent transporter, rather than passive lipid flip-flop, as the primary pathway for transbilayer movement. These data are consistent with either a broader degree of substrate specificity for the PS flippase than has been previously recognized or are suggestive of the presence of multiple ATP-dependent transporters with different structural specificities.

ACKNOWLEDGMENT

We thank Dr. Michael Zimmerman for initiating these studies and the mass spectrometry and NMR facility of the Department of Chemistry, Indiana University, Bloomington, IN, for analyzing the synthetic lipids.

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BI061333X