Phospholipid Flip-Flop and Phospholipid Scramblase 1 (PLSCR1) Co-localize to Uropod Rafts in Formylated Met-Leu-Phe-stimulated Neutrophils*

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Most resting cells appear to demonstrate marked asymmetry in the transbilayer distribution of phospholipids composing the plasma membrane with the majority of sphingomyelin and phosphatidylcholine (PC)1 localizing to the membrane outer leaflet and most phosphatidylethanolamine and nearly all phosphatidylserine (PS) localizing to the membrane inner leaflet (1, 2). This phospholipid asymmetry is thought to be maintained by both an aminophospholipid translocase activity that flips (inward) PS, and to a lesser extent phosphatidylethanolamine, from the outer leaflet back to the inner leaflet and a low spontaneous rate of phospholipid flip and flop (outward) across the plasma membrane bilayer (1, 3–5).

When cells die by programmed cell death, or apoptosis, PS exposure almost universally results. Recognition of exposed PS on the apoptotic cell by a stereospecific receptor on the phagocyte signals for both phagocytic engulfment and down-regulation of inflammatory mediator production (6). As such, apoptotic cells are cleared in an efficient and non-phlogistic manner (7). PS exposure has also been reported under certain conditions during activation of inflammatory cells and on capacitated spermatozoa (8). For example, PS is exposed on platelets stimulated by thrombin and collagen, on B cells with the B cell antigen receptor capped, on mast cells with cross-linked FcγRI, and most recently on neutrophils treated with dimeric galectin-1 after initial stimulation with fMLP (9–13). Externalization of PS on inflammatory cells is thought to play a role in the activation of both complement and coagulation cascades and supports the binding and activity of secreted phospholipases A₂ (see “Discussion”).

The mechanism(s) of enhanced exposure of PS during apoptosis has been shown variously to be either caspase-dependent or -independent and to require cytoskeletal alterations, calcium flux, or oxidation (14–16). Where it has been investigated, PS exposure is thought to result, at least in part, from loss of PS flip due to declining aminophospholipid translocase activity (14, 17). The mechanisms of PS exposure during cellular activation have received much less attention. Where it has been studied, PS exposure in activation, like apoptosis, is accompanied by enhanced staining with lipophilic dyes that indicate changes in membrane lipid packing and enhanced nonspecific

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uptake of phospholipids bearing various head groups including PC species (12, 14, 17–21).

Notably the mechanisms of phospholipid movement and the proteins involved in either outward movement of PS or inward movement of PC have not been unequivocally identified (see “Discussion”). Members of the P-type ATPase family have been proposed as candidate proteins mediating flip of PS from the outer leaflet to the inner leaflet and are credited with estab-
lishing and maintaining the basal asymmetric distribution of PS, but reverse function resulting in PS exposure has not been noted (1, 3–5). Other proteins of the ABC family have been proposed as mediating flip of phospholipids from inner leaflet to outer leaflet (e.g. ABCA1, ABCB1, and ABCB4) (for reviews, see Refs. 1 and 22) but have not been found to enhance inward flip of phospholipids from outer leaflet to inner leaflet. Also these proteins appear to be constitutively active with little evidence that they are activated within minutes of stimulation as has been described for PS exposure in inflammatory cells. Although controversial, the PLSCR1 is hypothesized to medi-
ate calcium-activated, bidirectional, nonspecific (with regard to head group) phospholipid movement that has been shown to accompany PS exposure in apoptosis and cellular activation (23–26) (see “Discussion”). However, where phospholipid movement has been associated with PLSCR1 expression, the precise mechanisms of movement have not been elucidated. Movement of phospholipids could involve either transbilayer flip-flop across the plasma membrane (27) and/or, given PLSCR1 local-
ization to recycling endosomes in epithelial cells (28), could involve bilayer mixing during vesicle-plasma membrane fission and fusion.

Here we have shown that stimulated neutrophils transiently expose PS during FMLP activation in the absence of evidence for apoptosis. PS exposure was accompanied by altered plasma membrane phospholipid packing and enhanced inward move-
ment of both alkyl- and acyl-linked choline-containing phospho-
lipids. As these phospholipid movements were not accompanied by evidence of either actin-dependent or actin-independent endo-
cytosis/pinocytosis, the data are strongly supportive of phospho-
lipid movement by flip-flop across the plasma membrane. Fur-
thermore we present evidence that phospholipid flip-flop and membrane packing changes occurred in “raft” membranes at the uropod of polarized neutrophils, and that plasma membrane PLSCR1 co-localized to these same domains.

EXPERIMENTAL PROCEDURES
Reagents—Cholera toxin B (CT-B)-Alexa 555, FM 1-43, annexin V-Alexa 488, fluorescein isothiocyanate-phalloidin, 6-(N-(7-nitrobenz-2-oxa-1,3-dioxo-4-yl)amino)hexanoylsphingosyl phosphocholine (NBD-sphingomyelin), and Lucifer Yellow were from Molecular Probes (Eugene, OR). 1-Palmitoyl-2-[12-(-7-nitro-2,1,3-benzoxadiazol-4-ylamino)hexanoylsn-glycero-3-phosphoserine (NBD-PS) and 1-palmitoyl-2-[(7-nitro-2-1,3-benzoxadiazol-4-ylamino)hexanoylsn-glycero-3-phosphocholine (NBD-PC) were from Avanti Polar Lipids (Alabaster, AL). Radiolabeled 1-O-hexadecyl-2,13-HNE(2/4-N-methylcarbamyl)phosphatidyl ethanolamine (cPAF) was synthesized by PerkinElmer Life Sciences. Anti-CD55 was from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-CD11b was from Dako Corp. (Carpenteria, CA). Anti-
CD45 clone BRA-55, fatty acid-free BSA, and FMLP were from Sigma, AEBSF, aprotinin, and leupeptin were from Calbiochem. Factor Va and anti-Factor Va were from Hematologic Technologies, Inc. (Essex Junction, VT). Rabbit PLSCR1 antisera raised against the 14 carbox-
yl-terminal amino acids of PLSCR1 (sequence CESTGSQEQKSGVW).

Cell Isolation and Culture—Using endotoxin-free reagents and plas-
tiwear, human neutrophils were isolated by the plasma Percoll method as described previously (29). Unless otherwise noted, all incubations were done in NBD buffer (157 mM NaCl, 2.7 mM KCl, 2 mM MgCl2, 5 mM glucose, 10 mM HEPES pH 7.4) or Krebs-Ringer phosphate-dextrose buffer (KRPD buffer) (9.0% saline, 4.8 mM KCl, 0.93 mM CaCl2, 1.2 mM MgSO4, 3.1 mM NaH2PO4, 12.5 mM NaHPO4, 5% dextrose) with designated amounts of fatty acid-free BSA. T-Rex-293 cells (Invitrogen) were cul-
tivated in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1-glutamine, 1% penicillin, 1% streptomycin, 1 mM sodium pyruvate at 37 °C in a 10% CO2 incubator.

cPAF Uptake—Uptake of radionabeled cPAF was carried out as de-
scribed previously (24) with the following modifications. Uptake of [3H]cPAF took place over the last 5 min of stimulation with FMLP at 37 °C. Samples were diluted with an equal volume of ice-cold 10% BSA in KRPD buffer and washed two more times with 5% BSA in KRPD buffer at 4 °C to remove outer leaflet lipid. The cells were resuspended in 500 μl of 1% Triton X-100. Uptake of labeled lipid was determined by scintillation counting.

NBD-PS, NBD-PC, NBD-sphingomyelin, Annexin V, and Factor Va Staining—NBD-PC (2.5 μg), NBD-PS (2.5 μg), or NBD-sphingomyelin (5.0 μg) was dried under nitrogen and resuspended in 20 μl of NBD buffer containing 180 μl of propidium iodide (50 μg/ml stock). Neutraets were stimulated as described at a concentration of 5 × 105/ml in NBD buffer with 1 mM CaCl2 and 0.25% BSA. Following stimulation, a final concentration of 1.25 μg/ml NBD-PC or NBD-PS or 2.5 μg/ml NBD-
sphingomyelin was added to 100 μl of cells at 37 °C for 5 min. Outer
leaflet lipid was removed by diluting cells with an equal volume of ice-cold 10% BSA in NBD buffer followed by two additional washes at 4 °C with 5% BSA in NBD buffer. Cells were resuspended in 500 μl of ice-cold NBD buffer and analyzed by flow cytometry.

For annexin V staining, 1 × 106 cells in 100 μl of NBD buffer with 2.5 mM CaCl2, annexin V-Alexa 488 (1:50), and 5 μg/ml propidium iodide were incubated for 15 min at room temperature, diluted with 400 μl of ice-cold NBD buffer with 2.5 mM CaCl2, and analyzed by flow cytometry. For activated Factor V (FVa) staining, 1 × 106 neutrophils in 100 μl NBD buffer with 1% BSA and 1 mM CaCl2 were transferred to 100 μl of FVs and propidium iodide (10 μg/ml and 5 μg/ml, respectively) or propidium iodide alone for the last 10 min of stimulation. Cells were then fixed for 10 min at room temperature with 5% paraformaldehyde, 3% sucrose in PBS, washed once, and incubated with 100 μl of anti-FVa antibody (final concentration of 10 μg/ml) for 10 min at room temperature.

Cells were washed once and incubated with 100 μl of anti-mouse IgG F(ab′)2 (1:100 in NBD buffer plus 1 mM CaCl2 and 0.1% BSA) for 30 min on ice, washed once, and analyzed by flow cytometry. For fluores-
cence microscopy, FVa staining was performed as for flow cytometry except the absence of propidium iodide and incubated for 15 min at room temperature with Cy3 goat anti-mouse IgG F(ab′)2, diluted 1:100. Cells were mounted on coverslips with DPDA (20 mg/ml o-phenylenedi-
amine dihydrochloride in 1 mM Tris base (pH 8.5) diluted 1:1 with glycerol) and viewed with a Leica DMRXA fluorescence microscope using a 63× oil (numerical aperture 1.32) Plan-Apo objective. Images were analyzed using Slidebook software (Intelligent Imaging Innovations, Inc., Denver, CO).

FM 1-43 and Lucifer Yellow Staining—Neutrophils at 10 × 106 cells/ml in NBD buffer with 0.05% BSA and 1 mM CaCl2 were stimu-
lated with FMLP for the times indicated. For the last minute of stimu-
lation, 5 × 105 cells were transferred to 450 μl of NBD buffer and FM 1-43 (final concentration of 1 μM) and allowed to incubate for an additional 1 min at 37 °C. Cells were centrifuged and resuspended in 500 μl of ice-cold NBD buffer and analyzed by flow cytometry. Lucifer Yellow staining was carried out as described previously (30).

Raft Membrane Isolation—Neutrophils (300 × 106) at 20 × 105/ml in KRPD buffer with 0.25% BSA were stimulated with FMLP for 15 min. Following stimulation, cells were centrifuged and resuspended in 1 ml of ice-cold lysis buffer (25 mM MES (pH 6.5), 150 mM NaCl, 1% Triton X-100, 1 mM AEBSF, 1 mM NaF, 5 μg/ml leupeptin, 5 μg/ml aprotinin) and sonicated for 1 min on ice. Unbroken cells, nuclei, and debris were removed by centrifugation at 1000 × g for 10 min at 4 °C. The supernatant was added to 2 ml of 80% sucrose in MES-buffered saline (25 mM MES, 50 mM NaCl, and the lyses were washed twice with a 10–40% continuous sucrose gradient for 18–24 h at 37,000 rpm at 4 °C. Following centrifugation, 1-ml fractions were harvested from the bottom by puncturing the tube with an 18-gauge needle. Protein concentration in each fraction were determined by the Bradford protein assay (31), and 10 μg of protein were run on 10% SDS-PAGE, trans-
ferred to nitrocellulose, and probed for PLSCR1 or CD55.

Alkaline Phosphatase Assay—Sucrose density fractions were mea-
ured for alkaline phosphatase activity to identify rafts. Fraction sam-
ples (20 μl) were added to a 96-well flat bottom plate and mixed with 200 μl of reaction buffer (5 mM p-nitrophenyl phosphate in 100 mM 2-amino-2-methyl-1-propanol (pH 10.0)). Reactions were incubated at 37 °C for at least 15 min, and the absorbance was read in a microplate reader at 405 nm.

Cloning and Transfection of PLSCR1—RNA isolated from Jurkat cells was synthesized to cDNA using a reverse transcriptase for PCR kit (Clontech Inc.) according to the manufacturer’s instructions. cDNA was
Activated Neutrophil Phospholipid Flip-Flop

Glycerophospholipids—To test whether the outward flop of exogenous PS was both specific for PS and unidirectional or alternatively was bidirectional as suggested by enhanced PS uptake observed in Fig. 1B, we tested whether exogenous phosphocholine-containing diradyl lipid probes presented to the activated neutrophil would be internalized. Using a non-hydrolyzable, alkyl-linked phosphocholine probe, c-PAF, fMLP stimulation resulted in enhanced phospholipid uptake that was rapid in onset, occurred as early as 5 min, and peaked at 15 min following stimulation (Fig. 1C). Enhanced phospholipid uptake did not result from stimulation of the PAF receptor by c-PAF as it occurred in the presence of the PAF receptor inhibitor WEB 2086 (data not shown) and was identical for the fluorescently labeled NBD-PC, an ester-linked phosphatidylethanolamine probe that does not stimulate the PAF receptor (Fig. 1D). As a positive control, neutrophils stimulated to undergo apoptosis with UV irradiation also showed increased phospholipid uptake of choline-containing probes (14, 17, 24). While phospholipid uptake during apoptosis was inhibited by the caspase 3 inhibitor DEVD phospholipid uptake during fMLP stimulation was not inhibited, again dissociating it from the process of apoptosis (data not shown) (24).

Phospholipid Uptake Was Independent of Endocytosis and Pinocytosis—In contrast to the enhanced uptake of choline-containing glycerophospholipids, internalization of NBD-sphingomyelin, which has been used as a lipid marker of endocytosis (35), was not enhanced during either stimulation or apoptosis (data not shown); this was despite equivalent staining for both NBD-sphingomyelin and NBD-PC of both control and unwashed samples.

To further investigate whether PC uptake was attributable to an endocytic/pinocytic pathway and whether PS exposure was the consequence of bilayer mixing during vesicle-plasma membrane budding and fusion during endosome recycling, the following experiments were conducted. First, uptake of the fluid phase marker Lucifer Yellow was determined in duplicate samples following stimulation of neutrophils with fMLP alone or fMLP with cytochalasins. Pretreatment with either cytochalasin B or D has been shown to inhibit actin assembly and actin-mediated endocytosis during fMLP stimulation but also to significantly enhance both degranulation and pinocytosis (30) while markedly stimulating NBD-PC uptake (Fig. 2B). Conversely pretreatment with either cytochalasin B (Fig. 2A) or D (not shown) prior to fMLP stimulation markedly enhanced uptake of Lucifer Yellow while having little effect on NBD-PC uptake over that seen with fMLP alone (Fig. 2B). Additionally inhibition of clathrin-mediated endocytosis by hyperosmolar sucrose (0.4 M) (36, 37) had no inhibitory effect on fMLP-stimulated uptake of NBD-PC (data not shown).

Movement of Phospholipids Was Accompanied by Altered Membrane Packing Associated with Transblayer Flip-Flop—It is known that calcium ionophore-treated erythrocytes demonstrate bidirectional, nonspecific, transbilayer flip-flop of phospholipid probes as well as enhanced staining with FM 1-43 or the related dye MC540, which are sensitive to phospholipid packing changes (38–40). Similarly cells undergoing apoptosis and activation have shown enhanced FM 1-43 (or MC540) dye staining (12, 14, 17–21, 41). Therefore, neutrophils were stimulated with fMLP for various lengths of time and stained with FM 1-43 for the last minute of stimulation. Enhanced staining was evident as early as 2 min following fMLP addition (Fig. 2C). Furthermore FM 1-43 staining was unaffected by the presence of cytochalasin B or D. Taken together, these data

RESULTS
PS Exposure on fMLP-stimulated Neutrophils—Using FVa as a sensitive probe to detect outer leaflet PS (34), we were able to demonstrate that fMLP stimulation resulted in transient PS exposure that was evident as early as 10 min and returned to baseline levels by 45 min (Fig. 1A). In contrast, this transient PS exposure was minimally detectable by the less sensitive annexin V binding (data not shown). PS exposure during fMLP stimulation was not accompanied by loss of aminophospholipid packing changes (38–40). Similarly cells undergoing apoptosis and activation have shown enhanced FM 1-43 (or MC540) dye staining (12, 14, 17–21, 41). Therefore, neutrophils were stimulated with fMLP for various lengths of time and stained with FM 1-43 for the last minute of stimulation. Enhanced staining was evident as early as 2 min following fMLP addition (Fig. 2C). Furthermore FM 1-43 staining was unaffected by the presence of cytochalasin B or D. Taken together, these data

used as a template to amplify full-length PLSCR1 using the forward primer 5′-AAGATCCGGACGACAGAATTTTCA-3′ and the reverse primer 5′-AAGATCCGGACGATTTTCA-3′, both of which have been engineered into an EcoRI restriction enzyme cloning site on the 5′ ends. The PCR product of the correct size was purified and cloned directly into the EcoRI sites of pcDNA3-T(Invitrogen), which allows the expression of the cloned gene in the presence of doxycycline. T-Rex-293 cells, expressing the tetracycline repressor, were transfected with PLSCR1 in the absence or presence of doxycycline to induce expression of PLSCR1 using FuGENE transfection reagent (Roche Applied Science) according to the manufacturer’s instructions. Cells were harvested 24 h after transfection into lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 0.25% deoxycholate, 1% Triton X-100). Protein concentrations were determined by the Bradford protein assay (31), and 10 µg of protein were run on 10% SDS-PAGE, blotted to nitrocellulose, and probed for PLSCR1.

PLSCR1, CD45, and Cholera Toxin B Staining—For PLSCR1 staining, neutrophils (7.5 × 10⁶) at 20 × 10⁶/ml were blocked for 15 min on ice with 1% BSA in KRBD buffer and then incubated with either PLSCR1 serum (1:50) or preimmune serum (1:50) from the same rabbit on ice for an additional 30 min. Primary antibody was removed, and neutrophils (in KRBD buffer with 0.25% BSA) were stimulated with fMLP for 10 min at 37 °C, fixed in 3% paraformaldehyde, 3% sucrose in PBS at room temperature for 10 min, washed twice with PBS, and incubated with Cy3 goat anti-Rabbit (Fab′)₂, diluted 1:100 for 30 min on ice. Cells were washed twice more with PBS and analyzed by flow cytometry. For CD45, neutrophils were preincubated as above except with 10 µg/ml mouse anti-human CD45 or isotype control antibody, and Cy3 anti-mouse Fab′/2 diluted 1:100 was used.

For fluorescence microscopy, cells were pretreated with antibody as above or with CT-B-Alexa 555 diluted 1:200 and either settled for 20 min at room temperature on poly-L-lysine-coated coverslips (pretreated with 100 µg/ml in PBS at 4 °C overnight) followed by fMLP stimulation at 37 °C or stimulated in suspension. Following stimulation, cells were fixed as above and incubated for 15 min at room temperature with secondary antibody, washed three times with PBS, and permeabilized with 0.2% Triton X-100 for 10 min at room temperature. Cells were washed twice more with PBS, stained with fluorescein isothiocyanate-phalloidin (1:250) and 5 µg/ml Hoechst in PBS for 15 min at room temperature, washed with PBS, mounted with OPDA, and viewed with a Zeiss fluorescence microscope using a 63 × oil (numerical aperture 1.4) Axiosvert 200M objective. Images were analyzed using Slidebook software.

Live Cell Imaging—Neutrophils (7.5 × 10⁶ cells at 5 × 10⁶/ml) in NBD buffer with 0.01% BSA, 1 mM EDTA, 300 µM EGTA, and either FM 1-43 or NBD-PC at a final concentration of 16 µM or 5 µM, respectively, were placed in a BSA (0.01%)-coated Delta T dish (Biotech Inc.) that was preheated to 37 °C. Neutrophils were stimulated with fMLP, and images were taken over 5 min with an inverted Olympus IX70 microscope equipped with a SenSys camera connected to TILLvision software (T.I.L.L. Photonics GmbH) every 3 s with a 500-ms exposure time for FM 1-43 and every 5 s with a 3-s exposure time for NBD-PC.

Subcellular Fractionation—Neutrophil granules were isolated by Percoll density gradients from control neutrophils as described previously (32). Plasma membrane and secretory vesicles from control neutrophils were isolated by free flow electrophoresis as described previously (33).

Testing for whether the outward flop of exogenous PS was both specific for PS and unidirectional or alternatively was bidirectional as suggested by enhanced PS uptake observed in Fig. 1B, we tested whether exogenous phosphocholine-containing diradyl lipid probes presented to the activated neutrophil would be internalized. Using a non-hydrolyzable, alkyl-linked phosphocholine probe, c-PAF, fMLP stimulation resulted in enhanced phospholipid uptake that was rapid in onset, occurred as early as 5 min, and peaked at 15 min following stimulation (Fig. 1C). Enhanced phospholipid uptake did not result from stimulation of the PAF receptor by c-PAF as it occurred in the presence of the PAF receptor inhibitor WEB 2086 (data not shown) and was identical for the fluorescently labeled NBD-PC, an ester-linked phosphatidylethanolamine probe that does not stimulate the PAF receptor (Fig. 1D). As a positive control, neutrophils stimulated to undergo apoptosis with UV irradiation also showed increased phospholipid uptake of choline-containing probes (14, 17, 24). While phospholipid uptake during apoptosis was inhibited by the caspase 3 inhibitor DEVD phospholipid uptake during fMLP stimulation was not inhibited, again dissociating it from the process of apoptosis (data not shown) (24).
demonstrate that PS exposure is accompanied by both nonspecific phospholipid uptake and membrane packing changes that are independent of both pinocytosis and actin-dependent endocytosis. These data strongly support activated, bidirectional, transbilayer flip-flop of phospholipids in the stimulated neutrophil.

Localization of PLSCR1 to Neutrophil Raft Membranes—While the role of PLSCR1 in mediating phospholipid flip-flop remains controversial (see the Introduction and “Discussion”), we sought to determine whether human neutrophils expressed PLSCR1 on the plasma membrane surface and whether its localization could be related to areas of phospholipid flip-flop and membrane packing changes. First, using an antibody raised against the 14 carboxyl-terminal amino acids of PLSCR1, a single band at 35 kDa was identified by Western immunoblot analysis of neutrophil whole cell lysates (Fig. 3A). This band was identical to PLSCR1 expressed endogenously and overexpressed in T-Rex-293 cells suggesting that this antibody does indeed recognize PLSCR1 protein. Additionally subcellular fractionation of neutrophil lysates revealed that PLSCR1 was localized mainly in the plasma membrane/secretory vesicle fraction but also to tertiary granules and to a lesser degree in secondary granules (Fig. 3B). It was absent from both the primary granules and the cytosol. Further investigation utilizing free flow electrophoresis supported the finding that PLSCR1 was localized in both the plasma membrane and, to a lesser extent, the secretory vesicles (Fig. 3B).

As the protein sequence analysis predicts PLSCR1 to be a Type II protein with a single transmembrane domain near the carboxyl terminus (27), we labeled non-permeabilized control and stimulated neutrophils at 4°C with the carboxyl-terminal domain antibody or preimmune serum from the same rabbit followed by flow cytometric analysis. As shown in Fig. 3C, we found evidence of surface staining for PLSCR1 on control neutrophils, and stimulation of cells with fMLP for 10 min resulted in little enhancement of surface staining for PLSCR1. By contrast, CD11b, also localized to secretory vesicles and secondary and tertiary granules, was increased following stimulation (Fig. 3D). These observations suggest that activated phospholipid flip-flop does not require increased PLSCR1 on the plasma membrane. Furthermore they also suggest the possibility that PLSCR1 localized to both the plasma membrane and labile secretory vesicles may be recycled between these compartments (see “Discussion”) reminiscent of the recycling endosomes of epithelial cells (28).

PLSCR1, a palmitoylated protein, has been localized to raft membrane domains in epithelial carcinoma KB cells (28). Isolation of detergent-resistant membranes from both control and fMLP-stimulated neutrophils followed by Western immunoblot analysis revealed that PLSCR1 was also enriched in neutrophil rafts (Fig. 4A) as determined by co-localization with CD55, a known raft protein (Fig. 4B), and alkaline phosphatase activity (Fig. 4C). Interestingly immunoblots for PLSCR1 from membranes prepared for raft isolation showed

![Figure 1](http://www.jbc.org/)

**Fig. 1.** Stimulated neutrophils transiently expose PS on their surface and take up phospholipid probes. Neutrophils were stimulated with 10 nM fMLP, and surface exposed PS was measured by FVa binding (A). Uptake of NBD-PS was used as a measure of aminophospholipid translocase activity in fMLP-stimulated or apoptotic neutrophils and compared with unstained control (CTR Unst), fMLP-stimulated (20′/H11032 Unst), or apoptotic (APO Unst) neutrophils (B). C, time course of uptake of radiolabeled cPAF in neutrophils stimulated with 10 nM fMLP. D, uptake of NBD-PC in fMLP-stimulated or apoptotic (UV-irradiated) neutrophils compared with unstained control (CTR Unst), fMLP-stimulated (20′/H11032 Unst), or apoptotic (APO Unst) neutrophils. "%, minutes.
two bands, one at the expected 35 kDa and a smaller one at 31 kDa. The presence of this smaller band, despite inclusion of protease inhibitors, remains unexplained but appeared to be related to the raft isolation procedure in that it was not apparent when whole cell lysates were prepared for Western blotting.

Analysis of PLSCR1 surface distribution on control non-permeabilized neutrophils by epifluorescence microscopy demonstrated that the protein was localized on the plasma membrane in a patchy distribution unlike CD45, a protein often excluded from rafts, which appeared evenly distributed on the membrane surface (Fig. 5A). Stimulation of neutrophils with fMLP to produce a polarized phenotype resulted in aggregation of these patches and redistribution of PLSCR1 to the uropod as shown in cells stained with anti-PLSCR1 antibody and phalloidin to identify the leading edge (Fig. 5B). CD45 staining, on the other hand, remained evenly distributed on the membrane despite the polarized phenotype.

It has been shown that migrating T cells exhibit a polarized phenotype with asymmetrically distributed raft domains: GM1-containing rafts localized to the uropod, and GM3-containing rafts localized to the leading edge (42). Similarly in neutrophils, it has been shown that chemoattractant receptors are redistributed to the front of the cell during migration (43) and that detergent-resistant membranes and the raft markers CD44 and CD43 localize toward the rear in polarized cells (44). Whether fMLP-polarized neutrophils asymmetrically distribute GM1-containing membrane rafts to the uropod was determined. Neutrophils were stained with CT-B to identify GM1-containing rafts and phalloidin to identify actin assembly at the leading edge. As shown in Fig. 5C, in unstimulated control cells (lacking F-actin), CT-B appeared to be patched and focally distributed similar to the pattern of staining observed with PLSCR1. Following fMLP stimulation, the leading edge was easily identified by phalloidin staining of F-actin, while GM1 stained with CT-B appeared to be excluded from the leading edge and aggregated exclusively at the uropod similar to that seen with PLSCR1. Direct co-localization could not be performed because CT-B binding appeared to interfere with PLSCR1 staining by flow cytometry (data not shown). Taken together, these data demonstrated that PLSCR1, a raft protein, associated with raft membranes at the neutrophil uropod following fMLP stimulation.

Phospholipid Flip-Flop Occurs in Neutrophil Rafts—We have shown that neutrophils exhibit enhanced nonspecific phospholipid flip-flop in response to fMLP and localized PLSCR1, a candidate protein proposed to play a role in mediating phospholipid flip-flop, to raft domains at the uropod. Therefore, we hypothesized that phospholipid flip-flop would occur in uropod raft membranes containing PLSCR1. As expected and shown in Fig. 6, staining of fMLP-polarized neutrophils with Factor Va showed bright staining of uropodia following stimulation demonstrating the uropod as the region of PS exposure. Additionally we hypothesized that the uropod was also the site of phospholipid packing changes and phospholipid uptake. Neutrophils were stimulated with fMLP in the presence of the dye FM 1-43 and visualized at 0 and 4 min. As demonstrated in Fig. 7A, FM 1-43 staining was brightest at developing uropodia in polarizing neutrophils stimulated with fMLP. Since enhanced FM 1-43 staining detects areas of altered phospholipid packing that have been associated with enhanced phospholipid flip-flop (21, 38, 39), we also stimulated neutrophils with fMLP in the presence of NBD-PC. As shown in Fig. 7B, incorporation of NBD-PC was also restricted to the uropod of the polarizing neutrophil. Taken together, the data strongly suggest that altered membrane packing, phospholipid flip, PS exposure (flop), and PLSCR1 all localize to raft membranes at the neutrophil uropod.
DISCUSSION

The movement of PS from inner to outer plasma membrane leaflet (flop) is demonstrated for nearly all cells undergoing apoptosis and in some cells following activation including the fMLP-stimulated neutrophil (Fig. 1). While PS exposure on apoptotic cells leads to efficient engulfment by phagocytes with anti-inflammatory consequences (6, 7), PS exposure during viable cell activation may or may not have similar consequences, likely dependent on quantity, localization, or duration of PS exposure required for recognition (10, 45) or the presence of “don’t eat me signals” (6, 7). Additionally activation of both the complement and coagulation cascades are well documented consequences of PS exposure and potentially result during inflammatory cell activation (2). Finally localized domains of exposed PS on inflammatory cells also significantly enhanced the binding of secreted phospholipases A2 (46). Indeed human embryonic kidney 293 cells overexpressing PLSCR1 have been reported to have both enhanced PS exposure and sensitivity to type IIa and type V secretory phospholipases A2 (26).

Growing evidence supports that outward phospholipid flop is not specific for PS but in fact is utilized to externalize other signaling phospholipids as well. Lyso-PC and PAF have been shown to be externalized and released in both activation and apoptosis (18, 39–49). In both cases, these lipid mediators are first synthesized within the cell, then moved across the plasma membrane bilayer, and released to acceptor proteins (e.g. albumin) before reaching target cells where they mediate effects through specific receptors.

Flop of phospholipids during neutrophil stimulation is accompanied by both phospholipid packing changes (Fig. 2) and inward flip of phospholipids (Fig. 1). As such, these findings are similar to observations made in apoptotic cells and in calcium ionophore-treated erythrocyte ghosts (14, 17, 39). The purpose of inward flip of phospholipids, other than the aminophospholipids in non-apoptotic cells, is not readily apparent, but compensatory movement of phospholipid between the bilayers may be required to maintain bilayer geometry (50). In contrast to activated flip-flop demonstrated here, investigation in unstimulated CHO-K1 cells suggested that NBD-PC can be internalized by endocytosis (51), and recent work in apoptosing HeLa cells suggested that while an alkyl-linked PAF-like lipid was also endocytosed, acyl-linked NBD-PC was internalized by flip-flop (52). Here, in fMLP-stimulated neutrophils, we have detected no differences in the activated uptake of sn-1 alkyl- and acyl-linked phosphorylcholine lipid species, and in both cases, phospholipid flip-flop rather than either endocytosis or pinocytosis appeared to be the route of entry (Figs. 1 and 2).

Exposed PS is described as punctate or patchy in distribution on activated cells (10, 11, 13) and has also been shown to be transiently localized to cell-cell contact areas during myotube formation (53) and to the apical region of capacitated spermatozoa (8). Data suggest that these localized domains of outer leaflet PS represent raft membranes: PS co-localized with caveolin in macrophages (54), co-capped with IgM in raft do-
mains following B cell stimulation (11), and co-localized with apical raft domains on capacitated spermatozoa (8, 41). In this study, we found in the fMLP-stimulated neutrophil that PS exposure and phospholipid packing changes also occurred in domains that co-localized with raft proteins as well as with PLSCR1 (Figs. 4, 5, and 6). Furthermore we found this co-localization at neutrophil uropodia, which along with the lamellipodia are known raft membrane locations in polarized neutrophils showing front-to-back segregation: while urokinase-type plasminogen activator receptor moves to lamellipodia (55), CR3, CD55, P-selectin glycoprotein ligand 1, intercellular adhesion molecule-3, CD44, and CD43 move to uropodia (44, 55, 56), and CD45 and HLA remain diffusely distributed on plasma membranes following stimulation (44). Other stimulated and polarized cells (myelocytic cells, T cells, and myeloma cells) have also shown similar raft protein segregation between lamellipodia and uropodia suggesting that these redistributions may be required for polarization and chemotaxis (42, 57–62).

Directional migration of neutrophils in response to fMLP requires phosphatidylinositol 3-kinases and protein kinases C, which recognize inner leaflet phospholipids (phosphatidylinositol and PS, respectively) (63). The recruitment of phosphatidylinositol 3-kinases, generation of phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate, and subsequent recruitment of pleckstrin homology domain-containing proteins (e.g. Akt/protein kinase B) and Rho GT-Pases to the leading edge are thought to transmit a shallow extracellular chemoattractant gradient into a steep intracellular gradient for the restricted assembly of F-actin at the lamellipodium (63–65). Thus spatial restriction of phosphatidylinositol 3-kinase-modified phosphatidylinositol, their catabolism (66, 67), and potentially their transbilayer movement would serve to inhibit actin assembly at locations other than the leading edge and enable directed migration. Similarly
adhesion molecules at the leading edge would be inhibited at the uropod (60, 61).

The role of PLSR1 in phospholipid flip-flop is controversial. Zhou et al. (27), who originally cloned the protein from erythroid leukemia cells, were able to show enhanced transbilayer movement of NBD-PC in PLSR1-reconstituted proteoliposomes. In earlier studies, we demonstrated enhanced cPAF uptake in CHO-K1 cells transfected with both PLSR1 and protein kinase Cδ, which was shown to phosphorylate PLSR1 (24). Finally, overexpression of PLSR1 (with or without calcium and ionophore stimulation) enhanced annexin binding in human embryonic kidney 293 cells (26), CHO-K1 cells (23), RBL-2H3 cells (69), and Raji cells (25), although other investigations have not shown enhanced activity with overexpression of the protein (28, 70, 71). On the other hand, recent studies using platelets from PLSR1−/− mice showed no difference in PS exposure, although direct measures of phospholipid movement using phospholipid probes were not reported, and the presence of other family member proteins raise the possibility of compensatory activity occurring in cells from knock-out mice (72). As a 35–37-kDa protein with one purported transmembrane domain, PLSR1 would seem an unlikely candidate to directly mediate transbilayer movement of phospholipids. However, as a palmitoylated raft membrane protein, it may either self-aggregate or associate with other partners or signal via phosphotyrosine-mediated associations within the raft platforms to enhance phospholipid movement across the bilayer (28, 73).

Recent studies in the PLSR1−/− mouse demonstrating defective myelopoiesis suggest a role for PLSR in neutrophil differentiation as well (72). Whether this or other functions of the protein, such as binding of secretory leukocyte protease inhibitor (74), enhancement of PS biosynthesis during apoptosis (23), or signaling to downstream c-Src (73), are of importance in phospholipid flip-flop remain to be determined. While our studies here in terminally differentiated human neutrophils place PLSR1 in the same location as phospholipid flip-flop and we hypothesize that PLSR1 is important in polarization and chemotaxis, its role in the actual process of phospholipid flip-flop requires future studies in functionally chemotaxing and genetically mutable cells for further elucidation.

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Phospholipid Flip-Flop and Phospholipid Scramblase 1 (PLSCR1) Co-localize to Uropod Rafts in Formylated Met-Leu-Phe-stimulated Neutrophils
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