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An Unrecognized Function of Cholesterol: Regulating the Mechanism Controlling Membrane Phospholipid Asymmetry

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Abstract

An asymmetric distribution of phospholipids in the membrane bilayer is inseparable from physiological functions, including shape preservation and survival of erythrocytes, and by implication other cells. Aminophospholipids, notably phosphatidylserine (PS), are confined to the inner leaflet of the erythrocyte membrane lipid bilayer by the ATP-dependent flippase enzyme, ATP11C, counteracting the activity of an ATP-independent scramblase. Phospholipid scramblase 1 (PLSCR1), a single-transmembrane protein, was previously reported to possess scrambling activity in erythrocytes. However, its function was cast in doubt by the retention of scramblase activity in erythrocytes of knockout mice lacking this protein. We show that in the human erythrocyte PLSCR1 is the predominant scramblase and by reconstitution into liposomes that its activity resides in the transmembrane domain. At or below physiological intracellular calcium concentrations, total suppression of flippase activity nevertheless leaves the membrane asymmetry undisturbed. When liposomes or erythrocytes are depleted of cholesterol (a reversible process in the case of erythrocytes), PS quickly appears at the outer surface, implying that cholesterol acts in the cell as a powerful scramblase inhibitor. Thus, our results bring to light a previously unsuspected function of cholesterol in regulating phospholipid scrambling.

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ASSOCIATED CONTENT

Supporting Information

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Three supporting figures (Figures S1–S3) included to confirm the expression of the PLSCR family in human and mouse erythroblasts, the amount of PLSCR1 reconstituted into liposomes, and the propriety of 2D PAGE analyses (PDF)

The human erythrocyte membrane is composed of several species of phospholipids, together with cholesterol, which comprises ~50 mol % of the total lipids in the bilayer.¹ The phospholipids are asymmetrically disposed between the membrane leaflets, with the aminophospholipids [predominantly phosphatidylserine (PS) and phosphatidylethanolamine (PE)] confined to the inner leaflet.² This asymmetry is largely maintained by an enzyme, a P-IV type ATPase, a flippase, which catalyzes ATP hydrolysis with rapid and selective translocation of PS and PE from the outer to the inner leaflet against their concentration gradients.³ Under physiological conditions, the intracellular ATP concentration of ~1 mM is sufficient to maintain the activity of the flippase, whose K_m is in the submillimolar range.^{3,4} We recently identified ATP11C, a member of a P-IV type ATPase, as a major source of flippase activity in human erythrocytes by analysis of a patient's cells, devoid of flippase activity.⁵ An overwhelming majority of these cells nevertheless show no exposure of PS on their outer surface. It would appear to follow that flippase activity is not primarily essential for the retention of PS within the inner leaflet under physiological conditions.

Restriction of PS to the inner leaflet is necessary for erythrocyte survival *in vivo*, because its exposure on the outer surface ensures that the cell will be recognized, phagocytosed by splenic macrophages, and eliminated from the circulation. An increased level of PS exposure has been shown to play a part in the diminution of erythrocyte life span in erythrocytes in sickle cell disease and in beta-thalassemia. Furthermore, interaction of PS at the inner membrane surface also exercises an important function by binding to the preponderant structural protein, spectrin, of the membrane skeleton, which governs the membrane mechanical stability, elastic properties, and shape of the cell;⁶ it also protects the spectrin from glycation, which otherwise reduces the deformability of the membrane skeleton and therefore that of the cells.⁷

Normally, phospholipid scrambling occurs only in senescent cells, where PS exposure has been described as an “eat-me signal” for senescent cell recognition, followed by phagocytosis by splenic macrophages.⁸ PS exposure is promoted by activation of scramblase activity caused by an increase in intracellular Ca^{2+} concentrations,^{9,10} an effect that is suppressed or minimized in healthy cells in which Ca^{2+} concentrations are maintained at a low submicromolar level by the Ca^{2+} -ATPase.¹¹ Scramblase bidirectionally translocates a wide variety of phospholipids in Ca^{2+} -dependent and ATP-independent manners.^{9,11} PLSCR1, a 35 kDa single-pass transmembrane protein, is the only scramblase that has been

isolated and cloned from human erythrocytes.^{9,12} Human PLSCR1 has a large cytoplasmic N-terminal domain and a very short (nine-amino acid) extracellular C-terminal domain.¹² Its scrambling activity is thought to be modulated by binding of Ca²⁺ to an EF-hand-like motif that is located in the juxta-transmembrane at the cytoplasmic domain.¹³ If now PLSCR1 is contained, as has been suggested, in cholesterol-rich lipid rafts,¹⁴ one may speculate that its function may be regulated by cholesterol. Cholesterol in the erythrocyte membrane is involved in restricting bilayer fluidity and regulating signal transduction through lipid rafts.¹⁵ Evidence of a role for cholesterol in asymmetric phospholipid distributions in human erythrocyte membranes is still lacking. Moreover, a scramblase function for PLSCR1 in erythrocytes has been questioned, on the grounds that erythrocytes from PLSCR1-knockout mice expose PS normally by high calcium concentrations.¹⁶

The objective of this study was to elucidate the regulatory mechanisms governing asymmetric phospholipid distributions at physiologically low Ca²⁺ concentrations in human erythrocyte membranes, with an emphasis on cholesterol- and PLSCR1-mediated scrambling. We also examined the basis for the apparent differences in PLSCR1 function in mice and humans.

EXPERIMENTAL PROCEDURES

Materials

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (phosphatidylserine, PS, 840034), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (phosphatidylcholine, PC, 850457), 1-palmitoyl-2-(6-NBD-hexanoyl)-*sn*-glycero-3-phospho-L-serine (NBD-PS, 810192), 1-palmitoyl-2-(6-NBD-hexanoyl)-*sn*-glycero-3-phosphocholine (NBD-PC, 810130), and cholesterol (700000) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). FITC-conjugated annexin V (FITC-annexin V), methyl- β -cyclodextrin (MBCD, C4555), cholesterol-MBCD (cholesterol-water-soluble, C4951), *n*-dodecyl β -D-maltoside (DDM, D4641), and dithionite (157953) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). *N*-Ethylmaleimide (NEM, 054-02063) was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), mouse polyclonal antiscramblase-1 (PLSCR1) (H00005359-A01) from Abnova (Taipei, Taiwan), mouse kidney QUICK-Clone cDNA from Clontech (Mountain View, CA), and Factor Xa from GE Healthcare Life Sciences (Buckinghamshire, England). *Eco*RI, *Sa*II, and the pMAL-c2X vector (N8076) were from New England Biolabs, Inc. (Ipswich, MA), and One Shot BL21-(DE3)pLysS (C6060) was from Life Technologies, Inc. (Carlsbad, CA). Other chemicals or drugs were reagent grade or of the highest quality available.

Preparation of Erythrocyte Membranes

After informed consent had been obtained, venous blood was collected from healthy volunteer donors to CPD blood bags (Terumo Corp., Tokyo, Japan), and murine blood was collected from the heart using a 25 gauge needle and a 2.5 mL syringe under anesthesia. The leukocytes and platelets were removed using an IMUGARD III-RC filter (Terumo Corp.). Erythrocytes were then washed three times by centrifugation at 1600g for 5 min at 4 °C with phosphate-buffered saline (PBS) [10 mM sodium phosphate and 150 mM NaCl (pH 7.4)].

Expression of Phosphatidylserine on Erythrocyte Exteriors

Ca^{2+} -dependent binding of annexin V with PS was conducted using FITC-annexin V using an Apoptosis Detection Kit (BV-K101-3, MBL International Corp., Woburn, MA).⁶ Briefly, erythrocytes were pretreated with or without 0.5 mM NEM (to inactivate flippase) for 15 min, followed by 5 mM MBCD for 20 min. The cells were next treated with 0.1–1.0 μM Ca^{2+} in the presence of 2 μM A23187; 1 mM ethylene glycol tetraacetic acid (EGTA) was used instead of Ca^{2+} in Ca^{2+} -free samples, and subsequently, 10^5 cells were washed once with PBS and resuspended in 85 μL of binding buffer [125 mM NaCl, 10 mM Hepes-NaOH, and 5 mM CaCl_2 (pH 7.4)]. Then, 10 μL of a FITC-annexin V solution was added. After a 30 min incubation at room temperature in the dark, the cells were transferred to ice, and the sample volume was increased to 0.5 mL with binding buffer. Membrane-associated fluorescence was quantitated using an EPICS XL/XL-MCL flow cytometer (Beckman Coulter, Inc., Brea, CA), and the results were analyzed with System II version 3.0.

PLSCR1 Peptides

Transmembrane peptides of PLSCR1, wild type containing a Ca^{2+} -binding site and C-terminal end (NH_2 -²⁷³DADBF GIQFP LDLDV KMKAV MIGAC FLI²⁹¹DF MFFES TGSQE QKSGV ³¹⁸W-COOH₂), and of PLSCR1 mutant whose ²⁹¹D was substituted with L were synthesized by Gene World Ltd. (Tokyo, Japan).

Preparation of a Recombinant Maltose-Binding Protein-Fused Full-Length PLSCR1

A cDNA clone of human PLSCR1 (hPLSCR1) was obtained from Invitrogen Corp. (Carlsbad, CA; GenBank accession number NM_021105). The open reading frame (954 bp) of hPLSCR1 was amplified by polymerase chain reaction (PCR) using a forward primer (5'-TCA GAA TTC GGA TCC ATG GAC AAA CAA AAC TCA CAG ATG-3') and a reverse primer (5'-GCT TGC CTG CAG GTC GAC CTA CCA CAC TCC TGA TTT TTG TTG TTC C-3'). For cloning of mouse PLSCR1 (mPLSCR1) mRNA (GenBank accession number NM_011636), the open reading frame (984 bp) of mPLSCR1 was amplified by PCR using a forward primer (5'-TCA GAA TTC GGA TCC ATG GAA AAC CAC AGC AAG CAA ACT-3') and a reverse primer (5'-GCT TGC CTG CAG GTC GAC TTA CTG CCA TGC TCC TGA TCT TTG C-3') by using mouse kidney QUICK-Clone cDNA as a template. The amplicons were digested with *EcoRI* and *SaI*, and the fragment was inserted into a pMAL-c2X vector. Then, the plasmid was finally transformed in One Shot BL21(DE3)pLysS to prepare maltose-binding protein (MBP)-hPLSCR1 or MBP-mPLSCR1 protein. For digestion of MBP-PLSCR1 proteins to obtain PLSCR1 proteins, 30 μg of MBP-PLSCR1 proteins was incubated with 0.6 unit of Factor Xa for 8 h at room temperature in the presence of 1 mM Ca^{2+} .

Purification of Recombinant PLSCR1 Protein

MBP-PLSCR1 protein, prepared as described above, was sonicated in TBS-T buffer [200 mM Tris-HCl (pH 7.4), 200 mM NaCl, 0.05% Tween 20, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), and 1 mM phenylmethanesulfonyl fluoride]. The protein was passed through a MBP Trap HP column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden, 28-9187-79) using a binding buffer [200 mM Tris-HCl (pH 7.4), 200 mM

NaCl, 1 mM EDTA, and 1 mM DTT] and eluted with an elution buffer (binding buffer containing 10 mM maltose). The eluate was then dialyzed in a buffer, containing 50 mM Tris-HCl (pH 8.0) and 100 mM NaCl, and used in experiments.

Detection of PLSCR1 Protein in Human and Mouse Erythrocyte Membranes

Prepared erythrocyte membranes were dissolved in Laemmli's sample buffer [final concentration, 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 500 mM DTT, and 10% glycerol] and incubated at 95 °C for 5 min. The proteins were separated by 10% SDS-PAGE and electrophoretically transferred from the gel onto a polyvinylidene difluoride (PVDF) membrane by the semidry blotting method. The blots were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) [10 mM Tris-HCl, 100 mM NaCl, and 0.05% Tween 20 (pH 7.5)] at 4 °C overnight. The blots were further incubated with the anti-PLSCR1 antibody and the corresponding secondary antibody labeled with an infrared fluorescent dye (LI-COR, Lincoln, NE). The bands of PLSCR1 captured on the membrane were quantified with an Odyssey CLx infrared imaging system (LI-COR). The amount of endogenous PLSCR1 in erythrocyte membranes was quantitated with reference to known amounts of Factor Xa-digested recombinant hPLSCR1 or mPLSCR1 protein used as a standard.

Reconstitution of Recombinant MBP-PLSCR1 or PLSCR1 Peptide into Liposomes

Reconstitution of proteoliposomes was conducted as described in a previous report with slight modifications.¹² For recombinant MBP-PLSCR1, a mixture of PC and cholesterol (5 μ mol each) was dried in an evaporator and resuspended in buffer A [100 mM Tris-HCl (pH 7.4), 100 mM KCl, 0.1 mM EGTA, and 93.2 μ M CaCl₂, providing 1 μ M free Ca²⁺] containing 0.5 nmol of MBP-PLSCR1 protein. Alternatively, 1.3 nmol of PLSCR1 peptide was added to a mixture of PC, PS, and cholesterol (4.5, 0.5, and 5 μ mol, respectively), and the mixture was dried in an evaporator and resuspended in buffer A. Both of these proteoliposome preparations were sonicated in a Branson Sonifier 450 (Branson Ultrasonics Corp., Danbury, CT) for 2 min.

Detection of MBP-PLSCR1 Protein Reconstituted into Liposomes

Prepared proteoliposomes were ultracentrifuged at 300000g for 30 min at 4 °C and washed once in buffer A. The resultant pellet was dissolved in Laemmli's sample buffer and incubated at 95 °C for 5 min. The proteins were separated by 10% SDS-PAGE and visualized by Coomassie Brilliant Blue (CBB) R-250 staining.

Measurement of Phospholipid Scrambling Activity in Proteoliposomes

Phospholipid scrambling activity was measured following slight modifications of a previously reported method.¹² Briefly, proteoliposomes were mixed with 100 μ g/mL NBD-PC or -PS, and the mixture was immediately diluted 10-fold by addition of buffer A. After incubation of various periods at 37 °C, proteoliposome samples were further diluted 5-fold with buffer A containing 4 mM EGTA and stirred in a fluorescence cuvette. The NBD-derived fluorescence of both the outer and inner leaflets was recorded by a fluorescence spectrophotometer (F-3010, Hitachi Co., Ltd., Tokyo, Japan) using excitation and emission

at 470 and 532 nm, respectively. The fluorescence of the inner leaflet was measured by quenching the outer leaflet NBD response via the addition of 40 mM dithionite during monitoring. The percentage of fluorescence intensity derived from the inner leaflet was calculated as a ratio with the fluorescence intensities from both leaflets.

Estimation of the Molecular Weight of PLSCR1 in Human Erythrocyte Membranes

Erythrocyte membranes were prepared by hypotonic hemolysis with 5 mM sodium phosphate buffer and 1 mM EDTA (5P1E; pH 7.4) and washed three times to remove cytoplasmic proteins. For removal of cytoskeleton and peripheral membrane proteins, the membranes were treated with 0.5 mM sodium phosphate buffer (pH 8.8) for 40 min at 37 °C and following three washes treated with 0.5 mM sodium phosphate buffer (pH 11, adjusted with NaOH) at room temperature for 20 min.¹⁷ The resultant membranes were solubilized by 5P1E including 5% DDM, nondenatured and nonionic detergent, overnight at 4 °C. Blue Native (BN)-PAGE was performed with a Native PAGE 3 to 12% Bis-Tris gel according to the manufacturer's instructions (Invitrogen). The lane with 40 µg of dissolved membrane proteins was excised from the native gel and equilibrated with SDS sample buffer, and the gel fragment was overlaid on the acrylamide gel composed of 4% stacking and 10% separating gels. Electrophoresis and immunoblotting using the anti-PLSCR1 antibody were performed as described above. PLSCR1 signals generated by HRP-conjugated secondary antibody (Dako, Glostrup, Denmark) were visualized by enzymatic chemiluminescence (ECL Prime Western Blotting Detection Reagent, GE Healthcare). To assign accurate molecular weights via BN-PAGE, the electrophoresed lane with a native marker (Invitrogen) was directly blotted to the PVDF membrane and CBB-stained bands were recorded with WesternSure Pen (LI-COR).

RESULTS

PS Exposure in Cholesterol-Depleted and Flippase-Inhibited Human Erythrocytes

The effect of cholesterol on the PS distribution in human erythrocyte membranes was investigated using FITC-annexin V, which does not penetrate the membrane and so binds to only PS in the outer leaflet (exposed on the cell surface). MBCD was used to deplete the cells of cholesterol: this pretreatment with 5 mM MBCD reduced the membrane cholesterol content by ~40%. Pretreatment of erythrocytes with 0.5 mM NEM completely inhibited NBD-labeled PS internalization from the outer to inner leaflet, confirming the absence of any discernible flippase activity (data not shown). Cells were also incubated with a series of Ca²⁺ concentrations (0.1–1.0 µM) in the presence of 2 µM A23187, a Ca²⁺ ionophore.

The proportion of erythrocytes displaying PS on their outer surface, as revealed by FITC-annexin V labeling, was quantified by flow cytometry. Untreated cells exposed to FITC-annexin V served as controls; these gave a baseline level of 10 units, which remained unchanged with external Ca²⁺ concentrations of up to 1.0 µM (Figure 1A, top panel, and Figure 1B). The abscissa value of 10 was accordingly taken to represent the normal unperturbed cells. These results indicated that PS remained in the inner leaflet as long as flippase was active and the Ca²⁺ concentrations kept lower than 1.0 µM.

As shown in Figure 1, inhibition of flippase by NEM does not result in any discernible escape of PS from the inner to the outer membrane leaflet, as long as calcium is excluded from the medium [chelated with 1 mM EGTA (Figure 1A, middle panel)]. With increasing Ca^{2+} concentrations, the proportion of PS-positive cells rose to a plateau value of 30% (Figure 1B), implying the existence in these flippase-deprived cells of a population possessing calcium-activated scrambling activity.

To examine the effect of cholesterol on scrambling activity, NEM-treated erythrocytes were depleted of cholesterol by MBCD treatment. In the absence of Ca^{2+} , <5% gave evidence of surface PS exposure (Figure 1A, bottom panel). At a Ca^{2+} level of 0.1 μM , this proportion rose to 50%, and at 0.5–1.0 μM , it rose to 70–80% (Figure 1B). We infer that a decreased membrane cholesterol content leads to activation of PS scrambling even at free Ca^{2+} concentrations in the physiological range.

The effect of cholesterol depletion is substantially reversible. Thus, NEM-treated erythrocytes, depleted of cholesterol as described above, were incubated with 11.6 mM cholesterol dispersed in MBCD to restore the membrane cholesterol content to almost the original, native level. As shown in Figure 2A, while a vast majority of cholesterol-depleted human erythrocytes showed evidence of surface PS exposure (consistent with data in Figure 1), restoration of cholesterol resulted in significant reductions in PS-positive cells at all Ca^{2+} concentrations tested (0.1–1.0 μM) (Figure 2B). This reversal of sensitivity to calcium of the scrambling activity following restoration of cholesterol implies that cholesterol itself has a major regulatory influence on the calcium-dependent aminophospholipid translocation in the erythrocyte membrane at physiologically relevant Ca^{2+} concentrations.

Expression of PLSCR1 in Human Erythrocyte Membranes

The validity of the identification of PLSCR1 as the scramblase in human erythrocytes¹² has been questioned on the grounds that erythrocytes of knockout mice lacking this protein exhibit unchanged PS exposure at high calcium concentrations.¹⁶ To resolve this apparent paradox, we examined the expression of PLSCR1 protein in both human and mouse erythrocyte membranes. Immunoblot analysis showed the prominent presence of PLSCR1 in human erythrocytes, but vanishingly little or none in those of mouse erythrocyte membranes (Figure 3). This observation is borne out by RNAseq data, which showed significantly less PLSCR1 mRNA in normal murine erythroblasts than in human erythroid cells (Figure S1).¹⁸ In contrast, there is a markedly higher level of expression of PLSCR3 in murine erythroblasts than in human erythroid cells. This may account for the lack of phenotype in the PLSCR1-knockout animals. The most plausible conclusion is that PLSCR1 is indeed the scramblase in human erythrocytes whereas PLSCR3 may serve this purpose in the mouse.

Role of PLSCR1 in Phospholipid Scrambling in Proteoliposomes

While the scramblase function of PLSCR1 in human erythrocytes now seems beyond doubt, no information regarding the role of cholesterol in modulating its activity is available. To answer this question, we have had recourse to proteoliposomes reconstituted with MBP-fused full-length human PLSCR1 (MBP-PLSCR1, 76 kDa) (Figure S2). Measured scrambling activity in these proteoliposomes will be solely due to PLSCR1 because no other

flippases or proteins are present in these liposomes. When NBD-labeled PC or PS was added to liposomes, as described in Experimental Procedures and NBD-PC or -PS in the outer layer was quenched by dithionite, the fluorescence intensity of NBD-PC or -PS in the inner leaflet was measured using a fluorescence spectrophotometer. With incubation times of up to 3 h, the fluorescence intensity of NBD-PC increased to 3% even in the absence of PLSCR1 and the cholesterol content did not affect this basal PLSCR1-independent NBD-PC movement (Figure 4A, top panel). In contrast, the presence of PLSCR1 fluorescence intensity doubled, indicating that PLSCR1 accelerated NBD-PC scrambling from the outer to inner leaflet of the liposomes. Strikingly, the presence of cholesterol in the liposomes abolished this PLSCR1-dependent scrambling, whether assayed with NBD-PC or NBD-PS (Figure 4A, bottom panel). These results indicated that PLSCR1 mediates scrambling of both PC and PS in reconstituted proteoliposomes. A nontagged PLSCR1 (produced by digestion of MBP-PLSCR1 by Factor Xa) provided results similar to those obtained for MBP-PLSCR1 (data not shown).

As PLSCR1 has a single transmembrane-spanning domain, we sought to determine whether this alone might be responsible for the scramblase activity. We therefore synthesized the transmembrane domain sequence of 46 amino acids [D273–W318, PLSCR1-pep (see Figure 6A)]. This synthetic peptide was incorporated into PC/PS liposomes with or without cholesterol and containing either NBD-PC or -PS. Phospholipid translocation was assayed as described above. The results (Figure 4A) showed that the transmembrane peptide retained, within experimental error, the full scramblase activity for PC and PS of the intact full-length recombinant PLSCR1 protein. As before, the scramblase activity was totally inhibited by the presence of cholesterol in the liposomes (Figure 4B). These results strongly suggested that PLSCR1's transmembrane domain alone is capable of phospholipid scrambling; this scrambling activity can be completely inhibited by constituent cholesterol.

Self-Association of PLSCR1 in Erythrocyte and Liposome Membranes and Lack of an Effect of Cholesterol

Pore-forming peptides such as magainins form multimers/oligomers in the membrane and facilitate lipid scrambling.¹⁹ We speculated that PLSCR1, a single-pass transmembrane protein, may similarly assemble into multimers to allow passage of the hydrophilic polar headgroup of phospholipids. To test this possibility, the molecular weight of native (endogenous) PLSCR1 extracted from erythrocyte membrane was estimated by two-dimensional (2D) electrophoresis using BN-PAGE in the first step, followed by SDS-PAGE in the second step (Figure 5). Endogenous PLSCR1 extracted in the native state by 5% DDM from erythrocyte membranes depleted of peripheral proteins was analyzed (Figure 5). As expected, PLSCR1 migrated at 35 kDa in a SDS-PAGE gel, whereas in the native gel, its migration corresponded to an apparent molecular mass of 170–280 kDa, corresponding to an oligomer of five to eight subunits. This result was unaffected by the presence of cholesterol in the erythrocytes (Figure 5) and liposomes (data not shown), nor was it affected by the DDM concentration of the protein extraction medium within the range of 0.5–2.5% (data not shown). Recombinant MBP-PLSCR1 (76 kDa) in solution was also detected as a multimer with five to eight monomers (Figure S3B).

Structural Basis for Scrambling of Phospholipids

The results described above suggest a possible feature in the mechanism of action of the scramblase in transporting phospholipids of differing structures between membrane leaflets. An examination of the hydrophobicity profile of the transmembrane segment, using the SOSUI program, showed a relatively hydrophilic region at its center (Figure 6A). Here a D → L substitution resulted in a reduced hydrophilicity relative to that of the native sequence, markedly impaired in the scramblase activity with respect to that of PC but not those of PS and PE (Figure 6B). Sphingomyelin was not translocated by either WT or mutant peptide (data not shown). We conclude that the hydrophilicity in the core of the trans-membrane segment of the scramblase is an important structural feature for the capture of the phospholipid to be translocated.

DISCUSSION

We have described here a previously unsuspected but essential function of cholesterol in the human erythrocyte membrane. We have found that cholesterol inhibits the activity of the phospholipid scramblase, PLSCR1, thereby preventing the exposure of PS on the outer membrane surface and, thus, by inference, the elimination of the cell from the circulation, *in vivo*, and its eventual destruction. We have further shown that this activity of cholesterol also operates in proteoliposomes containing the scramblase, and it seems likely therefore that it may apply to plasma membranes of eukaryotic cells in general. As PS localization in the inner leaflet has previously been shown to be essential for the maintenance of erythrocyte morphology and membrane stability⁶ and protection of spectrin glycation,⁷ these findings also imply that regulation of scramblase activity by cholesterol may have broader implications for red cell function.

The function of PLSCR1 in the erythrocyte membrane has been open to doubt. We have shown that the suggestion that it is a scramblase is correct, yet even when the endogenous flippase has been inhibited, there is still minimal PS translocation to the outer membrane leaflet, provided the intracellular calcium concentration is in or below the physiological range. The inference is that the scramblase activity of PLSCR1 is subject to inhibition by a membrane constituent, rather than merely offset in the native cell by the flippase. The translocation of PS observed in cells depleted of cholesterol implied that cholesterol is an endogenous scramblase inhibitor. It has been previously suggested that cholesterol and unsaturated phospholipids by creating a hydrophobic barrier can reduce the degree of water penetration in the model membrane,^{20,21} raising the possibility that cholesterol depletion per se might alter phospholipid movement. However, our findings clearly document no changes in phospholipid movement following cholesterol depletion in the absence of activation of the scramblase in either liposomes (Figure 4, white bars) or erythrocytes (Figure 1A, a and g, our unpublished data). Thus, our findings show that the observed effects of cholesterol are primarily due to regulation of scramblase activity by cholesterol.

A molecule previously suggested as a scramblase in human erythrocytes¹² was demonstrated to indeed possess phospholipid scrambling activity that was clearly inhibited by cholesterol in PLSCR1-reconstituted liposomes. Observed cholesterol dependency agreed well with the feature of scrambling activity in erythrocytes, strongly suggesting that PLSCR1 is indeed the

protein accounting for cholesterol-suppressed phospholipids scrambling in the human erythrocytes. Our evidence that PLSCR1 exists in the membrane in oligomeric form suggests that it functions as a pore, allowing passage of phospholipids into and out of the inner and outer membrane leaflet. Although PLSCR1 was demonstrated to be concentrated in cholesterol-rich rafts in the membrane,¹⁴ such segregation is clearly not required for phospholipid scrambling, in view of the strong scramblase activity of the protein in our reconstituted liposomes, which likely contain no rafts. We so far have not been able to establish whether cholesterol interacts directly with PLSCR1, as has been suggested,²² or inhibits its propensity to form oligomeric structure. However, it remains unclear how cholesterol inhibits the transfer of phospholipid across these membranes.

In this study, we could show that PLSCR1 formed an oligomer, suggesting that it functions by producing a cluster with an integral “hole” or “channel” that allows passage of phospholipid hydrophilic head regions. It is possible that cholesterol might prevent cluster formation or change the conformation of the cluster. However, we were unable to document differences in the oligomeric state of PLSCR1 in the presence or absence of cholesterol. It would be worth examining whether binding of cholesterol to the PLSCR1 cluster might prevent the interaction of phospholipid with PLSCR1. Indeed, a recent report suggested that PLSCR1 possessed a cholesterol recognition motif.²²

PLSCR1 was originally identified and characterized by studies in reconstituted liposomes as a high- Ca^{2+} -activated transporter of PS and PC.^{9,12} This single-pass transmembrane protein was found to contain a Ca^{2+} -binding site and palmitoylation sites in the cytoplasmic domain.^{13,23} We, by contrast, found as reported above that transfer of both NBD-PC and -PS occurs in liposomes reconstituted with full-length PLSCR1 and no cholesterol even at Ca^{2+} concentrations of $1\ \mu\text{M}$, equivalent to those in the normal human erythrocyte. We conclude that, while calcium is required for the activity of PLSCR1, the protein can function at concentrations much lower than those previously reported to be necessary in the absence of cholesterol. Palmitoylation seems to be unnecessary for translocation of PS and PC, because the PLSCR1 isolated transmembrane domain of the protein, which does not contain these sites, sufficed for phospholipid translocation at much the same rate as that of the intact protein. We cannot exclude the possibility that palmitoyl groups might play an unknown role in the interaction of the protein with the lipid bilayer. Analysis of hydrophobicity of the transmembrane region using the SOSUI program showed that the hydrophilic portion is present in the middle of the transmembrane domain and replacing D with L in this region, thereby reducing hydrophilicity, resulted in a loss of PC transfer activity. This finding implies the importance of the hydrophilic portion in the middle of the transmembrane domain for random unselective phospholipid scrambling.

We consider finally the apparent divergence between the lipid scrambling characteristics between human and mouse erythrocytes. In particular, those of knockout mice devoid of PLSCR1 display unexplained phospholipid scrambling activity at high Ca^{2+} concentrations.¹⁶ Our observation that immunoblots reveal only a vanishingly low level of PLSCR1 protein in mouse compared to human erythrocytes might afford an explanation, namely that scramblase activity in the mouse is dominated by a different member of the scramblase family of proteins. Scrutiny of recently published RNAseq data for human and

mouse erythroid cells offered further clarification.¹⁸ In humans, PLSCR1 mRNA was expressed throughout the terminal differentiation of erythroblasts, and the expression level rises from proerythroblast to polychromatophilic stages. In mice, by contrast, PLSCR3 expression predominates, and the low levels of PLSCR1 expression in proerythroblasts fall further during terminal erythroid differentiation (Figure S1). Thus, it is not surprising that the knockout mice did not show any defects in scramblase activity.

Ca²⁺ at very high (millimolar) concentrations has been reported to activate phospholipid scrambling⁹ in erythrocyte membranes. This, however, does not bear on the work described here, in which we have been concerned only with behavior under physiologic conditions at Ca²⁺ concentrations of $\sim 1 \mu\text{M}$.

It is not unreasonable conjecture that other molecules might possess scrambling activity in human erythrocytes. In other cells, for example, XKR8 and TMEM16F were clearly demonstrated to be scramblases, the former activated by caspase digestion during apoptosis²⁴ and the latter by Ca²⁺ concentrations sufficiently high to activate platelets.^{25,26} We cannot exclude the possibility that these scramblases might also function in human erythrocytes. Both these genes are in fact expressed, though at very low levels, in human erythroid cells (our unpublished data).

We have recently discovered that ATP11C, belonging to the P-IV ATPase family, is a major flippase in human erythrocytes. This study clearly demonstrates that membrane cholesterol plays a crucial role in suppressing PS exposure under physiological Ca²⁺ conditions by inhibiting PLSCR1-mediated phospholipid scrambling in human erythrocytes. The possible mechanisms of maintenance of the PS distribution by ATP11C and PLSCR1 are summarized in Figure 7.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CPD	citrate-phosphate-dextrose
FITC-annexin V	fluorescein isothiocyanate-labeled annexin V
K_m	Michaelis constant
MBCD	methyl- β -cyclodextrin
MBP	maltose-binding protein

NEM	<i>N</i> -ethylmaleimide
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PLSCR1	phospholipid scramblase 1
PS	phosphatidylserine
SM	sphingomyelin
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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Figure 1.

Augmented scrambling of PS in MBCD-treated erythrocytes. (A) Flow cytometric analysis of PS externalization in cholesterol-depleted erythrocytes. Washed cells were pretreated without (a–c) or with (d–i) 0.5 mM NEM for 15 min and further treated with 5 mM MBCD for 20 min (g–i). These cells were then treated with various concentrations of Ca^{2+} of 1 μM with 2 μM A23187. Cells with PS surface expression were labeled with FITC-annexin V and detected by flow cytometry. (B) Quantification of annexin V-positive cells. Percentages of annexin V-positive cells (termed PS-positive cells) were plotted vs Ca^{2+} concentration. With an increase in Ca^{2+} concentration, the level of PS-positive cells in untreated erythrocytes (none) slightly increased and increased to some extent in NEM- or MBCD-treated cells and dramatically increased by combined NEM and MBCD treatment (NEM/MBCD). ** $p < 0.01$, evaluated by a Student's t -test vs none, NEM, and MBCD ($n = 3$).

Figure 2.

Addition of cholesterol to MBCD-treated erythrocytes decreases the level of PS externalization. (A) Flow cytometric analysis of PS externalization in cholesterol-restored erythrocytes. Washed cells were pretreated with 0.5 mM NEM for 15 min and then 5 mM MBCD for 20 min (a–h), followed by 11.6 mM cholesterol-MBCD (e–h). Cells were further treated with various concentrations of Ca^{2+} of 1 μM in the presence of 2 μM A23187. Cells with PS surface expression were labeled with FITC-annexin V and detected by flow cytometry. (B) Quantification of annexin V-positive cells. Percentages of annexin V-positive cells (termed PS-positive cells) were plotted vs Ca^{2+} concentration. With an increase in Ca^{2+} concentration, the level of PS-positive cells in NEM-treated erythrocytes (NEM) slightly increased and dramatically increased after combined NEM and MBCD treatment (NEM/MBCD). Addition of cholesterol to these cells (NEM/MBCD+Chol) diminished levels of PS-positive cells in a manner similar to that of untreated cells. ** $p < 0.01$ [evaluated by a Student's *t*-test vs NEM and NEM/MBCD+Chol ($n = 3$)].

Human PLSCR 1

Murine PLSCR1

Figure 3.

Expression of PLSCR1 in human or murine erythrocytes. The PLSCR1 protein level in human (left) or murine (right) erythrocyte membranes was detected by immunoblotting. Ghost membrane proteins of human (#1, #2, and #3) or murine (#1 and #2) erythrocytes and recombinant PLSCR1 protein of human (hPLSCR1-WT) or murine (mPLSCR1-WT) were separated by SDS-PAGE, followed by immunoblot analyses using the mouse polyclonal anti-PLSCR1 antibody. Arrowheads indicate PLSCR1 protein.

Figure 4.

Phospholipid scrambling by recombinant MBP-PLSCR1 and the transmembrane domain peptide of PLSCR1 in reconstituted liposomes. Left panels show representative results of the time dependency of lipid scrambling (NBD-PC or -PS internalization) for up to 3 h at 37 °C in liposomes with or without cholesterol reconstituted with MBP-PLSCR1 (A) and the PLSCR1 transmembrane domain peptide (PLSCR1-pep) (B). At the indicated time, NBD-PC or -PS internalization was measured by the dithionite quenching method. Right panels represent the proportions of internalized NBD lipids in these liposomes at 1 h. Graphs show the mean values $\pm$ SD ($n = 3$). * $p < 0.05$; ** $p < 0.01$ [evaluated by A Student's t -test ($n = 3$)].

Figure 5. Multimer formation of PLSCR1 in human erythrocyte membranes. Membrane proteins were natively extracted from (A) untreated and (B) cholesterol-depleted ghosts with 5% DDM and then separated according to the molecular weight under native conditions using the BN-PAGE system (first dimension, 1D). The gel was sliced out, equilibrated in SDS sample buffer, and then applied to a 10% SDS-PAGE gel (second dimension, 2D). The PLSCR1 signals were detected by immunoblotting using the anti-PLSCR1 antibody.

Figure 6.

Hydrophobicity of the PLSCR1 transmembrane domain regulates scrambling activity. (A) Alignment of amino acid sequences and hydropathy profiles (obtained by SOSUI analysis) for the PLSCR1 transmembrane domain (TM). The PLSCR1 mutant peptide (PLSCR1-Mut) was synthesized as one amino acid substitution, Asp (D) to Leu (L), at residue 11 of TM. This substitution increased hydrophobicity at the center of the hydrophilic region of the wild type (PLSCR1-WT). (B) Scrambling activity for PC, PS, and PE in PLSCR-WT and PLSCR1-Mut-reconstituted liposomes. Internalized NBD-PC, -PS, or -PE was measured as described in the legend of Figure 4. *, # $p < 0.05$; **, $p < 0.01$; ***, ### $p < 0.001$ [evaluated by a Student's t -test vs control ($n = 3$)].

Figure 7.

Mechanisms for the maintenance of the PS asymmetric distribution. In human erythrocyte membranes, ATP11C, belonging to the P-IV ATPase family, plays a major role in the active transport of aminophospholipids, such as PS and PE, while the oligomer of PLSCR1 is responsible for scrambling of various phospholipids, including PS. Under physiological conditions with low Ca^{2+} concentrations, PLSCR1 activity is suppressed by membrane cholesterol, localizing PS in the inner leaflet of the lipid bilayer of erythrocyte membranes. At high Ca^{2+} concentrations like in senescent cells, PLSCR1 is activated to expose PS to the outer leaflet (regardless of ATP11C activity) that is recognized and phagocytized by macrophages.