Molecular Cloning of Human Plasma Membrane Phospholipid Scramblase

A PROTEIN MEDIATING TRANSBILAYER MOVEMENT OF PLASMA MEMBRANE PHOSPHOLIPIDS

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The rapid movement of phospholipids (PL) between plasma membrane leaflets in response to increased intracellular Ca2+ is thought to play a key role in expression of platelet procoagulant activity and in clearance of injured or apoptotic cells. We recently reported isolation of a ~37-kDa protein in erythrocyte membrane that mediates Ca2+-dependent movement of PL between membrane leaflets, similar to that observed upon elevation of Ca2+ in the cytosol (Basse`, F., Stout, J. G., Sims, P. J., and Wiedmer, T. (1996) J. Biol. Chem. 271, 17205–17210). Based on internal peptide sequence obtained from this protein, a 1,445-base pair cDNA was cloned from a K-562 cDNA library. The deduced “PL scramblase” protein is a proline-rich, type II plasma membrane protein with a single transmembrane segment near the C terminus. Antibody against the deduced C-terminal peptide was found to precipitate the ~37-kDa red blood cell protein and absorb PL scramblase activity, confirming the identity of the cloned cDNA to erythrocyte PL scramblase. Ca2+-dependent PL scramblase activity was also demonstrated in recombinant protein expressed from plasmid containing the cDNA. Quantitative immunoblotting revealed an approximately 10-fold higher abundance of PL scramblase in platelet (~104 molecules/cell) than in erythrocyte (~105 molecules/cell), consistent with apparent increased PL scramblase activity of the platelet plasma membrane. PL scramblase mRNA was found in a variety of hematologic and nonhematologic cells and tissues, suggesting that this protein functions in all cells.

The plasma membrane phospholipids (PL)1 are normally asymmetrically distributed, with phosphatidylcholine (PC) and sphingomyelin located primarily in the outer leaflet, and the aminophospholipids, phosphatidylserine (PS) and phosphati-

dylethanolamine restricted to the cytoplasmic leaflet (1, 2). An increase in intracellular Ca2+ due to either cell activation, cell injury, or apoptosis causes a rapid bidirectional movement of the plasma membrane PL between leaflets, resulting in exposure of PS and phosphatidylethanolamine at the cell surface (1, 3–5). This exposure of the plasma membrane aminophospholipids has been shown to promote assembly and activation of several key enzymes of the coagulation and complement systems, as well as to accelerate the clearance of injured or apoptotic cells by the reticuloendothelial system, suggesting that Ca2+-induced remodeling of plasma membrane PL is central to both vascular hemostatic and cellular clearance mechanisms (1, 6–9).

We recently reported isolation of a ~37-kDa integral membrane protein from human erythrocytes that when reconstituted into liposomes mediated a Ca2+-dependent, bidirectional scrambling of PL between membrane leaflets mimicking the action of Ca2+ at the endofacial surface of the erythrocyte membrane (10, 11). Evidence for protein(s) in platelet that mediates a similar “PL scramblase” function when incorporated into liposomes has also been reported (12). Here we report the cDNA cloning and deduced structure of the PL scramblase from human erythrocyte and show evidence that this same protein is expressed in human platelet and various other cell lines and tissues where plasma membrane PL scramblase activity has been observed.

EXPERIMENTAL PROCEDURES

Materials—Egg yolk PC, brain PS, and 1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-sn-glycero-3-phosphocholine (NBD-PC) were obtained from Avanti Polar Lipids. Expressed sequence tag (EST) clone with GenBank® accession number gb AA143023 was obtained through American Type Culture Collection (ATCC 962235). All restriction enzymes and amylose resin were from New England Bio- Labs, Inc. KlenTaq polymerase was from CLONTECH Laboratories, wheat germ agglutinin Sepharose was from Sigma, isopropyl-β-D-thiogalactopyranoside was from Eastman Kodak, factor Xa was from Hematologic Technologies, and Bio-Beads SM-2 were from Bio-Rad. N-Oc-
yl-β-D-glucopyranoside (OG) and Glu-Gly-Arg chloromethyl ketone were from Calbiochem. Sodium dithionite (Na,S,O3, Sigma) was freshly dissolved in 1 m Tris, pH 10, at a concentration of 1 m.

PL Scramblase Isolation and Amino Acid Sequencing—PL scramblase was purified as described previously (10, 11), with the following modifications. The active fraction eluting from Mono S was concentrated and exchanged into 20 mM Tris, 0.1 mM EGTA, 0.1% Nonidet P-40, and absorbed against 5 ml of wheat germ agglutinin-Sepharose to remove trace contaminating glycofenins. The breakthrough material was concentrated and exchanged into 20 mM Tris, 0.1 mM EGTA, 0.02% Nonidet P-40, pH 7.4, and subjected to SDS-PAGE under reducing conditions in a 10% NuPAGE gel (Novex, San Diego, CA). The band at ~37 kDa was visualized with 0.1% Brilliant Blue R-250 and excised for amino acid analysis and sequencing (University of Michigan Protein and Carbohydrate Structure Facility). 450 pmol of this protein was subjected to in situ cleavage with 10 mg/ml CNBr in 70% formic acid, the cleaved peptides were extracted into 60% acetonitrile, 10% trifluoroacetic acid, dried in a speed vacuum, and
resolved by SDS-PAGE and electroblotted onto sequencing grade polyvinylidene difluoride. Peptides were observed by staining with Coomasie Blue, excised, and subjected to microsequencing using Edman chemistry on a model 494 Applied Biosystems sequencer run with standard cycles, yielding the sequence \( \text{PAQPPILNCPGLEYLSQSLDQILHQQ-10} \). The scheme was confirmed upon generation of the \( 3 \) digest only. The sequencing was used to screen a cDNA library derived from human erythrocytic cell line K-562 in \( \text{gtg11} \) (CLONTECH). \( \text{Escherichia coli} \) strain Y1090w was transformed by K-562 cDNA library (4.86 \( \times \) 10\(^6 \) plaques/plate) and used to screen a cDNA library derived from human erythrocytic cell line K-562 in \( \text{gtg11} \) (CLONTECH). \( \text{Escherichia coli} \) strain Y1090w was transformed by K-562 cDNA library (4.86 \( \times \) 10\(^6 \) plaques/plate) and used to screen a cDNA library derived from human erythrocytic cell line K-562 in \( \text{gtg11} \) (CLONTECH). \( \text{Escherichia coli} \) strain Y1090w was transformed by K-562 cDNA library (4.86 \( \times \) 10\(^6 \) plaques/plate) and used to screen a cDNA library derived from human erythrocytic cell line K-562 in \( \text{gtg11} \) (CLONTECH). \( \text{Escherichia coli} \) strain Y1090w was transformed by K-562 cDNA library (4.86 \( \times \) 10\(^6 \) plaques/plate) and used to screen a cDNA library derived from human erythrocytic cell line K-562 in \( \text{gtg11} \) (CLONTECH). 

PL Scramblase Activity—PL Scramblase activity was measured as described previously (10, 11). Routinely, proteoliposomes labeled with NBD-PC were incubated for 2 h at 37 °C in Tris buffer in the presence or the absence of 2 mM Ca\(^{2+}\). Proteoliposomes were diluted 25-fold in Tris buffer containing 4 mM EGTA and transferred to a stirred fluorometer cuvette at 23 °C. Initial fluorescence was recorded (SLM Aminco 8000 spectrophotometer; 470 nm excitation, 532 nm, 20 mM dithionite was added, and the fluorescence was continuously monitored for a total of 120 s. 

Isoelectric Focusing of PL Scramblase—Isoelectric focusing was performed using FreeCal version 4.0 software (generously provided by Dr. Lawrence F. Brass, University of Pennsylvania, Philadelphia, PA).

Protein Concentration—Protein concentrations were estimated based upon optical density at 280 nm, using extinction coefficients (m \(^{-1}\) cm \(^{-1}\)) of 39,000 (PL scramblase), 64,500 (MBP), and 105,000 (PL scramblase-MBP fusion). 

RESULTS AND DISCUSSION

Cloning of PL Scramblase cDNA—PL scramblase was purified from human erythrocyte membranes and cleaved with 

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**Antibody against PL Scramblase C-terminal Peptide**—The peptide CESTGSEQEQKSGVW, corresponding to amino acids 306–318 of the predicted open reading frame of PL scramblase with an added N-terminal cysteine, was synthesized and conjugated to keyhole limpet hemocyanin (Protein Core Facility, Blood Research Institute). 

**Immunoprecipitation of PL Scramblase**—PL scramblase purified from human erythrocytes was \( 125^I \)-labeled with Iodogen (Pierce), free iodide removed by gel filtration, and the protein was incubated (4 °C, overnight) with either anti-306–318 or an identical quantity of preimmune rabbit IgG (1 mg/ml in 150 mM NaCl, 10 mM MOPS, 50 mM OG, pH 7.4) or no IgG as control. The IgG was precipitated with protein A-Sepharose-CLAB (Sigma). Peptide-specific antibody was isolated by affinity chromatography on UltraLink Iodoacetate beads (Pierce) to which peptide CESTGSEQEQKSGVW was conjugated. 

**Isolation of PL Scramblase cDNA by Plaque Hybridization**—The length of cDNA insert was examined by PCR with 32 strongly positive plaques at a density of 50–100 plaques/plate. Times with 0.2 g/ml of anti-306–318, or an identical quantity of preimmune rabbit IgG (1 mg/ml in 150 mM NaCl, 10 mM MOPS, 50 mM OG, pH 7.4) or no IgG as control. The IgG was precipitated with protein A-Sepharose-CLAB (Sigma). Peptide-specific antibody was isolated by affinity chromatography on UltraLink Iodoacetate beads (Pierce) to which peptide CESTGSEQEQKSGVW was conjugated. This affinity-purified antibody (anti-306–318) was used for immunoprecipitation and Western blotting of PL scramblase (see "Results and Discussion"). 

**Expression and Purification of PL Scramblase-MBP Fusion Protein**—To express PL scramblase as a fusion protein with maltose binding protein (MBP), cDNA encoding PL scramblase was cloned into pMAL-C2 vector digested with \( \text{EcoRI} \) and \( \text{SalI} \) and isolated by electrophoresis on 1\% low melting agarose gel and purification with Wizard kit (Promega). The amplified cDNA was cloned into pMAL-C2 vector digested with \( \text{EcoRI} \) and \( \text{SalI} \), immediately 3′ of MBP. This construct was amplified in \( \text{E.coli} \) strain TB1, and the sequence of the cDNA insert of plasmids from single colonies was confirmed. 

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cDNA Encoding the Plasma Membrane Phospholipid Scramblase

The possibility that PL scramblase function is mediated by a phosphoprotein has previously been suggested based on an observed decrease in PL scrambling activity in erythrocytes depleted of ATP (14).

To confirm that the cDNA we cloned from the K-562 cDNA library actually encodes the same protein purified as PL scramblase from human erythrocyte membrane, we raised a rabbit antibody against the deduced C terminus predicted on the deduced amino acid sequence (Fig. 1, underlined sequence). This peptide sequence, plus the anticipated methionine residue N-terminal to the predicted site of cyanogen bromide digestion of purified erythrocyte PL scramblase, are indicated by single underline. Also indicated are the residues comprising a predicted inside-to-outside transmembrane domain (Ala291–Gly309; double underline) and a protein kinase C phosphorylation site (Thr318; asterisk). See “Experimental Procedures” for details.

cyanogen bromide, and Edman degradation was performed on a 12-kDa peptide fragment to obtain 32 residues of peptide sequence (Fig. 1, underlined sequence). This peptide sequence, plus the anticipated methionine residue N-terminal to the predicted site of cyanogen bromide cleavage, was identified in the translation product of a 568-bp EST clone deposited in GenBank by the I.M.A.G.E. Consortium (clone identification number AF008445). The 32 residues of peptide sequence that were obtained from cyanogen bromide digest of purified erythrocyte PL scramblase are indicated by single underline. Also indicated are the residues comprising a predicted inside-to-outside transmembrane domain (Ala291–Gly309; double underline) and a protein kinase C phosphorylation site (Thr318; asterisk). See “Experimental Procedures” for details.

Analysis of the cDNA-derived protein sequence (Tmpred program, ISREC server, University of Lausanne, Epalinges, Switzerland) revealed a strongly preferred (p < 0.01) inside-to-outside orientation of the predicted 19-residue transmembrane helix, consistent with a type II plasma membrane protein. Most of the polypeptide (residues 1–290) thereby extends from the cytoplasmic membrane leaflet, leaving a short exoplasmic tail (residues 310–318). The predicted orientation of this protein is consistent with the anticipated topology of PL scramblase in the erythrocyte membrane, where lipid-mobilizing function is responsive to [Ca²⁺] only at the endosomal surface of the membrane (3, 5, 10, 11, 15, 16).

To confirm that the cDNA we cloned from the K-562 cDNA library actually encodes the same protein purified as PL scramblase from human erythrocyte membrane, we raised a rabbit antibody against the deduced C terminus predicted from the open reading frame of the cloned cDNA (codons 306–318). As shown in Fig. 2, this antibody precipitated the ~37-kDa red cell protein we tentatively identified as PL scramblase and also absorbed the functional activity detected in this isolated erythrocyte membrane protein fraction. As also evident from Fig. 2 (inset), we often observed the partial proteolysis of 37-kDa PL scramblase purified from human erythrocytes labeled with125I and precipitated with either anti-306–318 IgG (bar 1) or preimmune rabbit IgG (bar 2), and protein remaining in the supernatant was determined to be liposomes for measurement of residual PL scramblase activity. Data normalized to PL scramblase activity were measured for identical controls omitting antibody (100%; bar 3). Error bars denote the means ± SD (n = 4). Inset, erythrocyte PL scramblase was labeled with 125I and precipitated with anti-306–318 antibody, and the resulting pellet was analyzed by SDS-PAGE (lane 1). Matched controls incubated with either preimmune rabbit IgG (lane 2) or no IgG (lane 3) served as control. See “Experimental Procedures” for details. Data of single experiment are shown, representative of two so performed.
mobilizing activity of recombinant PL scramblase expressed and purified from *E. coli* was approximately 50% of that observed for the endogenous protein purified from the erythrocyte membrane, which is likely due to incomplete folding of the recombinant protein. Half-maximal [Ca^{2+}]_i required for activation was approximately 100–200 μM for recombinant protein purified from *E. coli* versus ~40 μM for the erythrocyte-derived protein, raising the possibility that altered folding or an unknown post-translational modification in mammalian cells affects the putative Ca^{2+} binding site (10, 11). In addition to activation by Ca^{2+}, the transbilayer migration of PL in erythrocytes is accelerated upon acidification of the inside leaflet to pH < 6.0 (in absence of Ca^{2+}), a response that is also observed in proteoliposomes containing PL scramblase purified from erythrocyte membranes (11). A similar acid-dependent activation of PL mobilizing function was also exhibited by proteoliposomes incorporating recombinant PL scramblase purified from *E. coli* (not shown).

**Platelet PL Scramblase**—In addition to the presumed role of PL scramblase in PS exposure following cell injury and upon repeated sickling of SS hemoglobin red cells, the capacity of activated platelets to rapidly mobilize aminophospholipids across the plasma membrane is thought to play a central role in the initiation of thrombin generation required for plasma clotting (17). Whereas incubation with Ca^{2+} ionophore causes a marked acceleration in transbilayer movement of plasma membrane PL in both platelets and erythrocytes, the apparent rate of transbilayer PL migration in platelet exceeds that in erythrocyte by approximately 10-fold, implying either a higher abundance of PL scramblase or the action of another component in platelet with enhanced PL scrambling function (18, 19). Zwaal and associates recently reported evidence for the existence of protein(s) in platelet with functional properties similar to that of PL scramblase we isolated from erythrocyte (10–12). To determine whether the protein we now identify in the erythrocyte membrane is also found in platelets, we probed platelets with antibody against PL scramblase residues 306–318 (Fig. 4). As shown in Fig. 4, this antibody blotted a single protein in platelet with similar mobility to the ~37-kDa PL scramblase in erythrocyte. Based on quantitative immunoblotting with anti-306–318, we estimate approximately 10^4 molecules/cell in platelet versus 10^5 molecules/cell in erythrocyte, consistent with the increased PL scramblase activity and procoagulant function observed for human platelets versus erythrocytes.

**Tissue Distribution**—In addition to platelet and red blood cell, PL scramblase activity has been observed in many other cells, and this Ca^{2+}-induced response is thought to be central to the rapid movement of PS and phosphatidylethanolamine from inner plasma membrane leaflet to the surface of perturbed endothelium and a variety of injured and apoptotic cells (17). The resulting exposure of PS at the cell surface is thought to play a key role in removal of such cells by the reticuloendothelial system, in addition to activation of both the plasma complement and coagulation systems (8, 9, 17). Whereas the molecular mechanism(s) in each circumstance remains unre-

**Fig. 3. Activity assay of recombinant PL scramblase.** Purified PL scramblase-MBP fusion protein (0–43 × 10^{-11} mol; abscissa) was reconstituted into liposomes (1 μmol of total PL), and MBP was proteolytically removed by incubation with factor Xa in presence of 0.1 mM EGTA. After digest to release MBP, the proteoliposomes were recovered by determination of PL scramblase activity, measured in the absence (○) or the presence (●) of 2 mM CaCl_2 as described under “Experimental Procedures.” Data are corrected for nonspecific transbilayer migration of NBD-PC probe in identically matched control liposomes containing either MBP or no added protein (not shown). Error bars denote the means ± SD (n = 3). Data of single experiment are shown, representative of two so performed. Similar results were also obtained for proteoliposomes containing intact PL scramblase-MBP fusion protein, omitting the factor Xa digest (not shown).

**Fig. 4. Immunoblotting of PL scramblase in human erythrocytes and platelets.** 2 × 10^9 platelets (lane 1) and ghost membranes from 2 × 10^9 erythrocytes (lane 2) were separated by SDS-PAGE, transferred to nitrocellulose, and Western blotted with anti-306–318 antibody as described under “Experimental Procedures.” Lane 3 contains 0.9 pmol of factor Xa cleaved recombinant PL scramblase, and lane 4 contains 0.5 pmol of PL scramblase purified from erythrocytes. Data of single experiment are shown, representative of three so performed.

**Fig. 5. Expression of PL scramblase in multiple human tissues and cancer cell lines.** Northern blotting with PL scramblase cDNA is shown for equal amounts of poly(A) RNA obtained from the human tissues indicated (A) and from the following human cancer cell lines (B): promyelocytic leukemia HL-60, epithelial cancer HeLa S3, chronic myelogenous leukemia K-562, lymphoblastic leukemia MOLT-4, Burkitt’s lymphoma Raji, colorectal adenocarcinoma SW480, lung carcinoma A549, and melanoma G361. Lower panels show results for β-actin. Blots were developed as detailed under “Experimental Procedures.” Data of single experiment are shown. kb, kilobases.
solved, evidence for a specific platelet membrane protein functioning to accelerate migration of PL between membrane leaflets at increased cytosolic [Ca$^{2+}$] has been reported (12), similar to the proposed role of PL scramblase in red blood cells (10, 11). It was thus of interest to determine whether mRNA for this protein is expressed in nucleated cells where PL scramblase-like activity has been observed. As shown by Fig. 5, Northern blotting with PL scramblase cDNA revealed transcripts of ~1.6 and ~2.6 kilobases in all tissues and cell lines tested. Some tissue-to-tissue and cell line variability in the relative abundance of these two transcripts is apparent, the significance of which remains to be determined. Also notable was markedly reduced expression in HL-60 and the lymphoma lines Raji and MOLT-4, whereas abundant message was detected in spleen, thymus, and peripheral leukocytes. In addition to the transformed cell lines shown, mRNA for PL scramblase was also confirmed in human umbilical vein endothelial cells (not shown). Whereas these data imply that the same protein identified as mediating accelerated transbilayer flip-flop of the erythrocyte membrane PL also plays a similar role in the plasma membrane of platelets, leukocytes, and other cells, actual confirmation for this role of PL scramblase awaits analysis of a cell line that is selectively deficient in this protein.

In Scott syndrome, a bleeding disorder related to an inherited deficiency of plasma membrane PL scramblase function, erythrocytes and other cells deficient in PL scramblase activity were found to contain normal amounts of the PL scramblase protein (11). Furthermore, despite the apparent deficiency in Scott syndrome cells of endogenous PL scramblase function, when PL scramblase protein from these cells was purified and reconstituted in proteoliposomes containing exogenous PL, it exhibited normal Ca$^{2+}$-dependent PL mobilizing activity (11). This suggests that in addition to the known regulation by intracellular [Ca$^{2+}$], the activity of PL scramblase in the plasma membrane is regulated by other as yet unidentified membrane or cytoplasmic component(s).

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