Palmitoylation of Phospholipid Scramblase Is Required for Normal Function in Promoting Ca^{2+}-Activated Transbilayer Movement of Membrane Phospholipids†

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ABSTRACT: Accelerated transbilayer movement of plasma membrane phospholipids (PL) plays a central role in the initiation of plasma clotting and in phagocytic clearance of injured or apoptotic cells. We recently identified a plasma membrane protein that induces rapid transbilayer movement of PL at elevated Ca^{2+}, and we presented evidence that this PL scramblase mediates the transbilayer movement of plasma membrane PL in a variety of cells and tissues exposed to elevated intracellular Ca^{2+} [Zhou, Q. et al. (1997) *J. Biol. Chem. 272*, 18240−18244]. Activation of PL scramblase entails coordination of Ca^{2+} by a 12 residue segment resembling an EF hand loop motif that is adjacent to the single transmembrane helix of the polypeptide. On the assumption that correct orientation of the Ca^{2+}-binding loop segment required a distal segment of the polypeptide to orient back toward the membrane, we considered the possibility of membrane anchoring through covalent fatty acid. Human Raji cells transformed with PL scramblase cDNA in the expression vector pEGFP-C2 were metabolically labeled with [^{3}H]palmitate, and fusion protein immunoprecipitated with antibody against GFP-PL scramblase was found to covalently incorporate ^{3}H, whereas no radioactivity was covalently associated with GFP. The identity of the covalently bound ^{3}H in PL scramblase as a thioester-linked [^{3}H]palmitate was confirmed by hydroxylamine cleavage and by thin-layer chromatography of the liberated fatty acid. Consistent with the assumption that activation by Ca^{2+} might require accessory site(s) of polypeptide attachment to the membrane, hydrolysis of thioester bonds in purified erythrocyte PL scramblase markedly reduced the Ca^{2+}-dependent activity of the membrane-incorporated protein.

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 phosphatidylethanolamine restricted to the cytoplasmic leaflet (*1*, *2*). An increase in intracellular Ca^{2+} due to 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 Ca^{2+}-induced remodeling of plasma membrane PL is central to both vascular hemostatic and cellular clearance mechanisms (*1*, *6*−*10*).

We recently identified an integral plasma membrane protein (PL scramblase) that mediates a Ca^{2+}-dependent, bidirectional scrambling of PL between membrane leaflets, mimicking the action of Ca^{2+} at the endofacial surface of the plasma membrane (*11*−*15*). PL scramblase was shown to be expressed in erythrocytes, platelets, endothelium, and a variety of other cells and tissues that are known to expose plasma membrane PS in response to elevated cytosolic $[Ca^{2+}]_c$. Furthermore, it was shown that when Ca^{2+} enters the cytosol, the level of expression of PL scramblase in the plasma membrane determines the extent to which PS becomes exposed at the cell surface (*15*).

The deduced structure of PL scramblase indicates that it is a 318 residue, type 2 plasma membrane protein with a single transmembrane domain near the carboxyl terminus and a low-affinity Ca^{2+} binding site within the cytoplasmic domain (*13*, *14*). This Ca^{2+} binding site has been localized to a 12 residue segment of the PL scramblase polypeptide with similarity to Ca^{2+}-binding loops identified in other proteins with known Ca^{2+}-binding EF hand motifs (*14*). Surprisingly, the Ca^{2+}-binding segment in PL scramblase (Asp^{273}-Asp^{284}) is very close to the single transmembrane domain (Ala^{291}-Gly^{309}), implying that formation of a Ca^{2+}-binding loop in this segment of the polypeptide would necessitate a second site for peptide insertion or attachment

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[1] Abbreviations: PL, phospholipids(s); PC, phosphatidylcholine; PS, phosphatidylserine; GFP, green fluorescent protein; NBD-PC, 1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-*sn*-glycero-3-phosphocholine; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); PCR; polymerase chain reaction; $[Ca^{2+}]_c$, cytosolic calcium concentration.

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to the membrane. As α-helical structure is not conserved in the segment of PL scramblase polypeptide that is immediately N-terminal to the Ca^{2+}-binding loop, and as no membrane intercalating segment N-terminal to Ala^{291} is predicted, we considered the possibility that this loop might form through membrane attachment mediated by lipid or fatty acid covalently bound to a distal segment of the polypeptide. Here we show that PL scramblase is palmitoylated at Cys residue(s) that are distal (N-terminal) to the metal binding site, and that this posttranslational modification is required for normal activation of the membrane-incorporated protein by Ca^{2+}.

EXPERIMENTAL PROCEDURES

Materials. Restriction enzymes were from New England BioLabs, Inc. (Beverly, MA). Klentaq polymerase and pEGFP-C2 vector were from CLONTECH Laboratories (Palo Alto, CA). OPTI-MEM and Geneticin were from Life Technologies (Gaithersburg, MD). Fetal bovine serum, RPMI 1640, protein A Sepharose-CL4B, and leupeptin were from Sigma Chemical Co. (St. Louis, MO). SuperSignal ULTRA Chemiluminescence Kit was from Pierce Chemical Co. (Rockford, IL). Egg yolk phosphatidylcholine (PC), brain phosphatidylserine (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. Bio-Beads SM-2 were from BioRad. Triton X-100 and *N*-octyl-β-D-glucopyranoside (OG) were from Calbiochem. All other chemicals were of reagent grade.

Cell Culture. Human Burkitt's lymphoma cell line Raji was from American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 containing 10% fetal bovine serum.

Antibodies. Anti-GFP, affinity-purified rabbit antibody, and murine monoclonal antibody against green fluorescent protein (GFP) were from CLONTECH Laboratories. Murine monoclonal antibody 11F4 is against human PL scramblase.

Mutagenesis of PL Scramblase. Human PL scramblase amino acid residue Cys^{297} was mutated to Ala with oligonucleotide-directed mutagenesis by two rounds of PCR, as previously described, and cloned back into pEGFP-C2 vector (*14*, *15*). After confirmation of correct DNA sequence, the Ala^{297} mutant PL scramblase-GFP was expressed at comparable levels to wild-type fusion protein, and cells were metabolically labeled with [^{3}H]palmitate for analysis of covalently incorporated radioactivity.

Expression of PL Scramblase in Transfected Raji Cells. Clonal populations of human Raji cells stably transformed with pEGFP-C2-PL scramblase (or pEGFP-C2 as vector-only control) were obtained as previously described (*15*). The pEGFP-PL scramblase transformants expressing the 62 kDa GFP-PL scramblase fusion protein were identified by Western blotting with anti-GFP and with anti-PL scramblase antibodies. Western blotting of pEGFP-C2 transformants (without insert) confirmed the presence of 27 kDa GFP. Clones expressing comparable amounts of GFP-PL scramblase or GFP were each expanded for assay. Expression level of GFP was quantified by flow cytometry using the FL1 channel (FACScan, Becton-Dickinson).

Metabolic Labeling with [^{3}H]Palmitate. Transfected Raji cell clones expressing comparable levels of GFP or GFP-PL scramblase were incubated overnight (10^7 cell/mL, 37 °C) with 200 μCi/mL [^{3}H]palmitate (Dupont NEN, specific activity 60 Ci/mmol) in RPMI-1640 containing 20% dialyzed fetal bovine serum and 5 mM sodium pyruvate. Cells were then harvested and washed, and membrane proteins (10^8 cells/mL) were extracted into 1% Triton X-100, 5 mM EDTA, 20 μM leupeptin, 20 mM *N*-ethylmaleimide, 10 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride in Tris-buffered saline, pH 7.4.

Immunoprecipitation and SDS−PAGE. Insoluble material was removed from the cell extracts by centrifugation, and the supernatants were precleared by incubation with 10 μg/mL normal rabbit IgG and 30 μL/mL protein A Sepharose. The GFP and GFP-PLS proteins were then precipitated by overnight incubation (4 °C)with 50 μg/mL affinity purified rabbit anti-GFP and 50 μL/mL protein A Sepharose. The beads were exhaustively washed with 1% Triton X-100 in Tris-buffered saline, pH 7.4, and bound protein eluted into 5% SDS−PAGE sample buffer by 3 min heating to 80 °C. Each sample (GFP or GFP-PL scramblase) was divided and incubated 1 h either with 1 M Tris-hydroxylamine-HCl, pH 7.2, or in 1 M Tris-HCl, pH 7.2, and each sample was subjected to polyacrylamide gel electrophoresis under non-reducing conditions (4−20% polyacrylamide; NOVEX).

Western Blotting. Following SDS−PAGE and transfer to nitrocellulose, the blocked membrane was incubated with either monoclonal 11F4 (against PL scramblase) or monoclonal anti-GFP (CLONTECH). The blots were developed with the horseradish peroxidase conjugate of goat anti-mouse IgG, using SuperSignal ULTRA chemiluminescence.

Fluorography. Fluorography of the SDS−PAGE gel was performed after fixation of the gel in 50% ethanol, 5% acetic acid, and soaking the washed gel in Enlightning scintillant (Dupont-NEN) before its drying and exposure to preflashed Kodak XAR-5 film (−80 °C, 4 days).

Thin-Layer Chromatography. The identity of the covalently bound ^{3}H in PL scramblase as a thioester-linked [^{3}H]palmitate was confirmed by hydroxylamine cleavage and thin-layer chromatography of the liberated fatty acid. The radioactive GFP-PLS protein band was sliced from the wet gel; the gel was soaked in methanol:water:acetic acid (30:60:10, v:v:v), washed exhaustively with 50% methanol, and lyophilized. To liberate covalently bound fatty acids, the dried gel was incubated overnight in 1.5 N NaOH. Following acidification with HCl (pH < 2), the released fatty acids (1 mL) were extracted by addition of 3.75 mL chloroform:methanol (1:2, v:v). To separate the fatty acids into a chloroform phase, 1.25 mL chloroform and 1.25 mL H_2O were then added, the tube centrifuged, and the bottom layer recovered and dried under nitrogen. The dried pellet was taken up in chloroform:methanol (2:1, v:v) and spotted onto KC18 silica gel (Whatman). The chromatograph was developed in acetonitrile:acetic acid (9:1, v:v) and air-dried. The dried plate was sprayed with Enhance (Dupont-NEN), and radioactivity was detected by fluorography on preflashed Kodak XAR-5 film (−80 °C, 4 days). [^{3}H]Palmitate served as standard.

Functional Assay. PL scramblase was purified from human erythrocyte membranes as previously described (*11*−*13*). The sample was divided and incubated 1 h at room temperature either in 1 M Tris-hydroxylamine-HCl, 25 mM *N*-octyl-β-D-glucopyranoside, pH 7.2, or in the identical

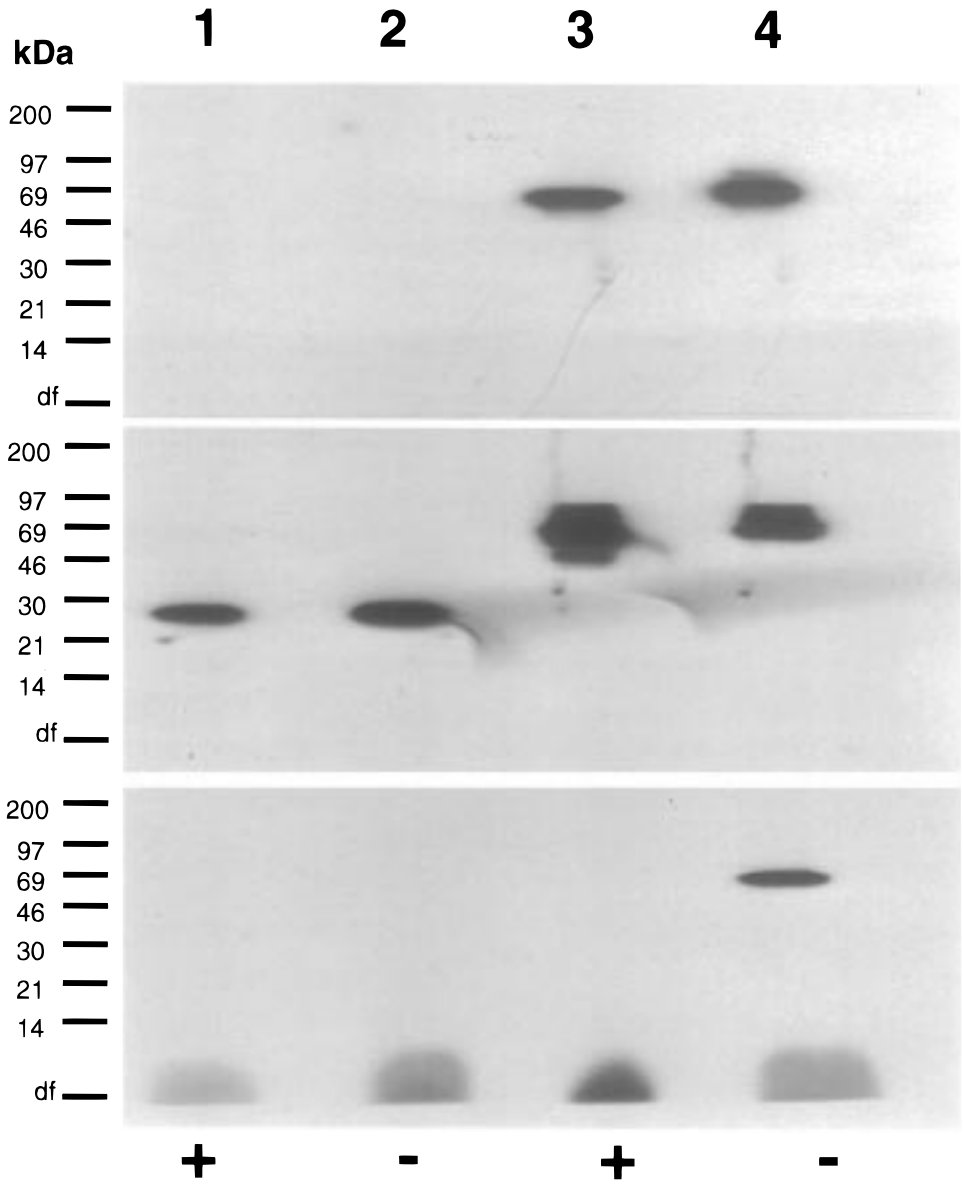

FIGURE 1: Metabolic labeling of PL scramblase with [3H]palmitate reveals covalent thioester-linked fatty acid. The presence of cysteinyl-linked palmitate in PL scramblase was confirmed by metabolic labeling of pEGFP-PL scramblase-transfected Raji cells (GFP-PLS) or identically treated vector-only controls (GFP) with exogenous [^{3}H]palmitate. GFP vector control (lanes 1, 2) and the GFP-PL scramblase fusion protein (lanes 3, 4) each eluted from protein A Sepharose with SDS−PAGE sample buffer and incubated 1 h with either 1 M Tris-hydroxylamine-HCl, pH 7.2 (+; lanes 1, 3) or 1 M Tris-HCl, pH 7.2 (−; lanes 2, 4) before SDS−PAGE under nonreducing conditions (see Experimental Procedures). Upper panel: Western blotting was performed with murine monoclonal antibody 11F4 against human PL scramblase. Middle panel: Western blotting performed with monoclonal antibody against GFP. Lower panel: shows fluorogram of radioactivity from ^{3}H. Note radioactivity in GFP-PL scramblase (see ∼62 kDa band of radioactivity, lane 4) that is released from GFP-PL scramblase by treatment with hydroxylamine (see radioactivity at dye front, lane 3). Data of single experiment, representative of three independent experiments.

solution omitting hydroxylamine. After incubation, samples were dialyzed exhaustively and reconstituted with exogenous phospholipids and NBD-PC for functional assay of PL scramblase activity according to dithionite fluorescence quenching assay as previously described (*11*, *12*).

RESULTS AND DISCUSSION

Metabolic Labeling with [^{3}H]Palmitate. The presence of covalently linked palmitic acid in PL scramblase was directly confirmed by metabolic labeling of the protein with [^{3}H]-palmitate. Human Raji cells, which we have shown to contain low amounts of endogenous PL scramblase, were transformed with plasmid encoding human PL scramblase as a fusion protein with green fluorescent protein (GFP) and metabolically labeled with [^{3}H]palmitate. GFP was employed to facilitate selection, FACS sorting, and cloning of transformants expressing plasma membrane PL scramblase, a type 2 protein that does not provide an accessible cell surface epitope (*13*, *15*). After extraction into detergent, the expressed proteins were precipitated with antibody to GFP and analyzed by polyacrylamide gel electrophoresis. As illustrated by data shown in Figure 1, these experiments revealed covalent incorporation of ^{3}H in the GFP-PL scramblase fusion protein, whereas no radioactivity was found associated with GFP. The identity of the covalently bound ^{3}H in PL scramblase as a thioester-linked [^{3}H]-

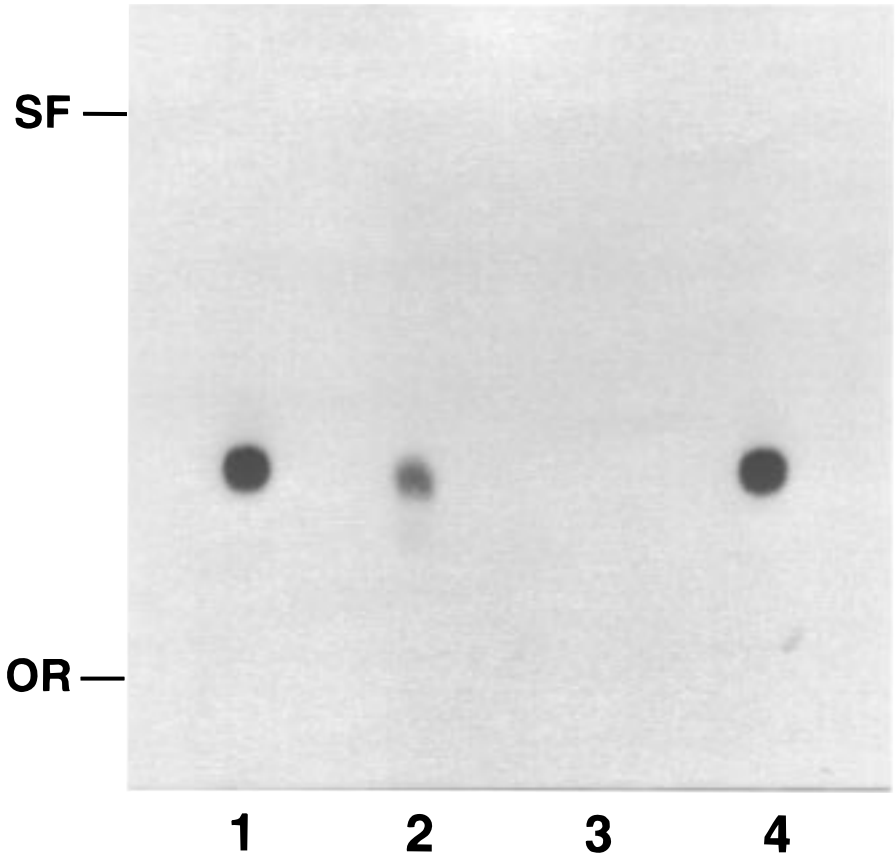

FIGURE 2: Thin-layer chromatography of [3H]fatty acid liberated from PL scramblase. The GFP-PLS protein band at ~62 kDa illustrated in Figure 1 was sliced from the wet gel and the gel slice soaked in methanol:water:acetic acid (30:60:10, v:v:v), and then washed exhaustively with 50% methanol. The covalently bound fatty acids were then liberated by base hydrolysis and analyzed by thin-layer chromatography and fluorography (see Experimental Procedures). In the fluorogram, radioactivity liberated from GFP-PLS (lane 2) is compared to matched gel slice from GFP vector control **(**lane 3), using [^{3}H]palmitate as standard (standards applied in lanes 1, 4). All samples were spotted at position OR; SF indicates position of the solvent front. Data of single experiment.

palmitate was confirmed by hydroxylamine cleavage and thin-layer chromatography of the liberated fatty acid (Figures 1 and 2).

A diverse group of membrane proteins are now known to be modified by attachment of long-chain fatty acids through thioesterification at one or more protein cysteinyls (*16*−*20*). In a very few of these proteins, dynamic cycles of palmitoylation/depalmitoylation have been shown to control protein attachment to the plasma membrane or to regulate specific protein function. Protein cysteinyl residues identified as potential acceptors for palmitoylation have been shown to fall into three groups: (1) cysteines located near the transmembrane cytoplasmic termini of transmembrane helices; (2) cytoplasmic cysteines distal to a transmembrane helix, and (3) cysteines located near preformed terminal *N*-myristoylglycyl- or *S*-prenylcysteinyl- residues(*16*, *18*, *19*, *21*−*24*). Purification of a peripheral erythrocyte membrane protein with protein-palmitoyl acyltransferase activity was recently reported, although the molecular identity, subcellular compartmentalization, and mechanism of this or potentially other enzymes involved in protein palmitoylation/depalmitoylation remain to be elucidated (*20*).

PL Scramblase Is Palmitoylated at Cytoplasmic Cysteinyl(s) Distal to the Ca^{2+}-Binding Loop. Human PL scramblase contains a single cysteine (Cys^{297}) within the single predicted transmembrane helix (Ala^{291}-Gly^{309}) that is also conserved in the murine protein (Figure 3) (*13*, *14*). There are no cysteine residues between the transmembrane helix and the cytoplasmic Ca^{2+} binding site (Asp^{273}-Asp^{284}), whereas distal (i.e., N-terminal) to this Ca^{2+}-binding loop segment the polypeptide contains 15 cytoplasmic cysteines that are conserved in both murine and human PL scramblase. We found that alanine substitution at Cys^{297} caused no reduction in the amount of [^{3}H]palmitate that was covalently bound to the GFP-PL scramblase, excluding this conserved cysteine within the transmembrane helix as the site of palmitoylation of the protein (data not shown). This implies that palmitoylation occurs at one or more of the conserved cytoplasmic cysteines, illustrated in Figure 3. Palmitoylation of the cysteine(s), all located distal to the transmembrane helix and Ca^{2+} -binding site, would presumably serve to anchor the polypeptide back to the membrane, thereby facilitating folding of segment Asp^{273}-Asp^{284} into the loop configuration that is required to complex the metal ion (*14*). The possibility that this posttranslational modification of PL scramblase might contribute to its Ca^{2+}-dependent activity had been suggested by the distinctly reduced activity and reduced affinity for Ca^{2+} exhibited by recombinant PL scramblase expressed and purified from E. coli, when compared to PL scramblase isolated from erythrocyte and other mammalian cells (refs *11*−*13*, and unpublished data).

Inactivation of Protein Function by Thioester Hydrolysis. To confirm that Cys-acylation of PL scramblase contributes to protein function, PL scramblase purified from human

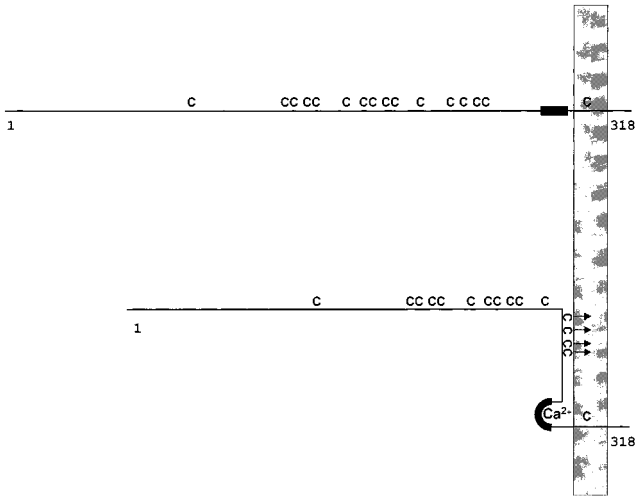

FIGURE 3: Positions of conserved cysteines in PL scramblase. Cartoon depicts positions of conserved cysteines (labeled C) within primary sequence of human PL scramblase in relationship to the conserved transmembrane domain (Ala^{291}-Gly^{309}) and Ca^{2+} binding site (Asp^{273}-Asp^{284}, bold line) of the polypeptide. Also depicted is how folding into the loop conformation of the Ca^{2+}-binding site might be facilitated by distal anchoring to the membrane through one or more palmitoylated cysteines. The conserved cysteinyls within the human PL scramblase cytoplasmic domain (residues 95, 148, 149, 153, 154, 181, 184, 185, 188, 189, 213, 234, 237, 239, and 240) and in the membrane-spanning helix (residue 297) are indicated. Not shown are the cysteines at positions 186 and 254 of human PL scramblase that are not conserved in murine PL scramblase. Domain structure taken from refs *13*, *14*. Mutagenesis indicates that intramembranous Cys^{297} is not the site of palmitoylation. The residues shown as potentially palmitoylated (↓) are illustrative, and the specific esterified cysteinyls remain to be identified (see Results and Discussion).

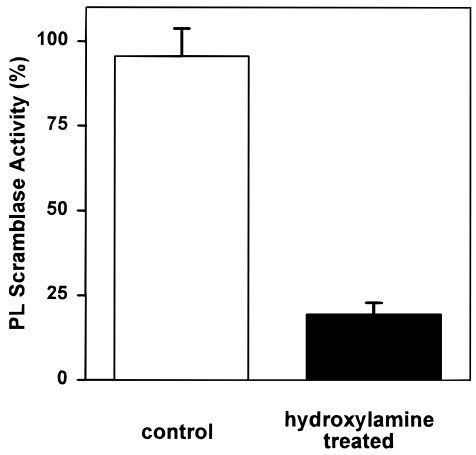

FIGURE 4: Inactivation of PL scramblase by thioester cleavage. Purified human erythrocyte PL scramblase was subjected to thioester hydrolysis at pH 7.2 and reconstituted in lipid vesicles containing NBD-PC for functional assay of PL scramblase activity. Ordinate represents relative NBD-PC flipped from outer to inner leaflet in samples incubated (2 h, 37 °C) in the presence of 2 mM Ca^{2+}, after correction for background measured for identical samples incubated without added Ca^{2+}. Activity of hydroxylamine-treated PL scramblase (solid bar) and that of match-pair control protein that was incubated in 1 M Tris-HCl omitting hydroxylamine (open bar) are each normalized to the activity of untreated PL scramblase. Error bars denote mean ± SD (n = 6). The Ca^{2+}-induced transbilayer movement of NBD-PC probe in those vesicles containing untreated PL scramblase (100% activity) represented a net specific transfer to the inner leaflet of between 20 and 25% of total NBD-PC initially added to the outer membrane leaflet. Data represent combined results of two separate experiments performed on different days.

erythrocyte membrane was subjected to thioester hydrolysis by treatment with hydroxylamine at neutral pH, and then assayed for its capacity to mobilize transbilayer redistribution of NBD-labeled PL when reconstituted into PC/PS-containing proteoliposomes. PL scramblase identically incubated with 1 M Tris-HCl served as matched control. As shown by data of Figure 4, treatment with hydroxylamine under conditions known to selectively hydrolyze thioester linkages resulted in an approximate 80% reduction in the PL transporting function of the erythrocyte-derived PL scramblase protein. Western blotting with antibody directed against the carboxyl-terminus of PL scramblase confirmed the absence of proteolysis under these conditions (data not shown). Whereas the possibility of a hydroxylamine-induced change in protein conformation (other than that resulting from thioester bond cleavage) cannot be excluded, these data imply that thioesterification of the PL scramblase polypeptide is required for its normal function in promoting Ca^{2+}-accelerated transbilayer movement of membrane PL. This is consistent with reduced function that was previously noted for bacterial-expressed protein lacking this posttranslational modification (refs *11*−*13*, and unpublished data). Presumably, membrane-insertion of the thioester-linked acyl chain(s) of PL scramblase serves to promote the correct conformation of the Ca^{2+} binding site within the Asp^{273}-Asp^{284} loop segment of the polypeptide, or these fatty acids directly participate in accelerating transbilayer movement of membrane PL, potentially as the consequence of an induced reorientation of the membrane-anchored segment and associated acyl chains when Ca^{2+} binds to the protein. To our knowledge, this is the first example of a membrane-spanning protein in which the functional activity induced by a bound metal ligand appears to depend on secondary attachment to the membrane, mediated by fatty acid membrane anchors that are linked to the polypeptide at site(s) distally separated from the transmembrane domain.

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