Identity of a Conserved Motif in Phospholipid Scramblase That Is Required for Ca^{2+}-Accelerated Transbilayer Movement of Membrane Phospholipids†

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ABSTRACT: Accelerated transbilayer movement of plasma membrane phospholipids (PL) upon elevation of Ca^{2+} in the cytosol plays a central role in the initiation of plasma clotting and in phagocytic clearance of injured or apoptotic cells. We recently identified a human erythrocyte membrane protein that induces rapid transbilayer movement of PL at elevated Ca^{2+}. We also presented evidence that this PL scramblase is expressed in a variety of other cells and tissues where transbilayer movement of plasma membrane PL is promoted by intracellular Ca^{2+} [Zhou, Q., et al. (1997) *J. Biol. Chem. 272,* 18240−18244]. We have now cloned murine PL scramblase for comparison with the human polypeptide. Both human and murine PL scramblase are acidic proteins (p*I* = 4.9) with a predicted inside-outside (type 2) transmembrane segment at the carboxyl-terminus. Whereas human PL scramblase (318 AA) terminates in a short exoplasmic tail, murine PL scramblase (307 AA) terminates in the predicted membrane-inserted segment. The aligned polypeptide sequences reveal 65% overall identity, including near identity through 12 residues of an apparent Ca^{2+} binding motif (D[A/S]DNFGIQFPLD) spanning codons 273−284 (human) and 271−282 (murine), respectively. This conserved sequence in the cytoplasmic domain of PL scramblase shows similarity to Ca^{2+}-binding loop motifs previously identified in known EF hand structures. Recombinant murine and human PL scramblase were each expressed in *Escherichia coli* and incorporated into proteoliposomes. Measurement of transbilayer movement of NBD-labeled PL confirmed that both proteins catalyzed Ca^{2+}-dependent PL flip-flop similar to that observed for the action of Ca^{2+} at the cytoplasmic face of plasma membranes. Mutation of residues within the putative EF hand loop of human PL scramblase resulted in loss of its PL mobilizing function, suggesting that these residues directly participate in the Ca^{2+}-induced active conformation of the polypeptide.

Plasma membrane phospholipids (PL)[1] are normally asymmetrically distributed, with phosphatidylcholine (PC) and sphingomyelin located primarily in the outer leaflet, and phosphatidylserine (PS) and phosphatidylethanolamine in the cytoplasmic leaflet of the membrane (*1, 2*). In resting cells, the rate of spontaneous exchange of PL between plasma membrane leaflets is very slow. An increase in intracellular Ca^{2+} due to cell activation, injury, or apoptosis causes rapid bidirectional movement of plasma membrane PL between leaflets, resulting in exposure of PS and phosphatidylethanolamine at the cell surface (*2*−*7*). Cell surface-exposed amino-PL have been shown to promote membrane assembly of several key enzymes of the coagulation and complement systems. They have also been implicated as a signal in the clearance of injured or apoptotic cells by the reticuloendothelial system. This suggests that Ca^{2+}-induced redistribution of membrane PL plays a central role in the regulation of coagulation and in cellular clearance mechanisms (*8*−*14*).

We recently reported the molecular cloning of human plasma membrane PL scramblase. This protein is ubiquitously expressed in plasma membranes and induces rapid Ca^{2+}-dependent bidirectional movement of PL between membrane leaflets upon addition of Ca^{2+}, suggesting that this PL scramblase is responsible for the reorganization of plasma membrane PL observed at elevated cytosolic Ca^{2+} (*15, 16*). Here we report the molecular cloning, expression, and functional reconstitution of the murine homologue of PL scramblase and demonstrate that a conserved Ca^{2+} binding motif of 12 amino acids (D[A/S]DNFGIQFPLD) in the cytoplasmic domain with similarity to known EF hand loop structures found in other proteins is involved in the regulation of PL scramblase activity by Ca^{2+} (*17, 18*).

EXPERIMENTAL PROCEDURES

Materials. Mouse fibroblast 5′-stretch plus cDNA library and KlenTaq polymerase were obtained from CLONTECH Laboratories. Expressed sequence Tag (EST) clone with GenBank accession number AA110551 was from American Type Culture Collection (ATCC 977052). [α-^{32}P]dCTP was

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[1] Abbreviations: PL, phospholipid(s); PC, phosphatidylcholine; PS, phosphatidylserine; NBD-PC, 1-oleoyl-2-[6-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-*sn*-glycero-3-phosphocholine; EST, expressed sequence tag; MBP, maltose binding protein; PAGE, polyacrylamide gel electrophoresis; bp, base pair(s); PCR, polymerase chain reaction.

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purchased from Dupont. Random Primed DNA Labeling Kit was from Boehringer Mannheim. Hybond-N Nylon membrane was from Amersham. Expression vector pMAL-C2, amylose resin, and all restriction enzymes were from New England Biolabs. Wizard Kit was from Promega. Qiagen Lambda Kit was from Qiagen. 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. Factor Xa was from Haematologic Technologies, and Bio-Beads SM-2 were from BioRad. *N*-Octyl β-D-glucopyranoside and Glu-Gly-Arg chloromethyl ketone were from Calbiochem. Sodium dithionite (Sigma) was freshly dissolved in 1 M Tris, pH 10, at a concentration of 1 M.

Labeling of DNA Probe. The DNA insert of EST clone GenBank AA110551 was released by digestion with *Eco*RI and *Apa*LI and purified by Wizard Kit. Four micrograms of purified DNA was labeled with 1 mCi of [α-^{32}P]dCTP. The specific radioactivity of the probe was 3.9×10^8 dpm/μg DNA.

Isolation of Mouse PL Scramblase cDNA by Plaque Hybridization. Escherichia coli strain Y1090r was transformed by mouse fibroblast cDNA library (6×10^5 pfu) and poured onto 30 plates (15 cm diameter, 20 000 pfu per plate). Plaques were lifted onto Hybond-N Nylon membranes. After denaturation, neutralization, and UV cross-linking, the membranes were first prehybridized in a solution composed of 5× Denhardt, 5× SSC, 1% SDS, and 200 μg/mL herring sperm DNA for 3 h at 68 °C, and then hybridized in the same solution containing 5 ng/mL ^{32}P-labeled probe for 16 h at 68 °C. The membranes were washed once with 2× SSC and 0.1% SDS, and then three times with 0.1× SSC and 0.1% SDS for 20 min at 65 °C, and exposed to X-ray film. Secondary plaque lifts and hybridization were carried out on eight positive plaques at a density of about 100 plaques/plate. Single positive and well-isolated plaques were picked and amplified. λDNA was purified with Qiagen Lambda Maxi Kit.

DNA Sequencing. DNA was sequenced on an ABI DNA Sequencer Model 373 Stretch (Applied Biosystems) using PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Perkin-Elmer).

Cloning of Mouse PL Scramblase into pMAL-C2 Expression Vector. To express mouse PL scramblase as a fusion protein with maltose binding protein (MBP), cDNA encoding mouse PL scramblase was cloned into pMAL-C2 expression vector. PCR was performed on a mouse scramblase clone using the primers 5′TCA GAA TTC GGA TCC ATG GAG GCT CCT CGC TCA GGA AC3′ with an *Eco*RI site before the ATG start codon and 5′GCT TGC CTG CAG GTC GAC CTA CAC ACA GCC TTC AAA AAA CAT G3′ with a *Sal*I site after the stop codon. KlenTaq polymerase was used to ensure high-fidelity amplification. The PCR product was digested with *Eco*RI and *Sal*I, isolated by electrophoresis, and cloned into pMAL-C2 immediately 3′ of MBP. *E. coli* strain TB1 was transformed, and sequence of the cDNA insert of plasmid from a single colony was confirmed.

Expression and Purification of Mouse PL Scramblase−MBP Fusion Protein. Mouse PL scramblase was expressed as fusion protein with MBP in *E. coli* TB1 and purified on amylose resin as previously described for human PL scramblase (*16*). The purified fusion protein was centrifuged at 106000*g* for 1 h at 4 °C to remove aggregated protein and was analyzed by SDS−PAGE.

Reconstitution and Functional Activity of PL Scramblase. Reconstitution, removal of MBP, and functional assay of PL scramblase were performed as previously described (*15*, *16*, *19*). Routinely, 420 pmol of protein was reconstituted with 1 μmol of PL. To remove MBP, proteoliposomes were incubated for 3 h at room temperature with 1/40 (w/w) factor Xa. The digest was terminated by addition of 100 μM Glu-Gly-Arg chloromethyl ketone. Proteoliposomes labeled with NBD-PC were incubated for 2 h at 37 °C in Tris buffer (100 mM Tris, 100 mM KCl, 0.1 mM EGTA, pH 7.4) in the presence or absence of $CaCl_2$, as indicated in figure legends, and diluted 25-fold in Tris buffer containing 4 mM EGTA. Initial fluorescence was recorded (SLM Aminco 8000 spectrofluorimeter; excitation at 470 nm, emission at 532 nm); 20 mM dithionite was added, and the fluorescence was continuously monitored for a total of 120 s. Scramblase activity was calculated according to the difference in nonquenchable fluorescence observed in the presence versus absence of $CaCl_2$. The concentration of Ca^{2+} was calculated using FreeCal version 4.0 software (generously provided by Dr. Lawrence F. Brass, University of Pennsylvania, Philadelphia, PA).

Protein Concentrations. Protein concentrations were estimated on the basis of 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 protein).

Mutagenesis of PL Scramblase. Human PL scramblase amino acid residues in EF hand Ca^{2+}-binding motif at positions of Asp^{273}, Asp^{275}, Phe^{277}, Ile^{279}, Phe^{281}, and Asp^{284} were mutated to Ala with oligonucleotide-directed mutagenesis by two rounds of PCR. PL scramblase−pMAL-C2 was selected as template, and the first round of PCR was performed with pairs of a complementary oligonucleotide primer containing the point mutation plus a primer complementary to a site near the ATG initial codon or TAG stop codon. PCR products were purified by Wizard Kit. Full-length mutated PL scramblase cDNA was obtained by overlapping PCR and cloned back into pMAL-C2 vector. After confirmation of correct DNA sequence the mutants were recombinantly expressed in *E. coli* as described above and analyzed by SDS−PAGE.

RESULTS AND DISCUSSION

Isolation of cDNA of Mouse PL Scramblase. Murine EST clones in GenBank containing putative PL scramblase sequence were identified by a Blast homology search using the human PL scramblase cDNA. Among several clones exhibiting significant homology, a 403-bp Stratagene mouse kidney clone (GenBank accession number AA110551) with 79% nucleotide sequence identity to human PL scramblase was selected, and this clone was used to probe a mouse fibroblast cDNA library. Eight positive clones were identified after two rounds of plaque hybridization. Two of the eight clones were sequenced yielding 1354 and 1529 bp, respectively. Alignment revealed 1261 bp of overlapping sequence that spanned an open reading frame of 921 bp and specified a total of 1622 bp of unique cDNA sequence (GenBank accession number AF015790).

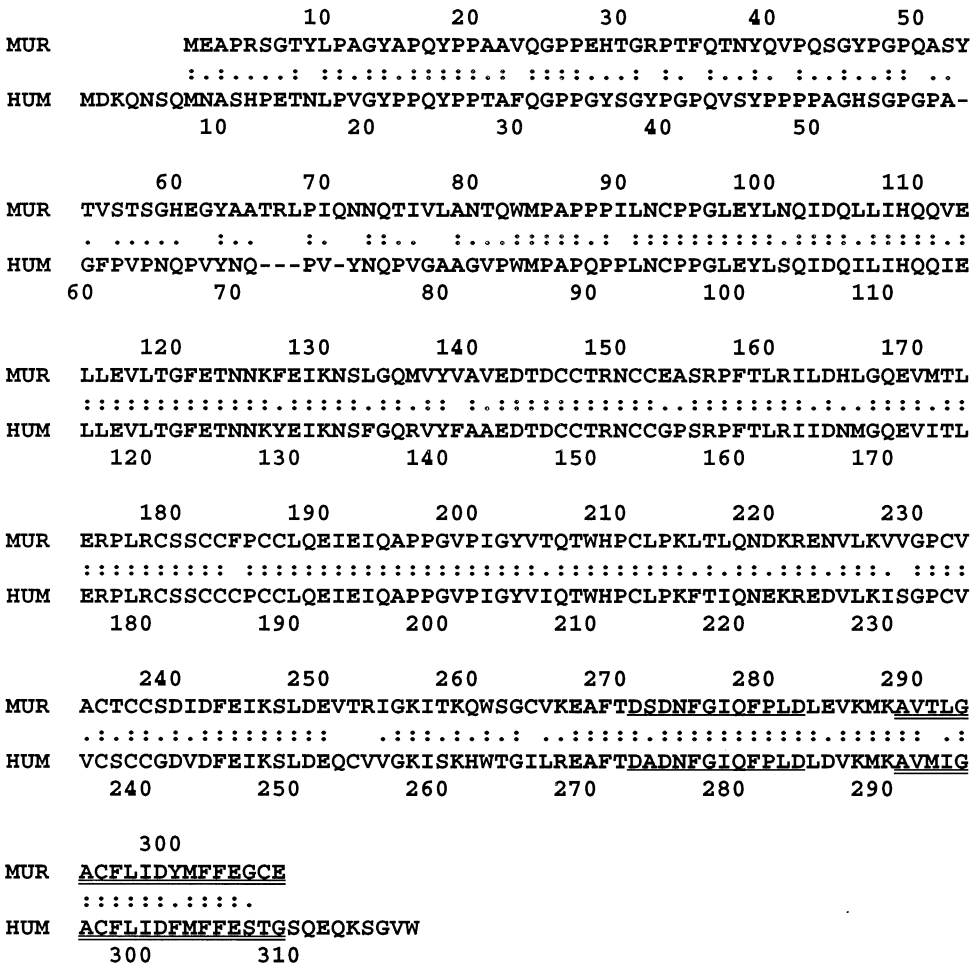

FIGURE 1: Alignment of protein sequences of mouse and human PL scramblase. Alignment between mouse (MUR) and human (HUM) PL scramblase was performed by FASTA program using the Smith−Waterman algorithm (23). GenBank accession numbers are AF015790 for mouse and AF008445 for human PL scramblase, respectively. Amino acid identities (:) or similarities (.) between the two sequences are indicated. Also indicated are the residues comprising a predicted inside-out transmembrane domain (MUR 289−307, HUM 291−309, double underline), and the 12 residues of the acidic loop of a putative EF-hand (MUR 271−282, HUM 273−284, single underline).

The deduced mouse PL scramblase cDNA encodes a 307-residue protein with a molecular mass of 33.9 kDa and a theoretical pI = 4.9, similar to values obtained for the human protein (318 residues, 35.1 kDa; pI = 4.9; ref *16*). The overall identity of the mouse and human PL scramblase is 64.8%, with the most divergent sequence generally contained in the N-terminal portion of the polypeptide (Figure 1). In both proteins, a single 19-residue transmembrane helix is predicted at the carboxyl terminus, exhibiting a strongly preferred inside-to-outside orientation. Whereas the mouse protein terminates immediately after this conserved transmembrane helix, the human PL scramblase contains an additional nine residues, implying that the short exoplasmic peptide in human PL scramblase is nonessential to function. Homology motifs conserved in both proteins include a potential site for protein kinase C phosphorylation (Thr^{159} in mouse, Thr^{161} in human) and a potential Ca^{2+}-binding EF hand loop motif adjacent to the transmembrane helix (residues Asp^{271}−Asp^{282} in mouse and residues Asp^{273}−Asp^{284} in human). The cytoplasmic orientation of this protein and the proximity of this putative Ca^{2+} binding domain to the segment of polypeptide that is inserted into the plasma membrane are consistent with the proposed activity of this protein in situ, where Ca^{2+} acting directly at the endofacial membrane surface is known to initiate the rapid transbilayer movement of plasma membrane PL (*3, 8, 15, 20, 21*).

Functional Activity of Recombinant Mouse PL Scramblase. To confirm that the cDNA identified as mouse PL scramblase encodes a protein of similar function to that identified in human, the human and mouse proteins were each expressed in *E. coli*, purified, and reconstituted in proteoliposomes for measurement of PL mobilizing activity. As shown in Figure 2, recombinant mouse PL scramblase mediated a Ca^{2+}-dependent transbilayer movement of membrane PL with a specific activity and affinity for Ca^{2+} indistinguishable from that observed for the recombinant human protein.

Mutational Analysis of a Putative Conserved EF-Hand Motif. As noted above, the deduced protein sequence of mouse and human PL scramblase reveals an extensive segment of highly conserved sequence extending through residue Glu^{306} (in human; corresponding to Glu^{304} in mouse; Figure 1). The predicted secondary structure through this portion of the protein reveals that it contains two short α-helical segments near the C-terminus that are separated by a 12-residue acidic loop. In both proteins (human and mouse), the C-terminal α helix represents a predicted transmembrane segment with a strongly preferred inside-to-outside orientation, whereas sequence contained within the

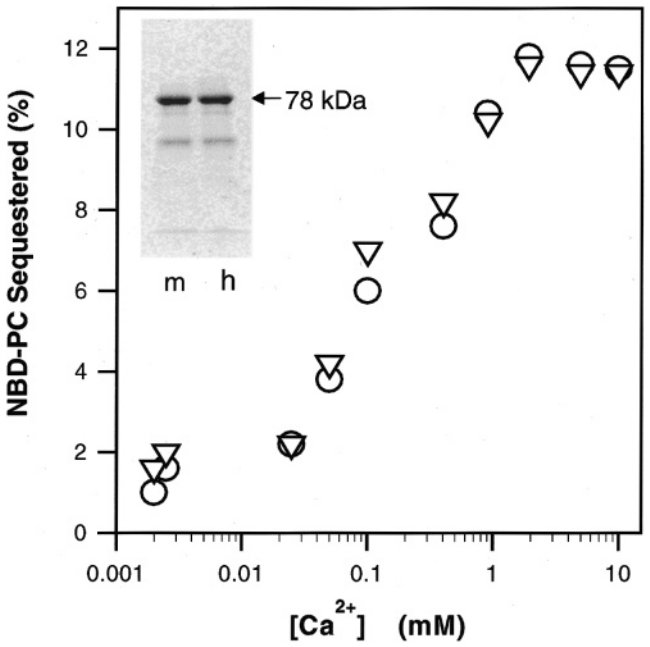

FIGURE 2: Ca^{2+} dependence of mouse and human PL scramblase. Mouse (○) or human (▽) PL scramblase−MBP fusion protein (420 pmol) was reconstituted into PC/PS liposomes (1 μmol of total PL), respectively; MBP was removed by digestion of the proteoliposomes with factor Xa, and PL scramblase activity was determined as described in Experimental Procedures and plotted as a function of external free [Ca^{2+}]. Inset: SDS−PAGE of the purified mouse (m) and human (h) PL scramblase−MBP fusion proteins used in these experiments. Data of single experiment.

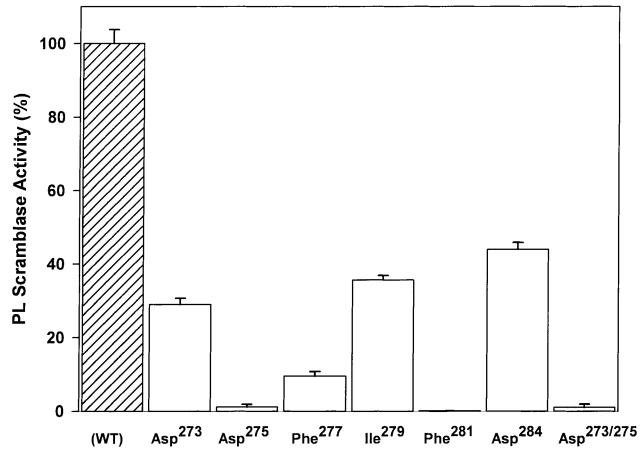

FIGURE 3: Mutational analysis of putative EF hand loop motif contained in human PL scramblase. Wild-type (WT) and mutant constructs of human PL scramblase were expressed as fusion proteins with MBP in *E. coli*, purified, and reconstituted in proteoliposomes. After release of MBP by incubation with factor Xa, PL scramblase activity was assessed (Experimental Procedures). For each mutant construct, the residues in human PL scramblase that were replaced by Ala are indicated on the abscissa. PL scramblase activity (ordinate) was measured in the presence of 2 mM $CaCl_2$, and in each case was normalized to the activity of WT human PL scramblase (11.76 ± 0.44% of total NBD-PC flipped), with correction for the nonspecific transbilayer movement of NBD-PC (0.20 ± 0.08% of total NBD-PC flipped) measured in PL vesicles lacking added protein. Error bars indicate mean ± SD of three independent measurements performed with each mutant construct. Data of single experiment, representative of two separate experiments so performed.

adjacent 12-residue acidic loop conforms, in part, to a consensus sequence that is characteristic of an EF hand Ca^{2+}-binding loop motif (*22*). In this motif, residues in positions

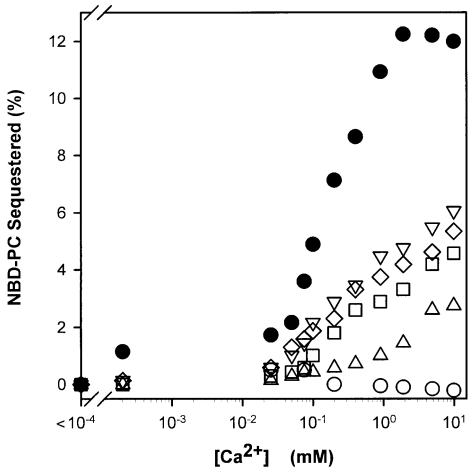

FIGURE 4: Ca^{2+} dependence of mutant human PL scramblase. PL scramblase activity of wild-type (WT) and selected mutant constructs of Figure 3 were determined as described in Experimental Procedures and plotted as a function of external free [Ca^{2+}]: WT (●), Asp^{273} (□), Phe^{277} (△), Ile^{279} (◇), Phe^{281} (○), Asp^{284} (▽). Data are corrected for nonspecific transbilayer migration of NBD-PC in the absence of free [Ca^{2+}]. Data of single experiment.

1, 3, 5, 7, 9, and 12 of the loop contribute to octahedral coordination of the Ca^{2+} ion, with the residues in position 1 (Asp), 3 (Asp, Asn, or Ser), and 12 (Asp or Glu) being those most highly conserved. To gain insight into whether this segment of the protein might be directly involved in the Ca^{2+}-dependent reorganization of membrane PL mediated by PL scramblase, we expressed mutant human PL scramblase with Ala substitutions at positions corresponding to residues 1 (Asp^{273}), 3 (Asp^{275}), 5 (Phe^{277}), 7 (Ile^{279}), 9 (Phe^{281}), and 12 (Asp^{284}) of this putative 12-residue EF hand loop. As illustrated by Figure 3, Ala substitution at any of these positions reduced PL scramblase function, with mutation at Asp^{275} and Phe^{281} resulting in complete inactivation of the Ca^{2+}-dependent response. In those mutant polypeptides showing partial retention of activity, reduced response to Ca^{2+} was related, in part, to an apparent reduction in avidity for Ca^{2+} (Figure 4). These results suggest that residues contained in the putative EF hand loop spanning Asp^{273}−Asp^{284} are critical to the function of PL scramblase, presumably for coordination of Ca^{2+} as required to induce the PL transporting state of the protein. It remains to be determined what conformational changes are induced in the polypeptide in the presence of Ca^{2+}, including potential reorientation of helical segments flanking the putative Ca^{2+} binding loop, that might contribute to the accelerated transbilayer movement of membrane phospholipids.

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