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Research Article

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Scott Syndrome Erythrocytes Contain a Membrane Protein Capable of Mediating Ca^{2+}-dependent Transbilayer Migration of Membrane Phospholipids

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Abstract

Phospholipid (PL) scramblase is a plasma membrane protein that mediates accelerated transbilayer migration of PLs upon binding Ca^{2+}, facilitating rapid mobilization of phosphatidylserine to the cell surface upon elevation of internal Ca^{2+}. In patients with Scott syndrome, a congenital bleeding disorder related to defective expression of membrane coagulant activity, circulating blood cells show decreased cell surface exposure of phosphatidylserine at elevated cytosolic [Ca^{2+}], implying an underlying defect or deficiency of PL scramblase. To gain insight into the molecular basis of this disorder, we compared PL scramblase in Scott erythrocyte membranes to those of normal controls. Whereas membranes of Scott cells were unresponsive to Ca^{2+}-induced activation of PL scramblase at neutral pH, apparently normal PL scramblase activity was induced at pH $<$ 6.0. After extraction with octylglucoside, a membrane protein was isolated from the Scott cells which exhibited normal PL scramblase activity when reconstituted in vesicles with exogenous PLs. Like PL scramblase from normal erythrocytes, PL scramblase from Scott erythrocytes was maximally activated either by addition of Ca^{2+} (at pH 7.4) or by acidification to pH $<$ 6.0, and similar apparent affinities for Ca^{2+} and rates of transbilayer transfer of PLs were observed. This suggests that the defect in Scott syndrome is related to an altered interaction of Ca^{2+} with PL scramblase on the endofacial surface of the cell membrane, due either to an intrinsic constraint upon the protein preventing interaction with Ca^{2+} in situ, or due to an unidentified inhibitor or cofactor in the Scott cell that is dissociated by detergent. (*J. Clin. Invest.* 1997. 99:2232–2238.) Key words: blood coagulation • phosphatidylserines • phosphatidylcholines • erythrocyte membrane • calcium

Introduction

The plasma membrane phospholipids (PL)[1] of erythrocytes (RBC), platelets, and vascular endothelium are normally asymmetrically distributed, with phosphatidylserine (PS) and phosphatidylethanolamine (PE) residing almost exclusively in the inner leaflet, and phosphatidylcholine (PC) and sphingomyelin enriched in the outer leaflet (1, 2). This asymmetric distribution of PL is maintained by an aminophospholipid translocase, an Mg^{2+}-dependent ATPase that transports PS and PE, but not PC, from outer to inner plasma membrane leaflet (2–5). Whereas the rate of spontaneous movement of PL between membrane leaflets is normally quite slow, a substantial increase in intracellular Ca^{2+} resulting either from agonist-induced activation, or secondary to apoptosis or immune injury, initiates rapid transbilayer migration of all plasma membrane PL, collapsing the normal PL asymmetry (1, 6–9). The resulting exposure of PS and PE at the surface of activated or injured blood cells or endothelium serves to promote blood coagulation by providing binding sites for coagulation enzyme complexes including factor VIIIaIXa (tenase) and factor VaXa (prothrombinase) (1, 10, 11). Surface exposure of plasma membrane PS and PE can also initiate complement activation (12), and signals the removal of injured or apoptotic cells by the reticuloendothelial system (13).

The mechanism(s) that gives rise to this transbilayer randomization or "scrambling" of plasma membrane upon cell activation or injury remains unresolved. Elevated cytosolic Ca^{2+} has been shown to initiate rapid bidirectional transbilayer redistribution of all plasma membrane PL (6–8, 14, 15), although evidence for concomitant selective and vectorial egress of PS and PE to the cell surface has also been reported (9, 16). Proposed mechanisms for this effect of intracellular Ca^{2+} on the movement of PL between plasma membrane leaflets include (*i*) Ca^{2+}-induced vesiculation of the plasma membrane (11, 17); (*ii*) calpain-mediated proteolysis of the submembrane cytoskeleton, liberating PL headgroups from interaction with endofacial proteins (18); (*iii*) loss of polyamine–membrane associations that might serve to stabilize PL asymmetry (15, 19); (*iv*) destabilization of the PL bilayer by the complex of Ca^{2+} and the polyanionic PL, phosphatidylinositol 4,5-bisphosphate (19, 20); (*v*) action of a Ca^{2+}-dependent membrane protein with PL scramblase activity. Recently, an as yet unidentified RBC membrane protein of $\sim$ 37 kD was shown to exhibit PL scramblase activity when reconstituted in proteoliposomes containing exogenous PL (21). This protein was shown to mediate Ca^{2+}-dependent bidirectional transbilayer movement of PL which mimicked the effects of Ca^{2+} interacting at the

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1. *Abbreviations used in this paper:* CV, column volume(s); IOV, inside-out RBC membrane vesicle(s); mS, milliSiemens; NBD-PC, 1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-*sn*-glycero-3-phosphocholine; NBD-PS, 1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-*sn*-glycero-3-phosphoserine; OG, *N*-octyl-β-D-glucopyranoside; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipid(s); PS, phosphatidylserine; RBC, red blood cell.

endofacial surface of the RBC membrane. Evidence for a platelet membrane protein of similar function has also been reported (22).

Scott syndrome is a rare bleeding disorder in which normal cellular PL scramblase activity of blood platelets is defective despite normal secretory and aggregation responses, resulting in markedly reduced mobilization of PS to cell surfaces and reduced expression of membrane catalytic surface for the tenase and prothrombinase enzyme complexes (23–28). The plasma membranes of these cells contain normal amounts of PS and other PLs, and exhibit normal aminophospholipid translocase activity (24, 29). Although Scott syndrome was originally described as an isolated platelet disorder, it is now clear that other blood cells, including RBC and lymphocytes, are also affected, and there is now evidence that this disorder is related to a defective gene that selectively affects membrane PL scrambling (28, 30, 31). The molecular defect giving rise to Scott syndrome remains to be elucidated. To gain further insight into the origin of the functional defect exhibited by Scott syndrome blood cells, we undertook the isolation of PL scramblase protein from Scott RBC membranes.

Methods

Materials. Egg yolk phosphatidylcholine (PC), brain phosphatidylserine (PS), 1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino] caproyl-*sn*-glycero-3-phosphocholine (NBD-PC) and 1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-*sn*-glycero-3-phosphoserine (NBD-PS) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). *N*-octyl-β-D-glucopyranoside (OG) was purchased from Calbiochem (La Jolla, CA). All other reagents were from Sigma Chemical Co. (St. Louis, MO).

Solutions. Solution A, 20 mM Tris, 0.1 mM EGTA, 50 mM OG, pH 7.4; Solution B, 50 mM HOAc, 0.1 mM EGTA, 50 mM OG, pH 5.0; Solution C, 100 mM Tris, 100 mM KCl, 0.1 mM EGTA, pH 7.4; Solution D, 100 mM citric acid, 100 mM KCl, 0.1 mM EGTA; Solution E, 50 mM Tris, 50 mM Bis-Tris, 100 mM KCl, 0.1 mM EGTA. Sodium dithionite ($Na_2S_2O_4$) was freshly dissolved in 1 M Tris pH 10 at a concentration of 1 M.

Erythrocytes. Blood, collected in acid-citrate-dextrose, was obtained from healthy volunteers and from Scott syndrome patient MS, and shipped at 4°C by air express to Milwaukee, WI. Immediately upon receipt, erythrocytes were washed and utilized for PL scramblase isolation or preparation of inside-out membrane vesicles (see below). For these experiments, blood from patient MS (and normal controls) was obtained on three separate occasions.

Preparation of erythrocyte inside out vesicles (IOV). Erythrocyte inside out vesicles were prepared essentially by methods described by Steck and Kant (32) with minor modifications (21). Assays for acetylcholinesterase (outside) and glyceraldehyde 3-phosphate dehydrogenase accessibility (inside) (32) confirmed that > 90% of these IOV were properly oriented and resealed.

PL scramblase activity in IOV. PL scramblase in IOV was measured as previously described (21, 29): IOV were suspended in solution C at 150 μg protein/ml. NBD-PS was added to a final 0.25 mol% of total IOV membrane PL. After incorporation of label for 3 min at room temperature, IOV were incubated in the presence of 0–1 mM $CaCl_2$ for 15 min at 37°C. For each sample, percentage of NBD-PS displaced from the external to inward-facing membrane leaflet was determined by the BSA back-exchange method as previously described (29). PL scramblase activity was evaluated by comparing the amount of NBD-PS moved to the inward-facing leaflet of IOV in the presence of Ca^{2+} to that observed in the presence of 0.1 mM EGTA. Due to the known transport of dithionite by the erythrocyte membrane anion exchanger (33), the transmembrane distribution of NBD-PS in IOV was measured using the method of back-extraction of external NBD-PS in 1% albumin. When directly compared, these two methods for monitoring transmembrane distribution of NBD-PS (dithionite quenching and albumin back-extraction) have been shown to yield equivalent results (14). For measurement of PL scramblase as a function of pH, IOV were suspended to 150 μg protein/ml in solution E adjusted to various pH as indicated. NBD-PS was incorporated as described above and the samples were incubated for 15 min at 37°C without $CaCl_2$. The percentage of NBD-PS displaced from the external to inward-facing membrane leaflet was determined as described above.

PL scramblase isolation. PL scramblase was purified by modification of a protocol previously described (21). Washed white erythrocyte ghosts in 5 mM sodium phosphate, 1 mM EDTA, pH 7.4 were prepared from 15 ml of packed erythrocytes (Scott syndrome or control) and membrane proteins extracted (30 min, 23°C) with solution A made 100 mM OG, 1 mM phenylmethylsulfonyl fluoride. The cytoskeleton and insoluble proteins were removed by centrifugation at 155,000 *g* at 4°C, the supernatant filtered through a 0.45 μm membrane (Millipore Corp., Bedford, MA), and applied to a 0.5 × 5.0-cm DEAE Sepharose Fast Flow column (Pharmacia Biotechnology Inc., Uppsala, Sweden) equilibrated in solution A. Flow rate was 0.5 ml/min. The column was washed with 30 column volumes (CV) of solution A and eluted with a 0–500 mM NaCl gradient in solution A over 10 CV and 0.5-ml fractions were collected. Column fractions were assayed for PL scramblase activity (described below). The peak of activity was pooled and diluted fourfold with solution A, and then applied to 0.5 × 5.0-cm Mono Q HR column (Pharmacia Biotechnology Inc.) equilibrated in solution A at 0.5 ml/min. After washing with 30 CV of solution A, proteins were eluted with a 0–500 mM NaCl gradient in solution A over 10 CV. Column fractions were assayed for PL scramblase activity and the peak of activity pooled. The sample was desalted and buffer-exchanged into solution B using a PD-10 column (Pharmacia Biotechnology Inc.). The sample was loaded onto 0.46 × 5.0-cm POROS 20 HS column (PerSeptive Biosystems, Framingham, MA) equilibrated in solution B at 0.5 ml/min. The sample flow-through containing PL scramblase was collected and used for functional studies. All column chromatography procedures were performed at room temperature on a BioCAD 20 perfusion chromatography workstation (PerSeptive Biosystems).

Reconstitution into proteoliposomes. A mixture of PC and PS (9:1 molar ratio) was dried under a stream of nitrogen and resuspended in solution C by vigorous vortexing. Protein samples were mixed with these liposomes at a final lipid concentration of 4 mg/ml in the presence of 60 mM OG. Detergent was then removed by dialysis overnight at 4°C against 200 vol of solution C containing 1 g/l SM2-Biobeads (Bio-Rad Laboratories, Hercules, CA). Alternatively, the detergent was removed by addition of SM2-Biobeads (200–300 mg/ml) directly to the protein-lipid sample and after mixing for 1 h at room temperature, the beads were allowed to settle by gravity, and proteoliposomes recovered. Equivalent results were obtained for proteoliposome reconstitution of PL scramblase by both methods and these methods were used interchangeably.

Labeling of proteoliposomes with NBD-PL. Proteoliposomes were labeled with fluorescent NBD-PC or NBD-PS (0.25 mol%) selectively incorporated into the outer membrane leaflet (29, 34) by addition of NBD-PL (in DMSO, final solvent concentration 0.25%) to the preformed proteoliposome suspension.

PL scramblase activity in proteoliposomes. Scramblase activity was measured as previously described (21). Routinely, proteoliposomes (0.4 mg/ml PL final concentration) labeled with NBD-PL (PC or PS) in the outer membrane leaflet were incubated for 1 h (PC) or 3 h (PS) at 37°C in solution C in presence or absence of $CaCl_2$ at concentrations indicated in figure legends. Proteoliposomes were diluted 25-fold in solution C containing 4 mM EGTA, and transferred to a stirred fluorescence cuvet at 23°C. Initial fluorescence was recorded (8000 spectrofluorimeter SLM Aminco, Urbana, IL; excitation at 470 nm, emission at 532 nm), and 20 mM dithionite added with fluorescence continuously monitored for total of 90–120 s. The difference in

residual (nonquenchable) fluorescence observed for samples preincubated at 37°C in presence vs absence of $CaCl_2$ was attributed to Ca^{2+}-induced change in NBD-PL located in the outer leaflet, and hence accessible to dithionite (21, 34). Maximum (100%) dithionite quenching was determined by addition of 1% Triton X-100. In all cases, NBD fluorescence was corrected for photobleaching (< 6%; measured in absence of dithionite). Ionized [Ca^{2+}] (see Figs. 1 and 6) was calculated using FreeCal version 4.0 software (generously provided by Dr. Lawrence F. Brass, University of Pennsylvania, Philadelphia, PA). For measurement of PL scramblase as a function of pH, proteoliposomes (0.4 mg/ml PL final concentration) labeled with NBD-PL in the outer membrane leaflet were incubated in the absence of $CaCl_2$ for 3 h at 37°C in solution C or solution D, adjusted to various pH as indicated. Proteoliposomes were processed as described above and assessed for the transbilayer movement of NBD-PL based on the residual (nonquenchable) fluorescence observed below pH 7.4. Lastly, a kinetic study of NBD-PLs at 2 mM $CaCl_2$ was performed on homogeneous proteoliposome samples by monitoring the fluorescence quenched by dithionite of aliquots removed from the samples over time as indicated in the figure legend.

Results

The circulating blood cells of patients with Scott syndrome have previously been shown to exhibit a marked defect in their capacity to express procoagulant properties reflecting impaired mobilization of PS to the cell surface through Ca^{2+}-accelerated transbilayer movement of plasma membrane PLs (23–28). Whereas the molecular defect responsible for this disorder remains unknown, the functional defect identified in Scott cells suggests either (*i*) a deficiency or defect in a specific protein that normally confers PL scramblase activity to the plasma membrane or, (*ii*) the expression in defective Scott cells of an inhibitor of the normal PL scramblase pathway. To gain insight into whether the PL scramblase defect that is characteristic of Scott RBC resides with a membrane component or, reflects a soluble factor in the cytosol, we measured PL scramblase activity in IOV after removal of soluble intracellular components. As indicated by Fig. 1, IOV prepared from Scott RBC exhibited the same defect in Ca^{2+}-induced PL scramblase activity that is characteristic of the intact RBC, platelets, and lymphocytes found in this disorder, implying that impaired PL scramblase function in Scott syndrome cells is an inherent property of the plasma membrane, and cannot be attributed to a soluble intracellular factor.

We recently reported that the normal PL scramblase activity of the RBC membrane can be reconstituted in proteoliposomes prepared from exogenous PLs and an RBC membrane protein fraction containing an as yet unidentified highly acidic integral membrane protein, with an apparent molecular mass of ~ 37 kD (21). The PL scramblase activity conferred by this protein in reconstituted lipid vesicles was shown to closely mimic the inherent response of the RBC membrane to elevated internal Ca^{2+}, with half-maximal activation of a rapid transbilayer PL movement observed in both cases at ~ 50 μM

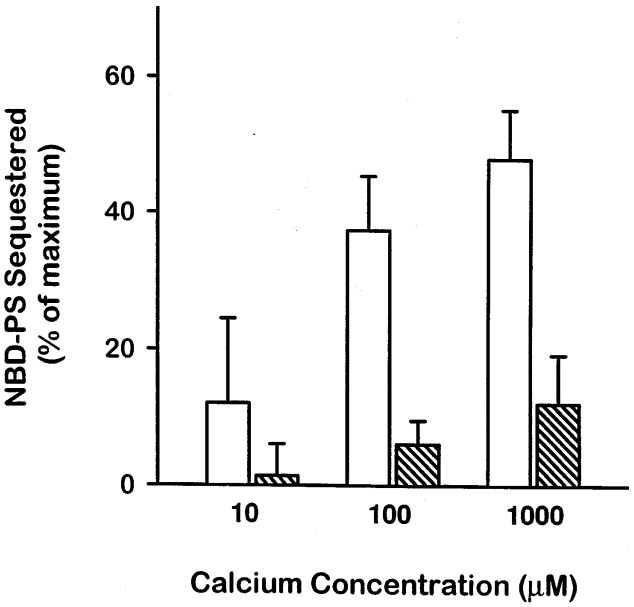

Figure 1. Effect of Ca^{2+} on PL scramblase activity in normal control and Scott IOV. IOV prepared from normal control (*open bars*) or Scott (*hatched bars*) erythrocytes suspended at 150 μg protein/ml were labeled with 0.25 mol% NBD-PS in the presence of 0, 10, 100, or 1,000 μM Ca^{2+} for 15 min at 37°C. The percentage of NBD-PS flipped to the inner membrane leaflet was determined by the BSA "back-exchange" method (see Methods). The amount of NBD-PS in the inward-facing leaflet of the IOV (ordinate) is expressed as the percentage relative to that at equilibrium of probe across the membrane. Data represent mean (±SD) from three independent experiments.

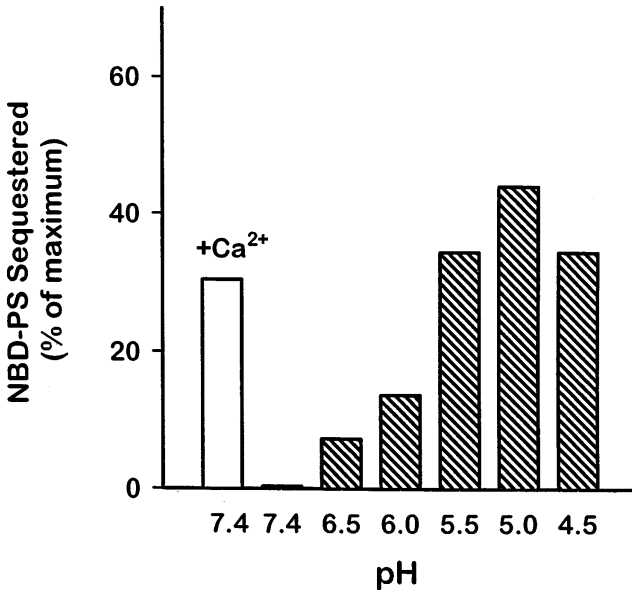

Figure 2. pH-dependent activation of PL scramblase. PL scramblase was isolated from normal RBC membranes and reconstituted into liposomes as described in Methods. The proteoliposomes were labeled with 0.25 mol% NBD-PS added initially to outer leaflet and incubated for 3 h at 37°C in solutions containing 0.1 mM EGTA (no added Ca^{2+}) at pH indicated by abscissa (*hatched bars*). PL scramblase activity was assessed by the amount of NBD-PS sequestered to the inner leaflet, deduced from the residual (nonquenchable) fluorescence after addition of 20 mM dithionite (see Methods). Data are expressed as the percentage of NBD-PS flipped to the inner membrane leaflet relative to that at equilibrium of probe across the membrane. All data are corrected for background leak of probe (< 5%) observed for proteoliposomes suspended in 0.1 mM EGTA (no added Ca^{2+}) at pH 7.4. Open bar denotes NBD-PS flipped when proteoliposomes were incubated in the presence of 2 mM Ca^{2+}, pH 7.4. Data of single experiment, representative of two so performed.

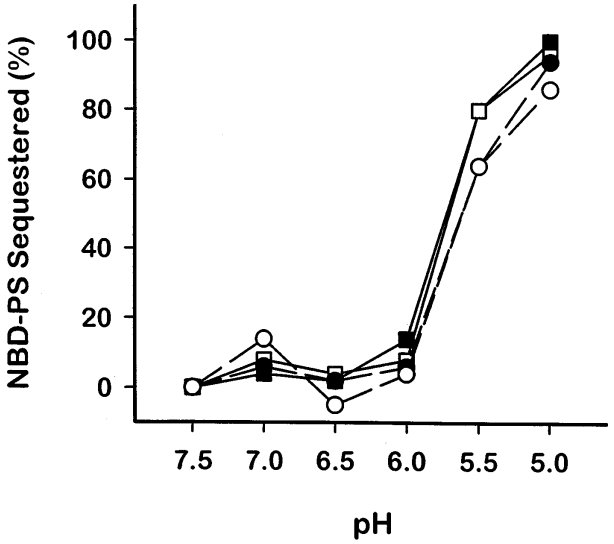

Figure 3. pH-dependent activation of PL scramblase in normal control and Scott IOV. Inside-out vesicles from normal control (□, ■) or Scott (○, ●) suspended at 150 μg protein/ml were labeled with 0.25 mol% NBD-PS added externally and amount of NBD-PS flipped to inward-facing leaflet of the IOV determined after 15 min at 37°C, using the BSA back-exchange method (see Methods). Experiments were performed at pH indicated (*abscissa*) always in presence of 0.1 mM EGTA (no added Ca^{2+}). Data are expressed as the percentage of NBD-PS flipped to the inner membrane leaflet relative to that at equilibrium of probe across the membrane. All data are corrected for background leak of probe observed for proteoliposomes suspended in 0.1 mM EGTA (no added Ca^{2+}) at pH 7.4 (< 5%). Data from two independent experiments are shown (*open and closed symbols*).

$[Ca^{2+}]$. In certain Ca^{2+}-binding proteins, evidence has been reported that conformational changes induced by bound Ca^{2+} can also be initiated by acidification below the pKa of the residues contributing to the metal ion binding site (35, 36). We therefore considered whether the accelerated transbilayer movement of PLs observed in PL scramblase-containing liposomes at elevated $[Ca^{2+}]$ can also be induced under conditions of acidic pH . As shown in Fig. 2, in the absence of Ca^{2+}, acidification < pH 6.0 resulted in an accelerated transbilayer PL migration in proteoliposomes reconstituted with PL scramblase. Under these conditions of altered pH , PL migration in vesicles omitting PL scramblase was unaffected (not shown). These results suggest that the conformational change in PL scramblase that is required for expression of its activity is achieved directly through addition of a proton to one or more residues in the protein, either at His, or, at a carboxylate (Glu, Asp) that might contribute to the putative Ca^{2+}-binding site. We next examined how acidification affected the rate of transbilayer movement of PLs in IOV prepared from Scott and normal erythrocytes. By contrast to the attenuated response of Scott versus control IOV to added Ca^{2+} (see Fig. 1), we observed marked induction of PL scramblase activity in both Scott and control IOV when pH was lowered below 6.0, resulting in equivalent rates of transbilayer movement of PL in both membranes (Fig. 3). These results suggest that the membranes of Scott cells contain a protein that is potentially capable of accelerating transbilayer movement of membrane PLs, but with a markedly reduced capacity for activation by Ca^{2+} interacting at the endofacial membrane surface.

To further explore the possibility that Scott RBC contain a protein with PL scramblase function, we next undertook detergent extraction, purification, and liposome reconstitution according to procedures used to isolate this protein from erythrocyte ghost membranes of normal controls. As illustrated by data of Figs. 4–6, we found that when Scott RBC membrane proteins were subjected to extraction into OG and chromatographic separation by ion exchange, we recovered a protein

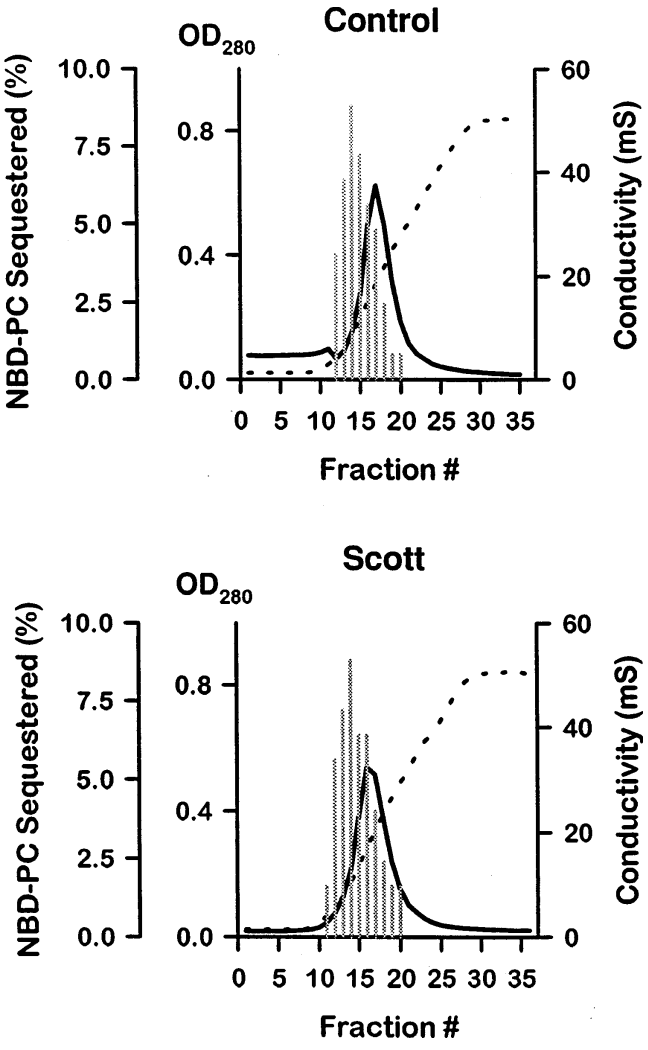

Figure 4. Initial fractionation of PL scramblase from Scott and normal control RBC. Chromatogram shows elution of erythrocyte membrane proteins from DEAE Sepharose Fast Flow performed as detailed in Methods. The solid black line represents the OD_{280} for the eluted proteins in each 0.5-ml fraction collected and the dashed line shows the conductivity of each fraction, expressed in milliSiemens (*mS*). PL scramblase activity (*gray bars*) was measured for each fraction after reconstitution of eluting proteins into proteoliposomes containing 0.25 mol% NBD-PC as probe added initially to outer leaflet (see Methods). PL scramblase activity is expressed as the amount of NBD-PC flipped to inner leaflet of proteoliposomes after 1 h, 37°C in presence of 2 mM Ca^{2+}, with correction for nonspecific leakage observed for identical matched-pair proteoliposomes maintained in 0.1 mM EGTA. Top panel shows the data for normal control RBC and bottom panel shows data for Scott syndrome RBC.

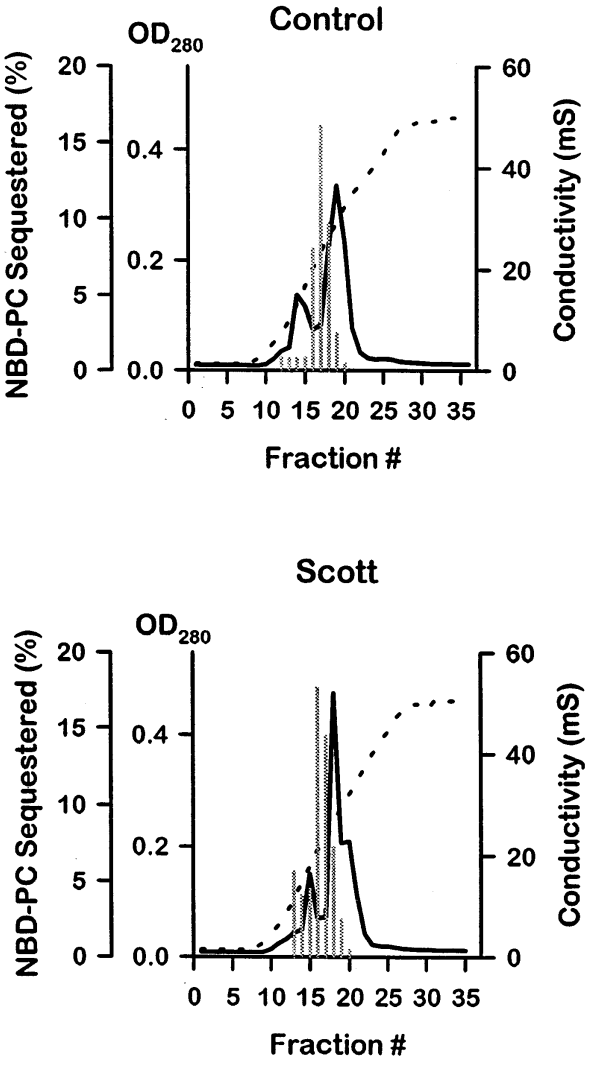

Figure 5. Purification of PL scramblase on Mono Q. Chromatography of PL scramblase extracted from normal control RBC (*top*) and Scott syndrome RBC (*bottom*) on Mono Q. The peak of PL scramblase activity from DEAE (Fig. 4) was applied to Mono Q and proteins eluted with NaCl gradient (see Methods). Eluting protein in each fraction (0.5 ml) was monitored for PL scramblase activity as described for Fig. 4.

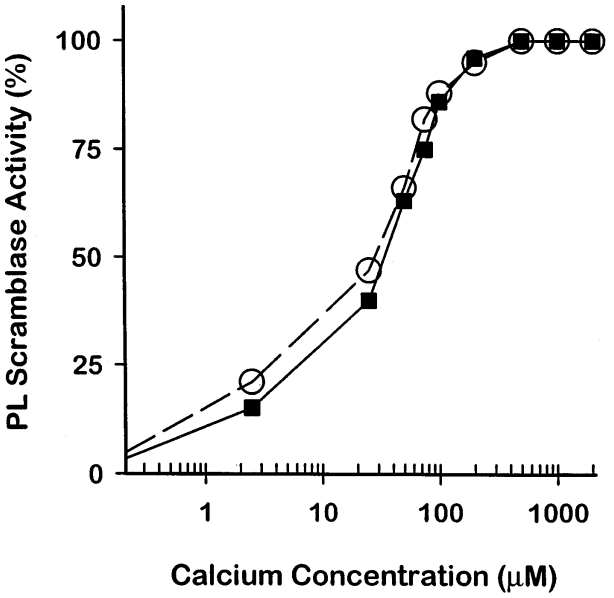

Figure 6. Ca^{2+}-dependent activation of PL scramblase isolated from normal control and Scott syndrome RBC. PL scramblase was partially purified from normal control or Scott RBC ghosts as described in Methods. PL scramblase from normal control (■) or Scott (○) was reconstituted into proteoliposomes labeled with 0.25 mol% NBD-PS added initially to outer leaflet, and suspended in solution C at pH 7.4 in presence of Ca^{2+} concentrations indicated (*abscissa*). After 3 h at 37°C, the amount of NBD-PS flipped to inner leaflet of the proteoliposome was determined, with correction for nonspecific leak observed in 0.1 mM EGTA (no added Ca^{2+}; see Methods). Data at each [Ca^{2+}] are normalized to activity observed at 2 mM Ca^{2+}(100%). Data of single experiment, representative of two so performed.

fraction exhibiting PL scramblase activity when reconstituted into proteoliposomes containing exogenous PL. Comparison of the active fractions isolated from Scott versus control membranes revealed no difference in the elution behavior of the protein through multiple steps of ion exchange chromatography (Figs. 4 and 5). When reconstituted in proteoliposomes, PL scramblase from Scott and normal erythrocytes were identically activated by Ca^{2+}, with half-maximal activation in both cases observed at 40–50 μM Ca^{2+} (Fig. 6) Comparison of the rate of transbilayer migration of NBD-PC and NBD-PS mediated by reconstituted PL scramblase isolated from normal or Scott RBC revealed that in both cases the rate of facilitated transbilayer movement of PC by activated PL scramblase was approximately twice that observed for PS (Fig. 7). Again, results for PL scramblase isolated from Scott erythrocytes were indistinguishable from those observed for the same protein isolated from normal cells. These data suggest that whereas the Scott cells exhibit a markedly impaired activation by Ca^{2+} of membrane PL scramblase in situ, extraction in detergent and reconstitution with exogenous PL restores normal activity to the protein, including affinity for Ca^{2+} that is characteristic of PL scramblase from normal RBC membranes.

Discussion

There is now considerable evidence to suggest that the impaired expression of the coagulant properties of activated or injured blood cells observed in Scott syndrome reflects a mutation that selectively affects Ca^{2+}-dependent activation of the plasma membrane PL scramblase pathway: in the two patients now reported with this syndrome, clinically significant bleeding occurred without any other detected abnormality of platelet or plasma hemostatic function, except a deficient expression of plasma membrane coagulant activity, as induced either through platelet activation by an agonist, membrane injury by complement C5b-9, or by exposure of these cells to Ca^{2+} ionophore (24, 28). In each case, deficient expression of membrane coagulant activity in response to elevated internal Ca^{2+} was found to correlate to deficient mobilization of PS to the platelet surface and a markedly decreased bidirectional scrambling of plasma membrane PC as well as PS. Decreased scrambling of plasma membrane PL of Scott cells at elevated intracellular Ca^{2+} was also associated with a marked decrease in vesicula-

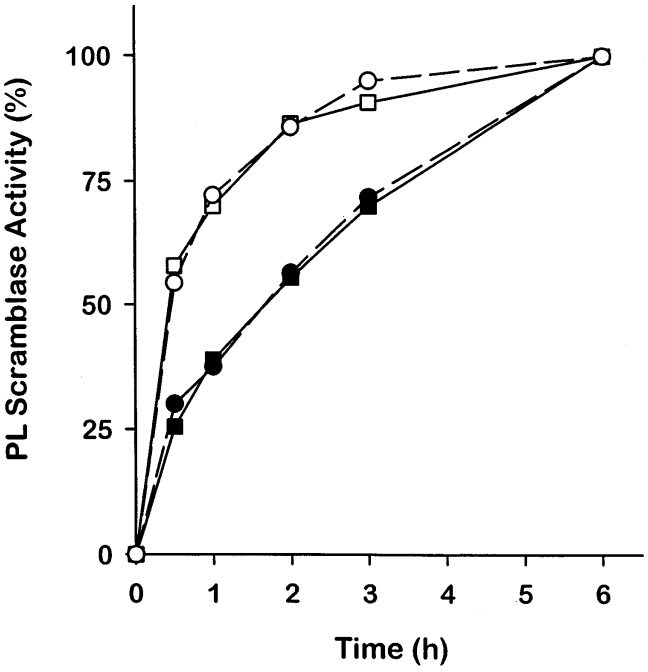

Figure 7. Kinetics of transbilayer movement of NBD-PC and NBD-PS by PL scramblase isolated from normal control and Scott syndrome RBC. PL scramblase was purified in OG from normal control (□, ■) or Scott syndrome (○, ●) RBC membranes and reconstituted into liposomes containing 0.25 mol% NBD-PC (*open symbols*) or NBD-PS (*closed symbols*) added initially to the proteoliposome outer leaflet (see Methods). The proteoliposomes were incubated at 37°C in presence of 2 mM Ca^{2+} and amount of NBD-PL probe flipped to inner leaflet was determined at times indicated (*abscissa*). Data were corrected at each time for nonspecific leakage of NBD-PL in presence of 0.1 mM EGTA, and are normalized to t = 6 h (100% flipped). Data of single experiment representative of two so performed.

tion of membrane microparticles from the surface of these cells. This implies that the stability of the plasma membrane is linked to the maintenance of the normal asymmetric distribution of PL between inner and outer membrane leaflets, and that vesiculation of the plasma membrane is causally related to activation of the PL scramblase pathway (7, 11, 17, 25, 37). The possibility of an autosomal recessive mode of inheritance of the Scott defect was suggested by recent evidence for partially reduced activity of PL scramblase in cells of other family members (28). In addition to platelets, this aberrant membrane phenotype was also demonstrated in erythrocytes and leukocytes obtained from these patients, and the aberrant Scott phenotype was shown to propagate in cultured lymphoblasts derived by EBV-transformation of the patients' B cells (28, 31). Consistent with evidence for an underlying recessive gene defect, hybridoma clones produced by fusion of defective Scott lymphoblasts with wild-type cells were all found to exhibit normal PL scramblase activity (31).

The results of the present experiments demonstrate: (*a*) In addition to known activation by Ca^{2+}, erythrocyte PL scramblase is also activated at pH < 6.0 (in absence of Ca^{2+}). (*b*) Whereas Scott syndrome cells exhibit aberrant insensitivity to activation of PL scramblase by Ca^{2+}, this pathway is activated in the Scott erythrocyte membrane at acidic pH, and such activation is indistinguishable from that observed for membranes of normal erythrocytes. (*c*) The peculiar insensitivity to the effects of Ca^{2+} that is characteristic of the PL scramblase pathway in Scott syndrome cells is not observed for PL scramblase protein purified from the membranes of these cells. This implies that PL scramblase within the membrane of Scott syndrome cells is normally unable to interact with Ca^{2+}, but that its capacity to bind and be activated by Ca^{2+} is restored through detergent extraction and reconstitution with exogenous PL. These data leave unresolved whether the unresponsiveness to Ca^{2+}, observed for PL scramblase expressed in the membranes of Scott cells reflects a mutation in PL scramblase itself that restricts its capacity to bind Ca^{2+} in situ, the action of a PL scramblase inhibitor or Ca^{2+}-dependent cofactor present in Scott cells that is removed from the protein during purification in detergent, or, another mechanism that restricts access of Ca^{2+} to the endofacial surface of the membrane of Scott cells.

Recent studies suggest that intracellular polyamines, including spermine and spermidine, are inhibitory to the Ca^{2+}-dependent activation of transbilayer PL migration across the erythrocyte membrane (15, 19). This raises the possibility that the Scott cell defect is related either to an abnormal increase in one or more polyamines, or, to an increased sensitivity of PL scramblase to inhibition by these compounds. Measurement of the spermine and spermidine concentration in Scott RBC revealed normal concentrations of these polyamines (unpublished data). Furthermore, as demonstrated by Fig. 1, normal PL scramblase activity can be demonstrated in washed erythrocyte IOV, whereas IOV from Scott erythrocytes retain the unresponsiveness to Ca^{2+} that is characteristic of the intact Scott cells. These data are consistent with recent evidence demonstrating that Ca^{2+}-activated PL movement across the plasma membrane reflects the activity of an integral membrane protein (21, 22). They also imply that the aberrant Scott phenotype is intrinsic to the membrane itself, and cannot be attributed to a soluble inhibitor in the cytosol. The results for IOV suspended in presence of Ca^{2+} are also consistent with earlier data demonstrating Ca^{2+} ionophore-initiated PL scramblase activity in ghosts of normal RBCs that were resealed to highly diluted hemolysate, whereas resealed ghosts prepared from Scott erythrocytes showed the same unresponsiveness to Ca^{2+} ionophore that is characteristic of intact Scott RBC (30). Although these results exclude a soluble inhibitor of membrane PL scramblase in the etiology of Scott syndrome, the possibility remains that PL scramblase in the defective Scott cells expresses an aberrant high-affinity binding site for a polyamine or other soluble PL scramblase inhibitor accounting for the persistence of the defect in ghost and washed IOV membranes. Alternatively, another component of the Scott cell membrane might prevent access of Ca^{2+} to its binding site in PL scramblase. After extraction into detergent, dissociation of PL scramblase from such putative inhibitor(s) would restore an apparently normal sensitivity to activation by Ca^{2+} (see Figs. 4–6).

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