The Distribution of Erythrocyte Phospholipids in Hereditary Spherocytosis Demonstrates a Minimal Role for Erythrocyte Spectrin on Phospholipid Diffusion and Asymmetry

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In the human erythrocyte membrane phosphatidylcholine and sphingomyelin reside mainly in the outer leaflet, whereas the aminophospholipids, phosphatidylethanolamine and phosphatidylserine, are mainly found in the inner leaflet. Maintenance of phospholipid asymmetry has been assumed to involve interactions between the aminophospholipids and the membrane skeleton, in particular spectrin. To investigate whether spectrin contributes to maintaining the phospholipid transbilayer distribution and kinetics of redistribution, we studied erythrocytes from hereditary spherocytosis patients whose spectrin levels ranged from 34% to 82% of normal. The phospholipid composition and the accessibility of membrane phospholipids to hydrolysis by phospholipases were in the normal range. Spin-labeled phosphatidylserine and phosphatidylethanolamine analogues that had been introduced into the outer leaflet were rapidly transported at 37°C to the inner leaflet, whereas the redistribution of spin-labeled phosphatidylcholine was slower. The kinetics of transbilayer movement of these spin-labeled phospholipid in all samples was in the normal range and was not affected by the level of spectrin. Although these erythrocyte membranes contained as little as 34% of the normal level of spectrin and were characterized by several physical abnormalities, the composition, distribution, and transbilayer kinetics of the phospholipids were found to be normal. We therefore conclude that spectrin plays, at best, only a minor role in maintaining the distribution of erythrocyte membrane phospholipid.

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duced spectrin levels) were evaluated for the distribution of endogenous phospholipids as well as the redistribution kinetics of spin-labeled phospholipids introduced into the outer monolayer. Our results indicate that the reduction in spectrin levels has no effect on phospholipid distribution or kinetics in hereditary spherocytosis and support the conclusion that the contribution of spectrin to the maintenance of phospholipid asymmetry, if it exists, is only a minor one.

MATERIALS AND METHODS

Erythrocytes. Human erythrocyte suspensions were prepared from fresh human venous blood collected in heparinized tubes after informed consent was obtained from individuals diagnosed with hereditary spherocytosis (HS) or from laboratory volunteers. Erythrocytes were pelleted by centrifugation; washed twice with 0.9% NaCl, and once with incubation buffer; and finally diluted in incubation buffer to the appropriate hematocrit level. The buffer used for the phospholipase experiments contained 50 mmol/L NaH₂PO₄, 4.95 mmol/L KCl, 1.25 mmol/L KH₂PO₄, 0.25 mmol/L MgCl₂, 0.5 mmol/L CaCl₂, 120 mmol/L NaCl, pH 7.4. The buffer for the spin labeling and incubation studies was composed of 10 mmol/L HEPES, pH 7.4, containing 144 mmol/L NaCl, 1 mmol/L MgCl₂, 0.5 mmol/L EGTA, 10 mmol/L glucose, 5 mmol/L KCl.

Lipid analysis. Erythrocyte membrane lipids were extracted according to the method of Rose and Oklander. Phospholipid classes were separated by two-dimensional thin layer chromatography according to the method of Roelofsen and Zwaal, and lipid phosphorus was determined according to the method of Rouser et al.

Phospholipase treatment of erythrocytes. The hydrolysis of membrane phospholipids by phospholipase A₂ from bee venom (Sigma, St Louis, MO) and sphingomyelinase C from Staphylococcus aureus (Sigma) was determined as described by Roelofsen and Zwaal. After the incubation with the combination of phospholipases, erythrocytes were pelleted by centrifugation, and the relative amount of hemoglobin in the supernatant was used to calculate the percentage of cells that hemolyzed during phospholipase treatment. Based on the assumption that all phospholipids in a cell become available for phospholipid hydrolysis and the calculation of the probe fraction in the outer monolayer that can be determined by a back-exchange technique using ankyrin, band 4.1, and other minor proteins were noted. Details are given in previous studies. ATP was extracted from the cells in 0.5% trichloroacetic acid, 2 mmol/L EDTA, and measured with the Pharmacia LKB luciferin-luciferase assay (Pharmacia, Piscataway, NJ).

RESULTS

Characterization of erythrocytes. The individuals with spherocytosis included in this study were deficient in spectrin (Table 1). Smaller reductions in ankyrin, band 4.1, and other minor proteins were noted. Details are given in previous studies of these patients. The designation of the samples (A through H) from the patients and control (N) was used to identify these samples in the tables and figures.

Composition and organization of endogenous phospholipids. The relative amounts of the major phospholipid classes of these erythrocyte membrane samples are given in Table 2. No significant differences were found between the phospholipid classes of these samples.

Table 2. Relative Abundance of the Major Phospholipid Classes in Membranes of Control Cells and Erythrocytes From Eight HS Patients

<table>
<thead>
<tr>
<th>Subject</th>
<th>PC</th>
<th>PE</th>
<th>PS</th>
<th>SM</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>28.5 ± 2.7</td>
<td>31.2 ± 2.8</td>
<td>13.1 ± 1.8</td>
<td>27.1 ± 2.0</td>
</tr>
<tr>
<td>B</td>
<td>28.0 ± 0.8</td>
<td>30.5 ± 0.4</td>
<td>13.0 ± 0.3</td>
<td>28.3 ± 0.4</td>
</tr>
<tr>
<td>C</td>
<td>28.6 ± 1.2</td>
<td>30.3 ± 1.8</td>
<td>13.4 ± 1.2</td>
<td>27.5 ± 0.3</td>
</tr>
<tr>
<td>D</td>
<td>27.4 ± 0.6</td>
<td>29.7 ± 0.8</td>
<td>13.4 ± 0.2</td>
<td>29.8 ± 0.1</td>
</tr>
<tr>
<td>E</td>
<td>31.2 ± 0.7</td>
<td>29.1 ± 0.7</td>
<td>11.8 ± 0.7</td>
<td>27.5 ± 0.1</td>
</tr>
<tr>
<td>F</td>
<td>28.0 ± 1.2</td>
<td>32.0 ± 0.8</td>
<td>14.8 ± 2.5</td>
<td>25.3 ± 0.8</td>
</tr>
<tr>
<td>G</td>
<td>31.0 ± 0.2</td>
<td>29.8 ± 0.6</td>
<td>11.8 ± 0.5</td>
<td>27.5 ± 0.6</td>
</tr>
<tr>
<td>H</td>
<td>27.8 ± 0.5</td>
<td>29.5 ± 1.5</td>
<td>13.6 ± 0.7</td>
<td>28.9 ± 0.1</td>
</tr>
<tr>
<td>N</td>
<td>30.0 ± 1.8</td>
<td>29.0 ± 0.7</td>
<td>12.4 ± 1.3</td>
<td>29.1 ± 2.3</td>
</tr>
</tbody>
</table>

The average of three determinations ± SD is given for samples A through H. The average of six determinations ± SD is given for control cells (N).

Determination of the trans membrane redistribution of spin-labeled lipids. The spin-labeled phospholipids used contained a short nitroxide-labeled fatty acyl group in the sn-2 position. The partial solubility of these probe molecules facilitates their incorporation into membranes and enables the measurement of lipid translocation by the determination of the probe fraction in the outer monolayer that can be extracted by bovine serum albumin (BSA). Before being labeled, erythrocytes (30% hematocrit) were incubated for 5 minutes in buffer at 37°C with 5 mmol/L (final concentration) di-isopropyl fluorophosphate to minimize the hydrolysis of the spin-labeled phospholipids. 1-palmitoyl-2-(4-doxyl-pentanoyl)-phosphatidylcholine (spPC), 1-palmitoyl-2-(4-doxyl-pentanoyl)-phosphatidylethanolamine (spPE), and 1-palmitoyl-2-(4-doxyl-pentanoyl)-phosphatidylyserine (spPS) were synthesized as recently described. The spin-labeled phospholipids were dried from a chloroform solution and resuspended in buffer. Labeling was performed at 30% hematocrit with label at a final concentration of approximately 10 μmol/L. Determination of the amount of spin label in the outer monolayer of the erythrocyte was performed by a back-exchange technique using BSA as described. Erythrocytes were separated from the spin-labeled BSA supernatant by a 1-minute spin at 7,600g in an Eppendorf (Madison, WI) centrifuge. Quantitation of the spin-label of the supernatants was performed on a Varian EPR spectrometer (Varian, San Fernando, CA).

ATP measurement. Intracellular ATP concentration was measured in a 30% cell suspension in the buffer used for the spinlabel studies. ATP was extracted from the cells in 0.5% trichloroacetic acid, 2 mmol/L EDTA, and measured with the Pharmacia LKB luciferin-luciferase assay (Pharmacia, Piscataway, NJ).
The distribution of endogenous PC (○), PE (□), and PS (△) between the inner and outer monolayers for control erythrocytes (N) and erythrocytes from eight individuals with HS (A through H).

A combination of phospholipase A₂ from bee venom and sphingomyelinase C from S. aureus was used to determine the distribution of the phospholipid in the bilayer. The erythrocytes from normal controls (sample N) and those containing reduced amounts of spectrin (samples A through H) exhibited only small differences in the accessibility of PC and PE to phospholipase A₂ hydrolysis (Fig 1). No PS degradation was observed in any of the samples, indicating that PS is exclusively located in the inner monolayer of the membrane. Approximately 70% to 78% of PC and 18% to 26% of PE in the membrane were degraded by phospholipase A₂, indicating that endogenous PC is mainly located in the outer monolayer, whereas most of the PE is located in the inner monolayer. Sphingomyelinase C treatment indicated that approximately 80% of SM is located in the outer monolayer. These results, similar to previous reports on normal erythrocytes, indicate that spectrin levels as low as 34% of normal have no effect on the asymmetric organization of endogenous phospholipids in the erythrocyte.

A correction on the results describing the phospholipid asymmetry as measured with phospholipases was needed as the fragility of the membrane of the spectrin-deficient erythrocytes led to substantial hemolysis of some samples during phospholipase treatment. The normal control samples (N) as well as the heterozygous control sample (H) exhibited less than 2% hemolysis after treatment with both enzymes. The spectrin-deficient samples, with the exception of A, showed 1% to 4.5% hemolysis after treatment with phospholipase A₂. This level of hemolysis was similar after sphingomyelinase treatment for samples C through E. However, the samples containing the lowest amount of spectrin (A and B) exhibited substantial hemolysis during combined phospholipase treatment (25% and 12%, respectively), whereas sample A also exhibited 20% hemolysis during treatment with phospholipase A₂ alone. On the assumption that all phospholipids in the bilayer of the hemolyzed cell fraction are available to phospholipase degradation, a correction for hemolysis, as indicated in the method section, was made on the data shown in Fig 1.

Transbilayer kinetics of spin-labeled phospholipid analogues. A typical result of the redistribution of spin-labeled...
phospholipids across the membrane bilayer of normal cells is shown in Fig 2. The lines connecting the experimental data points are derived from a least-square, exponential nonlinear regression fit. The half-times of equilibration across the bilayer, as calculated from these fits, are approximately 2, 40, and 120 minutes for PS, PE, and PC, respectively. The plateaus, as calculated from these fits, that were reached for the distribution across the bilayer for PE (79%) and PC (28%) closely resembled the asymmetric distribution found for these phospholipids based on their availability to phospholipase degradation as depicted in Fig 1. The redistribution kinetics of spin-labeled PS indicates that approximately 10% of the spPS introduced into the membrane resides in the outer monolayer at equilibrium and is extractable by BSA. In contrast, no endogenous PS could be hydrolyzed by phospholipases (Fig 1). These data on rate and equilibrium distribution of spin-labeled phospholipids are in agreement with published results, and the difference between the equilibrium distribution of spin-labeled PS and the availability of endogenous PS to phospholipases was noted previously.

The data on the kinetics of spin-labeled phospholipid redistribution of erythrocytes in association with varying degrees of spectrin deficiency are depicted in Fig 3. The redistribution of spPS was measured in all samples (Fig 3A), whereas the redistribution of spPC was determined in a limited number of samples because of the total sample size available from various individuals (Fig 3C). For each class of phospholipids the intersample differences that were detected were minor. No correlation was found between the level of spectrin and the redistribution of these spin-labeled probes.

The activity of the aminophospholipid translocase can be affected by ATP depletion. We therefore tested the intracellular ATP concentration of the samples used for the redistribution studies with the spin-labeled probes. The ATP level was similar in all HS samples (average value, 0.99 mmol/L ± 0.15 mmol/L) and did not differ significantly from the ATP levels determined in the normal control cells (1.03 mmol/L ± 0.08 mmol/L). No correlation was found between the ATP levels and the kinetics of transbilayer movement.

**DISCUSSION**

The asymmetric distribution of phospholipids in membranes was postulated 20 years ago by Bretscher and has been demonstrated in a wide variety of cells by a variety of techniques. All techniques allow distinction between both leaflets and between head groups of the different membrane phospholipid molecular species and include measurements of accessibility of phospholipids to chemical reagents, phospholipid transfer proteins, and phospholipases as well as measurements of redistribution of phospholipid probe mol-

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**Fig 3.** The kinetics of the inward translocation of three spin-labeled phospholipid classes. (A) Spin-labeled PS in erythrocytes from two normal individuals (♀, ♂) and eight HS patients: A (○), B (●), C (▲), D (■), E (+), F (△), G (●) and H (▪). (B) Spin-labeled PE in erythrocytes from two normal individuals (♀, ♂) and seven HS patients: A (○), B (●), D (■), E (+), F (△), G (●) and H (▪). (C) Spin-labeled PC in erythrocytes from two normal individuals (♀, ♂) and four HS patients: D (▲), E (+), G (●) and H (▪).
ecules. It became apparent that this asymmetric distribution is the equilibrium in a dynamic movement of phospholipids across the erythrocyte membrane bilayer. In contrast to pure lipid systems, which exhibit very slow transbilayer movement, the erythrocyte lipids show a wide range of transbilayer movement, with half-times on the order of a few minutes (for PS species) to more than 20 hours for certain PC species. In a normal erythrocyte, PC and sphingomyelin reside at equilibrium mainly in the outer leaflet, whereas the aminophospholipids PE and PS are primarily found in the inner leaflet.

Three separate factors have been implicated in this transbilayer movement and asymmetric distribution: (1) an ATP-independent, or “passive” transbilayer movement of all endogenous phospholipid molecular species in which integral membrane proteins have been implicated to play a role; (2) an ATP dependent, or “active” aminophospholipid translocation, a process that facilitates the transbilayer movement of PE and PS, with a high affinity for PS; (3) the interaction of phospholipids (in particular, PS) with membrane skeleton proteins. This role of the membrane skeleton in phospholipid distribution was supported by results that showed the interaction of spectrin with PS on monolayers and vesicles. In addition to spectrin, other membrane skeleton proteins such as protein 4.1 were found to interact with phospholipid. In other cells, proteins such as incubin, alpha-actinin, and profilin interacted with phospholipids, and modification of the membrane skeleton in platelets had a profound effect on the phospholipid distribution, although conditions that led to a loss of PS asymmetry in platelets did not coincide with a loss of interaction of PS with the platelet cytoskeleton.

Relatively few studies have assessed the importance of the phospholipid-membrane skeletal interaction in intact erythrocyte membranes. Membrane fragments released from RBCs as a result of heat treatment contain significantly lower amounts of spectrin but largely retain their ability to redistribute spin-labeled phospholipids introduced in the outer monolayer. No data are available on the distribution of endogenous phospholipids in these fragments. Similar treatment of erythrocytes at 50°C led to a loss of normal spectrin structure in the remnant cells, whereas the phospholipid distribution was not affected as indicated by phospholipase treatment and fluorescamine or merocyanine 540 labeling. In addition, a study that used photolabeling of spectrin with a photoactivable PE analogue did not support a model in which spectrin is involved in maintaining the asymmetric phospholipid distribution.

Sickled erythrocytes have been used as a model in which to study the effect the disruption of the normal interactions in the membrane has on phospholipid distribution as a result of the extreme shape changes under low oxygen tensions. Deoxygenated sickle erythrocytes exhibit an altered asymmetric distribution of endogenous phospholipids and a higher transbilayer movement of PC. Under these conditions, the activity of the aminophospholipid translocase is also decreased as measured by the redistribution of spin-labeled aminophospholipids, and spin-labeled PC also exhibits a faster transbilayer movement. This organizational altered bilayer was interpreted to be located in the spectrin devoid spicules formed during the sickling process. The combination of ATP depletion and shape change was suggested to be responsible for the loss of asymmetry. Other factors implicated in membrane abnormalities, such as oxidative damage, in particular to the aminophospholipid translocase, could also play a role in altering the phospholipid distribution in sickle cells.

In this study we postulated that using erythrocytes that contain significantly lower amounts of spectrin would provide a more direct approach to delineate this effect of the membrane skeleton protein on phospholipid distribution. Hereditary spherocytosis, a congenital hemolytic anemia, results in abnormally fragile erythrocytes that are more spherical than discoid. The membranes of these cells are reported to have a partial reduction in their total phospholipid, a condition that seems to be corrected by splenectomy. All spheroerycocytes in our study were obtained from splenectomized individuals (A through G). No significant differences in phospholipid composition were found between these samples and those from nonsplenectomized individuals (H and N). Hence, our data suggest that the loss of phospholipid as a result of splenic conditioning is a random process that is not selective for specific phospholipid classes. Furthermore, because no significant differences in the phospholipid distribution were found, our data suggest that splenectomy does not affect phospholipid distribution in erythrocytes. However, these conclusions are only indicative because we could not compare erythrocytes before and after splenectomy.

Previous analyses of erythrocyte membranes from this set of spheroerycocytes demonstrated a close correlation among spectrin content, relative membrane spectrin density, and several physical properties of the membrane, including a reduction in its shear modulus, bending stiffness, and viscosity, and an increased lateral redistribution of band 3 protein and glycoporphins. Because of the reduction in the ratio of surface area to volume of spherocytes as compared with discocytes, the spherocytes are more sensitive to hypotonic lysis. A correlation was noted between the sensitivity towards hypotonic lysis and the quantity of spectrin as well as deformability, as measured by ektacytometry. This instability of the membrane could be the basis for the increased hemolysis, which is correlated with the degree of spectrin deficiency, that was found during phospholipase treatment. Although these parameters indicate that the membrane organization is altered, the hydrolysis of endogenous phospholipids with phospholipase was not correlated with the level of spectrin. These data indicate that the increased ratio of lipid bilayer relative to membrane skeleton does not affect endogenous phospholipid asymmetry. The substantial level of hemolysis after phospholipase treatment in the samples with low levels of spectrin necessitated a correction of the data based on the assumption that all phospholipids were available to phospholipase hydrolysis as soon as hemoglobin was lost from the cell. The validity of this correction and the conclusion that phospholipid asymmetry is retained was confirmed by the measurement of the redistribution of the spin-labeled phospholipids across the bilayer. Also, in this set of experi-
mements the equilibrium reached was similar for the erythrocytes of the heterozygous individual (H), those with spectrin levels as low as 34% of normal (A through G), and those of the normal control (N). In addition, the rate of redistribution of each class of spin-labeled phospholipids was virtually identical in these samples, indicating that the activity of the aminophospholipid translocase is not affected in these erythrocytes.

In conclusion, the composition, distribution, and transbilayer kinetics of phospholipids in patients with spectrin-deficient hereditary spherocytosis were normal although the membranes contained as little as 34% of the normal level of spectrin, and previous studies of these patients had established that the disorder alters several physical properties of these membranes. Our data show that any aminophospholipid molecule that moves to the outer monolayer is rapidly transported back to the inner monolayer as long as the aminophospholipid translocase is active even if the membrane is destabilized. Although the interaction of phospholipids with the membrane skeleton could restrict the outward movement, thereby stabilizing the membrane and reducing the ATP needed to maintain the asymmetric equilibrium, the loss of almost 70% of the spectrin does not seem to affect membrane phospholipid organization. Our data do not exclude a role for spectrin in the stabilization of the phospholipid bilayer but indicate that this protein plays at best only a minor role in the maintenance of erythrocyte membrane phospholipid organization as long as the aminophospholipid translocase is active. The inactivation of the amino phospholipid translocase, whether by the absence of ATP or some other disturbance such as oxidation, would ultimately lead to loss of phospholipid asymmetry, driven by a concentration gradient.

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