Increased Rotational Mobility and Extractability of Band 3 From Protein 4.2–Deficient Erythrocyte Membranes: Evidence of a Role for Protein 4.2 in Strengthening the Band 3–Cytoskeleton Linkage

By Anne C. Rybicki, Robert S. Schwartz, Eric J. Hustedt, and Charles E. Cobb

Band 3 (anion-exchange protein 1 [AE1]) is the major integral membrane protein of human red blood cells (RBCs) and contains two distinct structural domains that differ in function. The 52-kD C-terminal domain includes the membrane-spanning portion of band 3 responsible for anion (Cl⁻/HCO₃⁻) transport and contains antigenic determinants for blood group specificity and senescent antibody recognition. The 40-kD N-terminal domain includes the cytoplasmic tail of band 3 and contains binding sites for the cytoskeletal proteins ankyrin, protein 4.1, and protein 4.2 (P4.2), as well as for some glycolytic enzymes and both native and denatured hemoglobin.

In human RBC membranes, band 3 exists as a heterogeneous population of self-associated states in the plane of the membrane. These populations can be differentiated to some extent using nonionic detergents to solubilize band 3 from the membrane, although it is generally accepted that the majority of band 3 exists as dimers and a smaller proportion of the total band 3 exists principally as tetramers with some higher-order oligomeric species. The rotational mobility of band 3 is restricted by self-aggregation or by direct interactions of band 3 with cytoskeletal proteins. Although the relationship between the self-association state of band 3 and its function is unresolved, it has been suggested that the tetrameric form preferentially associates with the cytoskeleton.

Although the role of ankyrin in linking band 3 to the cytoskeleton is well established, the role of P4.2 in this regard is still controversial. Some reports find no specific role for P4.2 in band 3–cytoskeleton interactions, whereas others suggest that P4.2 may participate in strengthening interactions between band 3 and ankyrin. One reason for these discrepancies may be the difficulty in selectively stripping P4.2 from RBC membranes and in its purification, since its interaction with the membrane is strong and requires potentially denaturing conditions to remove it.

To investigate if P4.2 is required for the normal band 3–cytoskeletal linkage, we took advantage of naturally occurring genetic mutations that result in human RBC P4.2 deficiency. The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1996 by The American Society of Hematology.

From the Division of Hematology, Albert Einstein College of Medicine–Montefiore Medical Center, Bronx, NY; and the Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, TN.

Submitted October 31, 1995; accepted May 28, 1996.

Supported by National Institutes of Health Grants No. HL38655 (to R.L. Nagel, R.S.S. for Project 2), HL34737 (to A.H.B.) and T32DK07186 (NIH Training Grant to E.J.H.).

Address reprint requests to Anne C. Rybicki, PhD, Division of Hematology, Albert Einstein College of Medicine–Montefiore Medical Center, 111 E 210th St, Bronx, NY 10467.
anemia. Band 3 of RBCs is a \(^{(40)}\) glutamic acid to \(^{(40)}\) lysine mutation in band 3 that results in RBCs with approximately 12% of the normal membrane content of P4.2; the RBCs are osmotically fragile and the patients have episodic hemolytic anemia. We tested the effect of these naturally occurring P4.2 deficiencies on the band 3–cytoskeleton linkage by measuring band 3 rotational mobility and the loss of band 3 from octyl-\(\beta\)-glucoside–treated membranes, the latter representing conditions that do not disturb band 3–cytoskeleton interactions. We find that P4.2 deficiency results in increased band 3 rotational mobility and increased octyl-\(\beta\)-glucoside extraction, suggesting that P4.2 is required for normal band 3–cytoskeleton linkage.

**MATERIALS AND METHODS**

P4.2-deficient RBCs. P4.2-deficient RBCs used in these studies have been described previously and are greater than 99%, and approximately 88%, deficient in membrane P4.2, respectively. In addition to P4.2 deficiency, P4.2-deficient RBCs are 15% to 20% deficient in band 3 (ref 17 and data reported herein), and band 3-deficient RBCs are approximately 30% deficient in membrane-associated glyceroldehyde-3-phosphate dehydrogenase (G3PD). When patient blood was shipped, control blood from a hematologically normal adult was also drawn at the same time and the samples were shipped together on wet ice by overnight courier.

**Band 3 rotational mobility.** RBCs were covalently labeled with the fluorophore eosin-5-maleimide (EMA) essentially as described by Cobb and Beth. RBCs were washed in 113 mmol/L citrate buffer, pH 7.4 (113cit7.4), and labeled with EMA (Molecular Probes, Eugene, OR) at a ratio of 1 vol 0.5 mg/mL EMA:5 vol RBCs at 50% hematocrit for 40 minutes at room temperature in the dark. The reaction was terminated with 113cit7.4 with 0.2% bovine serum albumin, and the labeled RBCs were washed extensively in hypotonic hemolysis. All procedures from this step were performed at 0°C to 4°C and in minimum light to avoid photo-oxidative cross-linking of membrane proteins. Labeling of RBCs with EMA by this protocol results in the specific labeling of band 3 at lysine 430, with no detectable EMA in the lipid bilayer.

Time-resolved phosphorescence emission anisotropy (TPA) data were collected on a single photon-counting instrument previously described by Cobb et al. For the P4.2-deficient sample and its matched controls the excitation wavelength was 532 nm and the instrument was excited as described. For the band 3-deficient sample and its matched controls, the excitation wavelength was 523 nm. For these latter measurements, a TRF523Q laser (7-nanosecond full width half maximum [FWHM] pulse) operating at 80 Hz was used (SpectraPhysics, Mountain View, CA). The decay data were collected into 640-nanosecond-wide bins (15,360 bins, for a total recorded decay time of 9,830 microseconds). Due to light-induced postgate artifact of the photomultiplier tube and gating circuit used to detect the phosphorescence emission, the earliest data bin used was the true zero time point. EMA-labeled ghost membranes were diluted with 5 mmol/L phosphate buffer, pH 7.4 (5P7.4), to less than 0.1 absorbance unit (AU) at the excitation wavelength (either 532 nm for P4.2-deficient or 523 nm for band 3-deficient). The samples were purged of molecular oxygen by blowing a stream of argon over the sample in the cuvette for 15 minutes. An enzymatic oxygen-removal system consisting of 100 \(\mu\)g/mL glucose oxidase, 15 \(\mu\)g/mL catalase, and 5 mg/mL glucose was also used. Glucose oxidase and catalase were obtained from Sigma Chemicals (St Louis, MO).

Sample temperature was maintained at 37°C. Phosphorescence emission decay curves were collected through a solution filter of saturated K-dichromate placed between the sample and emission polarizer and a 660-nm cut-on glass filter placed between the emission polarizer and gated photomultiplier tube. A detailed description of the instrumentation is provided in Cobb et al. The values for TPA (\(r\)) were calculated from the measured decay curves with the equation,

\[
r(t) = \frac{(I_0 - G)(1 - e^{-\alpha t})}{(I_0 + G)(1 - e^{-\alpha t})}
\]

where \(I_0\) and \(I_1\) are the phosphorescence emission intensities collected parallel and perpendicular to the excitation laser pulse, and \(G\) is the measured instrumentation polarization bias. The anisotropy decay data were then fitted by an iterative nonlinear least-squares routine to a sum of three exponential terms plus a residual anisotropy term,

\[
r(t) = a_1(e^{-\lambda_1 t}) + a_2(e^{-\lambda_2 t}) + a_3(e^{-\lambda_3 t}) + \alpha_s
\]

where pre-exponential \(a_s\) values are the preexponential amplitudes, \(\lambda_s\) values are the exponential decay constants, and \(\alpha_s\) is the residual limiting anisotropy. It was determined that three decay terms plus the residual anisotropy were the minimum number necessary to fit the data adequately as judged by the resulting \(x^2\) statistic from the fitting procedure; addition of a fourth decay term did not significantly improve the data fits.

**Quantitation of band 3.** RBC band 3 was quantified by three different methods: (1) steady-state eosin fluorescence emission of hemolyzed eosin-labeled RBCs, (2) radio-labeled diisothiocyanato-hydrostilbene disulfonate (\(\text{H}_2\text{DIDS}\) binding), and (3) digitalized fluorescence microscopy of eosin-labeled RBC ghosts. In the steady-state fluorescence emission experiments, intact RBCs were labeled with EMA as described earlier and the cells were hemolyzed with water. Eosin fluorescence emission images of the ghosts were collected on a Zeiss (Thornwood, NY) LSM 410 confocal microscope, using an excitation wavelength of 488 nm and an emission cutoff filter of 515 nm. For the digitalized fluorescence microscopy experiments, fluorescence intensity was measured by placing a “box” of the same size around each ghost, subtracting background, and integrating the intensity. The mean intensity ratios for 54 control and 63 P4.2-deficient (\(\text{H}_2\text{DIDS}\)) ghosts were calculated, resulting in an intensity ratio (P4.2-deficient:control) of 84%. In \(\text{H}_2\text{DIDS}\) experiments, RBCs were labeled with 50 \(\mu\)mol/L \(\text{H}_2\text{DIDS}\) (Research Corp, Hospital for Sick Children, Toronto, Ontario, Canada) for 30 minutes at room temperature, hemolyzed with water, and radioactive counting was by liquid scintillation counting.

**Band 3 anion transport activity.** RBC band 3 anion transport activity was measured by unidirectional disodium \(^{35}\text{SO}_4^{2-}\) uptake in the presence or absence of DIDS (Sigma). Control and P4.2-deficient RBCs were washed once in 113cit7.4 and twice in 5P7.4 uptake buffer (271 mmol/L sucrose, 27 mmol/L glycyglycine, 1 mmol/L KC1, 1.4 mmol/L MgCl2, and 1.4 mmol/L CaCl2, pH 7.4), and equal numbers of cells were then resuspended at 20% hematocrit for 3 hours in \(^{35}\text{SO}_4^{2-}\) uptake buffer before the start of the assay. \(^{35}\text{SO}_4^{2-}\) uptake was initiated by mixing an equal volume of RBCs (20% hematocrit) with \(^{35}\text{SO}_4^{2-}\) uptake buffer containing 1 to 30 mmol/L \(^{35}\text{SO}_4^{2-}\) (New England Nuclear, Boston, MA). After 2, 4, and 6-minute incubations at 20°C, the reactions were terminated by rapid transfer of 200 \(\mu\)L cell suspension into 5 mL cold DIDS stop solution (40 mmol/L DIDS, 27 mmol/L glycyglycine, and 139.5 mmol/L NaCl, pH 7.4). RBCs were washed once in DIDS stop solution and collected by centrifugation. The RBC pellet was hemolyzed with 0.5 mL water, and hemoglobin was precipitated by addition of 0.5 mL 1-mol/L perchloric acid. Accumulated \(^{35}\text{SO}_4^{2-}\) was measured by liquid scintillation counting. Initial rates of \(^{35}\text{SO}_4^{2-}\) uptake were
calculated by linear regression analysis and expressed as micromoles intracellular SO₂⁻ per milliliter packed cells per minute. The data were normalized to band 3 content per milliliter packed RBCs.

Extraction of band 3 by octyl-β-glucoside and separation of band 3 dimers and oligomers by size-exclusion high-performance liquid chromatography. RBC ghost membranes (2 mg/mL) were incubated for 10 minutes on ice with 10 vol 10 mmol/L Tris hydrochloride, pH 7.4 (10T7.4), containing 1% octyl-β-glucoside (Fischer, Rockford, IL). Insoluble membrane pellets were centrifuged at 20,000g for 15 minutes at 4°C and washed once in 10T7.4. Ghost membranes without octyl-β-glucoside and octyl-β-glucoside-extracted membranes (20 to 25 µg) were solubilized in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) solubilization buffer, and the proteins were separated by SDS-PAGE using the Laemmli buffer system. The protein bands were stained with Coomassie brilliant blue (BioRad Laboratories, Richmond, CA), and the stained bands were quantified by densitometry using a laser scanning densitometer (Biomed Instruments, Fullerton, CA) and gel analysis software.

Size-exclusion high-performance liquid chromatography (HPLC) of extracted band 3 was performed exactly as described previously. BrieÎîly, octyl-β-glucoside extracts were centrifuged at 100,000g for 30 minutes at 4°C, and 20 µL of the supernatant was applied to a TSK4000 SWXL, HPLC size-exclusion column (30 cm × 7.8 mm ID; Tosohaas, Tokyo, Japan). Protein eluting from the column was monitored at A,=0, and peaks were quantified using peak fitting/integration software (Dionex AI-450 chromatography software version 3.31; Dionex Corp, Sunnyvale, CA). Protein standards with a molecular weight unaffected by the presence of detergent (T, thyroglobulin [M, 669 kD]; T, apoferritin [M, 443 kD]; A, β-amylose [M, 200 kD]; and B, bovine serum albumin [M, 66 kD]) were included in each HPLC run to aid in peak identification. In some cases, HPLC peaks were collected, concentrated by centrifugation in a 10,000-molecular weight cutoff ultrafiltration unit (Centricon JO; Amicon, Beverly, MA), and subjected to SDS-PAGE according to the Laemmli-method.

RESULTS
Increased rotational mobility of band 3 in P4.2-deficient RBC membranes. The decay of TPA arising from the EMA-labeled band 3 in control, P4.2<sup>NIPPON</sup>, and band 3<sup>MONTFOIRE</sup> ghost membranes is shown in Fig 1. Band 3 molecules that are constrained either through direct interaction with the cytoskeleton or by self-aggregation have a hindered rotational freedom, which is manifested as a slower rate of TPA decay; upon removal of these constraints, the TPA decay rate increases due to the increased rotational reorientiation rate of band 3. Band 3 in both P4.2<sup>NIPPON</sup> (>99% P4.2-deficient) and band 3<sup>MONTFOIRE</sup> (88% P4.2-deficient) ghost membranes has an increased rotational freedom as compared with band 3 in control ghosts. In the fits to the anisotropy decay curves shown in Table 1, the primary effect is a shift from the slower-rotating and immobile fractions (β<sub>1</sub> and α<sub>1</sub>) to the more mobile, faster decay terms (β<sub>2</sub> and α<sub>2</sub>) for both P4.2-deficient phenotypes. For band 3<sup>MONTFOIRE</sup>, there was an additional effect of apparent faster-decay constants (β<sub>3</sub>), in addition to increased α<sub>2</sub>. Since P4.2<sup>NIPPON</sup> RBCs are also 15% to 20% deficient in band 3<sup>31</sup> (and the section below) most likely as a result of increased membrane vesiculation, as a control we examined ghost membranes from a patient with hereditary spherocytosis due to spectrin deficiency (20% to 30% less spectrin), for which loss of membrane surface is also expected. We found that band 3 rotational mobility was normal in these spectrin-deficient hereditary spherocytosis membranes (data not shown), which is in agreement with recent findings by Corbett et al., who reported that band 3 rotational mobility in RBC ghosts is unaffected by ghost spectrin content.

Band 3 anion transport is normal in P4.2-deficient RBCs. Band 3 is composed of two distinct domains that can be separated by mild trypsin treatment of the membrane. Previous studies indicated that the 52-kD transmembrane domain mediates anion transport independently of the 40-kD cytoplasmic domain. However, a recent report suggested a role for P4.2 in modulating band 3 anion transport through specific interactions with the cytoplasmic domain. We therefore reexamined band 3 anion transport in control and P4.2<sup>NIPPON</sup> RBCs. The results are a mean of three data sets for both control and P4.2<sup>NIPPON</sup> RBCs (Fig 2). The calculated maximal rate (V<sub>max</sub>) and K<sub>m</sub> for both control and P4.2<sup>NIPPON</sup> RBCs were determined by three independent methods: (1) by steady-state eosin fluorescence emission of hemolyzed EMA-labeled RBCs, band 3 content in P4.2<sup>NIPPON</sup> RBCs was 86% of control RBCs; (2) by labeling band 3 with <sup>3</sup>H-DIDS, band 3 content of P4.2<sup>NIPPON</sup> RBCs was 89% of control RBCs; and (3) by digitalized fluorescence microscopy of EMA-labeled RBCs, band 3 content of P4.2<sup>NIPPON</sup> RBCs was 84% of control RBCs. Using these three independent methods, it was determined that band 3 content of P4.2<sup>NIPPON</sup> RBCs was 85% of control RBCs, which is similar to our earlier finding of approximately 20% band 3 deficiency determined by SDS-PAGE. Normalizing the data sets for equivalent band 3 content results in sulfate uptake kinetics overlapping between control and P4.2<sup>NIPPON</sup> RBCs.

Increased octyl-β-glucoside extractability and enrichment of extracted band 3 oligomeric species from P4.2-deficient RBC ghost membranes. Previous studies demonstrated the existence in both intact RBCs and ghost membranes of multiple classes of band 3 molecules that differ in lateral and rotational constraints. There is general agreement that the oligomeric forms of band 3 (tetramers and larger oligomers) are complexed to other cytoskeletal proteins, which results in constraining the movement of this class of band 3 molecules, whereas the dimeric form of band 3 is uncomplexed and more freely mobile in the membrane. It is well established that ankyrin is the principal cytoskeletal protein responsible for linking band 3 to the cytoskeleton. However, it is not clear whether other cytoskeletal proteins participate in stabilizing or strengthening the ankyrin–band 3 linkage. In this regard, one earlier study found that ankyrin was released from spectrin–actin–depleted inside-out vesicles (IOVs) prepared from P4.2<sup>NIPPON</sup> RBCs, whereas ankyrin...
was not released from control IOVs, suggesting that P4.2 may strengthen the ankyrin-band 3 linkage.

To address the question of whether P4.2 is required for normal band 3-cytoskeleton linkage, band 3 was extracted from control and P4.2-deficient RBCs using the nonionic detergent octyl-β-glucoside. Octyl-β-glucoside selectively extracts band 3 that is not attached to the cytoskeleton, and has been used to distinguish free band 3 from cytoskeleton-bound band 3 in a case of hereditary spherocytosis due to a band 3 mutation. In two separate experiments, octyl-β-glucoside extracted 30% (average of two experiments) and 61% more band 3 from P4.2- and band 3-MONTFIGE ghost membranes, respectively, compared with controls (Table 2).

Previous studies have shown that band 3 in nonionic detergent-solubilized RBC ghost membranes could be separated into oligomeric and dimeric fractions by size-exclusion HPLC. As shown in Fig 3A, separation of the octyl-β-glucoside extracts by size-exclusion HPLC generally resulted in four predominant HPLC peaks. SDS-PAGE analysis of these peaks showed that peaks 1 and 2 contained band 3 (Fig 3B). Peak 3 contained a protein migrating at a position similar to glycophorin A, and no protein was detected by Coomassie blue staining in peak 4 (not shown). Peak 4 has previously been shown to contain lipid and detergent. The assignment of peak 1 as containing band 3 oligomeric species, and peak 2 as containing band 3 dimeric species was based on the following criteria: (1) the elution position of peaks 1 and 2 corresponded exactly as described previously, where peak 1 was determined to contain band 3 oligomeric species and peak 2 band 3 dimeric species; and (2) incubating the octyl-β-glucoside extracts at 37°C or at pH 5.8, which was shown previously to result in band 3 aggregation in detergent solution and an increase in the band 3 oligomeric peak, resulted in a shift in the elution pattern of peak 1, with most of peak 1 now eluting close to the column V₀ (Fig 3C). These results are consistent with band 3 aggregation and with the assignment of peak 1 as containing band 3 oligomeric species, and confirm earlier findings.

In addition to band 3, peak 1 also contained a protein migrating on SDS-PAGE close to the position of spectrin/ankyrin (which are not resolved using the Laemmli buffer...
Table 1. Rotational Mobility of Band 3 in Control and P4.2-Deficient RBC Ghost Membranes

<table>
<thead>
<tr>
<th>Fit Parameter</th>
<th>Control (n = 4)</th>
<th>P4.2N1PP0N (&gt;99%, n = 2)*</th>
<th>Band 3M0NTER0RE (−88%, n = 1)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha_1 )</td>
<td>0.030 ± 0.004</td>
<td>0.055, 0.056</td>
<td>0.045</td>
</tr>
<tr>
<td>A1</td>
<td>0.18 ± 0.01</td>
<td>0.32, 0.33</td>
<td>0.30</td>
</tr>
<tr>
<td>( \Delta )</td>
<td>+78%, +83%</td>
<td>+67%</td>
<td></td>
</tr>
<tr>
<td>( \phi_1 )</td>
<td>46 ± 23</td>
<td>59, 43</td>
<td>34</td>
</tr>
<tr>
<td>( \phi_2 )</td>
<td>0.038 ± 0.007</td>
<td>0.052, 0.049</td>
<td>0.041</td>
</tr>
<tr>
<td>( \alpha_2 )</td>
<td>0.24 ± 0.03</td>
<td>0.30, 0.29</td>
<td>0.26</td>
</tr>
<tr>
<td>( \Delta )</td>
<td>+25%, +21%</td>
<td>+9%</td>
<td></td>
</tr>
<tr>
<td>( \phi_4 )</td>
<td>442 ± 63</td>
<td>374, 335</td>
<td>256</td>
</tr>
<tr>
<td>( \phi_2 )</td>
<td>0.045 ± 0.004</td>
<td>0.031, 0.024</td>
<td>0.027</td>
</tr>
<tr>
<td>( A_3 )</td>
<td>0.28 ± 0.02</td>
<td>0.18, 0.14</td>
<td>0.17</td>
</tr>
<tr>
<td>( \Delta )</td>
<td>−36%, −50%</td>
<td>−39%</td>
<td></td>
</tr>
<tr>
<td>( \phi_4 )</td>
<td>2.877 ± 430</td>
<td>3.036, 2.351</td>
<td>1.761</td>
</tr>
<tr>
<td>( \alpha_4 )</td>
<td>0.047 ± 0.004</td>
<td>0.035, 0.040</td>
<td>0.043</td>
</tr>
<tr>
<td>( A_4 )</td>
<td>0.30 ± 0.04</td>
<td>0.20, 0.24</td>
<td>0.27</td>
</tr>
<tr>
<td>( \Delta )</td>
<td>−33%, −20%</td>
<td>−10%</td>
<td></td>
</tr>
</tbody>
</table>

The ghost membrane content of P4.2 as compared with normal controls is indicated in parentheses; n is the number of individual experiments. Anisotropy decay curves were fit to equation 2. The \( \alpha \) terms are raw anisotropy amplitudes, and A terms are fractional amplitudes normalized to the initial anisotropy at time 0. \( \Delta \) represents the change in A of the P4.2-deficient ghosts v controls. \( \phi \) terms are the anisotropy decay constants. Thus, the \( \Delta \) terms are band 3 molecules rotating at correlation times \( \phi_1 \) = \( \phi_2 \), respectively. For the control fits, the mean ± SD for 4 separately fit controls (1 matched to each P4.2-deficient phenotype, plus 1 unrelated control sample) are shown. For the P4.2N1PP0N fits, the actual values for each of 2 samples are shown. The amplitude of the most rapidly rotating population of band 3 (\( \alpha_1 \), correlation time \( \approx 30 \) to 60 microseconds) is increased 81% and 67% in P4.2-deficient ghosts (P4.2N1PP0N and band 3M0NTER0RE, respectively) v control ghosts. The amplitude of the population of band 3 rotating at intermediate speed (\( \alpha_2 \), correlation time \( \approx 200 \) to 500 microseconds) is increased 23% and 8% in P4.2-deficient ghosts (P4.2N1PP0N and band 3M0NTER0RE, respectively) v control ghosts, at the expense of the slowly rotating component (\( \alpha_4 \), correlation time \( \approx 2,000 \) to 3,000 microseconds; amplitude decreased 43% and 39% in P4.2N1PP0N and band 3M0NTER0RE, respectively) and immobile component (\( \alpha_4 \), immobile on this experimental time scale; amplitude decreased 26% and 10% in P4.2N1PP0N and band 3M0NTER0RE, respectively) of band 3.

* RBC membrane P4.2 deficiency,17,18
† In microseconds.

system) and small amounts of an unidentified protein migrating below the position of P4.2 (Fig 3B). Peak 2 also contained, in addition to band 3, proteins migrating on SDS-PAGE at the positions of P4.1 and P4.2 (Fig 3B).

The band 3-containing peaks (peaks 1 and 2) were quantified in control and P4.2-deficient RBC membrane octyl-\( \beta \)-glucoside extracts. The amount of the band 3 oligomer-containing peak (peak 1, including band 3 tetramers, oligomers, and aggregates) was two to three times larger in extracts from P4.2-deficient membranes compared with controls (Table 2 and Fig 3A). The amount of the band 3 dimer-containing peak (peak 2) was similar in extracts from both P4.2-deficient membranes and controls. These results demonstrate that P4.2-deficient octyl-\( \beta \)-glucoside extracts are enriched in band 3 oligomeric species. Since band 3 oligomers are the form of band 3 that associates with the cytoskeleton,6 the results are consistent with a weakened band 3–cytoskeleton linkage in P4.2-deficient RBC membranes.

DISCUSSION

This study was designed to investigate the hypothesis that P4.2 plays a role in strengthening the band 3–cytoskeleton linkage in human RBCs. Our approach was to examine the rotational mobility of band 3 in membranes and the extractability of band 3 from membranes by the mild nonionic detergent octyl-\( \beta \)-glucoside. Increases in either parameter are indicative of weakened or altered band 3–cytoskeleton interactions.6,9

We also investigated anion transport in a P4.2-deficient RBC sample (P4.2N1PP0N) and found it to be normal, suggesting that P4.2 is not required for band 3 anion transport activity. This result is in contrast to that reported by Malik et al.29 who found that P4.2 enhanced band 3 anion transport. Possible reasons for this difference are that our studies were done with intact RBCs, whereas their studies were done with model membranes and purified proteins.

We find that the amplitude of the most rapidly rotating population of band 3 (correlation time, \( \approx 30 \) to 60 microseconds) is increased 81% and 67% in P4.2-deficient ghosts (P4.2N1PP0N and band 3M0NTER0RE, respectively) compared with control ghosts. The amplitude of the population of band 3 rotating at intermediate speed (correlation time, \( \approx 200 \) to 500 microseconds) is increased 23% and 8% in P4.2-deficient ghosts (P4.2N1PP0N and band 3M0NTER0RE, respectively) of band 3.

Fig 2. Band 3 anion transport activity of control and P4.2-deficient (P4.2N1PP0N, > 99% P4.2-deficient†) (P4.2(−)) RBCs. RBC band 3 anion transport activity was measured by unidirectional disodium 56S04' uptake. Initial rates of 56S04' uptake were calculated by linear regression analysis and expressed as \( \mu \)mol intracellular SO4' /mL packed cells/min. The data were normalized to band 3 per mL packed RBCs and corrected for band 3 content determined by 3 independent techniques. 56SO4' uptake was similar for both control and P4.2(−) RBCs.

From www.bloodjournal.org by guest on September 24, 2017. For personal use only.
compared with control ghosts, at the expense of the slowly rotating component (correlation time, ≈2,000 to 3,000 microseconds; amplitude decreased 43% and 39% in P4.2-NIPPON and band 3MONTEFORI, respectively) and immobile component (immobile on this experimental time scale; amplitude decreased 26% and 10% in P4.2-NIPPON and band 3MONTEFORI, respectively) of band 3. Since the slowest-decay and immobile components of band 3 are believed to arise from populations with high-affinity attachments to the cytoskeleton, most likely via ankyrin, this result is consistent with a shift in band 3 from a strongly restricted state to a much less restrictive state. The role of P4.2, therefore, appears to be stabilization of the high-affinity band 3-cytoskeleton interactions, presumably those involving band 3-ankyrin, although our results do not exclude other possibilities. The same trend is seen in the band 3MONTEFORI ghosts, but in addition to the shifting of the amplitudes to the faster-decay terms, the correlation times are apparently faster as well. This may be the result of P4.2 deficiency combined with potential effects of the band 3 mutation (glutamic acid to lysine) in this molecule.

Our results are in contrast to those reported by Wyatt and Cherry, who concluded that P4.2 had no effect on the rotational mobility of band 3. Possible reasons for these differences may be that the studies made by Wyatt and Cherry were performed using stripped ghost membranes reconstituted with purified proteins. It is possible that the procedure used to strip the membranes, which included strong protein denaturants, may have caused some irreversible protein damage. Since our studies were made directly on ghosts prepared from intact RBCs, the possibility of artifact is greatly reduced. Moreover, the method used by Wyatt and Cherry to extract membrane proteins does not result in complete extraction of P4.2, whereas our studies used more than 99% P4.2-deficient RBCs.

The second assay of the strength of the band 3-cytoskeleton linkage was the extraction of band 3 from ghost membranes by the nonionic detergent octyl-β-glucoside. Octyl-β-glucoside at 1% has previously been shown not to disturb the association of tetrameric band 3 with the cytoskeleton. One percent octyl-β-glucoside extracted 30% (mean of two experiments) and 61% (mean of two experiments) more band 3 from P4.2-NIPPON (>99% P4.2-deficient) and band 3MONTEFORI (~88% P4.2-deficient) RBC ghosts, respectively, compared with control ghosts. Moreover, the extracted band 3 from both of the genotypically unrelated P4.2-deficient phenotypes was enriched in band 3 oligomeric species (including aggregates, oligomers, and tetramers) compared with controls. Since band 3 oligomers are the form of band 3 that is attached to the cytoskeleton, these results, in combination with the increased extraction of band 3 from P4.2-deficient RBC membranes by octyl-β-glucoside, suggest that P4.2 deficiency results in a weakened band 3-cytoskeleton linkage. Thus, we propose that P4.2 directly participates in strengthening the band 3-cytoskeleton linkage. Our experiments also demonstrate that band 3 oligomers remain stably associated in octyl-β-glucoside extracts, consistent with earlier reports, and suggest that although cytoskeletal proteins serve as attachment sites for band 3 oligomers, they are not required to maintain band 3 in the oligomeric state. Whether cytoskeletal proteins play a role in the organization of band 3 in the membrane is unclear at this time.

Alternative interpretations for the finding of an increased amount of band 3 oligomeric species in octyl-β-glucoside extracts from P4.2-deficient ghosts are (1) an increased tendency of untethered band 3 to oligomerize in these membranes, or (2) an increased tendency of band 3 to form large aggregates in these membranes. In the first case, one would expect to find increased fast-component anisotropy decay constants (φ₁ and φ₂ in our experiments) in P4.2-deficient ghosts, whereas we find these decay constants to be normal (for φ₁) or slightly decreased (for φ₂). In the second case, one would expect to find an increased slow-component anisotropy decay constant (φ₃ in our experiments) in P4.2-deficient ghosts, whereas we find this decay constant to be normal (for P4.2-NIPPON) or slightly decreased (for band 3MONTEFORI). Thus, the increased amount of oligomeric band 3 in octyl-β-glucoside extracts from P4.2-deficient ghosts is most consistent with a weakened association of band 3 oligomers with the cytoskeleton in these membranes.

It should be noted that although the band 3MONTEFORI RBC is 88% P4.2-deficient, its use as an example of P4.2 deficiency may have limitations in that the band 3 mutation in

---

**Table 2. Octyl-β-Glucoside Extraction of Band 3 From Control and P4.2-Deficient Ghost Membranes**

<table>
<thead>
<tr>
<th>RBC Ghost Sample</th>
<th>Expt I</th>
<th>Expt II</th>
<th>Oligomer Peak Area (× 10⁻¹²)</th>
<th>Dimer Peak Area (× 10⁻¹²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>1</td>
<td>45</td>
<td>39</td>
<td>2.38</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>50</td>
<td>46</td>
<td>2.85</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>44</td>
<td>44</td>
<td>2.23</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>49</td>
<td>ND</td>
<td>1.96</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>47 ± 3</td>
<td>43 ± 3</td>
<td>2.36 ± 0.32</td>
<td>1.33 ± 0.19</td>
</tr>
<tr>
<td>P4.2-NIPPON</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Band 3-OLIGOMERIC</td>
<td>(&gt;99%)</td>
<td>58</td>
<td>59</td>
<td>4.47</td>
</tr>
<tr>
<td>Band 3-MONTEFORI</td>
<td>(~88%)</td>
<td>68</td>
<td>76</td>
<td>6.03</td>
</tr>
</tbody>
</table>

Ghost membranes (2 mg/mL) were incubated with 10 vol. 10 mmol/L Tris hydrochloride, pH 7.4, containing 1% octyl-β-glucoside for 10 minutes on ice. Membrane pellets were collected by centrifugation at 20,000 × g for 15 minutes at 4°C and washed once in buffer without octyl-β-glucoside. Ghost and octyl-β-glucoside-extracted membranes (20 to 25 µg) were separated by SDS-PAGE and the protein bands quantified by densitometry. The amount of band 3 extracted from the ghosts is derived by comparison of the band 3 content of octyl-β-glucoside-extracted membranes and intact ghosts, and is presented as % of total ghost band 3 (ie, band 3 content of octyl-β-glucoside-extracted membranes/band 3 content of intact ghosts × 100). For band 3 molecular species analysis, octyl-β-glucoside extracts were centrifuged at 100,000 × g for 30 minutes at 4°C, and 20 µL of the supernatant was applied to a TSK4000 SWXL HPLC size-exclusion column (30 cm × 7.8 mm ID). HPLC fractions were monitored at A₂₈₀, and peak areas (arbitrary units) were quantified using integration software.

Abbreviation: ND, not determined.

* Membrane P4.2 deficiency.† Membrane P4.2 deficiency.
Fig 3. (A) HPLC size-exclusion chromatography (SEC) of octyl-β-D-glucoside extracts from normal and P4.2-deficient (P4.2(-)) RBC ghosts (P4.2NIPPON > 99% P4.2-deficient)\(^6\), band 3 MONTEFIORE -88% P4.2-deficient.\(^{18}\) A representative chromatogram is shown for control and P4.2(-) samples. Elution times for HPLC fractions varied < 2%. HPLC peak fractions were collected, and components in each fraction were identified by denaturing SDS-PAGE and by comparison to previous results.\(^6\) Location of the column void volume (Vo) and elution times of protein standards (T, thyroglobulin [669 kD]; F, apoferritin [443 kD]; A, β-amylase [200 kD]; B, and bovine serum albumin [66 kD] used to calibrate the column are indicated by arrows). The band 3 oligomer-containing peak (peak 1) eluted from the HPLC column between Vo and T, and the band 3 dimer-containing peak (peak 2) eluted between T and F. There was a marked increase in the amount of the band 3 oligomer-containing peak (peak 1) for both P4.2(-) phenotypes compared with controls. (B) SDS-PAGE analysis of HPLC peaks 1 and 2 from A. RBC ghosts are shown in lane 1. Peak 1 (lane 2) contained band 3 and protein migrating on SDS-PAGE at the position of spectrin/ankyrin (Sp 2.1) (which is not resolved using the Laemmli buffer system). There was also some unidentified protein migrating below the position of P4.2. Peak 2 (lane 3) contained band 3 and proteins migrating on SDS-PAGE at the position of P4.1 and P4.2. (C) HPLC SEC of octyl-β-D-glucoside extracts from control RBC ghosts, as in A. The extracts were subjected to incubation at 37°C for 3 hours or at pH 5.8 for 30 minutes at 0°C, which produces band 3 aggregation.\(^6\) and also resulted in a shift of peak 1 to earlier elution times, consistent with band 3 aggregation.

this patient (40 glutamic acid to 40 lysine)\(^{18}\) could also potentially affect the association of band 3 with other proteins, including itself. Notably, band 3 MONTEFIORE is 30% deficient in G3PD,\(^{18}\) which may result from the 40 glutamic acid to 40 lysine mutation within the acidic N-terminal of the band 3 cytoplasmic domain that contains the G3PD binding site.\(^{18}\) Thus, it is possible that increased band 3 rotational freedom in band 3 MONTEFIORE ghosts is caused by G3PD deficiency in these membranes. Arguing against this is the fact that the extent of increased band 3 rotational freedom is similar or even larger in P4.2 NIPPON membranes that have a normal G3PD content.\(^{15}\) Moreover, although there is no evidence for altered interactions of band 3 MONTEFIORE with cytoskeletal proteins—indeed, the membrane content of cytoskeleton proteins except for P4.2 is normal in these cells—\(^{18}\) we cannot rule out this possibility, and perhaps this could explain why band 3 is easier to extract from band 3 MONTEFIORE ghosts with octyl-β-glucoside, as compared with P4.2 NIPPON ghosts, where band 3 is structurally normal.\(^{17}\) Despite this limitation, the fact that the association of band 3 with the cytoskeleton is compromised in two genetically unrelated P4.2-deficient phenotypes argues strongly in favor of a role for P4.2 in strengthening the band 3—cytoskeleton linkage.
The mechanism whereby P4.2 strengthens the band 3–cytoskeleton linkage is not addressed in our studies. Several possibilities exist, the most likely being that P4.2 stabilizes or enhances the binding of band 3 to ankyrin, possibly by formation of an ankyrin–P4.2–band 3 ternary complex, in a manner analogous to the P4.1–spectrin–actin ternary complex. In this case, P4.2 deficiency could lead to weakened band 3–ankyrin binding, as previously found in IOVs from P4.2-deficient RBCs, and result in partial loss of band 3 rotational constraints and weakened association of cytoskeleton-bound band 3 with the membrane. It is generally accepted that ankyrin is the primary cytoskeleton high-affinity attachment site for band 3 in human RBCs, making it likely that the abnormality in the band 3–cytoskeleton association in P4.2-deficient RBCs is due to a weakened band 3–ankyrin association, although our results do not exclude other possibilities.

Although P4.2 and ankyrin bind to separate sites on the cytoplasmic domain of band 3, it is possible that these proteins could communicate with each other in situ and that the binding of one protein to band 3 could influence the binding of the other protein to band 3. A less likely possibility is that P4.2 may directly tether band 3 to the cytoskeleton. In this case, ankyrin deficiency in the presence of normal amounts of P4.2 might not be expected to alter band 3 rotational mobility. The finding that ankyrin deficiency is associated with increased band 3 rotational mobility makes this possibility unlikely. However, since ankyrin deficiency is often associated with P4.2 deficiency, it is difficult to unambiguously dissect the individual roles of these proteins in this interaction. Since band 3 rotational mobility is also affected by the state of band 3 self-association, it is further possible that P4.2 directly affects the state of band 3 oligomerization.

It could be argued that increased band 3 rotational mobility in P4.2-deficient RBCs results secondarily from small decreases in the spectrin content of these cells. Two lines of evidence argue against this: (1) the spectrin content of P4.2NIPPON and band 3OCTOPHORE RBCs is within normal limits, measured both by SDS-PAGE (for both) and by spectrin radioimmuno assay (for P4.2NIPPON); and (2) although spectrin deficiency results in increased band 3 lateral mobility, it has no effect on band 3 rotational mobility.

In summary, natural mutations that result in RBC P4.2 deficiency result in increased band 3 rotational mobility and increased band 3 extraction from ghost membranes by octyl-β-glucoside. These results suggest that P4.2 is required for normal band 3–cytoskeleton association, presumably by stabilizing or strengthening the band 3–ankyrin association. It is also possible that P4.2 directly influences band 3 oligomerization state. Our studies did not find any influence of P4.2 on band 3 anion transport (sulfate uptake). Since P4.2 deficiency is a heterogeneous genotype, it is possible, if not likely, that not all P4.2-deficient phenotypes will result in identical membrane abnormalities. However, the finding that two genetically distinct P4.2-deficient phenotypes (P4.2NIPPON and band 3OCTOPHORE) resulted in similar weakened associations of band 3 with the cytoskeleton argues strongly in favor of a role for P4.2 in strengthening the RBC band 3–cytoskeleton linkage.

ACKNOWLEDGMENT

We thank Dr A.H. Beth for contributions to this study, S. Musto for technical assistance with band 3 extraction studies, and S.M. Blackman for technical assistance with band 3 quantitation.

REFERENCES

13. Wyatt K, Cherry RJ: Both ankyrin and protein 4.1 are required to restrict the rotational mobility of band 3 in human erythrocyte membranes. Biochim Biophys Acta 1103:327, 1992
with hemolytic anemia and a homozygous glutamic acid-lysine substitution in the cytoplasmic domain of band 3 (band 3MONTPEORE).


Increased rotational mobility and extractability of band 3 from protein 4.2-deficient erythrocyte membranes: evidence of a role for protein 4.2 in strengthening the band 3-cytoskeleton linkage

AC Rybicki, RS Schwartz, EJ Hustedt and CE Cobb