Control of Band 3 Lateral and Rotational Mobility by Band 4.2 in Intact Erythrocytes: Release of Band 3 Oligomers from Low-Affinity Binding Sites

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ABSTRACT Band 4.2 is a human erythrocyte membrane protein of incompletely characterized structure and function. Erythrocytes deficient in band 4.2 protein were used to examine the functional role of band 4.2 in intact erythrocyte membranes. Both the lateral and the rotational mobilities of band 3 were increased in band 4.2-deficient erythrocytes compared to control cells. In contrast, the lateral mobility of neither glycophorins nor a fluorescent phospholipid analog was altered in band 4.2-deficient cells. Compared to controls, band 4.2-deficient erythrocytes manifested a decreased ratio of band 3 to spectrin, and band 4.2-deficient membrane skeletons had decreased extractability of band 3 under low-salt conditions. Normal band 4.2 was found to bind to spectrin in solution and to promote the binding of spectrin to ankyrin-stripped inside-out vesicles. We conclude that band 4.2 provides low-affinity binding sites for both band 3 oligomers and spectrin dimers on the human erythrocyte membrane. Band 4.2 may serve as an accessory linking protein between the membrane skeleton and the overlying lipid bilayer.

INTRODUCTION

Human erythrocyte band 4.2 is a 72-kDa protein that is bound to the inner membrane surface through interactions with the band 3 (anion channel) protein. Each normal erythrocyte contains approximately 250,000 band 4.2 molecules (reviewed in Cohen et al., 1993). Although the exact function of band 4.2 is not known, several examples of hemolytic anemia associated with band 4.2 deficiency demonstrate that the protein is essential for normal erythrocyte survival and function (see Cohen et al., 1993; Yawata, 1994, for reviews). Erythrocytes lacking or deficient in band 4.2 have a shortened lifespan and have shape abnormalities ranging from mild to pronounced spherocytosis or ovalocytosis (Yawata, 1994). Band 4.2 deficiency has been associated with both defects in the cytoplasmic domain of the band 3 protein (Jarolim et al., 1992; Rybicki et al., 1993) and mutations in the band 4.2 gene itself (Bouhassira et al., 1992; Hayette et al., 1995a,b; Iwamoto et al., 1993; Kanzaki et al., 1995a,b; Takaoka et al., 1994). In the latter cases membrane-associated band 4.2 is completely absent by Coomassie blue staining of sodium dodecyl sulfate (SDS) gels, although in some cases small amounts of 72 and 74 kDa immunoreactive species can be detected by Western blotting (reviewed in Cohen et al., 1993; Yawata, 1994).

The detrimental changes in the shape and survival of band 4.2-deficient erythrocytes suggest that band 4.2 may play an important role in membrane skeletal function. Electron microscopic analysis indicates that band 4.2 may have a direct effect on the organization of the skeletal network (Inoue et al., 1994). The band 4.2 protein does not behave like a typical membrane skeletal protein, however. Unlike other skeletal proteins, band 4.2 is readily extracted from erythrocyte membranes by the non-ionic detergent Triton X-100 (Korsgren and Cohen, 1986; Steck and Yu, 1973; Yu et al., 1973). Band 4.2 is retained on membranes from which all other membrane skeletal proteins have been eluted (Korsgren and Cohen, 1986; Steck and Yu, 1973; Yu et al., 1973). It has been suggested that band 4.2 may be involved in the stabilization of ankyrin binding to the inner surface of the erythrocyte membrane (Rybicki et al., 1988), but this has not been confirmed in other studies (Ideguchi et al., 1990; Korsgren and Cohen, 1988). Band 4.2 does bind to the cytoplasmic domain of band 3, which serves as a crucial anchorage site for several membrane-skeleton interactions. It is therefore possible that band 4.2 plays a role in promoting or stabilizing membrane-skeleton linkages. Here, we provide evidence that band 4.2 is involved in linking band 3 oligomers to the membrane skeleton, possibly through direct associations between band 4.2 and spectrin. Our results suggest that band 4.2 may be one of several proteins that stabilize linkages between the membrane skeleton and the erythrocyte membrane.

MATERIALS AND METHODS

Eosin maleimide was purchased from Molecular Probes (Eugene, OR). Glucose oxidase and catalase were obtained from Sigma Chemical Co. (St.

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Louis, MO). Fresh blood was collected by venipuncture, anticoagulated with acid/citrate/dextrose, and shipped on ice to Boston by overnight courier. Within 48 h of venipuncture the buffy coat was removed by aspiration, and erythrocytes were washed three times and stored at 4°C in high potassium phosphate-buffered saline (KPBS) (140 mM KCl, 15 mM $NaPO_4$, 10 mM glucose, pH 7.4). High potassium buffers were used to prevent possible cellular dehydration associated with deoxygenation and fluorescent labeling.

Specific fluorescent labeling of erythrocyte band 3

One hundred microliters of freshly washed packed erythrocytes was incubated with 40 μl of eosin maleimide, 0.25 mg/ml in KPBS, at room temperature for 12 min. Cells were then washed three times in KPBS with 1% bovine serum albumin (BSA). Under these labeling conditions >80% of the membrane associated fluorescence was covalently bound to band 3 (Golan, 1989).

Specific fluorescent labeling of erythrocyte glycophorins

One hundred microliters of freshly washed packed erythrocytes was incubated with 100 μl of $NaIO_4$, 2 mM in KPBS without glucose, at 4°C for 15 min. Cells were then washed twice in KPBS with 0.1 M glycerol and once with KPBS. One hundred microliters of oxidized erythrocytes was added to 100 μl of fluorescein thiosemicarbazide, 0.5 mg/ml in KPBS, at 4°C for 1 h. Labeled cells were washed three times in KPBS with 1% BSA. Under these conditions >80% of the membrane-associated fluorescence was covalently bound to sialoglycoproteins (glycophorins); of this amount 75% was bound to glycophorin A (Golan, 1989).

Incorporation of the fluorescent phospholipid analog fluorescein phosphatidylethanolamine into intact erythrocyte membranes

Twenty microliters of fluorescein phosphatidylethanolamine (Fl-PE), 1 mg/ml in chloroform, was dried, resuspended in 0.5 ml of KPBS, and sonicated for 20 min in a low-power bath sonicator. One hundred microliters of freshly washed packed erythrocytes was incubated with the Fl-PE solution at room temperature for 30 min. Cells were then washed three times in KPBS with 1% BSA.

Lateral mobility measurements

Fluorescence photobleaching recovery (Axelrod et al., 1976) was used to measure the lateral mobility of band 3, glycophorins, and Fl-PE in membranes of intact erythrocytes. A gaussian laser beam was focused to a spot on the upper membrane of a labeled erythrocyte in a fluorescence microscope. After a brief, intense photobleaching pulse, recovery of fluorescence was monitored by periodic low-intensity pulses. Fluorescence recovery resulted from the lateral diffusion of unbleached fluorophores into the bleached area. Nonlinear least-squares analysis (Bevington, 1969) of fluorescence recovery data yielded both the diffusion coefficient and the fraction of labeled band 3 molecules that were free to diffuse on the time scale of the experiment (Golan et al., 1986). The effects of cell shape on fluorescence recovery kinetics were negligible because intracellular hemoglobin effectively shielded the lower membrane from excitation laser light and/or emitted fluorescence (unpublished observations), and because erythrocytes from the two patients used in this study exhibited only a mild degree of ovalocytosis. Details of the electronics and optics used in our fluorescence photobleaching recovery apparatus have been published (Corbett and Golan, 1993). All measurements were performed at 37°C. Using algorithms developed by Yoshida and Barisas (1986), the local temperature rise during the bleaching pulse because of laser-induced heating of surface fluorophores was < 0.02°C, and that because of heating of intracellular hemoglobin was 0.03°C.

Rotational mobility measurements

Polarized fluorescence depletion (PFD) (Yoshida and Barisas, 1986) was used to measure the rotational mobility of eosin-labeled band 3 in membranes of intact erythrocytes. Eosin molecules were photoselected to the triplet state by an intense polarized laser pulse. The decay of induced anisotropy was measured by monitoring ground-state fluorescence oriented parallel and perpendicular to the photoselection pulse (Johnson and Garland, 1981; Matayoshi and Jovin, 1991; Yoshida and Barisas, 1986). By using our laser microscope photometer, two exponential components of anisotropy decay and a residual anisotropy could be resolved, so data were fitted by nonlinear least-squares analysis to the equation

$$r(t) = r(\infty) + \alpha \cdot \exp(-t/\tau_1) + \beta \cdot \exp(-t/\tau_2),$$

where $r(t)$ was the anisotropy at time t, $r(\infty)$ was the residual anisotropy, and α and β were the fractions of molecules with rotational correlation times τ_1 and τ_2, respectively. An enzyme oxygen scavenging system, consisting of 50 units/ml glucose oxidase, 20 mM glucose, and 10^4 units/ml catalase, served to eliminate oxygen from the sample and thereby prevent quenching of the excited triplet state of eosin (Johnson and Garland, 1981). Slides were prepared for PFD experiments by placing 3.2 μl of a 10% erythrocyte suspension on a BSA-coated glass slide and using vacuum grease to seal a BSA-coated coverslip over the sample. Details of the electronics and optics used in our PFD apparatus have been published (Corbett and Golan, 1993). All measurements were performed at 37°C.

Biochemical analysis of erythrocyte membranes

The spectrin content, band 3 content, spectrin/band 3 ratio, and band 4.2 content of band 4.2-deficient and control erythrocyte membranes were measured by densitometric scans of Coomassie blue-stained SDS-polyacrylamide Laemmli gels (Laemmli, 1970). Quantitative densitometry was performed using a Stratagene (La Jolla, CA) Eagle Eye II video densitometer and RFLP Scan software (Scanalytics, Billerica, MA).

Extraction of band 3 by Triton X-100

Ghost membranes were prepared from simultaneously drawn band 4.2-deficient and control erythrocytes, as described (Korsgren and Cohen, 1988). Ghost samples were diluted to 1 mg protein/ml in 5 mM sodium phosphate, 0.5 mM EGTA, pH 7.6, with or without 150 mM NaCl. Thirty microliters of dilute ghost sample was added to 30 μl Triton X-100 in the same dilution buffer, such that the final Triton concentration was 0, 0.1, 0.5, or 1% (v/v). Samples were incubated on ice for 30 min and centrifuged in a Sorvall SS34 rotor at 17,000 rpm for 20 min. The supernatants were withdrawn and saved. Both the supernatants and pellets were solubilized in gel sample buffer containing 4% SDS and electrophoresed on 9% acrylamide gels, according to the method of Laemmli (1970). Coomassie blue-stained gels of supernatants and pellets were scanned, and the areas under the band 3 and spectrin/ankyrin peaks were quantitated, as described above.

Binding of spectrin to band 4.2 in solution

Erythrocyte spectrin and band 4.2 were prepared as previously described (Cohen and Foley, 1984; Korsgren and Cohen, 1988). Spectrin and band 4.2 were iodinated by using ^{125}I-labeled Bolton-Hunter reagent (DuPont-New England Nuclear, Boston, MA). Binding of ^{125}I-spectrin to band 4.2 was assayed by immunoprecipitation, performed exactly as described in Korsgren and Cohen (1988), at a fixed band 4.2 concentration of 4 μg/ml.

Polyclonal rabbit anti-band 4.2 antiserum and Pansorbin (Calbiochem, San Diego, CA) were used to immunoprecipitate spectrin-band 4.2 complexes. ^{125}I-Spectrin was incubated in the absence of band 4.2, at spectrin concentrations spanning the range used in the binding experiment, to correct for nonspecific binding of ^{125}I-spectrin. Nonspecific binding was subtracted from total ^{125}I-spectrin binding in the presence of band 4.2. The amount of band 4.2 protein immunoprecipitated by the polyclonal antiserum and Pansorbin was determined by performing a control immunoprecipitation with ^{125}I-band 4.2 in the absence of spectrin. This control value was used to normalize the amount of bound spectrin to the amount of immunoprecipitated band 4.2. Binding data were analyzed using both Enzfitter (Elsevier Biosoft, Amsterdam, The Netherlands) and TableCurve for Windows (Jandel, San Rafael, CA) software.

Binding of spectrin to band 4.2 reconstituted membranes

Erythrocyte spectrin and band 4.2 were prepared as described above. Inside-out vesicles, alkaline (pH 11)-stripped membranes, and band 4.2 reconstituted alkaline-stripped membranes were prepared, and the binding protocols were performed, according to procedures described in Korsgren and Cohen (1988). Membranes were reconstituted with band 4.2 by incubating pH 11-stripped membranes (0.25 mg protein/ml) with band 4.2 (0.2 mg protein/ml) for 18 h at 25°C, as described (Korsgren and Cohen, 1988). The band 4.2 content of the reconstituted membranes was confirmed by SDS gel electrophoresis. ^{125}I-Spectrin binding to inside-out vesicles, pH 11-stripped membranes, and band 4.2 reconstituted pH 11-stripped membranes was performed by incubating ^{125}I-spectrin with membranes (25 μg protein/ml) in 300 μl of 120 mM KCl, 5 mM sodium phosphate, 0.5 mM EGTA, 0.5 mM dithiothreitol, 0.02% NaN_3, 1 mg/ml BSA, and 10 μg/ml phenylmethylsulfonyl fluoride (pH 7.6) at 4°C for 90 min. Two 100-μl aliquots were centrifuged in a 42.2 Ti rotor at 17,000 rpm for 20 min, the supernatants were carefully removed, and the pellets were counted for ^{125}I. Added spectrin was determined by counting two 20-μl aliquots of the reaction mixture. Control binding incubations were performed at each spectrin concentration in the absence of membranes, to correct for nonspecific sedimentation of ^{125}I-spectrin. Nonspecific sedimentation was subtracted from total ^{125}I-spectrin sedimented in the presence of membranes.

RESULTS

Lateral and rotational mobility

The lateral and rotational mobilities of band 3, glycophorins, and Fl-PE were measured in four different experiments on intact erythrocytes from two patients with a subset of hereditary spherocytosis in which erythrocytes were completely deficient in band 4.2 (Ata et al., 1989; Ghanem et al., 1990). The lateral diffusion coefficient of band 3 in membranes of band 4.2-deficient erythrocytes was approximately twofold greater than control values. Although we were able to obtain samples from only two individuals with band 4.2 deficiency for the present study, there was much less difference between the diffusion coefficients in the two band 4.2-deficient erythrocyte samples than there was between the diffusion coefficients in band 4.2-deficient compared to control cells. The band 3 fractional mobility in membranes of band 4.2-deficient erythrocytes was not significantly different from control values (Fig. 1, Table 1). Neither the lateral diffusion coefficient nor the fractional mobility of glycophorins and Fl-PE in membranes of band

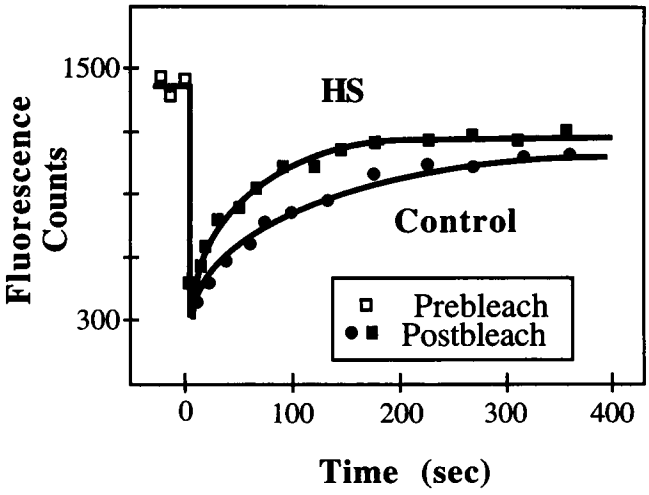

FIGURE 1 Typical fluorescence photobleaching recovery curves depicting the lateral mobility of band 3 in intact erythrocytes from patients with band 4.2 deficiency (HS, ■) and controls (Control, ●). Data points represent fluorescence counts at experimental times before and after the photobleaching pulse, which occurred at time 0. Band 4.2 deficient erythrocytes showed increased lateral diffusion coefficients relative to control erythrocytes, indicating increased freedom of band 3 lateral movement. Band 4.2 deficiency, diffusion coefficient = 3.9×10^{-11} cm^2 s^{-1}, fractional mobility = 65%. Control, diffusion coefficient = 1.4×10^{-11} cm^2 s^{-1}, fractional mobility = 58%.

4.2-deficient erythrocytes was significantly different from control values (Table 2).

Both band 4.2-deficient and control erythrocytes exhibited rapidly rotating, slowly rotating, and rotationally immobile populations of band 3. Compared to control erythrocytes, band 4.2-deficient erythrocytes manifested a significant shift from the slowly rotating and rotationally immobile populations of band 3 to the rapidly rotating

TABLE 1 Lateral mobility of band 3 in band 4.2-deficient and control erythrocytes

Sample	D ($\times 10^{11}$ cm^2 s^{-1})	f (%)	n
4.2 (−)-1	4.1 ± 2.3	52 ± 10	31
4.2 (−)-2	3.1 ± 1.3	63 ± 7	9
4.2 (−)-mean	3.8 ± 2.2*	55 ± 11‡	40
Control-1	2.0 ± 1.2	57 ± 12	31
Control-2	1.5 ± 0.7	53 ± 10	9
Control-mean	1.9 ± 1.2*	56 ± 12‡	40

Fluorescence photobleaching recovery was used to measure the lateral mobility of eosin labeled band 3 in intact erythrocytes from two patients with band 4.2 deficiency and hereditary spherocytosis and two controls with normal erythrocyte morphology. Experiments were performed at 37°C. D, diffusion coefficient; f, fractional mobility; n, number of measurements. Values represent mean ± standard deviation. The significance of differences between means for band 4.2-deficient and control samples was tested by using Student's unpaired two-tailed t-test (StatVeiw 512+, Abacus Concepts, Berkeley, CA). Values for control erythrocytes were statistically identical to results in a population of approximately 35 control individuals (see Corbett and Golan, 1993; Corbett et al., 1994).

* $p = 0.0001$.

‡ $p = 0.59$.

TABLE 2 Lateral mobility of glycophorins and FI-PE in band 4.2-deficient and control erythrocytes

Sample	D ($\times 10^{11}$ cm^2 s^{-1})	f (%)	n
4.2 (−)-1/GP	3.5 ± 1.0*	44 ± 8‡	11
Control-1/GP	4.2 ± 1.6*	40 ± 7‡	11
4.2 (−)-1/F1-PE	170 ± 50§	82 ± 9¶	14
Control-1/F1-PE	180 ± 30§	87 ± 6¶	14

Fluorescence photobleaching recovery was used to measure the lateral mobility of fluorescein labeled glycophorins (GP) and fluorescein-labeled phosphatidylethanolamine (F1-PE) in intact erythrocytes from one patient with band 4.2 deficiency and hereditary spherocytosis and one control with normal erythrocyte morphology. Experiments were performed at 37°C. D, diffusion coefficient; f, fractional mobility; n, number of measurements. Values represent mean ± standard deviation. The significance of differences between means for band 4.2-deficient and control samples was tested by using Student's unpaired two-tailed t-test (StatView 512+, Abacus Concepts, Berkeley, CA).

* $p = 0.20$.
‡ $p = 0.27$.
§ $p = 0.57$.
¶ $p = 0.09$.

TABLE 3 Rotational mobility of band 3 in band 4.2-deficient and control erythrocytes

Sample	α (%)	τ_1 (μs)	β (%)	τ_2 (ms)	$r(\infty)$ (%)	n
4.2 (−)-1	44 ± 4	281 ± 22	55 ± 4	2.4 ± 0.2	2 ± 0	2
4.2 (−)-2	36	247	52	1.6	12	1
4.2 (−)-mean	41 ± 5*	269 ± 25‡	54 ± 3§	2.2 ± 0.5¶	5 ± 6‖	3
Control-1	26 ± 1	162 ± 59	59 ± 7	2.0 ± 0.5	15 ± 7	2
Control-2	21	50	72	1.9	7	1
Control-mean	24 ± 3*	124 ± 77‡	63 ± 9§	2.0 ± 0.4¶	12 ± 8‖	3

Polarized fluorescence depletion was used to measure the rotational mobility of eosin-labeled band 3 in intact erythrocytes from two patients with band 4.2 deficiency and hereditary spherocytosis and two controls with normal erythrocyte morphology. Experiments were performed at 37°C. For each sample approximately 300 erythrocytes were illuminated by the laser beam, so the data represented the average rotational mobility of approximately 3×10^8 band 3 molecules per sample. Rotational mobility parameters were determined by nonlinear least-squares analysis, as described in Materials and Methods. χ^2 values ranged from 0.10 to 0.59 for the equations corresponding to the best-fit curves. α and β, fractions of band 3 molecules rotating with correlation times τ_1 and τ_2, respectively. $r(\infty)$, fraction of rotationally immobile band 3 molecules. n, number of samples. The significance of differences between means for band 4.2-deficient and control samples was tested by using Student's unpaired two-tailed t-test (StatView 512+, Abacus Concepts, Berkeley, CA). Values for control erythrocytes were statistically identical to results in a population of approximately 25 control individuals (see Corbett and Golan, 1993; Corbett et al., 1994).

* $p = 0.0079$.
‡ $p = 0.036$.
§ $p = 0.15$.
¶ $p = 0.67$.
‖ $p = 0.27$.

population. Unexpectedly, there was also a significant increase in the correlation time of the rapidly rotating band 3 population in band 4.2-deficient compared to control cells (Fig. 2, Table 3). Thus, compared to control erythrocytes, band 4.2-deficient erythrocytes showed an increased band 3 lateral diffusion coefficient, a shift to the rapidly rotating band 3 population, an increase in the rotational correlation time of the rapidly rotating band 3 population, and unchanged fractions of laterally and rotationally immobile band 3. We hypothesized that band 3 is both less constrained in its lateral and rotational mobility, due to removal of low-affinity binding sites, and more highly oligomerized, due to preferential loss of "free" band 3 dimers, in band 4.2-deficient erythrocytes. We tested this hypothesis further by measuring the band 3 content of control and band 4.2-deficient membranes.

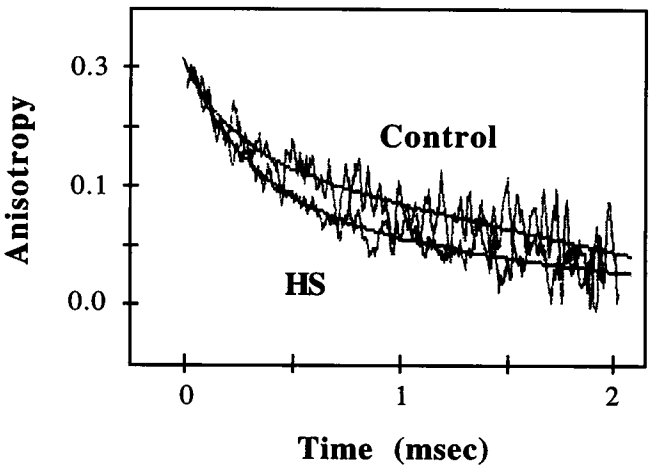

FIGURE 2 Typical polarized fluorescence depletion curves showing the rotational mobility of band 3 in intact erythrocytes from patients with band 4.2 deficiency (HS, *lower curve*) and controls (Control, *upper curve*). Data from approximately 300 cells were collected to obtain each curve. Band 4.2-deficient erythrocytes showed a shift of the band 3 population from the slowly rotating to the rapidly rotating fraction, indicating increased freedom of band 3 rotational movement. Band 4.2 deficiency, $\alpha = 46\%$, $\tau_1 = 296$ μs, $\beta = 52\%$, $\tau_2 = 2.3$ ms, $r(\infty) = 2\%$. Control, $\alpha = 27\%$, $\tau_1 = 203$ μs, $\beta = 64\%$, $\tau_2 = 2.4$ ms, $r(\infty) = 9\%$.

Biochemical analysis of band 4.2-deficient erythrocytes

Coomassie blue-stained SDS gels (Fig. 3) were examined by scanning densitometry. Compared to controls, the band 3/spectrin ratio in ghost membranes of cells from the two patients with band 4.2 deficiency was reduced by 18% and 14%, respectively. Similar results have been reported previously (Yawata, 1994). This reproducible finding suggested that, in the absence of band 4.2, a fraction of the band 3 population was lost from cells either during erythroid development in the bone marrow or during the circulation of mature erythrocytes. We hypothesized that this loss of band 3 protein, like the changes in band 3 lateral and rotational mobility, could be due to a reduction, in band 4.2-deficient erythrocytes, in low-affinity interactions between band 3 and the membrane skeleton. We tested this hypothesis further by measuring the extraction of band 3 by Triton X-100 from control and band 4.2-deficient membranes.

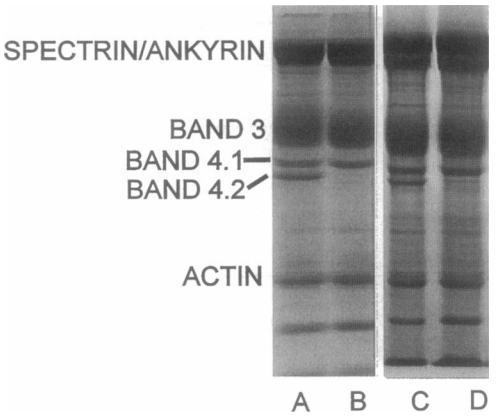

FIGURE 3 Coomassie blue-stained SDS-polyacrylamide gels of ghost membranes prepared from band 4.2-deficient and control erythrocytes. Membranes were solubilized in gel sample buffer containing 4% SDS and electrophoresed on 9% acrylamide gels. (*A, B*) Matched samples of control (*A*) and band 4.2 Nippon (*B*; Ata et al., 1989) erythrocytes. (*C, D*) Matched samples of control (*C*) and band 4.2 Tozeur (*D*; Ghanem et al., 1990) erythrocytes. In lane D, the faint band appearing to comigrate with band 4.2 failed to react with anti-band 4.2 antibodies (data not shown). Compared to controls, band 4.2-deficient membranes had a 14–18% decrease in the band 3/spectrin ratio.

Band 3 extractability

Treatment of ghost membranes with Triton X-100 removes the bulk of membrane lipids and integral membrane proteins, leaving behind a sedimentable membrane skeletal complex consisting of spectrin, actin, band 4.1, and skeleton-associated band 3 (Yu et al., 1973). Membrane skeletons were prepared from control and band 4.2-deficient erythrocytes at two different salt concentrations and three different concentrations of Triton X-100. From previous studies it was expected that only band 3 molecules that were either unattached or bound with low affinity to the skeleton would be extracted under low-salt conditions, and that most or all band 3 molecules, including those bound with high affinity to ankyrin, would be extracted under high-salt conditions (Yu et al., 1973). As shown in Fig. 4, skeletons from band 4.2-deficient cells retained a greater fraction of band 3 protein than did skeletons from control cells after low-salt (0 mM NaCl) extraction. In contrast, equal fractions of band 3 protein were retained in skeletons from control and band 4.2-deficient cells after high-salt (150 mM NaCl) extraction (Fig. 4). These results were consistent with the hypothesis that band 4.2-deficient erythrocytes are depleted specifically of band 3 molecules that are either unattached or bound with low affinity to the membrane skeleton.

Association of band 4.2 with spectrin

We next tested the hypothesis that band 4.2 affects band 3 mobility and skeletal retention through a direct binding interaction between band 4.2 and spectrin. We first used an immunoprecipitation binding assay to determine whether band 4.2 and spectrin could associate in solution. As shown

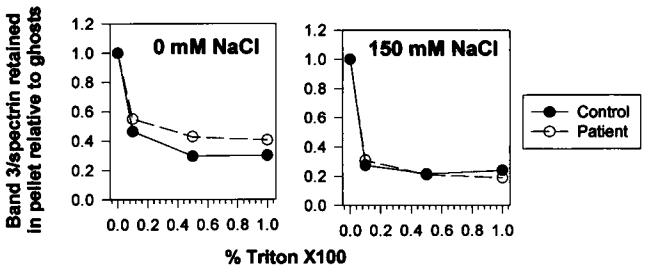

FIGURE 4 Triton X-100 extraction of band 3 from ghost membranes. Erythrocyte ghosts were treated with the indicated concentrations of Triton X-100 in the presence or absence of 150 mM NaCl. The amounts of band 3 retained in the membrane skeletal pellet were quantitated as described in Materials and Methods. Band 3 content was normalized to the sum of spectrin plus ankyrin because these proteins comigrated in the gel system used. Filled symbols, control membranes; open symbols, band 4.2-deficient membranes.

in Fig. 5, spectrin bound to soluble band 4.2 in an apparently saturable manner. The Scatchard plot of the binding data was concave (Fig. 5, *inset*), suggesting either negative cooperativity or multiple classes of binding sites. The data could be fitted by nonlinear regression analysis to a model incorporating two independent classes of binding sites: 1) a high-affinity ($K_d = 7.4 \pm 0.2 \times 10^{-9}$ M^{-1}, mean ± SD), low-capacity ($0.6 \pm 0.8 \times 10^{-9}$ M/liter) class of sites; and 2) a low-affinity ($K_d = 2.8 \pm 2.0 \times 10^{-7}$ M^{-1}), high-capacity ($5.8 \pm 1.0 \times 10^{-9}$ M/liter) class of sites. The total

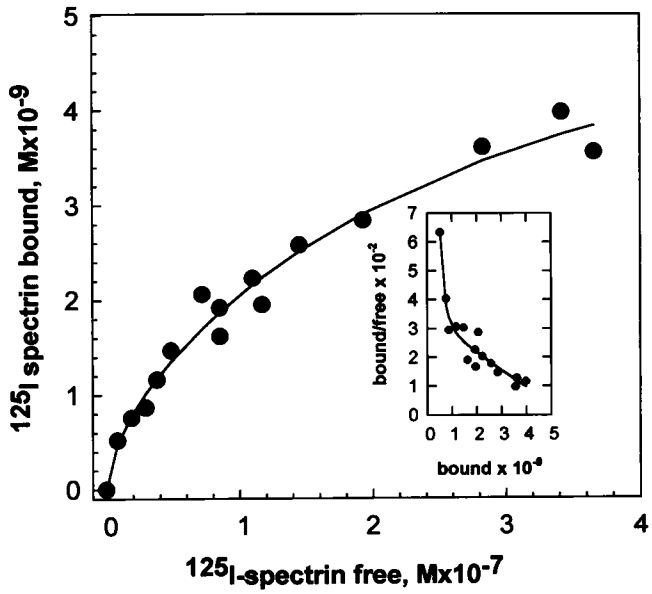

FIGURE 5 Binding of ^{125}I-spectrin to band 4.2 in solution. Binding of normal spectrin to normal band 4.2 was assayed as described in Materials and Methods. Spectrin was iodinated to a specific activity of 2840 cpm/μg. The inset shows a Scatchard plot of the data, which were fitted to a two-component model by using nonlinear regression analysis. The two binding sites had the following characteristics: Site 1, $K_d = 7.4 \times 10^{-9}$ M^{-1}, capacity = 0.6×10^{-9} M/liter; Site 2: $K_d = 2.8 \times 10^{-7}$ M^{-1}, capacity = 5.8×10^{-9} M/liter. Control binding experiments showed that the antiserum immunoprecipitated 4×10^{-8} M ^{125}I-band 4.2.

amount of bound spectrin was then compared with the amount of ^{125}I-band 4.2 immunoprecipitated under the conditions of the binding experiment. It was calculated that, at saturation, there was approximately one spectrin binding site per seven band 4.2 molecules.

To examine further the role of band 4.2 in skeleton-membrane interactions, we tested the ability of band 4.2 to promote spectrin binding to pH 11-stripped inside-out vesicles. As shown in Fig. 6, pH 11-stripped vesicles (which contained no ankyrin or other peripheral proteins) bound between 30 and 50 μg spectrin/mg vesicle protein. Reconstituting the vesicles with purified band 4.2 increased spectrin binding, at the three concentrations of spectrin tested, to within 50–80% of the capacity of inside-out vesicles (which contained normal amounts of ankyrin, band 4.2, and other membrane-associated proteins). It should be noted that the contribution of band 4.2 to the spectrin-binding capacity of the alkaline-stripped vesicles was accentuated in this experiment, because the membrane content of band 4.2 in vesicles reconstituted with band 4.2 was considerably greater than that of normal inside-out vesicles. In reconstituted vesicles the band 4.2/band 3 ratio was 0.45, whereas in normal inside-out vesicles the ratio was 0.15.

DISCUSSION

Constraints on band 3 lateral mobility in normal erythrocyte membranes include high-affinity binding interactions, low-affinity binding interactions, and steric hindrance interactions (Corbett and Golan, 1993; Fowler and Bennett, 1978; Golan and Veatch, 1980; Liu et al., 1990; Mohandas et al., 1992; Schindler et al., 1980; Tsuji et al., 1988; Tsuji and Ohnishi, 1986). High-affinity interactions between band 3 tetramers and ankyrin laterally immobilize approximately 40% of band 3 molecules in the normal erythrocyte membrane. Steric hindrance and low-affinity interactions between band 3 oligomers and the spectrin-based membrane skeleton slow the rate of lateral diffusion of mobile band 3 molecules by approximately 50-fold compared to the predicted diffusion rate of "free" band 3 in membranes devoid of a functional membrane skeleton (Corbett et al., 1994). Neither high-affinity binding of the cytoplasmic domain of band 3 to ankyrin nor the state of oligomerization of ankyrin-linked band 3 complexes appears to be altered in band 4.2-deficient erythrocytes, because the laterally immobile fraction of band 3 is similar in band 4.2-deficient and control cells. Although previous studies have shown that increased spectrin/band 3 ratios are associated with slowing of band 3 lateral diffusion (Corbett et al., 1994), the band 3 lateral diffusion rate is increased in band 4.2-deficient erythrocytes. Therefore, the increase in the band 3 lateral diffusion coefficient suggests that low-affinity binding interactions are significantly perturbed in band 4.2-deficient erythrocytes. Because normal band 4.2 binds to the cytoplasmic domain of band 3 (Korsgren and Cohen, 1986, 1988), it is likely that the absence of band 4.2 results directly in a decreased number of low-affinity binding sites for band 3 on the membrane skeleton.

Band 3 rotational mobility is constrained in normal erythrocytes by high-affinity and low-affinity binding interactions (Clague et al., 1989; Corbett and Golan, 1993; Liu et al., 1995; Matayoshi and Jovin, 1991; Nigg and Cherry, 1979, 1980; Tilley et al., 1991; Tsuji et al., 1988). In normal erythrocytes, the rotationally immobile fraction appears to consist of band 3 molecules bound with high affinity to ankyrin. The rapidly rotating band 3 population represents

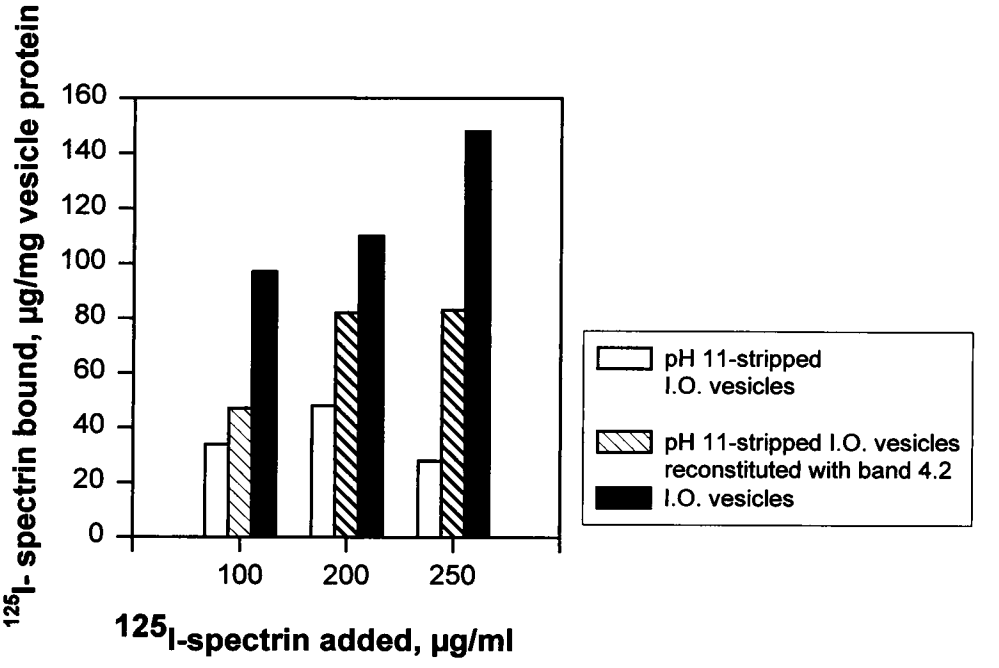

FIGURE 6 Binding of ^{125}I-spectrin to band 4.2 reconstituted membranes. pH 11-stripped membranes were prepared and reconstituted with purified band 4.2 as described in Materials and Methods. Binding of ^{125}I-spectrin (specific activity 12,096 cpm/μg) to membranes was corrected for nonspecific binding as described in Materials and Methods. The band 4.2/band 3 ratios on the membranes, determined by densitometry of Coomassie blue-stained SDS gels, were pH 11-stripped membranes, 0.02; inside-out vesicles, 0.15; band 4.2 reconstituted inside-out vesicles, 0.45. Data presented in this figure were representative of those obtained in five independent experiments using different preparations of spectrin, band 4.2, and membranes.

band 3 dimers, tetramers, and higher order oligomers that are free from constraints other than the viscosity of the lipid bilayer (Mühlebach and Cherry, 1985). The slowly rotating band 3 population appears to consist of band 3 oligomers that are rotationally restricted by low-affinity binding interactions between band 3 and either ankyrin-linked band 3 molecules or any of several membrane skeletal components. Steric hindrance interactions appear not to play a role in band 3 rotational mobility restraint (Corbett et al., 1994). The rotationally immobile fraction of band 3 is similar in band 4.2-deficient and control erythrocytes, suggesting that high-affinity binding of band 3 to ankyrin is unperturbed in band 4.2-deficient cells. Band 4.2-deficient erythrocytes manifest a significant shift from the slowly to the rapidly rotating fraction of band 3, consistent with the interpretation that low-affinity binding sites for band 3 are decreased on the membrane skeleton of such cells. Band 4.2-deficient erythrocytes also show a significantly increased rotational correlation time of the rapidly rotating band 3 fraction. These data suggest that there is a shift from a lower to a higher order state of band 3 oligomerization in band 4.2-deficient erythrocytes. This interpretation is consistent with the decreases, in band 4.2-deficient erythrocytes, in band 3/spectrin ratio and band 3 extractability, both of which could be caused by a preferential loss of "free" band 3 dimers from band 4.2-deficient cells. This interpretation is also consistent with the observed shift, in membranes from band 4.2-deficient erythrocytes, to a larger average size of intramembrane particles visualized by freeze fracture electron microscopy (Yawata, 1994).

At first glance, there appears to be an inconsistency between our interpretation that "free" band 3 is preferentially lost from band 4.2-deficient erythrocytes and our finding that the remaining band 3 molecules (which might have been expected to be more "bound") exhibit increased rather than decreased lateral and rotational mobility. One explanation for this apparent inconsistency could be that, in band 4.2-deficient cells, removal of 250,000 potential low-affinity band 3 binding sites from the inner surface of the membrane not only allows preferential loss of band 3 molecules (probably dimers) that are not attached with high affinity (either directly or indirectly) to ankyrin, but also allows more rapid lateral diffusion of the remaining (and more highly oligomerized) band 3 molecules. We note further that Triton extractability and band 3 mobility are likely to be differentially affected by removal of band 4.2. Triton extractability is governed mainly by the fraction of band 3 molecules that are bound with high affinity to the membrane skeleton, whereas the rate of band 3 lateral mobility is determined mainly by steric and low-affinity binding interactions between the cytoplasmic domain of band 3 and the membrane skeleton.

Our interpretation of the present results in terms of an effect of band 4.2 on band 3 mobility is predicated on the finding that, in both patients studied (Ata et al., 1989; Ghanem et al., 1990), the origin of the band 4.2 deficiency has been traced to a point mutation in the band 4.2 gene. In one of the patients (band 4.2 Tozeur; Hayette et al., 1995b) the cDNA encoding the entire cytoplasmic domain of band 3 has been sequenced and shown to be normal, ruling out a defect in the cytoplasmic domain of band 3. In the other patient (band 4.2 Nippon; Bouhassira et al., 1992) the limited tryptic digestion pattern of band 3 in inside-out vesicles is identical to that of a normal control, suggesting that there are no major structural abnormalities in the protein (data not shown).

The present studies suggest that band 4.2 may stabilize skeleton-membrane interactions by providing a direct link between band 3 and spectrin. Whereas previous studies have shown that band 4.2 does not play a direct role in ankyrin binding (Hargreaves et al., 1980; Korsgren and Cohen, 1988), to our knowledge the involvement of band 4.2 in spectrin binding has not been addressed. The stoichiometry of binding in solution (approximately seven band 4.2 molecules per spectrin dimer) suggests that two band 4.2 tetramers may be required to bind one spectrin dimer. Surprisingly, the K_d of spectrin binding to band 4.2 (2×10^{-7} M^{-1}) is only modestly higher than that for spectrin binding to ankyrin (between 10^{-7} and 10^{-8} M^{-1}; Bennett and Branton, 1977). The significance of the small number of high-affinity binding sites remains unclear, but could reflect the presence of a small pool of band 4.2, which is in a different physical state (e.g., through altered phosphorylation or self-association) than the bulk of the protein.

The increased lateral and rotational mobility of band 3 in band 4.2-deficient erythrocytes could indicate that band 4.2 serves to strengthen the band 3-ankyrin interaction, as has been suggested in one study (Rybicki et al., 1988). In this model, the absence of band 4.2 would weaken the band 3-ankyrin interaction and allow increased freedom of band 3 lateral and rotational movement. However, the observation here that spectrin interacts with band 4.2, together with previous observations that band 4.2 interacts with band 3 (reviewed in Cohen et al., 1993), suggests a more direct model in which the absence of band 4.2 leads to increased band 3 lateral and rotational mobility. Furthermore, it appears from our data that the amount of band 3 bound to the membrane skeleton with high affinity is the same in control and band 4.2-deficient erythrocytes, because the laterally and rotationally immobile fractions of band 3 molecules and the extractability of band 3 under high-salt conditions are identical in these two samples. Although our model does not require modification of the band 3-ankyrin interaction in band 4.2-deficient erythrocytes, the present data do not rule out such an effect.

Our results show that band 4.2 binds spectrin in solution and promotes spectrin binding to membranes. The absence of band 4.2 does not appear to result in spectrin deficiency, however, because the band 3/spectrin ratio in membranes from band 4.2-deficient erythrocytes is less than that in normal controls. This observation suggests that band 4.2 deficiency acts primarily to destabilize a fraction of band 3 molecules, resulting in loss of band 3 and possibly of membrane, rather than to destabilize the membrane skeletal

network itself. Thus, although band 4.2 is neither a classical membrane skeletal nor an integral membrane protein, it appears to promote interactions between these two classes of membrane proteins.

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