CONTRIBUTION OF THE BAND 3-ANKYRIN INTERACTION TO ERYTHROCYTE MEMBRANE MECHANICAL STABILITY

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In an effort to evaluate the role of the band 3-ankyrin linkage in maintenance of red blood cell membrane integrity, solution conditions were sought that would selectively dissociate the band 3-ankyrin linkage, leaving other membrane skeletal interactions intact. For this purpose erythrocytes were equilibrated overnight in nutrient-containing buffers at a range of elevated pHs and then examined for changes in mechanical stability and membrane skeletal composition. Band 3 was found to be released from interaction with the membrane skeleton over a pH range (8.4 to 9.5) that was observed to dissociate the band 3-ankyrin interaction in vitro. In contrast, all other membrane skeletal associations appeared to remain intact up to pH 9.3, after which they were also seen to dissociate. Whereas hemolysis of mechanically unstressed cells did not begin until ~pH 9.3, where the membrane skeletons began to disintegrate, enhanced fragmentation of shear stressed membranes was seen to begin near pH 8, where band 3 dissociation was first observed. Furthermore, the shear-induced fragmentation rate was found to reach a maximum at pH 9.4, i.e., where band 3 dissociation was essentially complete. Based on these correlations, we hypothesize that the band 3-ankyrin linkage of the membrane skeleton to the lipid bilayer is essential for red blood cell stability in the face of mechanical distortion but not for cellular integrity in the absence of mechanical stress.

EXPERIMENTAL PROCEDURES

Materials

Triton X-100 was purchased from Boehringer Mannheim (Indianapolis, IN) and diisopropylfluorophosphate was from Sigma (St Louis, MO). Fresh blood was obtained from the Central Indiana Regional Blood Bank or was drawn from healthy donors into acid citrate dextrose. Antibodies were raised in rabbits against purified human erythrocyte membrane proteins and characterized as described previously.17

Methods

Analytical procedures. Protein concentrations were determined according to the method of Lowry et al.14 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli10 on 5% to 10% acrylamide linear gradient gels. Parallel gel lanes were stained with Coomassie Blue or electrophoretically transferred to nitrocellulose for ankyrin.12 Another investigation has shown an inability of mouse erythrocytes partially deficient in ankyrin to undergo normal echinocytic or stomatocytic shape transformations.13 However, cells with highly defective ankyrin-band 3 linkages have still not been found in nature.

To more directly examine the importance of the band 3-ankyrin attachment to overall erythrocyte stability, we have explored the effect of artificially dissociating the interaction on membrane fragility. While recent studies have shown that dissociation of the two components can be promoted in vitro by certain sulfhydryl reagents14 and by competing antibodies,15 the most accessible method to disjoin ankyrin from band 3 was found to be achieved by simply equilibrating the pH to near 9.16 Thus, it was demonstrated that the ankyrin-band 3 interaction is controlled in vitro by structural transitions in band 3 at pH 7.2 and 9.2, and that the latter transition reversibly converts the anion transporter from a binding conformation to a nonbinding conformation. In this report we show that elevation of intracellular pH above 8.5 gradually releases band 3 from the membrane skeleton, leaving the remaining skeletal interactions intact, and that this disconnection of the membrane skeleton from the lipid bilayer renders the erythrocyte membrane mechanically unstable.
the overall deformability of the cell. Furthermore, during constant high-strength shearing the DI is reduced due to fragmentation of the cell into small spherical vesicles, which are resistant to shear deformation. Measurement of the half-time of this process provides a dependable assessment of the overall mechanical fragility of the cell.

Analysis of polypeptide composition of Triton X-100 shells (membrane skeletons) as a function of intracellular pH. Fresh whole blood, 50 mL, was centrifuged at 5,000g for 5 minutes at 4°C and the plasma anduffy coat were removed by aspiration. The resulting erythrocytes were washed twice in 5 mmol/L phosphate, 150 mmol/L NaCl, pH 7.4 and resuspended at 50% hematocrit in this same buffer. Disopropylfluorophosphate was added to 0.5 mmol/L, and the cell suspension was incubated 30 minutes at 37°C to inhibit endogenous proteases. Six 5-mL aliquots of the cell suspension were then taken and pelleted by centrifuging as described above, after which each pellet was resuspended in 40 mL of incubation buffer (20 mmol/L Na3HPO4, 20 mmol/L Na-hydroxylethylpiperazine-N’-2-ethane sulfonic acid (HEPES), 20 mmol/L glucose, 100 mmol/L KCl, 5 mmol/L glucose, 2 mmol/L inosine, 150 μg/mL penicillin G potassium salt, and 0.15 mg/mL streptomycin sulfate) at one of six pHs ranging from 7.4 to 10.4 and incubated for 15 hours at 23°C with mild agitation. Two 2-mL aliquots of the cell suspensions were then centrifuged and analyzed to determine both the final pH and the percent hemolysis after the overnight incubation. The final pH was measured with a Corning 130 pH meter directly on the previously described supernatant. The percent hemolysis was evaluated by comparing the hemoglobin absorbance at 562 nm of the supernatant with the hemoglobin absorbance of an equivalent volume of nonpelleted sample treated with 5% Triton X-100 to lyse all of the cells in the suspension. At 400 × magnification the erythrocytes appeared as biconcave discs at all pHs up to the point of hemolysis (∼pH 9.3).

The 36 mL of cell suspension not required in the above two assays was centrifuged as described above and the cells were resuspended in 5 mL of incubation buffer at ∼40% hematocrit at the appropriate pHs. Each suspension was then lysed by adding an equal volume of 4% Triton X-100 in incubation buffer at the same pHs, and 9 mL of the resultant solution was layered on 35% sucrose cushions in incubation buffer at pH 7.4 and centrifuged for 90 minutes at 200,000g. Pellets containing the Triton X-100 insoluble membrane skeletons and associated proteins (Triton X-100 shells) were washed once by resuspending in 10 mL of 5 mmol/L phosphate, 150 mmol/L NaCl, pH 7.4 and centrifuging at 40,000g for 20 minutes. Washed pellets were then resuspended in 500 μL of this same buffer and the protein concentration was determined. Samples of 35 μg were subjected to SDS-PAGE and Western blotted as described above.

Membrane stability measurements at various pHs. Washed RBCs were lysed in 40 vol of 5 mmol/L sodium phosphate, pH 7.4, pelleted by centrifugation at 24,000g for 5 minutes, and resuspended in 5 mmol/L NaHCO3, 1 mmol/L MgCl2, 100 mmol/L KCl, pH 7.4. The resulting pink ghosts were then resuspended in 10 vol of overnight incubation buffer (which had been adjusted to varying pHs with 0.1 N NaOH) and allowed to reseal in these buffers by incubating for 30 minutes at 37°C. The resealed ghosts were then repelleted (24,000g for 5 minutes) and resuspended in 35% isotonic dextran (400,000 M.) for ektacytometric studies. The stability of these membranes was then measured by subjecting the membranes to a continuous shear force of 575 dynes/cm². This force caused the membranes to fragment with time, generating small nondeformable spherical vesicles which led to a decrease in the DI. The time required for the DI to decrease to 0.35 of its original value was taken as a measure of the membrane’s stability.

RESULTS

pH Dependence of the Composition of Triton X-100 Skeletal Shells

To study the role of the band 3-ankyrin interaction in maintaining erythrocyte membrane stability, a method was required that would selectively dissociate the band 3-ankyrin linkage without significantly compromising the other essential associations in the membrane skeleton. Recent work by Thevenin and Low16 has demonstrated that band 3 undergoes a reversible conformational change near pH 9.2 that converts the protein from a binding conformation (below pH 9.2) to a form which exhibits no affinity for ankyrin (above pH 9.2). Because pH 9.2 seemed sufficiently low to leave most protein interactions intact, we decided to determine whether RBCs equilibrated to pHs near 9.2 might selectively release band 3 from their membrane skeletons without significantly jeopardizing the other major skeletal linkages. Therefore, whole erythrocytes were incubated overnight in nutrient-containing buffers adjusted to pHs between 7 and 10.5 and then extracted with the same buffers containing 2% Triton X-100 (see Methods). After sedimenting and washing the detergent-insoluble pellets in phosphate-buffered saline, the membrane skeletal fractions were examined by SDS-PAGE to determine the relative retention of each major polypeptide in the membrane skeleton. As shown in Fig 1A, the content of spectrin, actin, band 4.1, and band 4.9 remained essentially constant up to pH 9.3, suggesting that the stability of the linkages connecting the major membrane skeletal components was not significantly diminished by the high pH incubation. Likewise, ankyrin, which because of its cationization with spectrin had to be independently assayed by Western blotting (Fig 1C), displayed no decrease in content up through pH 9.3. Quantitative densitometry of the above gels and blots further confirmed the visual results (Fig 2), showing no significant reduction in the quantity of band 4.1, actin, spectrin, and ankyrin in the membrane skeletal complex. In contrast, at pH 9.7 little material could be isolated in the membrane skeletal pellet (Fig 1A, lane 7), suggesting that by this pH a major defect in the membrane skeleton had developed leading to dissociation into protein complexes too small to sediment through the 35% sucrose cushion.

When the behavior of band 3 as a function of pH was subjected to similar scrutiny, a notably different titration was observed (Fig 1A and B). Not only did the band 3 content of the membrane skeletons begin to decrease at pH 8.4, but by pH 9.3 more than 80% of the initial skeleton-associated band 3 had been extracted. Thus, densitometric scans of the Coomassie blue-stained gels showed a dramatic diminution of the anion transporter in the membrane skeletal pellet at moderate pHs, which extrapolated to near zero levels by pH 9.5 (Fig 3). Examination of the Triton X-100 supernatant by Western blotting with an anti-band 3 antibody15 also demonstrated that the soluble band 3 was completely intact (data not shown), indicating that proteolysis was not responsible for release of the anion transporter. Furthermore, band 4.2, an avid ligand of band 3, was also
Fig 1. Effect of equilibrium pH on the composition of Triton X-100 insoluble membrane skeletons. Erythrocytes that had been equilibrated for 15 hours at various pHs were extracted with 2% Triton X-100 and pelleted through a 35% sucrose cushion to remove any loosely associated proteins. The Triton-insoluble pellets were then washed and analyzed by SDS-PAGE and Coomassie Blue staining (A), or Western blotting with rabbit anti-band 3 antibody (B) or rabbit anti-ankyrin antibody (C). The identity of the lanes is as follows: ghosts (1), Triton X-100 shells extracted from cells at a final equilibrium pH of 7.3 (2), 8.4 (3), 8.7 (4), 9.0 (5), 9.3 (6), and 9.7 (7). The migration positions of major RBC membrane proteins are indicated in the margin.

seen to decrease over the pH range where loss of band 3 occurred (Figs 1A and 3). Taken together, these results indicate that by the pH where most of the skeletally associated band 3 had been lost from the spectrin-based skeleton, virtually none of the spectrin, actin, ankyrin, band 4.1, or band 4.9 had been released. This suggests that equilibration of erythrocytes at pHs up to and including 9.3 might be exploited to examine the contribution of the ankyrin-band 3 interaction to membrane fragility.

Effect of Equilibrium pH on Erythrocyte Membrane Stability

Two studies were conducted to evaluate the dependence of RBC membrane stability on equilibrium pH. First, because preparation of the aforementioned Triton X-100
more characteristic of the band 3-ankyrin interaction was obtained. Thus, ektacytometric measurements on ghosts adjusted to pHs between 7.4 and 9.3 showed rates of deformability index decrease that started to become significant at pH 8 and culminated in membranes unable to withstand shear stress at pH 9.4 (Fig 5). The midpoint of this pH transition for decreased mechanical stability oc-

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**Fig 2.** pH dependence of the retention of membrane proteins in Triton X-100 shells. Data from the Coomassie Blue-stained gel in Fig 1A were quantitated for the presence of actin (■) and band 4.1 (■■) by densitometry (A). Data from the Western blot of ankyrin in Fig 1C were quantitated as described in Methods (B). Results for ankyrin are reported as the peak area (measured in grams) of the densitometric scan of the ankyrin band in the blot.

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**Fig 3.** pH dependence of band 3 and protein 4.2 retention in Triton X-100 shells. Data from the Coomassie Blue-stained gel in Fig 1A were quantitated for the presence of band 3 (■) and band 4.2 (■■) by densitometric scanning (see Methods). The results are presented as the ratio of the amount of band 3 or band 4.2 to the amount of spectrin in each gel lane.

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**Fig 4.** RBC hemolysis as a function of equilibrium pH. Erythrocytes were incubated for 15 hours in incubation buffer (see Methods). The cells were then pelleted and the pHs of the supernatants were determined. This is reported as the final pH. The hemoglobin concentration of the same supernatant was determined from the absorbance at 562 nm, and the percent hemolysis was calculated by dividing this value by the hemoglobin concentration of a parallel sample of cells that were 100% hemolyzed with 5% Triton X-100 (see Methods). At the higher pH values, the final pH after equilibration was 0.5 to 1.0 pH U less than the pH of the initial suspending buffer.
BAND 3-ANKYRIN LINKAGE AND RBC STABILITY

The deformability index of resealed ghosts, prepared at the pHs indicated, was measured in the ektacytometer as described in Methods. (A) DI tracings with time at various pH values from one representative experiment. (B) A plot of the average of the DI data from three different experiments. The percent of the pH 7.4 control was calculated from the ratio of the time required to decrease to DI = 0.35 at a given pH versus the time to DI = 0.35 at pH 7.4 (control). Standard deviations between experiments varied between 9.3% at pH 8.1 to 4.9% at pH 9.3.

Fig 5. pH dependence of membrane stability in resealed ghosts. The deformability index of resealed ghosts, prepared at the pHs indicated, was measured in the ektacytometer as described in Methods. (A) DI tracings with time at various pH values from one representative experiment. (B) A plot of the average of the DI data from three different experiments. The percent of the pH 7.4 control was calculated from the ratio of the time required to decrease to DI = 0.35 at a given pH versus the time to DI = 0.35 at pH 7.4 (control). Standard deviations between experiments varied between 9.3% at pH 8.1 to 4.9% at pH 9.3.

curred at pH 8.7, slightly below the mid pH of the band 3 dissociation transition (pH 8.8, Fig 3), but well within the range of experimental variability. Importantly, the pH dependence of $^{125}$I-labeled ankyrin binding to inside-out erythrocyte membrane vesicles after a 26-hour incubation also yielded a pH dependence similar to the membrane mechanical stability changes, but vastly distinct from the unstimulated hemolysis curve. Thus, one hypothesis to explain the apparent correlation between band 3 extractability and membrane stability is that the band 3-ankyrin interaction is intrinsically involved in protecting the cell against fragmentation by mechanical stress, but not essential in preventing the hemolysis of mechanically unstressed cells that occurs at higher pH.

**DISCUSSION**

We have presented evidence that the predominant linkage of the lipid bilayer to the membrane skeleton can be gradually dissociated at pHs where the other major membrane skeletal interactions remain largely intact. Although both band 3 and band 4.2 are released from the membrane skeletons over this same pH range, we interpret the effect of pH on the release of band 3 to be due directly to dissociation of the band 3-ankyrin complex and not a consequence of the titration of some band 4.2 interaction. Our reasons for this contention are as follows. First, the dissociation of band 3 from the membrane skeleton closely followed the pH dependence of the band 3-ankyrin association at equilibrium in vitro and was therefore predicted from these model studies. Second, band 3 binds ankyrin ($K_d \approx 10^{-4} \text{ mol/L}$) independently of band 4.2 and, therefore, it is unlikely that loss of band 4.2 from the membrane skeleton could promote dissociation of band 3. Finally, the co-extraction of band 4.2 with band 3 as pH was elevated was anticipated from the known association of band 4.2 with band 3. Thus, as band 3 is released from its pH-sensitive interaction with ankyrin, the tightly associated band 4.2 would also be expected to extract.

Although no disappearance of spectrin, actin, or band 4.1 from the membrane skeletons was observed below pH 9.3, the complete loss of membrane mechanical stability before pH 9.3 (Fig 5B) could have been influenced by factors other than the pH-dependent dissociation of band 3. Thus, interactions among the skeletal components could have weakened without leading to fragmentation of any skeletal linkage. Also, dissociation of the band 3-ankyrin complex could have encouraged some spectrin dissociation via a positive cooperativity reported to exist in the band 3-ankyrin-spectrin linkage. Nevertheless, based on the strong correlation between dissociation of the band 3-ankyrin linkage and the membrane fragmentation pattern under shear stress, we suggest that the dominant defect responsible for membrane instability between pH 8 and 9.2 is the progressive dissociation of ankyrin from band 3.

The hypothesis that a strong membrane skeleton-bilayer junction may be essential for cell survival under mechanical deformation, but not for the maintenance of membrane integrity in the absence of shear stress, obviously requires further investigation. However, even in the absence of additional documentation, the proposal can still be argued largely on theoretical grounds. Thus, when two metastable layers are bonded together at regular intervals, a process termed "load transfer" can occur where a stress normally assumed by one of the layers is shared between both layers. Under these conditions a mechanical distortion or wave that might normally fragment one of the layers would be rapidly attenuated in the linked system. By analogy, whereas the membrane skeleton alone may be sufficient to maintain membrane integrity in the absence of shear stress, multiple junctions to the adjacent bilayer may be necessary to resist hemolysis in the face of mechanical deformation. Therefore, the significance of the band 3-ankyrin linkage may be to protect the RBC against hemolysis during tight passage through the narrow capillaries of the circulatory system.

In this regard it is interesting to note that absence of another linkage between the bilayer and the membrane skeleton (that of glycoophorin C with protein 4.1) has also...
been shown to result in decreased mechanical stability. The present data, in conjunction with this finding, imply that vertical linkages between the bilayer and skeletal complex are crucial for maintaining the mechanical integrity of the erythrocyte membrane. While much of the attention to date has been focused on spectrin-spectrin self-association and spectrin-actin-protein 4.1 interaction in regulating the structural integrity of the membrane, our data suggest that the band 3-ankyrin interaction may also play a crucial regulatory role.

REFERENCES

Contribution of the band 3-ankyrin interaction to erythrocyte membrane mechanical stability

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