

# Molecular Defect in the Sickle Erythrocyte Skeleton

## Abnormal Spectrin Binding to Sickle Inside-out Vesicles

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### Abstract

Although functional abnormalities of the sickle erythrocyte membrane skeleton have been described, there is little quantitative data on the function of the proteins that compose the skeleton. We have examined the association of spectrin, the major skeletal protein, with ankyrin, its high-affinity membrane binding site, and found sickle erythrocytes to have markedly reduced binding. Binding is assayed by incubation of purified  $^{125}\text{I}$ -spectrin with spectrin-depleted inside-out vesicles (IOVs) and measurement of the label bound to IOVs. Sickle IOVs bind ~50% less ankyrin than do control IOVs ( $P < 0.001$ ). Control experiments show that this reduced binding is not a function of faulty composition or orientation of sickle IOVs, or of reticulocytosis per se. Our least symptomatic patient has the highest binding capacity, suggesting that this abnormality may be related to clinical severity. This trend is supported by experiments showing that asymptomatic subjects with sickle trait, sickle cell anemia and high fetal hemoglobin, and sickle  $\beta^+$ -thalassemia have normal binding, whereas a symptomatic patient with sickle  $\beta^0$ -thalassemia has abnormal binding.

In contrast to what we see with ankyrin in situ on the IOV, when isolated and studied in solution, sickle ankyrin binds normally to spectrin. This discrepancy may be related to preferential purification of the normal ankyrin species or to an abnormal topography of the membrane near the spectrin attachment site. We hypothesize that sickle hemoglobin or perhaps the metabolic consequences of sickling damage the protein skeleton. This damage may alter the surface of the erythrocyte and result in abnormal cell-cell interactions which may be related to clinical severity.

### Introduction

The protein skeleton of the erythrocyte membrane influences cell shape, membrane flexibility, endocytosis, lipid organization, and lateral diffusion of integral membrane proteins (1). Abnormalities of all of these membrane properties have been described in erythrocytes from patients with sickle cell anemia, suggesting that the sickle cell skeleton is defective. The most graphic evidence is that skeletons of irreversibly sickled cells (ISCs)<sup>1</sup> retain the distorted sickle shape despite the absence of

detectable hemoglobin (2). ISC membranes are also rigid, and exhibit abnormal plastic deformation (3, 4). Endocytosis, a process that involves rearrangements of membrane proteins, is markedly reduced in sickle erythrocyte membranes (5). 25–30% of the phosphatidyl ethanolamine and phosphatidyl serine, which are normally sequestered in the cytoplasmic half of the bilayer, possibly through an interaction with spectrin (6, 7), are reversibly exposed on the outer surface of reversibly sicklable cells (RSCs) and permanently exposed on ISCs (8, 9). Hebbel et al. demonstrated that negative charges on the surface of sickle erythrocytes (presumably the glycoporphins) are abnormally clustered (10).

The skeletal proteins on the cytoplasmic side of the membrane modulate the outside surface characteristics of the cell through interactions with integral proteins and lipids. These surface characteristics—charge density, antigen presentation, and phospholipid distribution—may affect the interaction of erythrocytes with other cells and plasma proteins. For example, when phosphatidyl serine and phosphatidyl ethanolamine are flipped to the outer leaflet, they appear to activate coagulation proteins and become thrombogenic (11). As Hebbel et al. demonstrated, the change in charge distribution on the sickle erythrocyte is associated with a profound change in its interaction with endothelial cells—the erythrocyte becomes more adherent. Furthermore, they showed that the degree of endothelial adherence correlates with clinical severity (12). Although the major abnormality of the sickle erythrocyte is obviously the presence of the highly aggregated sickle hemoglobin, defects in the membrane skeleton may be involved along the pathophysiological pathway from hemoglobin gelation to tissue damage and thus be related to clinical expression.

We began a study of the sickle skeleton by focusing on the link between the skeletal proteins and the membrane. The protein meshwork is tied to the membrane by the specific association of spectrin with ankyrin (13–20), which in turn is bound to protein 3, a protein that spans the lipid bilayer and forms the channel for erythrocyte anion exchange (21–23). We examined binding of spectrin to ankyrin in sickle erythrocytes and found it to be markedly reduced.

### Methods

**Subjects.** The phenotype of individuals with sickle syndromes was determined by hemoglobin electrophoresis on cellulose acetate and citrate agar (24). The hemoglobin composition of these patients was: homozygous sickle cell anemia (SS) (8 patients): >90% hemoglobin S (Hb S), 0% hemoglobin A (Hb A), <3.5% hemoglobin A<sub>2</sub> (Hb A<sub>2</sub>), <5% hemoglobin F (Hb F); sickle cell trait (1 patient): 41% Hb S, 57% Hb A, 2% Hb A<sub>2</sub>; sickle  $\beta^0$ -thalassemia ( $S\beta^0$ ) (1 patient): 89% Hb S, 6% Hb A<sub>2</sub>, 5% Hb F; sickle  $\beta^+$ -thalassemia ( $S\beta^+$ ) (1 patient): 63% Hb S, 23% Hb A, 4% Hb A<sub>2</sub>, 10% Hb F; SS high Hb F (1 patient): 80% Hb S, 3% Hb A<sub>2</sub>, 17% Hb F. Informed consent was obtained. Control blood samples were obtained from normal individuals, and one non-splenectomized patient with pyruvate kinase deficiency and 32% reticulocytes.

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1. **Abbreviations used in this paper:** G3PD, glyceraldehyde 3-phosphate dehydrogenase; Hb A, Hb A<sub>2</sub>, Hb F, and Hb S, hemoglobins A, A<sub>2</sub>, F, and S, respectively; IOV, inside-out vesicle; ISC(s), irreversibly sickled cells; PAGE, polyacrylamide gel electrophoresis; ROV, rightside-out vesicle; RSC, reversibly sicklable cells;  $S\beta^0$ , sickle  $\beta^0$ -thalassemia;  $S\beta^+$ , sickle  $\beta^+$ -thalassemia; SS, homozygous sickle cell anemia.

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**Preparation of membranes and membrane proteins.** Venous blood was collected in citrate-phosphate-dextrose and stored at 4°C for up to 5 d. Erythrocyte membranes were prepared by hypotonic hemolysis (25). Spectrin-depleted inside-out vesicles (IOVs) were separated from low-ionic strength extracts by sedimentation (19). Rightside-out vesicles (ROVs) were produced by resealing of erythrocyte membranes in  $10^{-4}$  M  $MgSO_4$  (26).

Spectrin dimer was extracted from erythrocyte membranes at 37°C in 0.1 mM sodium phosphate, pH 8, purified by gel-filtration chromatography (25) and stored at 0°C in buffer of the following composition: KCl (130 mM), NaCl (20 mM), EDTA (1 mM),  $NaN_3$  (0.5 mM), dithiothreitol (1.0 mM),  $NaCl_2$  (1 mM),  $NaPO_4$  (10 mM), pH 7.5. Spectrin dimer was used within 5 d and was shown by nondenaturing polyacrylamide gel electrophoresis (PAGE) (27), to be uncontaminated by tetramer.

Ankyrin was purified by a modification of the procedure of Bennett and Stenbuck (17). A 1.0-M KCl extract of Triton-X 100 erythrocyte skeletons was applied to a DEAE-cellulose (DE-52; Whatman Chemical Separation Inc., Clifton, NJ) column. Ankyrin contaminated with protein 4.1 was eluted with 180 mM KCl and discarded. Purified ankyrin was eluted with 0.3 M KCl. Residual ankyrin bound to the column was removed with a 0.5 M KCl elution step. Purified ankyrin was stored at 0°C in 20% sucrose for up to 2 wk.

Spectrin and ankyrin were radioiodinated with Bolton-Hunter reagent (New England Nuclear, Boston, MA) (20).

**Assays of membrane protein interactions.** Rebinding of  $^{125}I$ -spectrin to spectrin-depleted IOVs was measured as described by Goodman and Weidner (19). Samples of  $^{125}I$ -spectrin were heat denatured (70°C, 10 min) and tested at every  $^{125}I$ -spectrin concentration in each experiment. Such measurements showed that only 1–5% of the total spectrin was nonspecifically bound. Specific binding was calculated as total binding minus nonspecific binding. All spectrin binding experiments are presented with each point representing the mean of duplicates.

The effect of dithiothreitol treatment of SS IOVs in the spectrin binding assay was examined in one experiment. Before use in the assay, SS IOVs were incubated for 60 min at 37°C, pH 8.0, in a solution of the following composition: dithiothreitol (100 mM),  $NaPO_4$  (5.0 mM), diisopropyl fluorophosphate (1 mM), phenylmethylsulfonyl fluoride (20  $\mu$ g/ml). As a control, SS IOVs were incubated under the same conditions without dithiothreitol.

Binding of  $^{125}I$ -ankyrin to spectrin in solution was performed as described by Tyler et al. (20), by the use of rabbit antispectrin IgG and *Staphylococcus aureus* bearing protein A (The Enzyme Center, Inc., Malden, MA) to precipitate the  $^{125}I$ -ankyrin-spectrin complex. Nonspecific components of binding were determined by the omission of spectrin but not  $^{125}I$ -ankyrin or *S. aureus* from the assay. Nonspecific binding represented ~45% of the counts per minute bound and was subtracted to give specific binding. The ankyrin binding experiments are presented with each point representing the mean of duplicates.

**Assessment of membrane sidedness.** The sidedness of IOV and ROV preparations was assessed by two methods. Limited  $\alpha$ -chymotrypsin digestion of IOVs and ROVs was accomplished by incubation in  $\alpha$ -chymotrypsin (1  $\mu$ g/ml) for 30 min at 0°C. Glyceraldehyde 3-phosphate dehydrogenase (G3PD) activity was measured (in duplicate) spectrophotometrically before and after the addition of 0.2% Triton X-100 (26), in three SS IOV, three control IOV, and one control ROV preparation.

**Other techniques.** Protein concentration was estimated according to the method of Lowry et al. (28). Sodium dodecyl sulfate (SDS)-PAGE was performed by the method of Steck (29). The amounts of ankyrin and protein 3 in IOVs were quantitated by the cutting out of band 2.1 and band 3 from Coomassie Brilliant Blue-stained SDS-PAGE slabs, elution overnight in 1 ml pyridine (25%), and reading of optical density at 605 m $\mu$  (30). This assay was performed in four SS preparations and five control preparations, each in triplicate or more. Density separation of SS cells was done by centrifugation, as previously described (2).

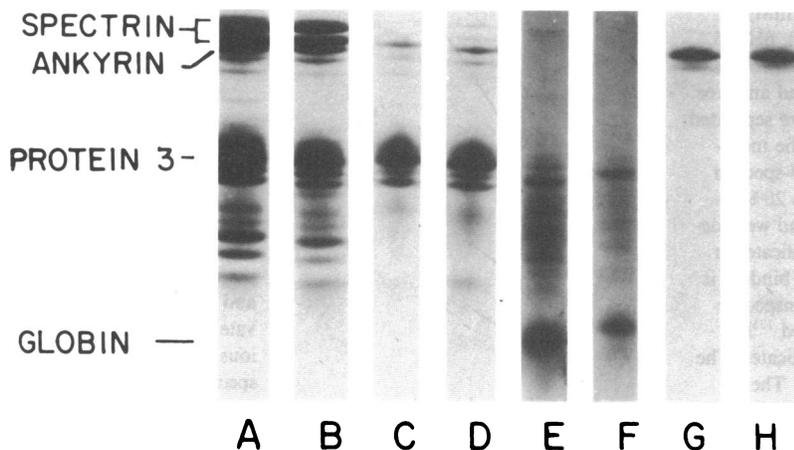
**Statistical methods.** The difference in spectrin binding between SS IOVs and control IOVs was assessed by linear regression of log binding on log concentration and sickle status (31). In addition, the amounts of spectrin bound to SS IOVs and control IOVs at each spectrin concentration were compared by a two-sample *t* test (31).

The compositions of SS IOVs and control IOVs were compared by application of the two-sample *t* test to the logarithms of the ankyrin/protein 3 ratios.

The sidedness of SS and sidedness of control IOVs as measured by change in G3PD activity, after addition of Triton, were compared by use of linear regression (31).

## Results

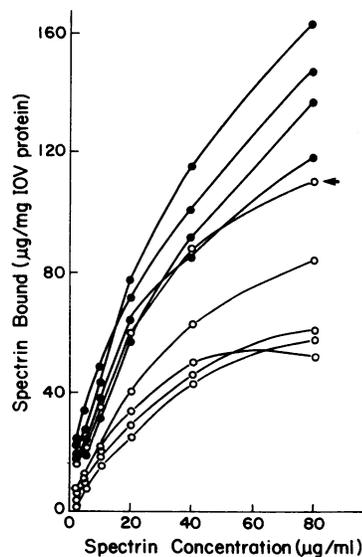
**Composition of membranes, vesicles, and protein preparations.** SDS-PAGE of SS and control erythrocyte membranes does not reveal any obvious difference in the proportion of the major polypeptide bands (Fig. 1, lanes A and B). There is a slight increase in the amount of hemoglobin and catalase in the SS membranes, as has been demonstrated in other hemolytic anemias (32), but the amount of hemoglobin does not exceed 1% of the total membrane protein. The composition of spectrin-depleted IOVs from control and SS membranes is comparable (lanes C and D). The hemoglobin content of IOVs does not exceed 0.5%. The ankyrin/protein 3 ratios of SS and control IOVs varied between 0.15 and 0.23, with a mean of 0.19. There was no difference between the ratios in SS IOVs and control IOVs, *P* = 0.8. The sidedness of these vesicles is confirmed, as  $\alpha$ -chymotrypsin digestion of both control and



**Figure 1.** SDS-PAGE of control (A) and SS (B) erythrocyte membranes; control (C) and SS (D) IOVs; control (E) and SS (F) IOVs after incubation with 1  $\mu$ g/ml  $\alpha$ -chymotrypsin for 30 min at 0°C; control (G) and SS (H) ankyrin eluted at 0.3 M KCl from DE-52. Approximately 40  $\mu$ g protein of erythrocyte membranes were applied (lanes A and B), 30  $\mu$ g protein of vesicles (lanes C–F), and 10  $\mu$ g protein of eluted fractions (lanes G and H).

SS IOVs results in complete loss of ankyrin (lanes E and F), whereas ROVs treated with  $\alpha$ -chymotrypsin maintains ankyrin (not shown). By the G3PD assay, the SS IOV preparations varied between 89 and 98% inside-out, with a mean of 94.2%. The control IOVs varied between 84 and 94% inside-out, with a mean of 90.2%. There was no difference in sidedness between the SS and control preparations,  $P = 0.9$ . By the same assay, the ROV preparation was 30% inside-out. Spectrin (not shown) extracted from control and SS membranes is identical in SDS-PAGE mobility, gel-filtration chromatography elution pattern, and yield. Ankyrin extracted from control and SS erythrocyte skeletons and eluted from DEAE-cellulose with 0.2 M KCl is identical in yield ( $\sim 20\%$ ) and SDS-PAGE mobility as shown in lanes G and H. A second elution step with 0.5 M KCl results in release of spectrin alone in the control preparation but of spectrin contaminated with ankyrin ( $<5\%$  of the ankyrin recovered from the column) in the SS preparation.

*The spectrin binding sites of SS membranes are abnormal.* The membrane binding sites for spectrin were examined in situ by comparison of the capacities of control and SS IOVs to bind normal  $^{125}\text{I}$ -spectrin. The results in Fig. 2 indicate that SS IOVs bind less spectrin than do controls. By regression analysis, the difference between SS and control binding was significant,  $P < 0.001$ . A comparison between SS and control binding at different spectrin concentrations is shown in Table I. The differences in spectrin bound are significant ( $P < 0.05$ ) in five of the six spectrin concentrations. Scatchard analysis of these data indicates that the binding capacity of control IOVs is  $177 \pm 15$   $\mu\text{g}$  spectrin dimer/mg IOV protein. This is significantly different from SS IOVs, which bind  $95 \pm 25$   $\mu\text{g}$  spectrin dimer/mg IOV protein ( $P < 0.001$ ). The binding affinities are not significantly different: control,  $5 \pm 2 \times 10^{-8} \text{ M}^{-1}$  and SS,  $6 \pm 3 \times 10^{-8} \text{ M}^{-1}$ . These control data are consistent with reported values (11, 14, 17); the SS data indicate a 50% decrease in the



**Figure 2.** The binding of control spectrin dimer to SS (○) and control (●) IOVs. Various concentrations of  $^{125}\text{I}$ -spectrin dimer were incubated for 90 min at  $0^\circ\text{C}$  in a 0.225 ml volume containing KCl (130 mM), NaCl (20 mM), EDTA (1 mM),  $\text{NaN}_3$  (0.5 mM), dithiothreitol (1.0 mM),  $\text{MgCl}_2$  (1 mM),  $\text{NaPO}_4$  (10 mM), pH 7.5, and 16.65 g IOV protein. Membrane-bound and free radioactivity were separated by pelleting of the membrane-bound  $^{125}\text{I}$ -spectrin dimer through a 20% sucrose cushion and were determined in duplicate for each point. The binding is corrected for nonspecific

components by subtraction of the values for heat-denatured  $^{125}\text{I}$ -spectrin. The points are presented as the mean of the duplicates. The data from four controls and five SS patients are presented. The SS patient indicated by the arrow is the only one who has been free of clinical vasoocclusive episodes for 5 yr.

**Table I. Binding of Control Spectrin Dimer to Control and SS IOVs\***

Spectrin concentration $\mu\text{g/ml}$	Mean spectrin bound $\mu\text{g/ml}$ IOV protein		Difference of means	P value
	Control	Sickle		
2	$20.38 \pm 3.11$	$6.35 \pm 2.50$	$14.03 (9.2-18.8) \ddagger$	$<0.001$
5	$27.77 \pm 6.31$	$19.93 \pm 5.67$	$7.84 (1.1-14.6)$	0.03
10	$39.29 \pm 4.31$	$28.4 \pm 10.8$	$10.89 (-0.5-22.3)$	0.06
20	$67.3 \pm 10.6$	$41.3 \pm 13.2$	$26.0 (13.6-38.4)$	$<0.0001$
40	$97.8 \pm 14.0$	$61.7 \pm 18.2$	$36.1 (19.3-52.9)$	$<0.0001$
80	$141.1 \pm 18.1$	$75.3 \pm 24.8$	$65.8 (43.2-88.4)$	$<0.0001$

\* Data from Fig. 2.

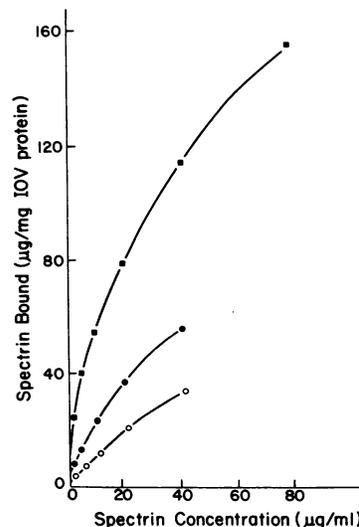
‡ 95% confidence intervals.

number of high-affinity spectrin binding sites. Pretreatment of the SS IOVs with 100 mM dithiothreitol does not correct the defect in spectrin binding (data not shown).

A high reticulocyte control with IOVs prepared from a patient with pyruvate kinase deficiency has normal spectrin binding (Fig. 3). In two experiments where IOVs are prepared from density-fractionated SS cells, the top layer (65% reticulocytes, 1% ISC) binds more spectrin than does the bottom layer (2% reticulocytes, 73% ISC) (Fig. 3). It appears that reticulocytosis alone is not responsible for the reduced spectrin binding and that the abnormality of spectrin binding in SS membranes is exaggerated in the most dense cell fraction.

It is interesting that of the five SS patients whose IOVs are studied in Fig. 2, the one with the highest binding capacity (see arrow) is the only patient considered clinically mild, i.e., having had no demonstrable sickle-related events in 5 yr. To avoid the vagaries of such clinical classifications within the SS group, we examined four other sickle syndromes with generally accepted clinical severity rating. As seen in Fig. 4, the asymptomatic individuals with sickle cell trait,  $\text{S}\beta^+$  and sickle cell anemia with high fetal hemoglobin have normal binding, whereas the symptomatic patient with  $\text{S}\beta^0$  has binding in the high SS range.

*The ankyrin binding site of SS spectrin is normal.* The



**Figure 3.** The binding of control spectrin dimer to reticulocyte-rich, ISC-poor SS IOVs (●), reticulocyte-poor, ISC-rich SS IOVs (○), and high reticulocyte pyruvate kinase IOVs (■). Various concentrations of  $^{125}\text{I}$ -spectrin were incubated with the above IOVs as described in Fig. 2.

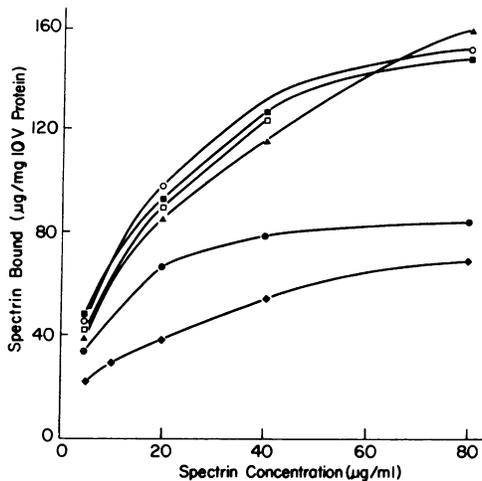


Figure 4. The binding of control spectrin dimer to control (■), SS (◆), SS high HbF (□), Sβ<sup>+</sup> (○), and Sβ<sup>0</sup> (●) IOVs. Various concentrations of <sup>125</sup>I-spectrin were incubated with the above IOVs as described in Fig. 2.

ankyrin binding site of spectrin is examined by comparison of the binding of SS and control <sup>125</sup>I-spectrin to control IOVs. No difference in binding is observed (Fig. 5), indicating that the ankyrin binding site on SS spectrin is normal.

The spectrin binding site of purified SS ankyrin is normal in solution. The membrane binding site for spectrin, ankyrin, is examined in solution by comparison of the capacities of purified control and SS <sup>125</sup>I-ankyrin to bind normal spectrin. The results in Fig. 6 indicate that purified SS ankyrin binds spectrin normally. The reduced capacity to bind spectrin of SS ankyrin in situ on the vesicle is no longer demonstrated when the SS ankyrin is purified and studied in solution.

## Discussion

There is considerable circumstantial evidence that the membrane skeleton of the sickle erythrocyte is defective. Careful quantitative studies of the sickle skeletal proteins have failed to demonstrate any abnormalities except for a slight increase in hemoglobin and catalase, a pattern seen in other hemolytic anemias (32). We therefore chose to study the qualitative

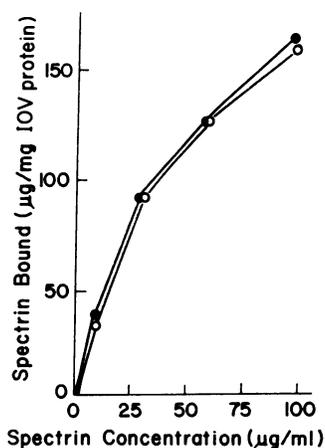


Figure 5. The binding of control (●) and SS (○) spectrin dimer to control IOVs. Various concentrations of the <sup>125</sup>I-spectrin dimers were incubated with control IOVs as described in Fig. 2.

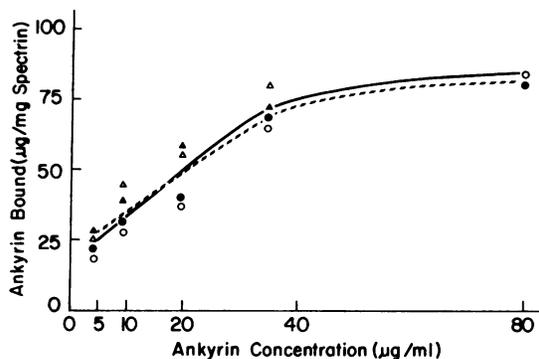


Figure 6. The binding of control (●, ▲) and SS (○, △) purified ankyrins to control spectrin dimer. Various concentrations of the <sup>125</sup>I-ankyrins were incubated for 90 min at 0°C in a 0.2 ml volume containing: KCl (130 mM), NaCl (20 mM), EDTA (1 mM), NaN<sub>3</sub> (2.0 mM), dithiothreitol (0.2 mM), Triton X-100 (0.1%), bovine serum albumin (1 mg/ml), NaPO<sub>4</sub> (5 mM), and purified control spectrin dimer 10 µg, pH 7.6. <sup>125</sup>I-ankyrin-spectrin dimer complex was precipitated by means of antispectrin IgG antibody and *S. aureus* bearing protein A. The binding is corrected for nonspecific components determined by omitting spectrin from the reaction mixture. The points represent the mean of duplicate determinations. Presented are the results of two experiments using ankyrins purified from two different controls and two different SS patients.

aspects of sickle skeletal structure, starting with the important interaction that links the skeleton to the membrane: spectrin-ankyrin binding.

We found significant a defect in spectrin binding to SS IOVs. SS IOVs bound ~50% less spectrin than did control IOVs, without an appreciable difference in the binding affinity. This consistent reduction in spectrin binding could not be attributed to increased hemoglobin in SS IOVs, contamination of SS IOVs with ROVs, or proteolysis and loss of SS ankyrin. The control and SS vesicles contained ~0.5% hemoglobin, not enough to change the binding capacity by inflating the IOV protein measurement. SS and control IOVs have similar ankyrin/protein 3 ratios and are >90% inside-out. Thus the ankyrin on the SS IOV appears normal in quantity and size, is accessible to large proteins such as α-chymotrypsin (which is smaller than spectrin), yet functions abnormally in its ability to bind spectrin.

It is not known whether defective spectrin binding is due to an abnormality in ankyrin, the primary binding site for spectrin, or to other sites of attachment such as protein 4.1 (33) or lipids (34). However, this is almost certainly a secondary defect that is acquired during the life of the erythrocyte, as indicated by the exaggeration of the defect in the dense ISC-rich population of erythrocytes as compared to the light reticulocyte-rich population (Fig. 3). We envision that ankyrin and/or its immediate environment is normal in the young erythrocyte and is subsequently damaged by sickle hemoglobin or by the sickling process and its attendant cation and water loss, calcium accumulation, and membrane distortion. Normal amounts of spectrin are bound to the membrane during its initial assembly and remain associated with the defective membrane throughout its life despite the acquired defect in spectrin rebinding.

Sickle hemoglobin may interfere with spectrin binding to ankyrin by direct steric hindrance or by oxidative damage at

or near the spectrin binding site. Although the putative membrane attachment site for hemoglobin S is in the immediate neighborhood of ankyrin, at the cytoplasmic portion of protein 3 (35–37), it is unlikely that steric hindrance plays an important role in our assay, since each of our IOVs contains ~100,000 ankyrins and only 15,000 hemoglobins. Unless there is clustering of the ankyrins so that one hemoglobin can interfere with several ankyrins, it is difficult to imagine that 15,000 hemoglobins could cause a 50% reduction in spectrin binding sites.

Sickle erythrocytes contain increased amounts of membrane-bound hemichrome and can spontaneously generate twice-normal amounts of superoxide, peroxide (H<sub>2</sub>O<sub>2</sub>), and hydroxyl radical (38). These oxidant species can oxidize membrane polyunsaturated fatty acids (39) and possibly such proteins as calcium ATPase (40). It is tempting to speculate that these species may also damage ankyrin or its neighbors and produce the defect we describe. Preincubation of the SS IOVs in 100 mM dithiothreitol did not reverse the ankyrin defect, indicating that if oxidation is indeed the damaging process, simple reduction is not adequate to reverse the damage. Sulfhydryls may not be involved or may not be accessible to the reagent under these conditions.

Metabolic defects associated with sickling such as calcium accumulation, dehydration, and ATP depletion may facilitate ankyrin damage and in fact may be a common pathway for cell destruction in other hemolytic anemias and possibly even in the senescence of normal erythrocytes. Calcium-activated proteases, for example, may clip critical peptides and affect skeletal integrity (40).

Although we have demonstrated a defect in ankyrin function in situ on SS IOVs, when we isolate SS ankyrin, it binds to spectrin normally in solution (Fig. 6). There are several possible explanations for this observation. One possibility is that the ankyrin that is isolated is in fact the normal species and that the defective protein is preferentially lost during the purification process. Under the best circumstances, only 18% of the initial ankyrin in the Triton X-100 skeletons is usually recovered after the 0.3 M KCl elution step (17). Our observation that a trace amount (<5%) of the SS ankyrin and none of the control ankyrin is further eluted with a second 0.5 M KCl step suggests that this preferential isolation issue may be relevant. What remains to be seen is whether the trace amount of ankyrin which is eluted with high salt has different functional properties. Another possibility is that the abnormal ankyrin maintains an abnormal configuration in the hydrophobic environment of the IOV but can refold normally in aqueous solution. Alternatively, the defect may not lie within the ankyrin molecule at all but in a neighboring protein or lipid species that can interfere with spectrin binding by altering either the topography or function of the ankyrin. An analogous observation has been made by Agre et al. (41) who demonstrated a defect in the binding of ankyrin to protein 3 in two kindreds with a congenital hemolytic anemia. In their study normal ankyrin bound poorly to patient protein 3 when the protein 3 was left in situ on the IOV. However, when both the ankyrin and the purified ankyrin-binding portion of protein 3 were studied in solution, no defect was demonstrable.

A defect in the association between spectrin and ankyrin such as we have demonstrated here might be responsible for the observed alterations in the surface characteristics of sickle erythrocytes. Precipitation or aggregation of spectrin in mem-

branes causes immobilization and clustering of integral membrane proteins directly over the spectrin aggregates (42–45). Spherocytic mice with congenital absence of spectrin have increased lateral mobility of integral proteins (46). Specifically weakening the spectrin-ankyrin interaction by incubation of membranes with the proteolytic fragment of ankyrin that binds to spectrin causes increased lateral mobility of integral proteins (47, 48). These pieces of evidence support the hypothesis that the spectrin-ankyrin interaction is important in dampening the lateral movement of the major charge-bearing protein, glycophorin. Clustering of charges on sickle cells, as demonstrated by Hebbel, is associated with increased endothelial adherence and correlates closely with the clinical severity of vasoocclusive events (12). Although others have confirmed increased endothelial adherence in sickle cells (49), the abnormal charge distribution was not confirmed when different conditions and techniques were used (50). Our small survey of sickle syndromes suggests that defective spectrin binding may also relate to clinical severity. The observation that our asymptomatic homozygous SS high Hb F patient had normal binding, whereas the symptomatic heterozygous S $\beta^0$  patient had abnormal binding, suggests that this observation is not simply related to a polymorphism associated with the S gene. We will need further studies of sickle cell anemia patients with graded clinical severity and concurrent measurement of endothelial adherence, integral protein mobility, and spectrin binding in order to understand the possible interrelationships of these observations.

Considering the variety of membrane abnormalities in sickle erythrocytes, we expect that there are other acquired abnormalities of sickle skeletons that remain to be identified. Further investigation of skeletal lesions in sickle erythrocytes may reveal clues to clinical heterogeneity as well as skeletal structure-function relationships.

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