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Spectrin's E2/E3 Ubiquitin Conjugating/Ligating Activity is Diminished in Sickle Cells

Tsui-Ling Chang,[1] David G. Kakhniashvili,[1] and Steven R. Goodman[1,2*]

[1] Department of Molecular and Cell Biology, UTD Sickle Cell Disease Research Center, Institute of Biomedical Sciences and Technology, The University of Texas at Dallas, Richardson, Texas

[2] Department of Cell Biology, UT Southwestern Medical Center, Dallas, Texas

Erythrocyte spectrin contains E2/E3 ubiquitin conjugating/ligating activity in its α subunit. Ankyrin is a target of spectrin's E2/E3 ubiquitin conjugating/ligating activity in vitro and in vivo. We compare the ubiquitination levels of ankyrin mediated by control and sickle cell spectrin using a biotinylated ubiquitin cell-free assay. Sickle cell spectrin has diminished ability to transfer ubiquitin from an intermediate spectrin–ubiquitin thioester adduct (α′ spectrin) to ankyrin, which may be due to glutathiolation of spectrin's E2 and/or E3 active site cysteines. There is also a diminished ability of the sickle cell ankyrin to serve as target of spectrin's E2/E3 activity, probably due to oxidative damage to ankyrin. A direct correlation exists between the α′/α spectrin ratio and spectrin's ability to ubiquitinate ankyrin. There is also an inverse correlation between severity of the disease and the α′/α spectrin ratio in SS erythrocytes. These results suggest that reduced spectrin E2/E3 activity is an important determinant of sickle cell severity. Am. J. Hematol. 79:89–96, 2005. © 2005 Wiley-Liss, Inc.

Key words: spectrin; ankyrin; E2 conjugating activity; E3 ligating activity; ubiquitin; sickle cell disease; sickle cell disease severity

INTRODUCTION

Sickle cell anemia patients have moderate to severe hemolytic anemia and frequent painful vaso-occlusive crises. Homozygous sickle cell anemia (SS) affects about 72,000 Americans, predominantly those of African ancestry. Physicians and researchers have sought explanations of the variability associated with the clinical expression of sickle cell disease (SCD). The most likely causes of this variability are disease-modifying factors. These factors include the hemoglobin F (HbF) content, red-cell membrane abnormalities, and increased adhesion to endothelium and white blood cells [1].

Sickle red blood cells (RBCs) contain a single point mutation causing substitution of a valine instead of a glutamic acid in residue 6 of β-globin. This leads to Hb S polymerization under deoxy conditions [2,3]. In addition to polymerization of hemoglobin S, hemoglobin S is unstable [4–6]. Heme and iron are released from hemoglobin S and deposit on the cytoplasmic surface of the red blood cell membrane [7–9]. SS RBCs produce membrane-associated hemin secondary to repeated formation of sickle hemoglobin polymers. This hemin (free ferriprotoporphyrin IX (FP) moieties) in oxyhemoglobin is oxidized from the Fe(II) state to the Fe(III) state with the consequent production of a half-molar equivalent of H_2O_2—a potentially toxic molecule [10,11]. Alternative detoxication pathways, including the binding to FP-binding proteins, reaction with glutathione, and FP degradation, may also contribute to FP detoxification [12]. However, if even a small amount (e.g., 0.5%) of

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*Correspondence to: Steven R. Goodman, 2601 N. Floyd Rd., FO 3.702, Richardson, TX 75080. E-mail: sgoodmn@utdallas.edu

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the FP escapes the neutralization process, membrane-associated hemin could cause redox damage and can oxidize membrane lipids (lipid peroxidation) and proteins [12,13]. Intact sickle RBCs spontaneously generate approximately twice the normal amount of superoxide [7]. The tripeptide glutathione (GSH) is the most prominent antioxidant to maintain the redox equilibrium. The reduced glutathione (GSH) is below detectable levels in the highest-density sickle cells, which are primarily irreversible sickle cells [14]. A high GSSG/GSH ratio causes glutathiolation of cysteines of several proteins including α spectrin [15; Shah and Goodman, unpublished results].

Ubiquitin is a small protein of 8.6 kDa molecular mass. When polyubiquitin is attached to target proteins, they are tagged for destruction by cytoplasmic organelles called proteasomes [16]. We now know that ubiquitination of target proteins also regulates functions as diverse as the sorting of proteins to different intracellular destinations, cell signaling, cell division, gene transcription, and protein–protein interactions [17]. The ubiquitination of target proteins requires a cascade of enzymes: E1—ubiquitin activating enzyme; E2—ubiquitin conjugating enzyme; and E3—ubiquitin ligating enzyme [18]. The activity of E1, E2, and E3 enzymes are downregulated by increased GSSG/GSH redox states due to glutathiolation of active site cysteines [19,20].

The membrane-associated cytoskeleton is a two-dimensional network located on the cytoplasmic surface of the plasma membrane [21–29]. It consists mainly of flexible rod-shaped tetramers of spectrin that are joined by actin protofilaments to form five- or six-sided polygons. There are two ubiquitination domains within α spectrin: one in repeat 17 (domain IV) and the other in repeats 20/21 (domain V) [30–33]. The α 20/21 repeat is a nucleation site for heterodimer formation and is associated with protein 4.1 and adducin-binding sites on β spectrin. Protein 4.1 and adducin form ternary complexes with spectrin and actin, and diminished ubiquitination of spectrin leads to slow dissociation of these ternary complexes [34,35].

Ankyrins are protein adaptors that bridge between spectrin and transmembrane proteins involved in ion transport [36], cell adhesion [37], and membrane trafficking [38]. One ankyrin R molecule associates a spectrin $\alpha_2\beta_2$ tetramer to band 3 [36,39–43].

We have demonstrated that erythrocyte spectrin possesses chimeric E2/E3 ubiquitinating activity in its α subunit and can ubiquitinate itself [33] as well as ankyrin [44] and band 3 [45]. We have furthermore demonstrated that the E2/E3 cysteines are residues 2071 and 2100, both of which can accept ubiquitin from an E1 enzyme and transfer it to a site within the C-terminal domain of α spectrin [46, 47]. As an E2 enzyme, α spectrin forms an intermediate DTT-sensitive ubiquitin adduct (α′ spectrin) and transfers ubiquitin to the target proteins. We found that SS erythrocytes have substantially diminished levels of α′ spectrin [48]. To understand the basis for reduced ubiquitination of SS erythrocyte membrane proteins in these studies, we compared (i) the ability of normal and sickle erythrocyte spectrin to ubiquitinate ankyrin; (ii) the ability of normal and sickle erythrocyte ankyrins to be a target for spectrin's ubiquitinating activity; and (iii) studied the correlation between the ubiquitinating activity and the content of α′ spectrin in normal and sickle erythrocytes from SS patients of varying severity.

MATERIALS AND METHODS

Red Blood Cell Membranes, Inside-Out Vesicles, and Purification of Spectrin

Human red blood cell membranes were prepared from seven healthy donors (control, AA) and 10 homozygous (SS) sickle cell patients. Blood samples were obtained at the UT Southwestern Comprehensive Sickle Cell Center after receiving informed consent. RBC membrane preparation and isolation of purified spectrin was performed as described [33]. Human red blood cell inside-out vesicles (IOV), depleted of spectrin and actin, were prepared as described [49]. The isolated spectrin was homogenous as indicated by SDS-PAGE [50].

Biotinylated–Ubiquitination Assay

The 60 μL incubation mixture including 75 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 6.0 μg spectrin, 0.1 μM E1 enzyme (Calbiochem, La Jolla, CA), 30 μg human RBC IOVs, 8.3 μM biotinylated ubiquitin, an ATP-generating system (3 mM ATP, 10 mM creatine phosphate, and 10 units/mL creatine phosphokinase), 1 μM leupeptin, 1 μM pepstatin, and 0.2 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) was incubated at 37°C for 2 hr. The incubation mixture was solubilized in loading buffer (1% SDS, 1 mM EDTA, 10% sucrose, 0.4 mg/mL pyronin Y, 10 mM Tris-HCl, pH 8.0) in the presence of 32 mM DTT for 20 min at 37°C. The solubilized proteins were analyzed by SDS-PAGE on a 5.5% polyacrylamide gel (14 × 16 cm) followed by Coomassie blue staining or electroblotting onto a nitrocellulose membrane. For detecting target protein (ankyrin) conjugated to biotinylated ubiquitin, streptavidin-horseradish peroxidase (HRP) (Amersham-Pharmacia, Buckinghamshire, England) was used at 1:3,000. The blot was then stripped with 50 mL of glycine buffer (200 mM glycine, 0.1% SDS, 0.01% Tween-20, pH 2.2) for 2 hr at room temperature and reprobed with antibody against the target protein

at 1:2,000 (anti-ankyrin, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Secondary antibody was used at 1:2,000 (sheep anti-mouse immunoglobulin-HRP). Proteins were detected by enhanced chemiluminescence (ECL) in a ChemiDoc XRS system (Bio-Rad, Hercules, CA). Each ubiquitinated ankyrin band, indicated by an arrowhead on the streptavidin-HRP blot, was quantified with ImageQuant, v 5.0 (Molecular Dynamics, Sunnyvale, CA). The total ankyrin was quantified in the same membrane area on the anti-ankyrin blot as ubiquitinated ankyrin was quantified on the streptavidin-HRP blot. The ratio of ubiquitinated ankyrin to total ankyrin was calculated.

Determination of α′/α Spectrin Ratio of Purified Spectrin

Spectrin (24 μg) was solubilized in loading buffer in the absence of DTT for 20 min at 37°C. The solubilized spectrin was analyzed by SDS-PAGE on 5.5% polyacrylamide gels (5.5 × 8 cm) followed by Coomassie blue staining. The α′ and α spectrin bands were quantified with a laser densitometer (Model LKB 2222–020 UltraScan XL, LKB, Bromma, Sweden), and the α′/α spectrin ratio was calculated. The values are the means of three different measurements ± standard error.

Determination of α′/α Spectrin Ratio of RBC Membranes

Human red blood cell membranes were prepared from 6 healthy donors and 14 sickle cell patients. RBC membrane proteins (15 μg) from control and sickle cell patients were run on 7% polyacrylamide SDS-PAGE minus reducing agent. After the gel was stained with Coomassie blue, the α′/α spectrin ratio was quantified with a laser densitometer (Model LKB 2222–020 UltraScan XL, LKB).

Definition of Sickle Cell Severity Index

Potential subjects for this study were evaluated at the Southwestern Comprehensive Sickle Cell Center by two different severity rating scales. Both scales rank individuals as having either mild, moderate, or severe SCD. The subjective scale involves clinical judgment and was performed by a panel of three experienced sickle cell disease clinicians (one pediatric hematologist and two nurse practitioners). This panel used personal experience with the patient, knowledge of psychosocial factors, and the review of medical records to derive the subjective ranking. The objective scale incorporates three observations: (1) steady-state hemoglobin concentration, (2) lifetime and recent vaso-occlusive complication rates, and (3) the presence of SCD-related chronic organ damage. Two experienced SCD clinicians (pediatric hematologists) rank potential subjects using the objective scale. All subjects involved in this study demonstrated agreement between the subjective and objective scales. Subjects were ineligible for the study if they had received any red blood cell transfusions or the medication hydroxyurea in the previous 4 months. The basis for these severity rating scales can be found in Quinn et al. [51].

Statistical Analysis

Data presented for each sample are the mean of five different experiments ± standard error (SE) in the biotinylated ubiquitin assay and the mean of 3 different experiments ± standard error for the α′/α spectrin ratio. We used Student's unpaired *t*-test to characterize the differences between two sets of values. The significance level of $P < 0.05$ was accepted.

RESULTS

We have demonstrated that ankyrin is a target of spectrin's E2/E3 ubiquitin conjugating/ligating activity [44]. In this study, we wanted to determine whether (i) there is a difference between sickle cell and normal spectrin's E2/E3 activity as measured by the transfer of ubiquitin to normal ankyrin; (ii) there is a difference in the ability of AA spectrin to transfer ubiquitin to AA versus SS ankyrin; (iii) there is a relationship between the α′/α spectrin ratio and the ability of spectrin to transfer ubiquitin to ankyrin; and (iv) there is a relationship between the α′/α spectrin ratio in the RBC membranes and sickle cell severity.

The ubiquitination assay was carried out as described in Materials and Methods [44]. We used biotinylated ubiquitin as a source of ubiquitin. Spectrin and IOVs derived from different samples of control (normal) and sickle erythrocytes were used as sources of ubiquitinating enzyme and target protein (ankyrin), respectively. Quantity of ubiquitinated ankyrin normalized to total ankyrin was used as a measure of ubiquitinating activity. A typical experiment is presented in Fig. 1.

Sickle Cell Spectrin Has Little E2/E3 Activity

We investigated ubiquitinating activity of sickle spectrin in comparison with control spectrin. The activity of spectrins derived from 10 different patients was analyzed. IOVs derived from control RBCs were used as a source of target protein (ankyrin). The results are presented in Fig. 2. The value for each sample is the mean of five experimental values. The activity in the mixture with control IOVs and spectrin (lane 1) was taken as 100%. Activities of all sickle

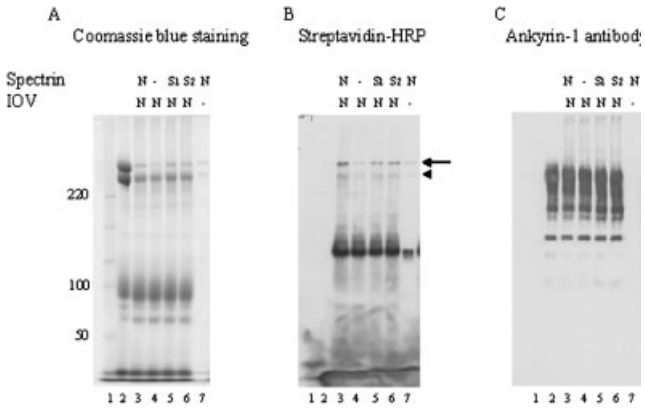

Fig. 1. Ubiquitination of ankyrin by normal and sickle cell spectrin in vitro. In addition to standard components (see Materials and Methods), 60 μL of incubation mixture contained 30 μg of IOVs and/or 6 μg of spectrin derived from different samples of normal or sickle erythrocytes (S1, S2) as indicated. After incubation for 2 hr at 37°C, the reaction products were analyzed by SDS-PAGE on a 5.5% polyacrylamide gel followed by Coomassie staining (A) or Western blotting (B,C). The blot was probed with streptavidin–HRP at 1:3,000 dilution (B) to detect ankyrin conjugated to biotinylated ubiquitin; then the blot was stripped and reprobed with anti-ankyrin at 1:2,000 dilution (C). The secondary antibody was used at 1:2,000 dilution. Lane 1, molecular weight standards; lane 2, RBC membrane proteins; lane 3, incubation mixtures containing normal IOVs and spectrin; lane 4, normal IOVs; lanes 5 and 6 respectively, normal IOVs and spectrin from two different S1 and S2 sickle cell samples; lane 7, normal spectrin. Proteins were detected by ECL and quantified with ImageQuant. The ratio of ubiquitinated ankyrin to total ankyrin was calculated. The arrow indicates the ubiquitinated α spectrin band. The arrowhead indicates ubiquitinated ankyrin.

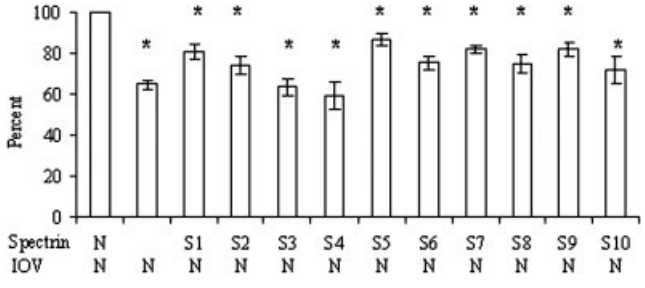

Fig. 2. Ubiquitination of ankyrin by sickle cell spectrin. Ubiquitination was carried out as described in Fig. 1. Lane 1, control spectrin plus control IOVs; lane 2, only control IOVs; lane 3–12, control IOVs plus spectrin from ten different samples (S1–S10) of sickle cell RBCs. The values for each mixture are the means of 5 different measurements ± standard error (SE). The asterisk (*) indicates values where difference from control value (lane 1) was statistically significant at $P < 0.05$.

spectrins (lanes 3–12) were decreased and varied from 86.9% ± 3.0% to 59% ± 6.5% of the control value. The differences were statistically significant at $P < 0.05$. Background activity in the mixture without spectrin (lane 2) was 64.9% ± 2.2% of control value (probably due to residual spectrin in control IOVs). Two samples (S3 and S4) are lower than control IOV minus spectrin (lane 2) at 63.5% ± 4.1% and 59% ± 6.5%. We can conclude that the ubiquitinating activity of sickle spectrin is significantly diminished as compared to control spectrin. Therefore, SS spectrin has diminished E2/E3 ubiquitin conjugating/ligating activity.

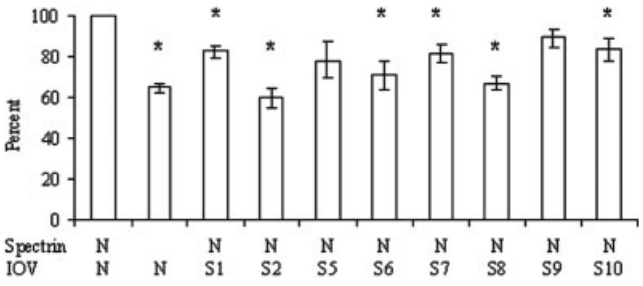

Fig. 3. Ubiquitination of sickle cell ankyrin by control spectrin. Ubiquitination was carried out as described in Fig. 1. Lane 1, control spectrin plus control IOVs; lane 2, only control IOVs; lane 3–10, normal spectrin plus IOVs from eight different samples of sickle cell RBCs. The values for each mixture are the means of five different measurements ± SE. The asterisk (*) indicates values where difference from control value (lane 1) was statistically significant at $P < 0.05$.

Sickle Cell Ankyrins Have Little Ability to Be Ubiquitinated by Spectrin

We investigated the ability of SS ankyrin to serve as a target for spectrin's ubiquitinating activity. IOVs derived from different samples of control and sickle erythrocytes were used as a source of the target protein ankyrin. Control spectrin was used as the ubiquitinating enzyme. The results are presented in Fig. 3. The values for each sample are the mean of five experimental measurements. The activity in the mixture with control IOVs and spectrin (lane 1) was taken as 100%. Activities in mixtures containing sickle IOVs (lanes 3–10) varied from 89.1% ± 4.0% to 59.6% ± 5.1% of control value. Decreases of the activity in all but two sickle cell subjects samples (lanes 5 and 9, samples S5 and S9) were statistically significant at $P < 0.05$. The activity in the mixture with control IOVs but without spectrin (lane 2) was 64.9% ± 2.2% of the control value.

In a similar series of experiments, a ubiquitination mixture contained both IOVs and spectrin derived from the same sample of control or sickle cell erythrocytes. These experiments were carried out to assess the impact of both sickle cell IOVs and spectrin on ubiquitinating activity. The results are presented in Fig. 4. The ubiquitination of ankyrin in control

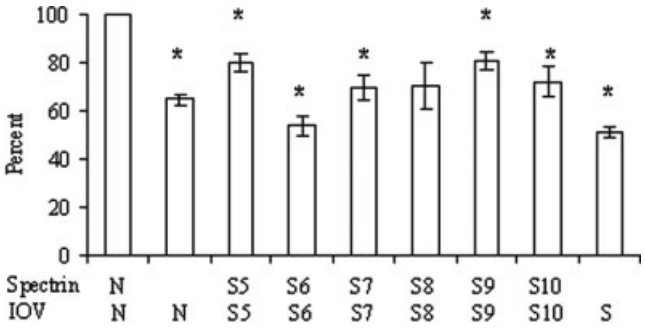

Fig. 4. Ubiquitination of sickle cell ankyrin by sickle cell spectrin. Ubiquitination was carried out as described in Fig. 1. Lane 1, control spectrin plus control IOVs; lane 2, only control IOVs; lanes 3–8, IOVs and spectrin from six different samples of sickle cell RBCs; lane 9, only sickle cell IOV. The values for each mixture are the means of five different measurements ± SE. The asterisk (*) indicates values where difference from control value (lane 1) was statistically significant at P < 0.05.

mixture (lane 1, normal IOVs and spectrin) was taken as 100%. The activity in the mixtures with sickle cell IOVs and spectrin (lanes 3–8) was decreased and varied from the 80.8 ± 3.7% to 53.9 ± 3.9% of the control value. The decrease in all sickle cell samples was statistically significant at $P < 0.05$. The background activity in the mixtures with normal (lane 2) or sickle cell (lane 9) IOVs alone were 64.9 ± 2.2% and 51.4 ± 2.0%, respectively.

In Fig. 5, we summarize the data presented in Figs. 2–4 for statistical purposes. The mean of all ubiquitinating activities obtained in the control mixtures with normal IOVs and normal spectrin (lane 1) was taken as 100%. The ubiquitinating activities in other mixtures were as follows: normal IOVs alone (lane 2), 64.9% ± 2.2%; normal IOVs plus sickle cell spectrin (lane 3), 75.1% ± 2.7%; sickle cell IOVs plus normal spectrin (lane 4), 76.6% ± 3.4%; sickle cell IOVs plus sickle cell spectrin (lane 5), 71.2% ± 4.0%; and sickle cell IOVs alone (lane 6), 51.4% ± 2.0%. Comparison of lane 3 (sickle cell spectrin plus control IOVs) and lane 4 (control spectrin plus sickle cell IOVs) to lane 1 (control spectrin plus control IOVs) revealed that the mean activity levels in both mixtures were significantly decreased; the difference was statistically significant at $P < 0.05$. This data shows that SS spectrin has very little E2/E3 activity and that SS ankyrin has little ability to accept ubiquitin from spectrin. It is important to note that, when spectrin and/or IOVs were derived from SS patients, the spectrin's E2/E3 activity was only slightly above the baseline values obtained from control IOVs alone (64.9%) (which is based on the roughly 5% residual spectrin which is not extracted from IOVs). Therefore, the E2/E3 activity of SS spectrin is minimal.

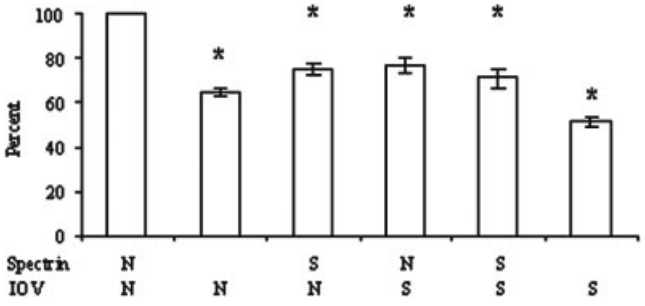

Fig. 5. Summary of ubiquitination of control or sickle cell ankyrin by control or sickle cell spectrin. The ubiquitination data obtained for IOVs and spectrins from different samples of control and sickle RBCs (presented in Figs. 2–4) were summarized. Lane 1, control IOVs plus control spectrin ($n = 7$); lane 2, control IOVs ($n = 7$) alone; lane 3, sickle cell spectrin ($n = 10$) plus control IOVs; lane 4, sickle cell IOVs ($n = 8$) plus control spectrin; lane 5, spectrin plus IOVs from the same sickle sample ($n = 6$); lane 6, sickle cell IOVs alone ($n = 3$). n is the number of analyzed samples. For each of n samples, 5 experimental measurements were obtained and the means of $n \times 5$ values were calculated. The control mean value (lane 1) was taken 100%. An asterisk (*) indicates the statistically significant difference at P < 0.05 as compared to control (lane 1) value.

Correlation Between the α′/α Ratio and Spectrin's Ability to Ubiquitinate Ankyrin

As a ubiquitin conjugating E2 enzyme, α spectrin forms an intermediate ubiquitin thioester adduct before ubiquitin is transferred to a target protein. We know homozygous (SS) sickle cells have diminished levels of α′ spectrin containing DTT-sensitive ubiquitin adduct [48]. We also demonstrated above that sickle spectrin has diminished ubiquitinating activity. Therefore, one would expect correlation between the α′/α spectrin and spectrin's ubiquitinating activity.

We determined the α′/α ratio and ubiquitinating activity of spectrin derived from different samples of control and sickle erythrocytes. The results are presented in Fig. 6A. Panel B presents the relationship (linear regression) between spectrin's ubiquitinating activity and α′/α ratio. There is direct correlation between the two variables with $r^2 = 0.8229$. We concluded that the ubiquitinating activity correlates with the α′/α spectrin ratio.

Correlation Between Erythrocyte α′/α Spectrin Ratio and Severity of Sickle Cell Disease

We determined the α′/α spectrin ratio in normal ($n = 6$) and SS RBCs, collected from patients with mild ($n = 3$), moderate ($n = 6$), and severe ($n = 5$) SS syndrome. The data are presented in Fig. 7. Values of the α′/α spectrin ratios obtained for samples with mild (0.72 ± 0.03), moderate (0.65 ± 0.03), or severe (0.50 ± 0.03)

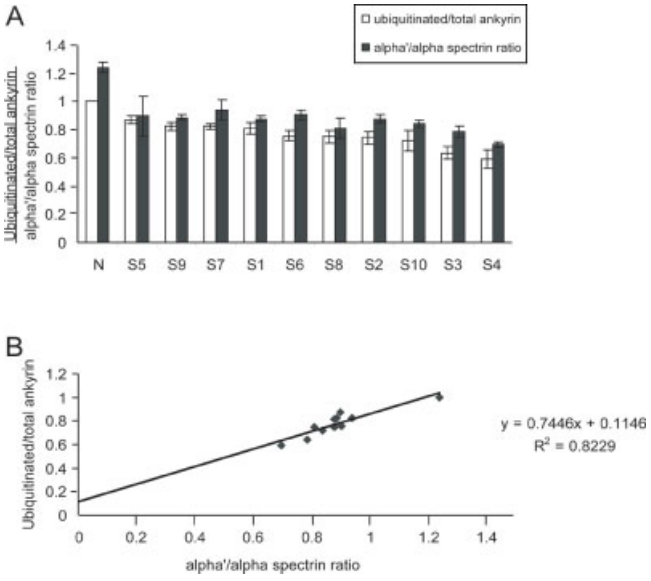

Fig. 6. Correlation between the α′/α spectrin ratio and spectrin's E2/E3 activity. (A) The ubiquitination assay was carried out as described in Fig. 1. In addition to control IOVs, mixtures contained control spectrin (lane 1), or spectrin from 10 different samples of sickle RBCs (lanes 2–11). The values for each mixture were the mean of 5 measurements. Control IOVs and spectrin (lane 1) was taken as 1. 24 μg of each spectrin sample was separated by SDS-PAGE, α′and α spectrin bands were quantified, and the α′/α spectrin ratio was calculated as described in Materials and Methods. The α′/α spectrin ratio for each spectrin sample is the mean of three measurements. (B) Linear regression analysis of data presented in panel A.

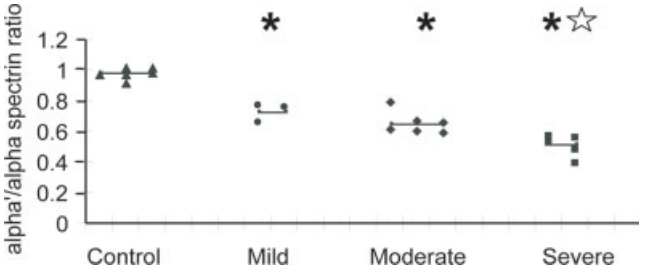

Fig. 7. Correlation between the α′/α spectrin ratio of membrane proteins and sickle cell severity. Membranes were prepared from blood samples collected from different healthy controls (*n* = 6, triangles), as well as patients with mild (*n* = 3, circles), moderate (*n* = 6, diamonds), and severe (*n* = 5, squares) sickle cell disease syndrome. The membrane proteins were separated by SDS-PAGE, α′ and α spectrins were quantified, and the α′/α spectrin ratio was calculated as described in Materials and Methods. *n* is the number of analyzed samples. For each of *n* samples, three measurements of α′/α ratio were obtained. The asterisk (*) indicates a statistically significant difference at *P* < 0.05 from control values. The star indicates a statistically significant difference at *P* < 0.05 from mild and moderate values.

SS syndrome were compared with each other as well as with control (0.97 ± 0.01) values at a significance level of $P < 0.05$. All sickle cell samples showed statistically significant decreases in the α′/α spectrin ratio as compared to control values. Samples with moderate SS syndrome showed lower levels of α′/α spectrin compared to mild SS syndrome, although the difference was not statistically significant. The lowest α′/α spectrin ratio was found in samples with severe SS syndrome. The difference between severe and mild or moderate SS syndrome was statistically significant.

Thus, (i) a decrease in the α′/α spectrin ratio is characteristic to sickle cell erythrocytes and (ii) severe sickle cell subjects have significantly decreased α′/α ratios as compared to mild and moderate SS clinical severity.

DISCUSSION

We demonstrated in this study that SS spectrin has diminished E2/E3 ubiquitin conjugating/ligating activity. The ubiquitin–spectrin E2/E3 thioester linkages occur through cysteine residues in α spectrin repeat 20 [33,46]. Sickle cells have very high GSSG/GSH ratios [14] resulting in increased glutathiolation of cysteines within RBC membrane proteins including α spectrin (Shah and Goodman, unpublished data), although we have not yet determined whether spectrin's chimeric E2/E3 cysteines (cys 2071 and 2100) are glutathiolated [46,47]. Therefore, the reduced SS spectrin's E2/E3 activity could be due to glutathiolation of the E2/E3 cysteines [29]. This may provide a rational explanation for the diminished α′/α spectrin ratios in SS erythrocytes [34,35,48].

We further demonstrate that SS ankyrin is a less efficient target for spectrin's E2/E3 activity than AA ankyrin. This is probably due to lower accessibility of the target lysines, which could also be linked to the increased oxidative stress within the cytoplasm of SS erythrocytes.

Thirdly, we demonstrated that reduced α′/α spectrin ratios correlate with reduced spectrin's E2/E3 activity. As α′ spectrin represents the ubiquitin–spectrin adduct, where ubiquitin is linked by a thioester linkage, it is entirely consistent that the lower amounts of α′ spectrin in SS erythrocytes would lead to diminished capacity to transfer ubiquitin to targets including itself and ankyrin.

Finally, we demonstrate an inverse correlation between the α′/α spectrin ratio and sickle cell severity. The more clinically severe sickle cell patients [51] have the lowest α′/α spectrin ratios and therefore the lowest spectrin's E2/E3 ubiquitin conjugating/ligating activity. We have recently demonstrated that reduced spectrin ubiquitination leads to tightened spectrin–4.1–actin and spectrin–adducin–actin ternary complexes that

dissociate poorly at 37°C [34,35]. This means that the most severe SS patients have the lowest levels of spectrin ubiquitination and, therefore, spectrin membrane skeletons that cannot disassemble or reassemble well at 37°C. In other words, the most severe SS patients would have SS RBCs that are the least flexible and capable of navigating the circulatory system. The result would be increased vaso-occlusion and related crisis.

In this regard, it is very interesting that *N*-acetylcysteine (NAC) can raise intracellular GSH levels in sickle cells and decrease the formation of irreversibly sickle cells and dense cells in vitro and in vivo [52,53]. In the phase II human NAC trial, 2,400 mg of NAC per day lowered the crisis rate by >60% [53]. One of the actions of NAC, via decreasing the GSSG/GSH ratio, would be to increase the ubiquitination of spectrin and other targets of its E2/E3 activity. The result would be a normalized membrane skeleton and an SS RBC with increased flexibility. This should also contribute to fewer vaso-occlusive events.

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REFERENCES

1. Hebbel RP, Boogaerts MAB, Eaton JW, Steinberg MH. Erythrocyte adherence to endothelium in sickle-cell anemia: a possible determinant of disease severity. N Engl J Med 1980;302:992–995.
2. Pauling L, Itano HA, Singer SJ, Wells IC. Sickle cell anemia, a molecule disease. Science 1949;110:543–548.
3. Ingram VM. A specific chemical difference between the globins of normal human and sickle cell hemoglobin. Nature 1956;178: 792–794.
4. Bunn HF, McDonald MJ. Electrostatic interactions in the assembly of haemoglobin. Nature 1983;306:498–500.
5. Mrabet NT, McDonald MJ, Turci S, Sarkar R, Szabo A, Bunn HF. Electrostatic attraction governs the dimer assembly of human hemoglobin. J Biol Chem 1986;261:5222–5228.
6. Shaeffer JR. Evidence for a difference in affinities of human hemoglobin beta A and beta S chains for alpha chains. J Biol Chem 1980;255:2322–2324.
7. Hebbel RP, Eaton JW, Balasingam M, Steinberg MH. Spontaneous oxygen radical generation by sickle erythrocytes. J Clin Invest 1982;70:1253–1259.
8. Hebbel RP, Morgan WT, Eaton JW, Hedlund BE. Accelerated autoxidation and heme loss due to instability of sickle hemoglobin. Proc Natl Acad Sci U S A 1988;85:237–241.
9. Hebbel RP. The sickle erythrocyte in double jeopardy: autoxidation and iron decompartmentalization. Semin Hematol 1990;27:51–69.
10. Browne P, Shalev O, Hebbel RP. The molecular pathobiology of cell membrane iron: the sickle red cell as a model. Free Radic Biol Med 1998;24:1040–1048.
11. Papalexis V, Siomos MA, Campanale N, et al. Histidine-rich protein 2 of the malaria parasite, *Plasmodium falciparum*, is involved in detoxification of the by-products of haemoglobin degradation. Mol Biochem Parasitol 2001;115:77–86.
12. Becker K, Tilley L, Vennerstrom JL, Roberts D, Rogerson S, Ginsburg H. Oxidative stress in malaria parasite-infected erythrocytes: host–parasite interactions. Int J Parasitol 2004;34:163–189.
13. Rank BH, Carlsson J, Hebbel RP. Abnormal redox status of membrane-protein thiols in sickle erythrocytes. J Clin Invest 1985;75:1531–1537.
14. Shartava A, McIntyre J, Shah AK, Goodman SR. The Gardos channel is responsible for CDNB-induced dense sickle cell formation. Am J Hematol 2000;64:184–189.
15. Mawatari S, Murakami K. Different types of glutathionylation of hemoglobin can exist in intact erythrocytes. Arch Biochem Biophys 2004;421:108–114.
16. Hershko A, Ciechanover A. The ubiquitin system. Annu Rev Biochem 1998;67:425–479.
17. Marx J. Ubiquitin lives up to its name. Science 2002;297: 1792–1794.
18. Haas AL, Siepmann TJ. Pathways of ubiquitin conjugation. FASEB J 1997;11:1257–1268.
19. Jahngen-Hodge J, Obin MS, Gong X, et al. Regulation of ubiquitin-conjugating enzymes by glutathione following oxidative stress. J Biol Chem 1997;272:28218–28226.
20. Sangerman J, Kakhniashvili D, Brown A, Shartava A, Goodman SR. Spectrin ubiquitination and oxidative stress: potential roles in blood and neurological disorders. Cell Mol Biol Lett 2001;6: 607–636.
21. Palek J, Lambert S. Genetics of the red cell membrane skeleton. Semin Hematol 1990;27:290–332.
22. Bennett V, Gilligan DM. The spectrin-based membrane skeleton and micron-scale organization of the plasma membrane. Annu Rev Cell Biol 1993;9:27–66.
23. Goodman SR, Shiffer K. The spectrin membrane skeleton of normal and abnormal human erythrocytes: a review. Am J Physiol 1983;244:C121–C141.
24. Goodman SR, Zagon IS. The neural cell spectrin skeleton: a review. Am J Physiol 1986;250:C347–C360.
25. Goodman SR, Shiffer K, Coleman DB, Whitfield CF. Erythrocyte membrane skeletal protein 4.1: a brief review. Prog Clin Biol Res 1984;165:415–439.
26. Goodman SR, Zagon IS, Riederer BM. Spectrin isoforms in mammalian brain. Brain Res Bull 1987;18:787–792.
27. Goodman SR, Krebs KE, Whitfield CF, Riederer BM, Zagon IS. Spectrin and related molecules. CRC Crit Rev Biochem 1988;23:171–234.
28. Goodman SR, Zimmer WE, Clark MB, Zagon IS, Barker JE, Bloom ML. Brain spectrin: of mice and men. Brain Res Bull 1995;36:593–606.
29. Goodman SR. The irreversibly sickled cell: a perspective. Cell Mol Biol 2004;50:53–58.
30. Bruce LJ, Beckmann R, Ribeiro ML, et al. Identification of ubiquitinated repeats in human erythroid alpha-spectrin. Eur J Biochem 2000;267:2812–2819.
31. Galluzzi L, Nicolas G, Paiardini M, Magnani M, Lecomte MC. Identification of ubiquitinated repeats in human erythroid alpha-spectrin. Eur J Biochem 2000;267:2812–2819.
32. Galluzzi L, Paiardini M, Lecomte MC, Magnani M. Identification of the main ubiquitination site in human erythroid alpha-spectrin. FEBS Lett 2001;489:254–258.

33. Kakhniashvili DG, Chaudhary T, Zimmer WE, Bencsath FA, Jardine I, Goodman SR. Erythrocyte spectrin is an E2 ubiquitin conjugating enzyme. Biochemistry 2001;40:11630–11642.
34. Ghatpande SS, Goodman SR. Ubiquitination of spectrin regulates the erythrocyte spectrin–protein 4.1–actin ternary complex dissociation: implication for the sickle cell membrane skeletons. Cell Mol Biol 2004;50:67–74.
35. Mishra R, Goodman SR. Ubiquitination of spectrin regulates the dissociation of the spectrin–adducin–F-actin ternary complex in vitro. Cell Mol Biol 2004;50:75–80.
36. Bennett V, Stenbuck PJ. The membrane attachment protein for spectrin is associated with band 3 in human erythrocyte membranes. Nature 1979;280:468–473.
37. Lokeshwar VB, Fregien N, Bourguignon LY. Ankyrin-binding domain of CD44(GP85) is required for the expression of hyaluronic acid-mediated adhesion function. J Cell Biol 1994;126: 1099–1109.
38. Devarajan P, Stabach PR, Mann AS, Ardito T, Kashgarian M, Morrow JS. Identification of a small cytoplasmic ankyrin (AnkG119) in the kidney and muscle that binds beta I sigma spectrin and associates with the Golgi apparatus. J Cell Biol 1996;133:819–830.
39. Batrukova MA, Betin VL, Rubtsov AM, Lopina OD. Ankyrin: structure, properties, and functions. Biochemistry (Mosc) 2000;65: 395–408.
40. Bennett V, Baines AJ. Spectrin and ankyrin-based pathways: metazoan inventions for integrating cells into tissues. Physiol Rev 2001;81:1353–1392.
41. Bennett V, Stenbuck PJ. Human erythrocyte ankyrin. Purification and properties. J Biol Chem 1980;255:2540–2548.
42. Cianci CD, Giorgi M, Morrow JS. Phosphorylation of ankyrin down-regulates its cooperative interaction with spectrin and protein 3. J Cell Biochem 1988;37:301–315.
43. Yu J, Goodman SR. Syndeins: the spectrin-binding protein(s) of the human erythrocyte membrane. Proc Natl Acad Sci U S A 1979;76:2340–2344.
44. Chang TL, Cubillos FF, Kakhniashvili DG, Goodman SR. Ankyrin is a target of spectrin's E2/E3 ubiquitin-conjugating/ligating activity. Cell Mol Biol 2004;50:59–66.
45. Chang TL, Cubillos FF, Kakhniashvili DG, Goodman SR. Band 3 is a target protein of spectrin's E2/E3 activity: implication for sickle cell disease and normal RBC aging. Cell Mol Biol 2004;50: 171–177.
46. Hsu YJ, Zimmer WE, Goodman SR. Erythrocyte spectrin's chimeric E2/E3 ubiquitin conjugating/ligating activity. Cell Mol Biol 2004 (in press).
47. Hsu YJ, Goodman SR. Spectrin and ubiquitination: a review. Cell Mol Biol 2004 (in press).
48. Monteiro CA, Gibson X, Shartava A, Goodman SR. Preliminary characterization of a structural defect in homozygous sickled cell alpha spectrin demonstrated by a rabbit autoantibody. Am J Hematol 1998;58:200–205.
49. Tyler JM, Hargreaves WR, Branton D. Purification of two spectrin-binding proteins: biochemical and electron microscopic evidence for site-specific reassociation between spectrin and bands 2.1 and 4.1. Proc Natl Acad Sci USA 1979;76:5192–5196.
50. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–685.
51. Quinn CT, Rogers ZR, Buchanan GR. Survival of children with sickle cell disease. Blood 2004;103:4023–4027.
52. Gibson XA, Shartava A, McIntyre J, et al. The efficacy of reducing agents or antioxidants in blocking the formation of dense cells and irreversibly sickled cells in vitro. Blood 1998;91:4373–4378.
53. Pace BS, Shartava A, Pack-Mabien A, Mulekar M, Ardia A, Goodman SR. Effects of *N*-acetylcysteine on dense cell formation in sickle cell disease. Am J Hematol 2003;73:26–32.