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Preliminary Characterization of a Structural Defect in Homozygous Sickled Cell Alpha Spectrin Demonstrated by a Rabbit Autoantibody

Carlos A. Monteiro, Xunda Gibson, Archil Shartava, and Steven R. Goodman*
Department of Structural and Cellular Biology and USA Comprehensive Sickle Cell Center, University of South Alabama College of Medicine, Mobile, Alabama

We have identified a rabbit autoantibody that strongly reacts with the core membrane skeleton of control red blood cells, and does not react with low- or high-density sickle cell core skeletons upon indirect immunofluorescence. Western blot analysis of red blood cell membrane proteins, utilizing this autoantibody, indicated no reactivity to any protein when SDS-PAGE was conducted in the presence of the reducing agent, dithiothreitol. However when SDS-PAGE was performed on control red blood cell membrane proteins separated in the absence of dithiothreitol, the autoantibody specifically reacted with a high molecular weight polypeptide (apparent $M_r \cong 310$ kD) representing a DTT sensitive form of control α spectrin, which we refer to as α' spectrin. There was no staining of high density or low density sickle cell α or α' spectrin. This autoantibody should be an excellent tool for the fine mapping of structural change(s) in control vs. sickle cell α spectrin, and determination of whether the structural alteration effects spectrin dimer-tetramer interconversion and/or the spectrin-actin interaction. The modification in α spectrin, detected by this antibody, is very specific for homozygous SS α spectrin because sickle cell β^+ thalassemic α spectrin and sickle cell trait α spectrin react intensely with the autoantibody. Am. J. Hematol. 58:200–205, 1998. © 1998 Wiley-Liss, Inc.

Key words: sickle cell disease; membrane skeleton; spectrin; red blood cell

INTRODUCTION

Blood obtained from homozygous sickle cell (SS) patients can be separated by density gradient sedimentation into morphologically and physiologically distinct red blood cell (RBC) classes [1]. The highest density class of RBCs include primarily irreversibly sickled cells (ISCs) (60–85%) that retain a sickled shape in well-oxygenated blood where the sickle cell hemoglobin (HbS) is depolymerized. These ISCs represent up to 45% of the circulating RBCs in SS blood and they are poorly deformable, of short life span, and have lower levels of fetal hemoglobin (HbF) than do the reversibly sickled cells (RSCs) [2]. During the course of vasoclusion, the highest density class of RBCs are selectively trapped in the microvasculature [3,4]. The ISCs appear to block the narrowed lumen of vessels lined primarily with the more adherent lower density RSCs [5,6], a process that is thought to contribute to the painful sickle cell crisis.

Twenty years ago Lux and colleagues demonstrated that most red blood cells ghosts and membrane skeletons derived from ISCs retain the sickled shape [7]. The spectrin membrane skeleton is a two-dimensional meshwork of proteins that covers the cytoplasmic surface of the erythrocyte, and is responsible for its biconcave shape and properties of elasticity and flexibility essential for its circulatory travel [8]. The core components of the spectrin membrane skeleton, which are minimally required to maintain shape are spectrin, f-actin, and protein 4.1 [9,10]. RBC spectrin is primarily an $(\alpha/\beta)_2$ tetrameric flexible rod formed by head-to-head linkage of two α/β heterodimers [11]. cDNA sequencing of the α subunit

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*Correspondence to: Dr. Steven R. Goodman, Department of Structural and Cellular Biology, University of South Alabama, MSB 2042, Mobile, AL 36688.

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[12] and β subunit [13] have indicated molecular weights of 280 kD (α) and 246 kD (β), with ~106 amino acid repeat units throughout most of the length of the spectrin subunits. Spectrin tetramers bind actin filaments at both tail regions, thereby cross linking f-actin [14–16]. The actin binding domain is localized to an N-terminal region (alanine 47 to lysine 186) of β spectrin [17]. RBC actin protofilaments are 33–37 nm in length in control (AA) membrane skeletons, equivalent to a double-stranded helix with 14 actin monomers [16–20]. The membrane skeleton viewed by negative staining and electron microscopy, is primarily a hexagonal lattice [19] with actin protofilaments at the center and six corners of the hexagons, interconnected by spectrin tetramers. The spectrin-actin interaction is strengthened by the binding of protein 4.1 to the tails of the spectrin tetramers [20–22]. The spectrin membrane skeleton is attached to the membrane bilayer in two ways. Ankyrin binds to βspectrin ~20 nm from the junction of the heterodimers and also binds to the transmembrane protein-band 3 [23–27] and protein 4.1 binds to glycophorin C [28,8].

We have demonstrated that membrane skeletons derived from ISCs dissociate more slowly than do skeletons isolated from AA erythrocytes, and RSCs, at all temperature tested from 24° to 37°C [29,30]. We demonstrated that the slow dissociation of the ISC spectrin membrane skeleton was due to a post-translational modification in β-actin in which a disulfide bridge was formed between cysteine 284 and cysteine 373 [29]. Furthermore, we have isolated the cystine peptide KC (373) F-C (284) DVDIR from a tryptic digest of ISC β-actin [31]. Recently we have demonstrated that this structural defect in β-actin leads to an altered ISC actin-actin interaction, where actin forms aggregates when placed under polymerizing conditions [32]. The inability of the core skeleton to disassemble due to this modification in β-actin, suggests a reasonable basis for the slow remodeling of the ISC shape [33].

Two indirect pieces of evidence suggested that their may also be a functional modification in ISC spectrin. Liu et al. [34] have suggested that the spectrin dimer-tetramer equilibrium may play a role in permanent deformation of irreversibly sickled cells. They based their conclusion on the fact that RSCs could be converted to ISCs in vitro at 37°C, a temperature at which spectrin tetramer-dimer interconversion occurs in solution, but RSCs were not converted to ISCs at 13°C , a temperature where the tetramer-dimer interconversion does not occur. However, Platt and Falcone [35] have found SS spectrin self-association to be normal. During the course of our studies on the slow dissociation of the ISC membrane skeleton, we found that a ternary complex formed from ISC spectrin—AA 4.1—AA actin dissociated less efficiently at 37°C than the control ternary complex [29]. We concluded, at that time, that there was probably a defect in ISC spectrin in addition to the well-documented defect in ISC β-actin [29]. In the current study, we utilize a rabbit autoantibody to directly demonstrate a structural difference between control and sickle cell α spectrin. This modification is observed in α spectrin derived from both low- and high-density SS red blood cells, and therefore is probably found in both RSCs and ISCs.

METHODS

Preparation of Density Separated RBCs, Ghosts, and Core Skeletons

Blood (20–30 ml) was obtained by venipuncture from homozygous SS subjects and AA control subjects in vacutainer tubes containing 143 USP units of lithium heparin. SS blood was density separated on a Percoll-Renografin gradient as previously described [29] and cells taken from the 45% Percoll layer (light density) and 65–70% Percoll layer (high density) were removed without cross-contamination and washed two times in 10 mM $NaPO_4$, 150 mM NaC1, pH 7.6 (PBS). Packed RBCs were lysed in 30 ml of ice cold lysis buffer (5 mM $NaPO_4$, 1 mM EDTA, pH 7.6) and ghosts sedimented at 31,000*g* for 15 min at 4°C. This procedure was repeated until the pellet became white or light pink. Freshly prepared ghosts (1 vol) were incubated on ice for 1 hr in 9 volumes of 10 mM $NaPO_4$, 0.6 M KCl, 1 mM ATP, 1 mM DFP, 1% Triton X-100, pH 7.6, to prepare core skeletons.

Immunofluorescent Images of Core Skeletons

Skeletons were fixed, applied to polylysine coated slides, and immunofluorescence performed as previously described [29]. Blood was removed and serum prepared from a New Zealand white rabbit expressing autoantibody. Skeletons were stained with rabbit serum diluted 1:500 (with PBS) for 15 min at room temperature. After washing three times in 1% BSA in PBS, FITC-goat anti-rabbit IgG (1:100) was applied for 15 min at room temperature. Non-bound antibody was removed by three washes with PBS + 1% BSA. For double immunofluorescence, staining of membrane skeletons with Texas-Red phalloidin was performed according to the manufacturer's protocol (Molecular Probes, Inc., Eugene, OR). Briefly, fixed skeletons were incubated with 200 μl of a 1:40 dilution of Texas Red-X Phalloidin in PBS containing 1% BSA for 20 min at room temperature. The fluorescent skeletons were mounted and observed using a Zeiss (Thornwood, NY) 1M35 fluorescent microscope.

SDS-PAGE and Western Blotting

SDS-PAGE was performed according to Laemmli [36], utilizing a 9% polyacrylamide separating gel. Samples for SDS PAGE were solubilized in SDS solution (1% SDS, 10 mM Tris-CI, 1 mM EDTA, 10% sucrose, 200 μg/ml Pyronin Y pH 8.0) ± 32 mM DTT for

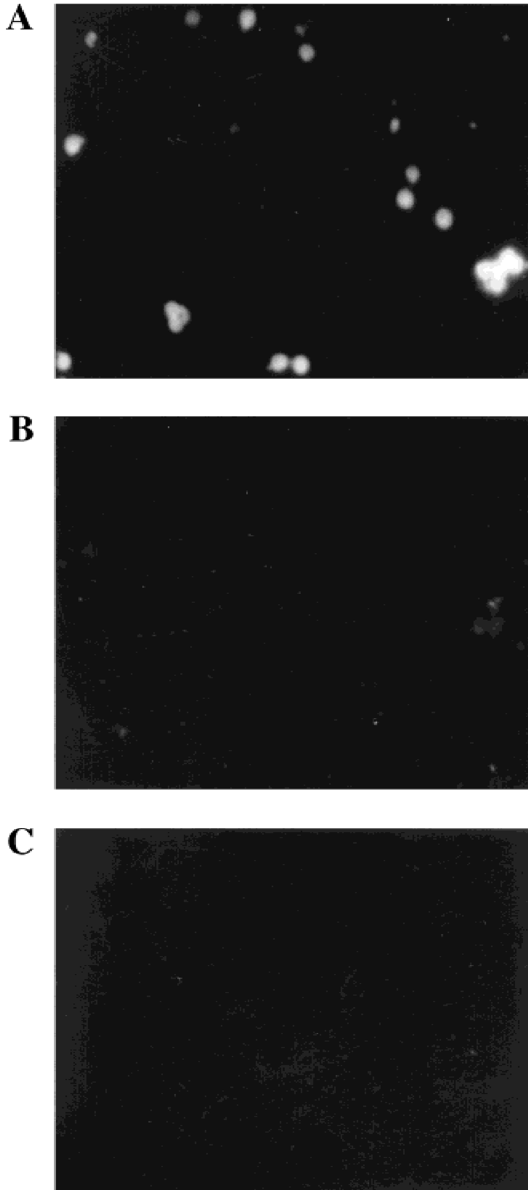

Fig. 1. Indirect Immunofluorescence of red blood cell core skeletons with the rabbit auto-antibody. RBC core skeletons associated with polylysine coverslips were stained with a 1:500 dilution of the rabbit autoantibody followed by FITC-goat antirabbit IgG at 1:100 dilution. A: Control AA core skeletons. B: Light-density SS core skeletons. C: High-density SS core skeletons. All skeletons were prepared in parallel.

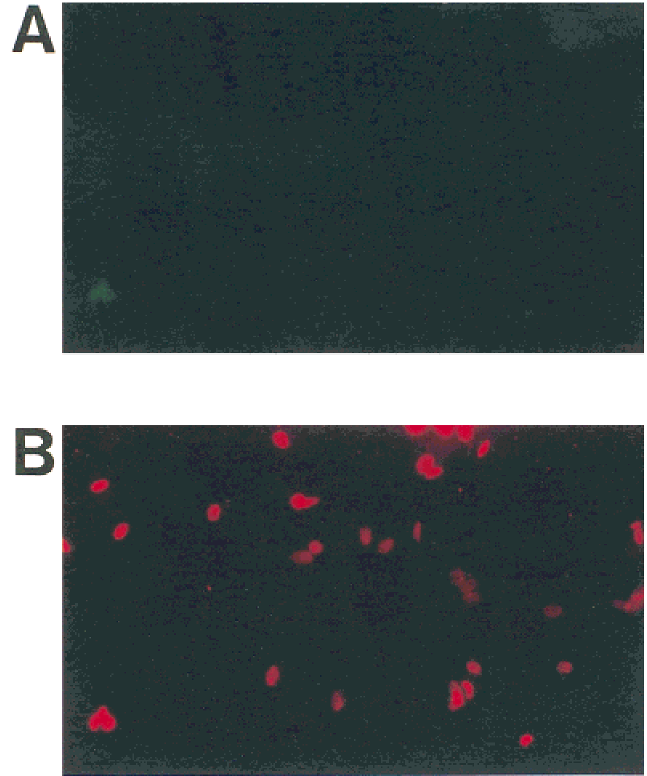

Fig. 2. Double immunofluorescent images of high-density SS core skeletons. High-density SS core skeletons were stained with a 1:500 dilution of rabbit autoantibody followed by FITC-goat anti rabbit IgG (1:100) and Texas Red phalloidin (0.1 μM). Filters were utilized that allowed observation of the FITC (A) and Texas Red (B) fluorescence of skeletons on the coverslip.

20 min at 37° C. Western blotting was performed by transferring protein from the gel to nitropure supported nitrocellulose paper (MSI, Westborough, MA) using a semi-dry system (Ideas Scientific, Minneapolis, MN) at 24 V for 6 hr. The membrane was rinsed in TBS (100 mM Tris, 150 mM NaCl, pH 8.0), and blocked in TBS + 2.5% milk + 0.1% triton X-100 for 2 hr at room temperature. The nitrocellulose membrane was then incubated overnight at 4°C with serum diluted 1:500 in blocking solution, washed three times with TBS + 0.1% triton X-100 and two times in blocking solution (30 min per wash). The blot was then incubated with 2.5 uCi I^{125} protein A (NEN Dupont, Wilmington, DE) in 25 ml of blocking solution. After a 2-hr incubation at room temperature, the membrane was washed as described above. Autoradiographs were prepared by 1-hr exposure at room temperature utilizing Kodak (Rochester, NY) Xomat-AR film.

RESULTS

During routine screening of preimmune serum from a New Zealand white rabbit, that we had intended to utilize for β-actin peptide specific antibody production, we discovered that the rabbit was producing autoantibodies that intensely reacted with control human AA core skeletons (Fig. 1A), and did not react with low- or high-density SS core skeletons (Fig. 1B and C). Double immunofluorescence experiments with the preimmune serum and FITC goat anti-rabbit IgG and Texas red phalloidin demonstrated bright staining of actin filaments in the high den-

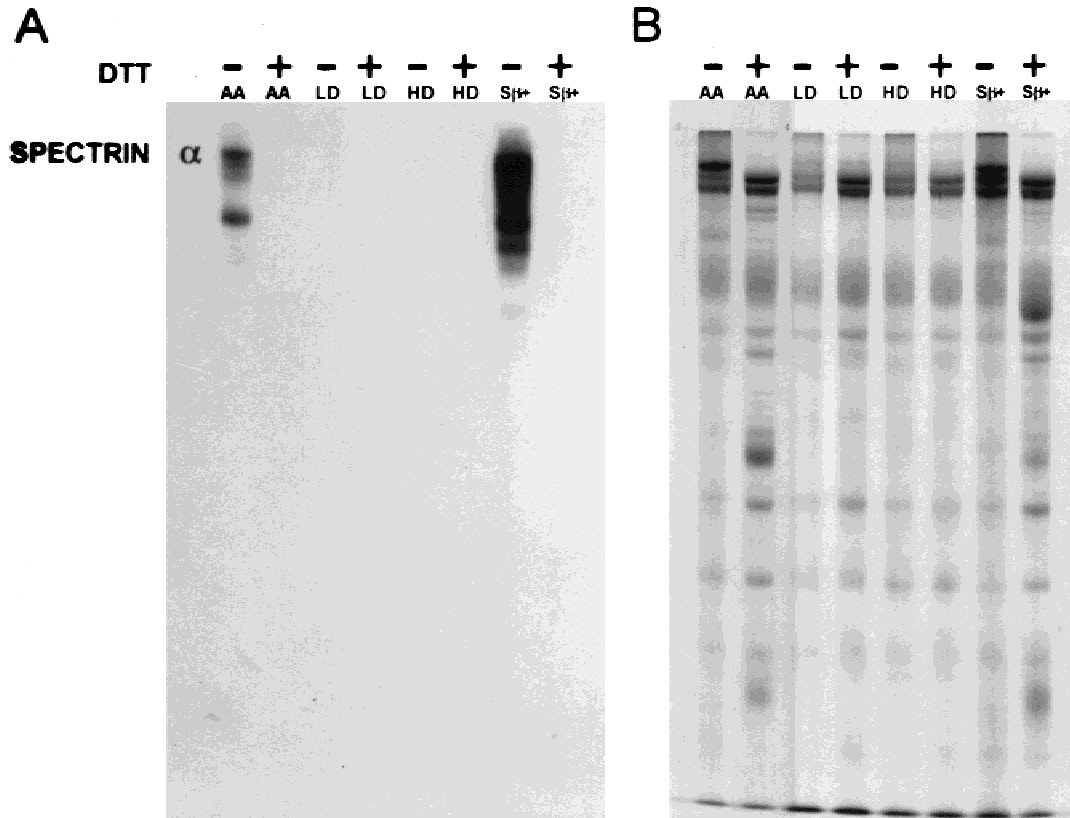

Fig. 3. Western blot analysis of red blood cell membrane protein stained with the rabbit auto-antibody. Erythrocyte membrane proteins (50 μg) were separated by SDS PAGE on a 9% polyacrylamide gel and proteins were stained with Coomassie blue (B) or the rabbit autoantibody at a 1:100 dilution plus ^{125}I protein A (A). Ghosts were solubilized in SDS buffer in the presence (32 mM) or absence of DTT. The lanes contain AA control ghosts, LDSS ghosts, HDSS ghosts, and Sβ^{+} ghosts from sickle cell β thalassemic patients. All ghost samples were prepared and analyzed in parallel.

sity SS core skeletons (Fig. 2B) but no staining with the rabbit autoantibody (Fig. 2A). This result demonstrated that the high-density SS core skeletons (Fig. 2B) were not staining with the rabbit autoantibody. High-density cells derived from five independent SS subjects all demonstrated no reaction with the rabbit autoantibody.

To determine the protein being recognized by the rabbit autoantibody, we performed Western blotting of rbc ghost protein derived from AA erythrocytes, low-density SS erythrocytes, and high-density SS erythrocytes (Fig. 3). To our surprise, when the SDS sample buffer contained 32 mM DTT, the autoantibody demonstrated no reaction with AA or SS ghost protein (Fig. 3A, + DTT). However, when the SDS sample buffer contained no DTT, there was a strong reactivity with a ~310 kD band that migrates slightly slower than AA α spectrin. The ~310 kD polypeptide derived from AA ghosts and sickle cell β thalassemic (Sβ^{+}) ghosts was stained intensely with the rabbit autoantibody (in the absence of reducing agent) (Fig. 3). Intense staining of this polypeptide was also observed upon Western blotting of sickle cell trait erythrocyte membranes with the rabbit autoantibody (data not shown). However, low- and high-density SS ghost protein showed no reactivity. To identify the ~310 kD polypeptide, we isolated control (AA), high-density (HD), and low-density (LD) SS erythrocyte membranes (Fig. 4). When the membrane proteins were electrophoresed by SDS PAGE in the absence of DTT, the ~310 kD polypeptide was present in the control sample (Fig. 4, lane D), but only to a minor extent in the HD and LD SS erythrocyte samples (see Fig. 3). Western blotting with antibodies against α spectrin (Fig. 4, lane A) and β spectrin (Fig., 4 lane B) demonstrate that the ~310 kD α$'$spectrin is derived from α spectrin. The rabbit autoantibody reacts exclusively with the control α$'$ spectrin (Fig. 4, lane C). We, therefore, conclude that we have a rabbit autoantibody, which recognizes an epitope present in control AA, Sβ^{+} and sickle cell trait alpha spectrin, which is not present in high- and low-density SS α spectrin. In addition, this epitope is sensitive to reduction of α$'$ spectrin by DTT.

DISCUSSION

We have utilized a rabbit autoantibody to demonstrate a structural difference between control α spectrin and SS

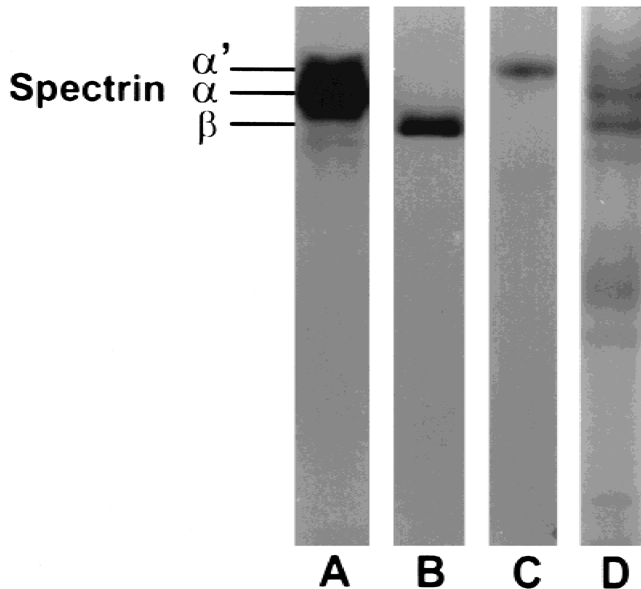

Fig. 4. Demonstration that the 310 kDa protein represents a modified version of α spectrin. Control erythrocyte ghosts, prepared in the absence of reducing agent, were solubilized in SDS buffer without DTT. SDS PAGE was performed on a 7% polyacrylamide gel in the absence of reducing agent. The protein was transferred to nitrocellulose paper and Western blotting was performed with (A) rabbit anti-human RBC α spectrin (1:5,000), (B) rabbit-anti human RBC β-spectrin (1:5,000), and (C) rabbit preimmune serum (1:500 dilution), which is reactive against control α′ spectrin. D: Coomassie blue stained lane of control erythrocyte membrane proteins in the absence of DTT. Note that α spectrin antibody stains both α′ spectrin and α spectrin (lane A), while β spectrin antibody reacts only with β spectrin (lane B).

α spectrin. In the absence of reducing agent, the autoantibody recognizes a DTT sensitive derivative of control α spectrin (called α′ spectrin) but not SS α or α′ spectrin. In the presence of reducing agent, the antibody no longer recognizes the control α spectrin. It is clear that a DTT sensitive modification of α spectrin exists in control erythrocytes, which is not present in sickle cell spectrin. The lack of this posttranslational modification occurs only in homozygous SS sickle cell disease, because heterozygous state $S\beta^+$ (for this patient HbS = 71%, HbA = 25%, HbA_2 = 4%) and sickle cell trait contain normal levels of α′ spectrin. It will be of substantial interest to determine whether the structural difference in SS α spectrin occurs in the 80 kD α-I N-terminal domain of spectrin and, therefore, influences the dimer-tetramer interconversion as suggested by Liu et al. [34]. Alternatively, the change could reside at the C-terminal tail end of α spectrin and influence the formation of the spectrin-4.1-f-actin ternary complex. While the actin binding domain resides within the region of 140 amino acids between alanine 47 and lysine 186 of β spectrin [17], the binding of protein 4.1 appears to require both subunits [8]. Therefore, a C-terminal structural change in SS α spectrin could impact upon protein 4.1 binding. This would be consistent with Shartava et al's [29] observation that ternary complex's formed from ISC spectrin, AA 4.1, and AA f-actin demonstrated slow dissociation when compared to the control ternary complex.

It will be of interest to determine the post-translational modification in control α spectrin, which leads to α′ spectrin. We know some facts concerning the modification that may prove helpful in its identification. The post-translational modification is eliminated by reducing agents (demonstrated in this publication), by boiling in SDS containing buffer, and by freeze thawing (Montiero, unpublished results). Our future studies will focus on identifying the modification, and determining the functional significance of its absence in SS erythrocytes.

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