

α -spectrin represents evolutionary optimization of spectrin for red blood cell deformability

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ABSTRACT Spectrin tetramers of the membranes of enucleated mammalian erythrocytes play a critical role in red blood cell survival in circulation. One of the spectrins, α I, emerged in mammals with enucleated red cells after duplication of the ancestral α -spectrin gene common to all animals. The neofunctionalized α I-spectrin has moderate affinity for β I-spectrin, whereas α II-spectrin, expressed in nonerythroid cells, retains ancestral characteristics and has a 10-fold higher affinity for β I-spectrin. It has been hypothesized that this adaptation allows for rapid make and break of tetramers to accommodate membrane deformation. We have tested this hypothesis by generating mice with high-affinity spectrin tetramers formed by exchanging the site of tetramer formation in α I-spectrin (segments R0 and R1) for that of α II-spectrin. Erythrocytes with α II β I presented normal hematologic parameters yet showed increased thermostability, and their membranes were significantly less deformable; under low shear forces, they displayed tumbling behavior rather than tank treading. The membrane skeleton is more stable with α II β I and shows significantly less remodeling under deformation than red cell membranes of wild-type mice. These data demonstrate that spectrin tetramers undergo remodeling in intact erythrocytes and that this is required for the normal deformability of the erythrocyte membrane. We conclude that α I-spectrin represents evolutionary optimization of tetramer formation: neither higher-affinity tetramers (as shown here) nor lower affinity (as seen in hemolytic disease) can support the membrane properties required for effective tissue oxygenation in circulation.

SIGNIFICANCE Spectrin tetramers, made by the association of pairs of $\alpha\beta$ -spectrin dimers, are essential for red blood cell function. α I β I-spectrin tetramer is a unique feature of highly deformable enucleated mammalian red blood cells; α II β I is a more stable tetramer found in other tissues. Here, we have developed a mouse model to investigate the connection between low-affinity red cell spectrin tetramer formation and cell deformability. Mice expressing the high-affinity α II-spectrin binding site for β I-spectrin have erythrocytes with lower deformability than wild-type. Lability of the α I β I-spectrin tetramer at the self-association site enables membrane skeleton remodeling in intact red blood cells and is an essential component of the red cell deformability.

INTRODUCTION

Mammalian red blood cells are extremely deformable; this is a requirement for effective tissue oxygenation and to survive the large deformations experienced as they transverse the vasculature. Structurally, the red blood cell membrane is a composite material in which the spectrin-based membrane skeleton is anchored to the lipid bilayer via two major

protein complexes: an ankyrin-based complex (containing, among other proteins, Band 3, Rh/RhAG, and protein 4.2) and an actin-based complex (containing, among other proteins, 4.1R, adducin, dematin, Band 3, and glycophorin C) (1–3). The deformability of the red blood cell is a combination of mechanical properties of the cell membrane and excess surface area relative to its volume. Although the structural organization of the various components of the red blood cell membrane is well described, the structural basis for its deformability is not fully understood (4).

Red cells undergo extensive elastic deformations during passage through the microvasculature while maintaining

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constant membrane surface area; the area dilation is energetically highly unfavorable. The principal resistance to area dilation (stretching) and bending is provided by the lipid bilayer, whereas the resistance to shear deformation is provided by the membrane skeleton that is anchored to the lipid bilayer (5).

Spectrin tetramers are the main structural component of the red blood cell membrane skeleton, consisting of two heterodimers of α I- and β I-spectrin interacting head to head at the dimer self-association site. Spectrins are polypeptides of multiple triple-helical repeats, 20 1/3 for α I-spectrin and 16 2/3 for the shorter β I-spectrin (6,7). At the self-association site, the single helix of the R0 repeat at the N-terminus of α I-spectrin interacts with the two helices of the R17 repeat at the C-terminus of β I-spectrin, forming a complete triple-helical repeat (8–10).

α I and β I erythrocyte spectrins are members of a family of proteins encoded by multiple genes in mammals; two encode α -spectrin, and five encode β -spectrin. All spectrins can form tetramers ($\alpha_2\beta_2$) by self-association of spectrin $\alpha\beta$ dimers (11). The relative strength of spectrin dimer interaction at the self-association site differs markedly between the erythroid α I- β I-spectrin and the abundant α II- β II-spectrin found in solid tissues, with α II- β II-spectrin tetramers being significantly more stable (11). The significance of the reduced strength of erythroid dimer self-association remains unclear; one current hypothesis is that it allows rearrangement of the membrane skeleton under the shear force experienced in the circulation (4).

Through phylogenetic analysis, Salomao et al. (12) showed that the gene encoding α I-spectrin is the result of duplication of the ancestral α -spectrin common to all animals. The duplication event was coincident with the emergence of mammals. One of the resulting pair, α II-spectrin, retained the ancestral characteristics of high thermal and mechanical stability (11), together with high-affinity binding to spectrin β -chains (11). The other of the pair, α I-spectrin, was neofunctionalized for highly deformable, enucleated erythrocytes; it has a more flexible structure deriving from the lower stability of some of its triple-helical repeats (12) and lower-affinity binding to β -chains (11). The precise nature of the physiological benefits of this neofunctionalization, the lower affinity for β -chains, and how they relate to the advantages of enucleated erythrocytes are not clear.

In this study, we explored the contribution of the binding strength of $\alpha\beta$ dimer at the self-association site to the biophysical properties of the red blood cell membrane. To achieve this, we generated a genetically modified mouse model in which the α I-spectrin self-association domain was exchanged for the α II-spectrin self-association domain. These mice are viable and do not present with significant anemia. Functional characterization of the genetically modified red cells showed that increasing the strength of the spectrin dimer self-association results in significantly reduced

membrane deformability, with marked reduction in deformation-induced remodeling of the spectrin skeleton of intact red cells. We conclude that α I-spectrin represents evolutionary optimization of spectrin dynamics to support the unique membrane requirements of mammalian erythrocytes in circulation.

MATERIALS AND METHODS

Generation of α II-spectrin knock-in mice

α II-spectrin knock-in (α II β I) mice were generated by the Ingenious Targeting Laboratory (Ronkonkoma, NY). Exons 1, 2, and 3 and part of exon 4 of the *Spta1* gene spanning an \sim 5.9 kb region were replaced with the exons 2 and 3 and part of exon 4 of *Sptan1*, spanning an \sim 4.9 kb region. The mice were initially generated as a 129 \times C57 hybrid and backcrossed for at least 10 generations into a C57BL/6 background. In experiments involving wild-type (WT) mice, pure C57BL/6 inbred mice were used. All animals were at least 3 months of age. Both sexes were used, and we did not observe differences between males and females. The mice were maintained at the animal facility of New York Blood Center under specific-pathogen-free conditions according to institutional guidelines. Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee.

Genotyping

Genomic DNA was extracted from mouse tails. Genotyping was performed by multiplex PCR. The α I-spectrin allele was amplified using primers: α I forward 5'-ATG GAA ACT CCA AAG GAA ACT GTG AGT A-3' and α I reverse 5'-GTC ATT CCA CAA GAG CAC AAT TTT TAG T-3'. Similarly, the α II-spectrin allele was amplified using primers: α II forward 5'-CAT TAT ACG AAG TTA TGG TAC CTG CAG-3' and α II reverse 5'-TCA ATT CCT AGT ACT CTG TCT GCC TCC-3'.

Generation of anti-spectrin antibodies

Polyclonal antibody against α I-spectrin N-terminus, α I-spectrin C-terminus, or α II-spectrin N-terminus was raised in rabbit using recombinant α I N-terminus or α I 20-C- or α II N-terminus as antigen. The antibodies were affinity purified on Sulfolink Coupling Resin (Pierce Biotechnology, Waltham, MA). Antibody specificity was examined by Western blotting using the corresponding recombinant protein.

Characterization of α -spectrin in α II β I mice red cells

The red blood cells (RBCs) of the α II β I mice were characterized by native gels and Western blot analysis. Ghosts prepared by lysis of RBCs in ice-cold hypotonic buffer A (5 mM Tris, 5 mM potassium chloride (pH 7.4)) were twice washed in the same buffer and twice more with extraction medium (buffer B: 0.25 mM sodium phosphate (pH 7.4)). Spectrin was extracted by overnight dialysis at 4°C in buffer B and recovered in the supernatant after centrifugation for 20 min at 20,000 \times g. Protein concentration was determined spectrophotometrically, taking E(280 nm; 1 mg mL⁻¹; 1 cm) = 1.07. The extracted spectrin was then examined by gel electrophoresis in the native state in a Tris-bicine buffer system, run in the cold (13) and stained with GelCode Blue. Membrane proteins from whole RBC ghosts separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 4% polyacrylamide gels and were transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA). Membranes were blocked for 1 h in PBS-T (PBS + 0.1% Tween 20) containing 4% nonfat milk and 1% BSA, followed by 1-h incubation with relevant α -spectrin primary

antibodies. After washing with PBS-T, the nitrocellulose membrane was incubated with the horseradish-peroxidase-conjugated secondary antibody (anti-rabbit). Immunoblots were visualized using the G:BOX gel imager (Imgen Technologies, New City, NY) by the enhanced chemiluminescence method.

Preparation of recombinant α II-spectrin fragment

The cDNA encoding the N-terminal region of nonerythroid α II-spectrin (amino acids 1–149) was expressed from the vector pGEX-2T, as described previously (11). The fragment was purified on a glutathione-Sepharose column, dialyzed, and concentrated using Centricon Plus-70 centrifugal filter units (Sigma-Aldrich, St. Louis, MO).

Incorporation of α II-spectrin fragment into erythrocyte ghosts

Total blood was obtained from WT or α II β I mice by cardiac puncture. RBCs were washed three times in ice-cold isotonic buffer and then lysed in 35 volumes of ice-cold buffer A to obtain ghosts. After three washes in 35 volumes of buffer A, ghosts (5×10^9 /mL) were incubated with the indicated concentrations of α II-spectrin fragment for 10 min on ice. Isotonicity was restored by adding 0.1 volume of 1.5 M potassium chloride (pH 7.4) for 10 min on ice, and ghosts were resealed by incubating them at 37°C for 40 min.

Extraction and analysis of spectrin from resealed ghosts

After resealing, the ghosts were lysed again in 35 volumes of buffer A. 10 μ L of packed ghosts was washed three times in isotonic saline and subjected to SDS-PAGE and Western blot with an anti-GST (anti-glutathione-S-transferase) antibody to confirm incorporation of the α II-spectrin fragment. The remaining ghost population was washed two additional times in ice-cold buffer A, and spectrin was then extracted in buffer B overnight at 4°C. After centrifugation ($20,000 \times g$, 20 min), the spectrin tetramer, dimer, and their complexes with the α II-spectrin fragment were resolved by gel electrophoresis in a Tris-bicine buffer system run in the cold (13).

Measurements of red cell deformability by ektacytometry

Blood (50–200 μ L) was drawn from mice via tail, submandibular vein (survival), or cardiac puncture (nonsurvival). RBC deformability was measured using an ektacytometer as previously described (14). RBCs were suspended in 4% polyvinylpyrrolidone (PVP, 360 kDa) PBS solution (5 mL PBS-PVP + 25 μ L whole blood) and deformed in a Couette viscometer, in which the outside cylinder is spun to obtain defined values of applied shear stress. The change in the laser diffraction pattern of the erythrocytes was recorded as they were subjected to increasing values of applied shear stress (0–30 Pa). The ratio of principal axes of the elliptically shaped diffraction pattern designated deformability index (DI) is a measure of the extent of cell deformation. The biomechanical origin of the shape of the DI curve is complex, but the reproducibility and certain characteristics are useful measures of the deformability of the red cell. At DI_{max} , the value of the DI attained at the maximal value of applied shear stress of 30 Pa is a measure of cellular deformability. Decreased values of DI_{max} reflect decreased surface area and hence increased sphericity of RBCs. As the rate of change of the DI at lower shear rates is a good indicator of the red cell membrane stiffness, illustrated best in the Eadie-Hofstee transformation of the DI curve (15), linear fitting of the Eadie-Hofstee transformed data allowed the maximal deformability

index (DI_{max}) and shear stress at half maximal deformation ($SS_{1/2}$) to be extracted (15,16).

Rheoscope imaging

Deformation of RBCs from WT and α II β I mice in Couette flow were observed and imaged in a transparent counter-rotating cone and plate rheoscope (17) (model MR-1; Myrenne Instruments, Rancho Cordova, CA) with a cone angle of 2°, mounted on a Leitz Diavert inverted microscope using a Leitz long-distance 32 \times (NA 0.4) objective (Leica, Wetzlar, Germany). 5 μ L whole blood in 1 mL 8% PVP-PBS solution was used, and a series of observations were made across the full speed range 1–43 RPM (revolutions per minute) of the instrument. The images were taken using a Nikon DS-Qi camera (Tokyo, Japan) with a 1-ms exposure.

Osmotic fragility analysis

5 μ L of packed RBCs was suspended in 2 mL of NaCl solutions of varying osmolarities and incubated for 20 min at room temperature. After low-speed centrifugation ($200 \times g$, 3 min), the absorbance of the supernatant was measured at 540 nm to quantify the released hemoglobin. The absorbance at 540 nm for the different osmolarities was normalized against complete red blood cell lysis using water (18).

Thermal sensitivity

Whole blood was washed three times in PBS, and 5 μ L of packed RBCs was suspended in 1 mL of PBS and 100 μ L aliquoted into 10 PCR tubes. The tubes were heated using a gradient capable PCR thermal cycler, and the block was heated to 45°C for 1 min and 45–50°C gradient for another 15 min. Heated cells were fixed by adding 100 μ L of 2% glutaraldehyde in PBS to each tube. The samples were examined for echinocytic and spherocytic cells by phase contrast microscopy and the numbers of normal and abnormal cells counted (19).

Blood analyses

Whole blood was collected from WT or α II β I mice using an EDTA-coated capillary from the retro-orbital sinus. Complete blood counts were obtained using the ADVIA 120 Hematology System (Siemens, Munich, Germany).

Optical microscopy

High-resolution images of intact RBCs from WT and α II β I mice were acquired using a Nikon DS-Qi camera mounted on a Nikon Ti-U inverted microscope with a Sorret band filter (413 nm, 10 nm) (Edmund Optics, Barrington NJ) (20).

Fluorescence-imaged micropipette deformation

The density distribution of proteins within the red blood cell membrane during deformation was measured using the combination of fluorescent imaging and micropipette aspiration as described by Discher et al. (21), with some additional modifications. Whole blood was washed three times in mouse-PBS (MPBS) (10 mM NaCl, 155 mM KCl, 10 mM glucose, 1 mM MgCl₂, 2.5 mM KHPO₄ (pH 7.4)) (22). Band 3 was chemically labeled using methods previously described (23). Briefly, 5 μ L of packed RBCs was incubated in 200 μ L of 80 μ g/ μ L Alexa Fluor 488 C₅ Maleimide (Life Technologies, Grand Island, NY) in MPBS for 40 min. They were washed three times in MPBS and then suspended at 1 μ L packed cells per mL MPBS + 0.1% bovine serum albumin. The labeled RBC suspension was injected into an imaging chamber consisting of a microscope slide and

coverslip separated by four layers of parafilm. Individual cells were aspirated into glass micropipettes with internal diameters between 1 and 2 μ m. The micropipettes were pulled from glass capillary tubing using a Sutter P-1000 micropipette puller (Sutter, Novato, CA) and cut to the required diameters using a home built microforge. The micropipettes were back filled using a Microfil capillary (World Precision Instruments, Sarasota, FL) with MPBS and connected to a manometer to control the aspiration pressure. The applied pressure was sufficient to aspirate the cell to the extent that the aspirated portion of the cell was maximal for the available surface area, and the proportion of the cell membrane outside the pipette was a sphere.

In this study, the aspiration length was adjusted by exchanging the suspending solution while individual cells were held in the micropipette. By using computer-controlled syringe pumps exchanging the PBS buffer between solutions of 250 and 150 mOsmol, we enabled the imaging of a single cell through a range of aspirated tongue lengths. Fluorescent images of the aspirated RBCs were acquired using a Nikon DS-Qi camera mounted on a Nikon Ti-U inverted microscope. All image analysis was done in ImageJ (24) and semiautomated with custom plugins. From measurements of the normalized aspiration length (L/R_p), the outside sphere radius (R_s), the normalized intensity profile along the aspiration, and the entrance intensity (ρ_e) and cap intensity (ρ_c) were computed.

Statistics

These data are obtained from at least three independent experiments using blood from at least three individual mice. Statistical significance was determined by two-tailed *t*-tests for unpaired samples with $\alpha = 0.05$. Data are expressed as the mean \pm SD (standard deviation); *p*-values > 0.05 were considered statistically significant and indicated by asterisks (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

RESULTS

Generation and characterization of α II β I knock-in mice

To assess the contribution of the binding strength of $\alpha\beta$ dimer at the self-association site on the mechanical properties of the red blood cell membrane, a knock-in strain of mice was generated in which the labile α I- β I-spectrin tetramer was replaced by a much higher-affinity α II- β I-spectrin tetramer by exchanging exons 2–4 of α I-spectrin (*Spta1*) with the corresponding exons of α II-spectrin (*Sptan1*). This results in mouse RBCs that express hybrid α -spectrin protein in the erythrocyte that has the N-terminus domains R0 and R1 from α II-spectrin in place of the N-terminus domains R0 and R1 from α I-spectrin. The R0 and R1 N-terminus domain interacts with the C-terminal domain of β -spectrin to form the heterodimer and one half of the spectrin tetramer, as illustrated in Fig. 1 A.

The genotypes of all mice were determined by PCR. Amplification with the primer pairs used produced a 340 bp fragment in WT mice and 700 bp fragment in the α II β I knock-in mouse. Although the 340 bp fragment was a feature of WT mice, the homozygous knock-in mice showed only the 700 bp fragment, whereas, as expected, heterozygous knock-in mice showed both fragments (Fig. S1).

Further validation of the knock-in of the hybrid α -spectrin was shown by SDS gel and Western blot analysis of red

blood cell membrane ghosts from WT and homozygous α II β I mice (Fig. S2). The overall patterns of proteins revealed in Coomassie blue-stained SDS gels (Fig. 1 B, left panel) appeared essentially identical between the strains, indicating that the knock-in does not result in any major change to overall protein composition of the membranes. The antibody to the N-terminus of α I-spectrin, but not the antibody to the N-terminus of α II-spectrin, recognized the spectrin of RBCs of WT mice (Fig. S2). In contrast, and as expected, only the antibody to the N-terminus of α II-spectrin, but not the antibody to the N-terminus of α I-spectrin, recognized the spectrin of RBCs of homozygous α II β I mice. Importantly, the antibody to the C-terminus of α I-spectrin recognized the spectrin in RBCs of both WT and α II β I mice. Thus, the knock-in strategy we used successfully generated a full-length hybrid α I-spectrin with the self-association domain of the N-terminus of α II-spectrin in the RBCs of α II β I mice.

Complete blood counts with an Advia 120 hematology analyzer (Siemens) demonstrated normal indices with no significant differences between WT and α II β I mice (Table S1). Although statistically significant differences in RBC count, hemoglobin concentration, and hematocrit are observed between the WT and α II β I mice, the difference is small and well within normal parameters for normal healthy mice and would only be considered to be very mildly anemic (25). In addition, both WT and α II β I mice presented similar erythrocyte morphology, both appearing as normal biconcave discocytes, as shown in the representative microscopy images in Fig. 1 B.

Previous studies have reported altered erythrocyte membrane deformability despite normal red cell parameters (26). The formation of spectrin dimers, tetramers, and higher-order oligomers in intact membranes was assessed by extracting the spectrin from isolated membranes under conditions that preserve its native state, i.e., using very low ionic strength media at 4°C. The resulting extracts were analyzed by nondenaturing gel electrophoresis (Fig. 1 C). As expected, the spectrin extracts from WT mice showed mainly tetramers with some dimers but without indication of higher-order oligomers. Extracts from α II β I mice showed similar tetramer composition but with no measurable dimers, instead showing a band indicating enhanced higher oligomer formation. Note also that the tetramer in the α II β I mice extracts has a faster electrophoretic mobility than WT, consistent with the genetic modification leading to a small change in the overall conformation of the tetramer.

Univalent fragments of α -spectrin, containing the dimer self-association site, are known to bind to spectrin on the membrane as tetramers undergo dynamic association and dissociation (5,12). If spectrin tetramers in intact membranes are more dynamic in WT than α II β I, then an exogenous α II-spectrin peptide fragment should incorporate more readily into WT than α II β I membranes. To test this, the α II-peptide was added to ghosts from WT or α II β I cells; the

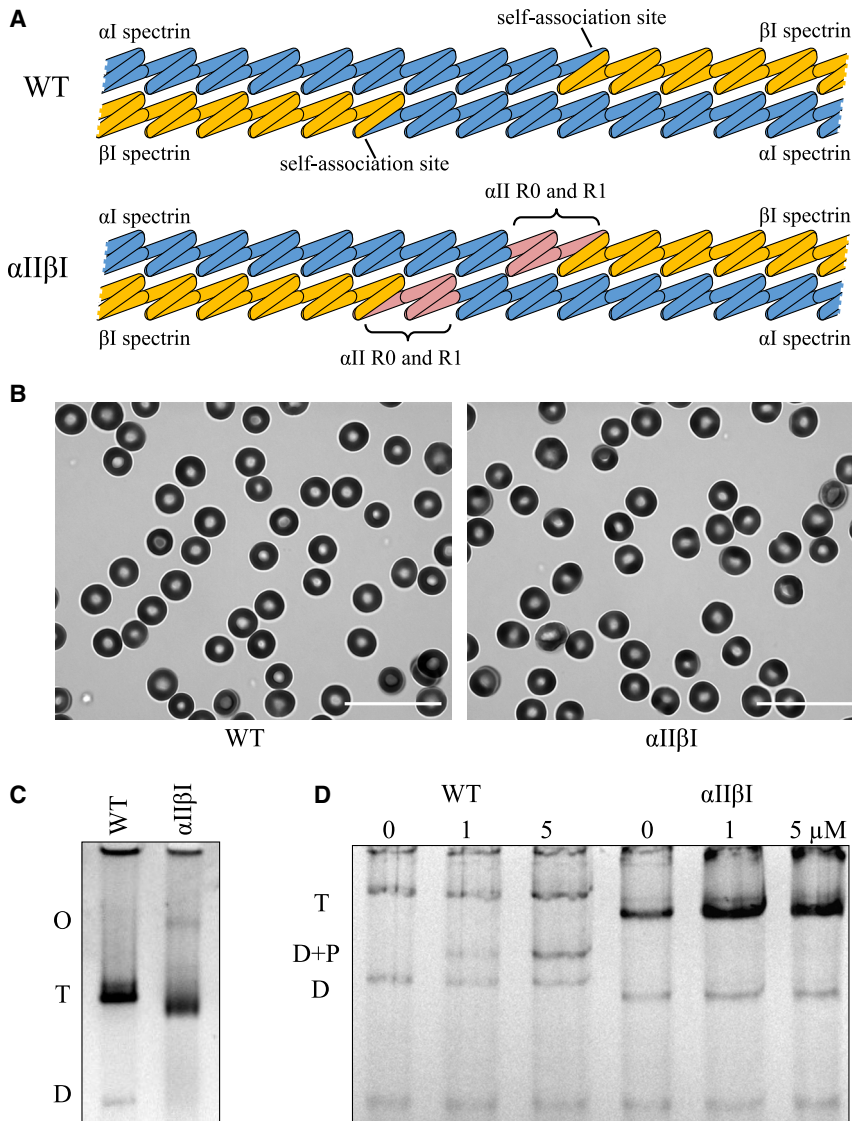


FIGURE 1 Development and initial analysis of the α II β I mouse model. (A) Partial domain structures of spectrin in WT and α II β I knock-in mouse showing self-association sites and the location of the substituted α II repeats. (Upper) WT spectrin with α I-spectrin on the left (blue) and β I-spectrin on the right (yellow). The interaction of α - with β -spectrin occurs between α -R0 and β I-R17. (Lower) α II β I knock-in mouse spectrin; red indicates α -spectrin R0 and R1 from α II, and R2–R4 from α I-spectrin are in blue. β I-spectrin is unchanged in this mouse model. (B) Brightfield microscopy of RBCs from WT and α II β I mice imaged using a Soret bandpass filter (413 nm, 10 nm). Scale bars, 20 μ m. (C) Analysis of the oligomerization state of spectrin in each mouse strain. Spectrin was extracted in low ionic strength medium at low temperature from erythrocytes from each mouse strain. The extracts were separated by nondenaturing gel electrophoresis and stained with Coomassie. The positions of spectrin dimers (D), tetramers (T), and higher oligomers (O) are indicated. (D) Increasing concentrations of α II-peptide resealed into RBC ghosts from WT and α II β I mice. T, tetramer; D + P, dimer-peptide complex; D, dimer. To see this figure in color, go online.

ghosts were resealed and incubated at 37°C to promote incorporation of the peptide. Fig. 1 D shows significant incorporation of the fragment into the spectrin of WT ghosts; by contrast, spectrin from α II β I incorporated little to none. This result is consistent with α II β I tetramers being much less labile than WT.

Surface area of α II β I and WT RBCs

The surface area of RBCs was measured geometrically using the micropipette aspiration method (27,28). For α II β I cells, it was $102.2 \pm 7.6 \mu\text{m}^2$, an $\sim 10\%$ loss compared to that of WT mice, $111.5 \pm 6.2 \mu\text{m}^2$ (Fig. 2 A). The osmotic fragility of RBCs was measured by exposure to hypotonic stress by suspending them in low ionic strength buffer solutions (Fig. 2 B). RBCs from α II β I mice show significantly less resistance to hemolysis with 50% lysis at 150 mOsm/

kg in contrast to 50% lysis at 130 mOsm/kg of RBCs from WT mice. Resistance to hemolysis is primarily defined by the cell surface area/volume ratio (cell sphericity) (29,30), and we conclude that increased osmotic fragility arises from the increased sphericity of α II β I cells, consistent with their decreased cell surface area.

Thermal sensitivity of α II β I knock-in and WT RBCs

Erythrocytes have been shown to have particular sensitivity to temperature (19,31). Although human RBCs maintain their discoid morphology upon heating to 48°C, increasing the temperature to 49–50°C induces rapid shape transition with membrane fragmentation and generation of spherocytes (19). Diluted suspensions of RBCs from α II β I and WT mice were heated to temperatures between 45 and 50°C. The RBCs from WT mice began to transform to

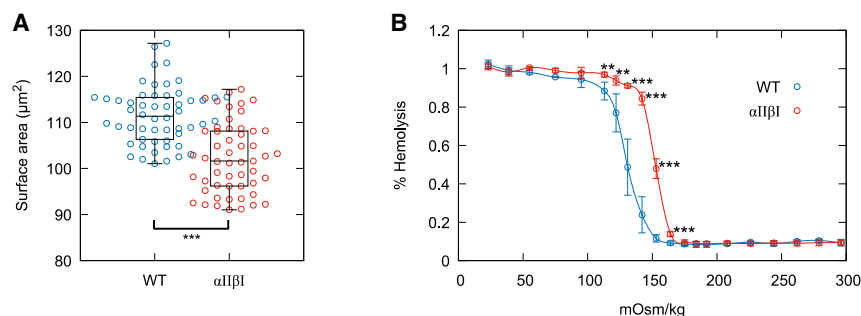


FIGURE 2 Effect of modified α II β I on membrane stability. (A) Surface area measured geometrically by micropipette aspiration. WT mean surface area = $111.5 \pm 6.2 \mu\text{m}^2$ ($n = 53$ cells), and α II β I = $102.2 \pm 7.6 \mu\text{m}^2$ ($n = 44$ cells), $\sim 8.3\%$ loss of surface area. (B) Osmotic fragility curves of WT ($n = 12$ mice) and α II β I ($n = 5$ mice) RBCs; error bars represent the SD and $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$ by unpaired Student's t -test. To see this figure in color, go online.

echinocytes (crenated) at 47°C , with significant crenations between 48 and 49°C , and were 100% spherocytic at 49°C (Fig. 3 A), with 50% of RBCs crenating at 48.4°C . In contrast, RBCs of α II β I mice did not start to crenate until $48\text{--}49^\circ\text{C}$, with significant crenation not occurring until $>48.5^\circ\text{C}$, with 50% crenated RBCs occurring at 49.5°C . At 50°C , α II β I RBCs did not reach 100% spherocyte cells. Shown in Fig. 3 B are brightfield images of heat-induced morphological changes in RBCs of both WT and α II β I samples between 47 and 49°C . These findings imply that the more stable dimer-dimer interaction of α II- β I bestows increased thermal resistance to RBCs of α II β I knock-in mice.

Deformability of RBCs as assessed by Couette flow

The effect of the modified α II- β I dimer on the deformability of RBCs was measured by ektacytometry (14). The maximal deformability index at the highest shear stress (DI_{max}) in the higher-suspending medium viscosity reached a plateau defined as DI_{max} , which is less for the α II β I mice samples ($DI_{\text{max}} 0.67 \pm 0.015$) compared to the WT samples ($DI_{\text{max}} 0.75 \pm 0.015$), as shown in Fig. 4 A (Student's t -test, $p = 4.2 \times 10^{-5}$). As the maximal DI should be only limited by the membrane surface area of the RBCs, provided the shearing does not result in cell fragmentation and loss of membrane area, the noted decrease in DI_{max} is the result of $\sim 10\%$ decrease in surface area of α II β I mice RBCs. Importantly, the peak DI of the α II β I cells did not reach a plateau at the maximal applied shear stress of the instrument in the suspending medium of lower viscosity (indicated in the Eadie-Hofstee transformed data shown in Fig. 4 B). The α II β I cells clearly need significantly greater shear force to deform to the same extent as the WT cells. For example, the WT cells reach half their maximal deformation ($SS_{1/2}$) at a shear stress of 1.13 ± 0.097 Pa, whereas the α II β I samples needed a shear stress of 8.54 ± 0.51 Pa (Student's t -test, $p = 2.9 \times 10^{-6}$) to reach the same extent of deformation, implying decreased membrane deformability (32). Additionally, at low shear stresses < 1 Pa, the α II β I cells did not undergo tank threading but instead showed DI values consistent with cell tumbling (33,34).

This behavior is further demonstrated in the images of the RBCs of deformed by the rheoscope in Fig. 4 C. In those, α II β I RBCs have significant resistance to shear deformation compared to WT mice. WT RBCs tank tread even at very low shear stress 0.4 Pa, whereas the α II β I RBCs require shear stress > 2 Pa to start tank treading.

Membrane skeleton density of deformed α II β I and WT as assessed by fluorescence-imaged microdeformation

Fluorescence-imaged microdeformation, developed by Discher et al. (21,23), is an effective way to determine the connectivity of membrane proteins such as Band 3 to the underlying membrane skeleton. The method typically involves imaging many cells suspended in isotonic buffer aspirated into micropipette with increasing aspiration pressures to determining the entrance (ρ_e) and cap (ρ_c) densities as a function of the aspirated cell length (Fig. 5 A). Changing the osmolality of the buffer in the chamber while the cell is aspirated in the micropipette enables the measurement of ρ_e and ρ_c at many aspiration lengths for each cell (Video S1). The ratio of ρ_e/ρ_c for Band 3 in the α II β I cells using this strategy showed a significantly steeper gradient than that seen for WT cells (Fig. 5 B). As the measured Band 3 density correlates to the membrane skeletal density distribution, this finding implies that increased affinity of spectrin interaction at the self-association site results in greater resistance to membrane skeleton remodeling. By fitting Eq. 1 below for the Band 3 density data obtained for each aspirated cell, where K is the area dilation modulus, μ the shear modulus, R_p the micropipette radius, and L the aspiration length, a value for the parameter (K/μ) can be derived. For the α II β I cells, the ratio of the area stretch/shear moduli (K/μ) was determined to be 1.10 ± 0.22 ($n = 14$) compared to 1.99 ± 0.32 ($n = 8$) for WT cells (Student's t -test, $p = 4.4 \times 10^{-5}$) (21,23).

$$\frac{L}{R_p} = \frac{K}{\mu} \left\{ \left(\frac{3}{4} \right) \left[1 - \left(\frac{\rho_c}{\rho_e} \right)^2 \right] + \left(\frac{\rho_c}{2\rho_e} \right) \ln \left(\frac{\rho_e}{\rho_c} \right) \right\} + \ln \left(\frac{\rho_e}{\rho_c} \right). \quad (1)$$

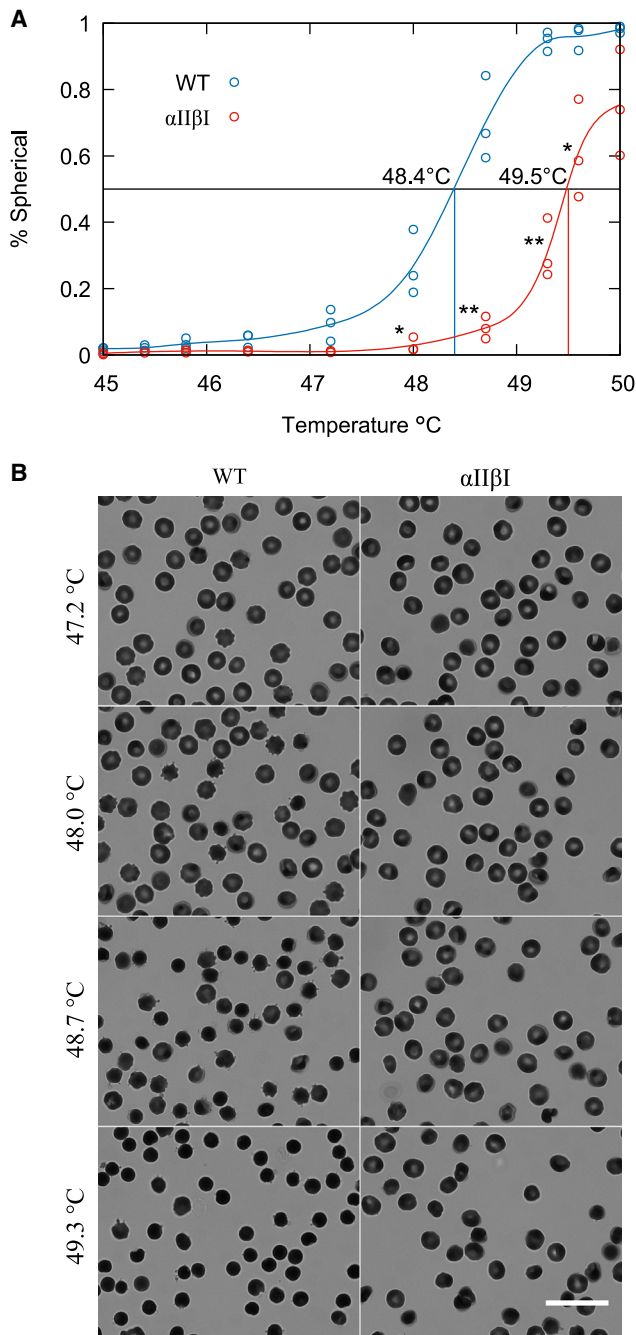


FIGURE 3 Thermal stability of red blood cells from WT and $\alpha\text{II}\beta\text{I}$ mouse. (A) The fraction of heat-induced spherical erythrocytes at various temperatures from 45 to 50°C. Dashed lines show the temperature at which 50% of red blood cells are spherical. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ by unpaired Student's t -test. (B) Brightfield microscopy of red blood cells from WT and $\alpha\text{II}\beta\text{I}$ mice imaged using a Soret bandpass filter (413 nm, 10 nm) after heating erythrocytes. Scale bars, 20 μm . To see this figure in color, go online.

In the absence or reduction of remodeling, the membrane skeleton undergoes a larger shear deformation before breakage of the spectrin self-association site, and this will be reflected by higher ρ_e intensity. Indeed, the intensity at

the micropipette entrance ρ_e is increased for all aspirated $\alpha\text{II}\beta\text{I}$ red cells compared to the WT cells, whereas the ρ_e intensity showed a much smaller difference between the two cell types (Fig. 6 A). This is a result of the difference in the dominant type of deformation of the membrane skeleton at the pipette entrance and cap of the aspirated cell. At the micropipette entrance, skeletal deformation is mainly due to shear deformation, whereas at the cap, the deformation is dominated by area dilation. ρ_e shows only a small difference intensity between the WT $\rho_e = 0.622 \pm 0.050$ compared to 0.525 ± 0.072 for the $\alpha\text{II}\beta\text{I}$ cells at $L/R_p = 6$ (Student's t -test, $p = 0.0016$). This indicates the maximal area dilation of the membrane skeleton is approximately the same, suggesting the maximal area extension of the membrane skeleton is constant, consistent with a spectrin tetramer length invariance between the two cell types. By contrast, ρ_e was significantly different between WT and $\alpha\text{II}\beta\text{I}$ cells; at $L/R_p = 6$, WT ρ_e intensity was 1.71 ± 0.054 , compared to 2.15 ± 0.098 for the $\alpha\text{II}\beta\text{I}$ cells (Student's t -test, $p = 0.0025$). This is consistent with a changed rate of remodeling spectrin tetramers in $\alpha\text{II}\beta\text{I}$ cells.

Estimates of the principal stretch ratios of the membrane skeleton can be determined from the relative membrane skeleton density using methods described previously (21,23,35). Briefly, by conservation of mass and considering the aspirated tongue to be an axisymmetric deformation of a portion of a spherical surface radius R_0 with uniform density, Eq. 2 can be used to estimate the principal stretch ratio λ_2 and then λ_1 .

$$\lambda_1 = 1/(\bar{\rho}\lambda_2)$$

$$\lambda_2(z') = \frac{r(z')}{R_p} \left\{ \left(\frac{R_0}{R_p} \right)^2 - \left[\left(\frac{R_0}{R_p} \right) - \left(\frac{1}{R_0} \right) \int_{pole}^{z'} \rho_{net} dz' \right]^2 \right\}^{-1/2}. \quad (2)$$

Because the intensity gradients of the aspirated portions of the cells are nearly perfectly linear, the mean ρ_e and ρ_c for a give aspiration length ($L = 6$) for each sample are used to model the ρ intensity profile to calculate the principal stretches shown in Fig. 6 B. Using this method, we find in WT cells $\lambda_1 = 2.18 \pm 0.069$, $\lambda_2 = 0.268 \pm 0.006$ at the pipette entrance and $\lambda_1 = \lambda_2 = 1.27 \pm 0.054$ at cap, whereas in $\alpha\text{II}\beta\text{I}$ cells $\lambda_1 = 1.86 \pm 0.080$ (Student's t -test, $p = 1.5 \times 10^{-10}$), $\lambda_2 = 0.251 \pm 0.008$ (Student's t -test, $p = 4.8 \times 10^{-7}$) at the pipette entrance and $\lambda_1 = \lambda_2 = 1.38 \pm 0.11$ (Student's t -test, $p = 0.003$) at cap. Findings from all these analyses are consistent with the hypothesis that spectrin tetramers undergo less remodeling in $\alpha\text{II}\beta\text{I}$ membranes than WT and that remodeling requires making and breaking tetramers. WT membranes are more

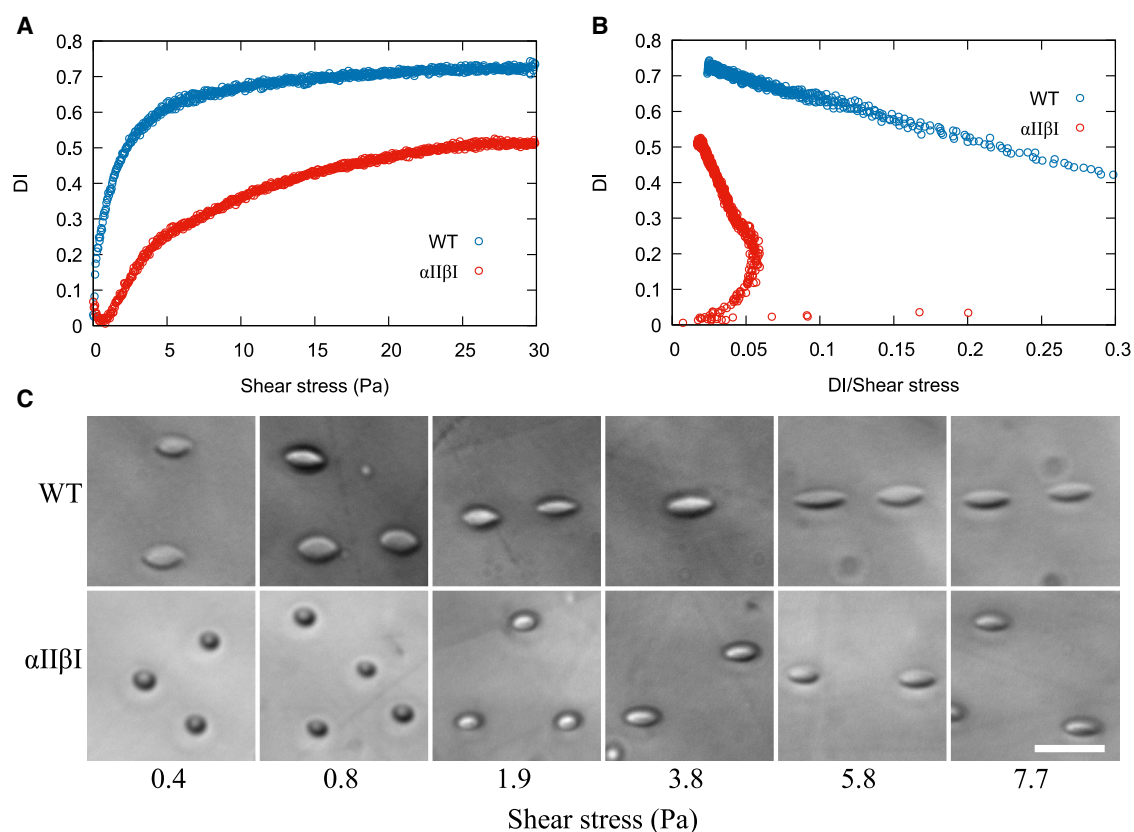


FIGURE 4 The deformability of WT and α II β I mouse red blood cells as a function of shear stress as measured by ektacytometry. WT sample is shown in blue and the α II β I shown in red. (A) The deformability index (DI) as a function of shear stress. (B) Eadie-Hofstee transformation of the ektacytometry data in (A), with the DI as a function of DI/(shear stress). (C) Rheoscope images of WT and α II β I red blood cells subject to increasing shear stress. Scale bars, 20 μ m. To see this figure in color, go online.

susceptible to deformation than α II β I membranes because remodeling can accommodate larger strain.

DISCUSSION

α I-spectrin emerged with the appearance of mammals and apparently coincident with the processes that allow enucleation of erythrocyte precursors and the circulation of mature enucleated erythrocytes. In earlier work, with fewer genomes than are now available, we noted the absence of α I-spectrin from all other vertebrates sequenced at that time (12). Subsequent sequencing of genomes of many more animals confirms this thesis. In particular, mammals are amniotes; the other amniotes are the birds and reptiles. Large numbers of bird and reptile genomes are now available; none of them encode α I-spectrin, although all encode the ancestral α -spectrin, which is retained in mammals as α II-spectrin.

These observations pose several questions about the physiological advantages obtained through the neofunctionalization of α I-spectrin. For example, is it part of, or required for, the process of enucleation? Alternatively (or in addition), is the low-affinity interaction with β -spectrin essential for the physiology of mature enucleated erythrocytes? In our study, we address this by exchanging the low-affinity binding site

for β -spectrin in α I-spectrin for the high-affinity binding site in α II-spectrin. The resulting α I/ α II chimeras clearly retain the ability first to form dimers with β -spectrin by side-to-side association and secondly to form tetramers by head-to-head association of dimers. Mature enucleated red cells form in the chimeric mice, indicating no indispensable role for α I-spectrin in the process of enucleation.

In broad terms, the overall properties of the chimeric spectrin are not grossly altered. The modification of spectrin does not result in phenotypes associated with anemia in humans. The α II β I mice are not deficient in spectrin, and membrane loss is small (<10%), which results in increased osmotic fragility, probably a result of reduced remodeling in the α II β I mice. The change in osmotic fragility is also minor compared to the more substantial increase in fragility seen in hereditary spherocytosis (HS) patients (36). Also, the osmotic fragility curve for the α II β I mice does not have the shoulder seen for HS samples because the heterogeneity of sphericity of the HS red cells is much broader than that noted in α II β I mice. Severe forms of hereditary elliptocytosis arise from mutations in α I- and β I-spectrin that inhibit tetramer formation (37,38); elliptocytes were absent in the α II β I mice. A clinically severe form of elliptocytosis, hereditary pyropoikilocytosis (HPP), arises from mutations at the

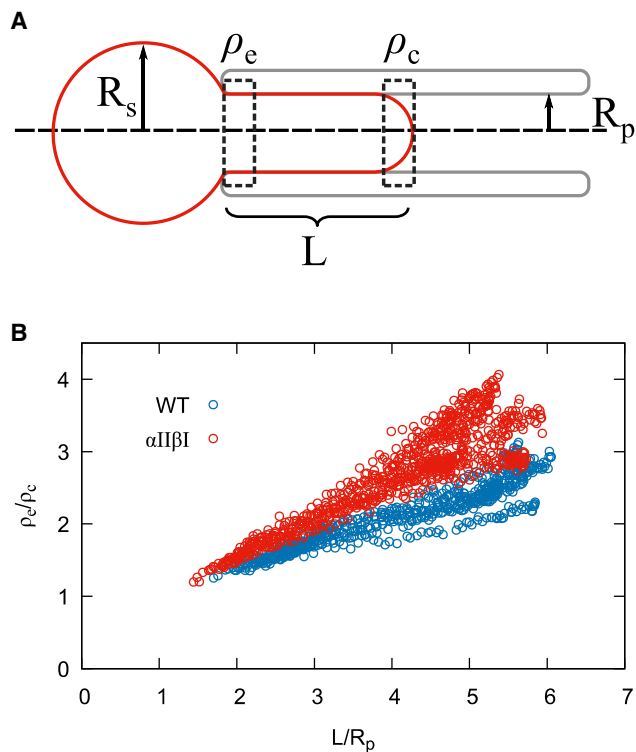


FIGURE 5 Fluorescence-imaged microdeformation. (A) Illustration of the aspirated red blood cell; the red cell membrane is indicated by the red line, and the gray indicates the micropipette. R_s , radius of spherical portion of cell outside micropipette; L , the length of aspirated portion of red cell; ρ_e , the fluorescent intensity at pipette entrance; ρ_c , the fluorescent intensity at cap of aspirated portion; R_p , the internal radius of the micropipette. (B) The ratio of ρ_e/ρ_c of Band 3 in WT (blue) ($n = 8$ cells) and α II β I (red) ($n = 14$ cells) as a function of the normalized aspiration length L/R_p . To see this figure in color, go online.

self-association site that markedly weakens spectrin dimer self-association and enhances the heat sensitivity of red cells (39,40). The weakened self-association site in HPP causes RBCs to fragment at 45°C compared to 50°C for normal human red cells (40). In marked contrast, red cells of the α II β I mice show increased thermal stability; shape changes occur at 49.5°C, 0.9°C higher than that noted for WT normal red cells. Additionally, electrophoresis by nondenaturing gels of HPP patient samples shows a shift from spectrin tetramers to dimers (39), whereas α II β I red cells show the opposite, with a shift to higher oligomers.

As measured by ektacytometry, there is a marked change in membrane deformability of α II β I red cells; however, the difference in DI_{max} is small, consistent with the relatively minor loss of membrane area. Membrane deformability as reflected by the $SS_{1/2}$ parameter is 7.6 \times greater for the α II β I red cells compared to normal red cells, clearly demonstrating the importance of the strength of dimer self-association to red cell membrane deformability. Distinctly different from the ektacytometry of HS red cells (in which DI_{max} is markedly decreased), that decrease in DI_{max} is much less for red cells of α II β I mice. However, although

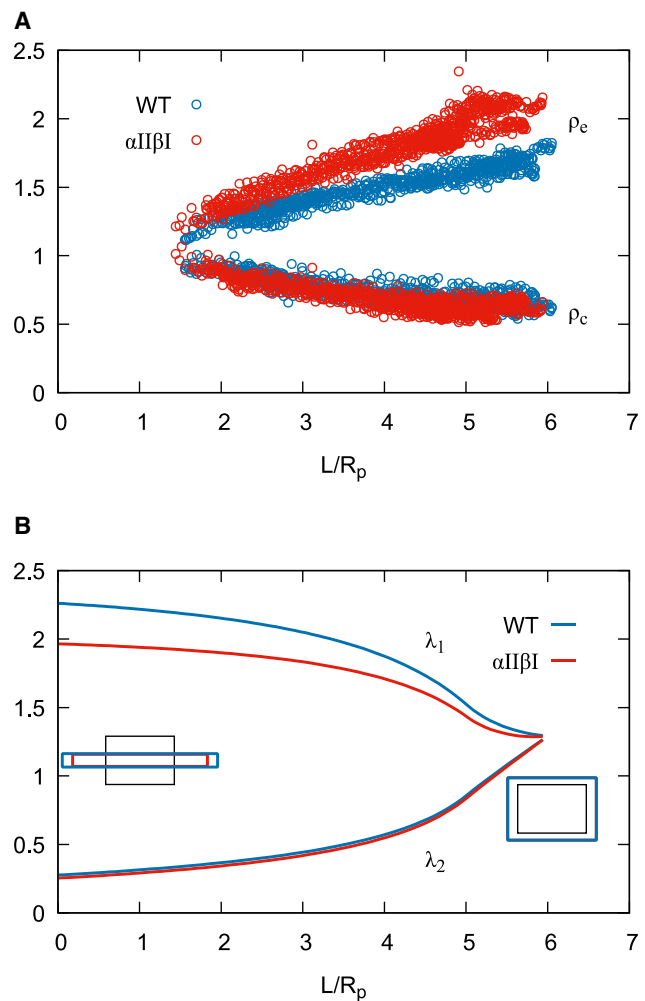


FIGURE 6 Fluorescence-imaged microdeformation. (A) ρ_e and ρ_c as a function of the normalized aspiration length L/R_p for aspirated red cells from both WT (blue) ($n = 8$ cells) and α II β I (red) ($n = 14$ cells) mice. (B) The computed principal components of membrane skeleton deformation as a function L/R_p for a cell with an aspiration length $L = 6$. Insets show the change in shape of a unit area of membrane skeleton for the entrance of the micropipette (left) and the cap (right). To see this figure in color, go online.

the rates of change in DI or $SS_{1/2}$ for HS and normal human red cells are very similar, the rate of change in DI is significantly less for α II β I red cells compared to normal red cells at low shear rates 0–10 Pa (41–43). At low shear stresses <1 Pa, the deformability profiles of α II β I red cells are consistent with red cell tumbling and swinging, and not tank treading (33,34,44). This is consistent with the images of the deformed red cells in the rheoscope showing the resistance of α II β I red cells to deformation. This has been previously observed in more rigid cells (33,34) and theoretical models of such behavior (45,46).

The K/μ ratio measured by fluorescence-imaged microdeformation of α II β I mice red cells was \sim 80% less than those from normal WT mice. In computer simulations of erythrocyte membranes, Boal showed $K/\mu = 2$ for sixfold

networks of simple harmonic springs (47); similarly, modeling by Hansen et al. (48) revealed that decreasing the connectivity of the network increased K/μ . The simplest interpretation of our K/μ measurements is that α II β I mice have increased connectivity in the cytoskeletal network, arising from enhanced interaction of spectrin dimers at the self-association site.

Electron microscopy studies of α II-spectrin and α I-spectrin have shown α II-spectrin to be a stiffer rod-like molecule compared to α I-spectrin (49,50). Because the spectrin modification in α II β I mice is small (1 1/3 repeats of the 20 1/3 repeats), the modified polypeptide structure is largely unchanged, and the flexibility of α I-spectrin is retained. If modeled as a simple harmonic spring network, the spring constant would be essentially same in both the α II β I and the WT. Therefore, the differences in membrane skeleton μ shear and K area dilation moduli observed between the α II β I and the WT are best explained by membrane skeleton remodeling (i.e., by making and breaking tetramers) resulting in reduced connectivity in the WT compared to the α II β I model.

The higher oligomers observed in the α II β I mice could also contribute to the change in the mechanical properties of the membrane skeleton. The significant difference in the incorporation of α II-spectrin into the membrane clearly indicates that the change in binding strength at the self-association site is the dominant factor in the observed change in mechanical properties of the membrane skeleton in the α II β I mice.

Almost all the computational and theoretical models of membrane biophysical properties ignore spectrin remodeling coming from breaking and reforming tetramers, and it is often absorbed into the shear modulus terms; this is largely due to the complexity of including this feature in the models. Recent attempts to model red blood cell membranes (51,52) have begun to include this contribution and, while giving improved understanding of red cell properties, the data presented here can be incorporated into theoretical models to refine them further. This will inform future understanding of cell fragmentation and life span in clinical settings, as well as understanding of evolutionary processes underlying the biology of blood circulation.

In relation to the evolution of spectrin gene function, it appears the ancestral α -spectrin underwent duplication at about the time mammals emerged, followed by selective adaptation of each, especially α I-spectrin. Gene pairs may become subfunctionalized, for example, by alterations to expression. In the case of mammalian α -spectrins, α II-spectrin is expressed in the vast majority of cell types other than erythrocytes; in erythrocytes, α I alone carries out the α -spectrin function.

Subfunctionalization is often the precursor to neofunctionalization (53), as functions can subsequently be adapted separately. In the case of α I-spectrin, we argued in earlier work (12) that the rapid make and break of spectrin tetra-

mers in erythrocyte membranes represents a new function. The data presented here extend and bring new, to our knowledge, insight to this concept and demonstrate that the ancestral high-affinity spectrin tetramers cannot provide the erythrocyte plasma membrane with the deformability under shear stress required physiologically. We view the promotion of erythrocyte deformability as a new function of α I-spectrin that was not provided by the ancestral (α II) spectrin; hence, α I-spectrin is neofunctionalized.

Although neofunctionalization was the end point, the simplest path to getting there may have been neutral drift of one of the pair of duplicates after separation of expression (i.e., asymmetric evolution (54)), to the point at which any further reduction of affinity for β I-spectrin impaired red cell survival. The point at which neutral drift resulted in weaker but optimal affinity of α I-spectrin for β I-spectrin represented the point of neofunctionalization. Once this point had been reached in an early mammal, it appears to have been strongly favored in subsequent evolution; every amino acid in α I-spectrin at the interface with β I-spectrin (PDB: 3LBX) has been conserved since the last common ancestor of humans and platypus, around 180 million years ago.

CONCLUSIONS

In conclusion, our findings reveal that spectrin tetramers are dynamic at the self-association site in intact red blood cells and that remodeling of the membrane skeleton is an essential component of the normal mechanical properties of red blood cells. Mammalian erythrocytes have a form of spectrin optimized for rapid make and break of tetramers and cannot tolerate either higher- or lower-affinity spectrin tetramers for normal biophysical function.

SUPPORTING MATERIAL

Supporting material can be found online at <https://doi.org/10.1016/j.bpj.2021.07.027>.

AUTHOR CONTRIBUTIONS

J.H., X.A., W.G., A.B., and N.M. designed research. J.H., X.G., E.G., J.P., and L.B. performed research. J.H., L.B., W.G., A.B., C.D.H., and N.M. analyzed data. J.H., C.D.H., W.G., A.B., and N.M. wrote the manuscript.

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Supplemental information

**α -spectrin represents evolutionary optimization of spectrin for red
blood cell deformability**

John Hale, Xiuli An, Xinhua Guo, Erjing Gao, Julien Papoin, Lionel Blanc, Christopher D. Hillyer, Walter Gratzer, Anthony Baines, and Narla Mohandas

Supplementary Information for

α I-spectrin represents evolutionary optimization of spectrin for red blood cell deformability.

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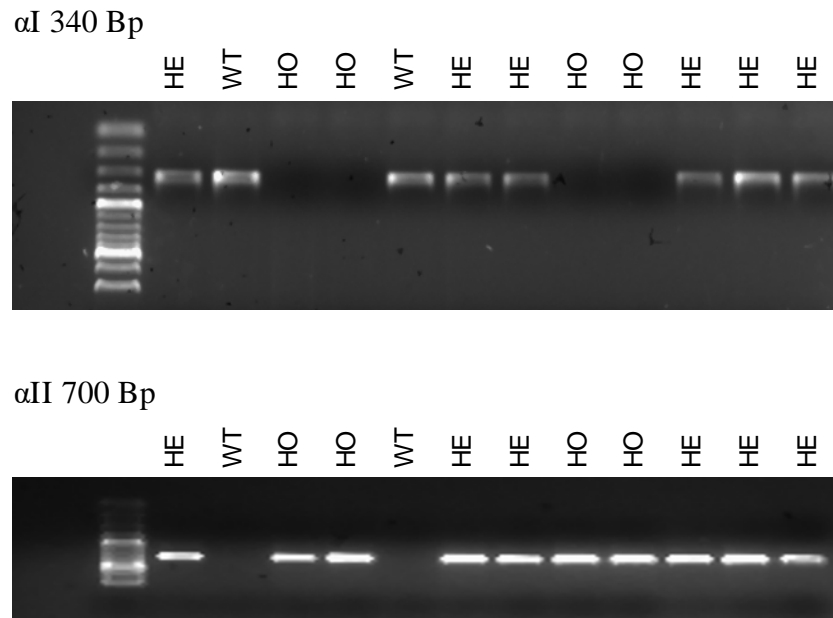


Figure S1. Genotyping by PCR of litter mates: (WT) wild-type mice, (HE) heterozygous and (HO) homozygous knock-in mice. 340-bp indicates presence of α I-spectrin and 700-bp indicates presence α II-spectrin.

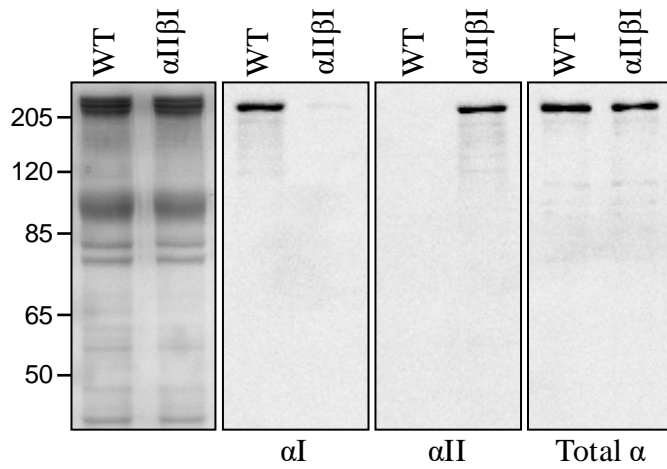


Figure S2. SDS gel electrophoresis and immunoblot analysis of WT and knock-in red cell membranes. Equal protein loadings of ghosts from either WT or $\alpha II\beta I$ were separated by SDS gel electrophoresis and either stained with Coomassie brilliant blue (left panel) or transferred to PVDF and probed with antibodies specific for αI , αII or total α -spectrin as indicated. The positions of standard marker proteins (kDa) are indicated on the left edge.

| | WT (n=9) | α II β I (n=7) |
|-----------------------------------|----------------------|-----------------------------|
| WBC (x10 ³ / μ l) | 7.76 \pm 1.57 | 7.07 \pm 3.45 |
| RBC (x 10 ⁶ / μ l) | 9.93 \pm 0.36 | 8.69 \pm 0.21 *** |
| HGB (g/dl) | 14.28 \pm 0.49 | 12.9 \pm 0.41 *** |
| HCT (%) | 44.92 \pm 1.81 | 40 \pm 0.96 *** |
| MCV (fl) | 45.32 \pm 0.73 | 46 \pm 0.43 * |
| MCH (pg) | 14.38 \pm 0.17 | 14.84 \pm 0.16 *** |
| MCHC (g/dl) | 31.77 \pm 0.5 | 32.26 \pm 0.5 |
| CHCM (g/dl) | 30.81 \pm 0.32 | 31.27 \pm 0.71 |
| CH (pg) | 13.93 \pm 0.15 | 14.39 \pm 0.23 ** |
| RDW (%) | 13.46 \pm 0.99 | 13.03 \pm 0.31 |
| HDW (%) | 1.81 \pm 0.13 | 1.94 \pm 0.05 * |
| PLT (x 10 ³ / μ l) | 1217.56 \pm 216.16 | 1233.57 \pm 273.23 |
| MPV (fl) | 7.08 \pm 0.73 | 7.03 \pm 0.96 |
| Retic (%) | 3.17 \pm 0.36 | 4.29 \pm 0.58 ** |

Table S1. The hematological parameters of WT (n=9 mice) and α II β I (n=7 mice) red blood cell samples as measured by AVIDA 120. WBC indicates white blood cell count; RBC red blood cell count; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin content; CHCM, corpuscular Hemoglobin concentration mean; CH, corpuscular hemoglobin content; RDW, red cell distribution width; HDW, hemoglobin distribution width; PLT, platelet count; MPV, mean platelet volume; Retic, reticulocyte count. Data is expressed as the mean \pm SD (standard deviation), P \leq 0.05, ** P \leq 0.01, *** P \leq 0.001).

allbl-FIMD.avi

Movie S1 (separate file). Fluorescence imaged micro-deformation: Video of the captured image sequence of the micropipette aspirated α II β I RBC as the buffer is exchanged adjusting the RBC volume and thereby changing the aspiration tongue length.