Membrane Protein Interactions in Sickle Red Blood Cells: Evidence of Abnormal Protein 3 Function

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The pattern of membrane abnormalities in sickle red blood cells suggests that sickle hemoglobin damages membrane proteins. We have previously shown a functional defect in sickle ankyrin, poor spectrin-binding ability. Here we examine the other major binding interactions of sickle membrane proteins including spectrin self-association, binding of ankyrin and protein 4.1 to protein 3, and the formation of the spectrin-actin-protein 4.1 complex. We found that sickle spectrin was normal in self-association and ability to participate in the spectrin-actin-protein 4.1 complex. Sickle protein 4.1 bound normally to protein 3 and formed normal complexes with actin and spectrin, even when sickle spectrin was used. The only major abnormality we found was a reduced ability of sickle protein 3 to bind ankyrin. This functional defect could not be explained experimentally on the basis of cysteine modification or enhanced tyrosine phosphorylation. We conclude that damage of sickle membrane proteins is not a diffuse scattershot process, but is largely confined to regions near membrane-associated hemoglobin, the spectrin-binding domain of ankyrin and the ankyrin-binding domain of protein 3. The mechanism and consequences of this damage continues to be investigated.

The pathophysiology of sickle cell anemia is dictated by the primary genetic defect—the presence of sickle hemoglobin. As seen in other genetic diseases, however, additional factors modulate clinical expression. Not all patients with sickle cell anemia follow the same clinical course. Some are more anemic than others, the frequency of vasoocclusive events vary, and life expectancy differs. There are several reasons why the red blood cell (RBC) membrane is likely to be one of the most important modulators of clinical severity. First, it is the structure most intimately associated with sickle hemoglobin and is, therefore, vulnerable to damage by its prodding and noxious byproducts. Second, its transport properties influence the concentration of sickle hemoglobin in the cell. And third, the membrane is the face of the cell that is recognized by proteins and cells in the blood, along the vasculature, and in the reticuloendothelium. The protein framework of the membrane affects cell shape, lipid organization, endocytosis, membrane flexibility, and diffusion of integral proteins. Because all of these characteristics are abnormal in sickle RBCs, the membrane proteins stand out as likely targets of sickle hemoglobin damage.

RBC membrane proteins are bathed in a hemoglobin solution of ~300 mg/mL throughout the life of the cell. At these concentrations, hemoglobin binds to the cytoplasmic domain of protein 3 and influences spectrin self-association. The damage that abnormal hemoglobins may create is an important issue in understanding the pathophysiology of the hemoglobinopathies and the process of erythrocyte senescence.

We have previously reported a functional defect in sickle ankyrin—normal spectrin bound poorly to sickle inside-out vesicles. We also studied a group of patients with β unstable hemoglobins and showed a similar, but quantitatively more severe ankyrin defect. A similar lesion could be provoked by incubating normal cells with the oxidant acetylphenylhydrazine, indicating that such a lesion may arise from the presence of denatured hemoglobin without necessarily invoking a distortion/sickling effect. In addition to the ankyrin defect, the unstable hemoglobinopathy cells showed an impairment in spectrin’s ability to form a complex with protein 4.1 and actin. This functional defect, and the appearance of an abnormal band on nondenaturing polyacrylamide gel electrophoresis (PAGE) of spectrin, was typical of spectrin exposed to diamide oxidation.

Shinar et al described a sickle-like ankyrin defect in α (but not β) thalassemia, a defect that could be reproduced in vitro by incubating normal membranes with isolated β globin chains. Interestingly, the β thalassemia RBCs exhibited a functional defect in protein 4.1 that was not found in the α thalassemia cells. A pattern emerged, supported by the work of Schrier et al that denatured α and β chains cause distinct membrane protein lesions.

The mechanism(s) by which abnormal globins apparently target and damage specific membrane proteins is poorly understood. Recent reviews by Hebbel et al detail the evidence supporting the hypothesis that this is the result of the combined effects of the enhanced tendency of sickle hemoglobin to undergo auto-oxidation and the proclivity for different toxic iron species to associate with different membrane compartments. The N-terminal, cytoplasmic domain of protein 3 is a particularly attractive target, as it is the site of not only normal hemoglobin, but also hemichrome/Heinz body binding. In this report, we examine the major interactions of sickle spectrin, ankyrin, protein 4.1, and protein 3. We describe a defect in protein 3 function and explore cysteine modification and tyrosine phosphorylation as possible mechanisms for this dysfunction.

MATERIALS AND METHODS

Subjects. Blood samples from patients and controls were obtained with consent. All experiments were performed using RBCs from at least three different patients with homozygous sickle cell anemia unless otherwise specified.

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ABNORMAL PROTEIN 3 IN SICKLE RED BLOOD CELLS

Preparation of membranes, vesicles, and membrane proteins. Venous blood was collected in citrate-phosphate-dextrose and stored at 4°C for up to 5 days. Blood samples from patients with sickle cell anemia were stored for up to 48 hours. Density separation of sickle RBCs was done by centrifugation, as described.32 Erythrocyte membranes were prepared by hypotonic hemolysis.31 Membrane shells were prepared from membranes by Triton X-100 extraction in buffers ranging in NaCl concentration from 75 mmol/L to 600 mmol/L.32 Composition of membrane ghosts and shells was determined by sodium dodecyl sulfate (SDS) PAGE using a 3.5% to 17% acrylamide gradient Steck system. In each lane, the total shell pellet obtained from an equivalent amount of ghost starting material was used to estimate their relative proportions.33 Inside-out vesicles (IOVs) were separated from low ionic strength membrane extracts by centrifugation and purified by dextran sedimentation.34 IOVs stripped of ankyrin and protein 4.1 (K-IOVs) were prepared by incubation in 1 mol/L KCl or KI.35 Spectrin dimer was extracted from erythrocyte membranes at 37°C in 0.1 mmol/L sodium phosphate, pH 8, and purified by gel-filtration chromatography.31 Spectrin was also extracted from erythrocyte membranes at 0°C and subjected to PAGE under non-denaturing conditions to estimate the degree of spectrin self-association in the native membrane.32 Ankyrin was purified by a modification of the procedure of Bennett and Stenback,37 as described.39 Protein 4.1 was extracted from erythrocyte membranes depleted of glyceraldehyde-3-phosphate dehydrogenase (protein 6), spectrin, and actin, using 1 mol/L KCl.32 Purified protein 4.1 was eluted from a column of DEAE-650S (Supelco Inc, Bellefonte, PA) with a continuous gradient of 0 to 5 mol/L NaCl. Conductivity was measured in each collected fraction (Radiometer, Copenhagen, Denmark). Rabbit muscle G-actin was prepared and polymerized to F-actin as described.38 Spectrin, ankyrin, and protein 4.1 were determined spectrophoto metrically. Spectrin, ankyrin, and protein 4.1 were radiiodinated with Bolton-Hunter reagent (New England Nuclear, Boston, MA).41

Assays of membrane protein interactions. Binding of 125I-ankyrin and 125I-protein 4.1 to K-IOVs was measured as described.38 For each experiment the degree of ankynin and protein 4.1 extraction in normal and sickle K-IOVs was comparable as judged by PAGE. Samples of 125I-protein were heat denatured (70°C, 10 minutes) and tested at various 125I-protein concentrations. Specific binding was calculated as total binding minus nonspecific binding. Binding of spectrin to F-actin was assessed in the presence and absence of protein 4.1, generally as described,44 or in two assays with the following modifications: binding of 125I-spectrin to F-actin with and without protein 4.1, as a function of total spectrin concentration was measured after incubation in a total volume of 50 μL, for 60 minutes, at 25°C, sedimentation (250,000 g, 5 minutes, TLA-100 rotor, Beckman), and analysis of the F-actin pellet by gamma counting. Concentrations of actin (4.65 × 10⁻⁶ mol/L) and protein 4.1 (2.5 × 10⁻⁷ mol/L) were in excess and kept constant. Nonspecific sedimentation of 125I-counts was determined in blanks containing 125I-spectrin, but no F-actin or protein 4.1 at each spectrin concentration and was subtracted from the counts in the complete binding mixture. Similarly, binding was assessed in one experiment where the 125I-spectrin concentration was held constant (1.1 × 10⁻⁷ mol/L) and protein 4.1 concentration was varied. In this experiment, 125I-protein 4.1 was used in increasing concentrations, and unlabeled spectrin was held constant (1.1 × 10⁻⁷ mol/L). The points on these binding curves represent the mean of duplicates after nonspecific binding is subtracted. Binding of 125I-protein 4.1 to spectrin immobilized on Sepharose 4B beads (Pharmacia, Piscataway, NJ) was determined as described.45

For all binding experiments, a representative assay is presented with each point representing specific binding, the mean of duplicates.

Assays of protein 3 characteristics. Cytosine modification of protein 3 was determined by examining the cleavage products that were generated by 2-nitro-5-thiocyanobenzozate (NTCB) digestion of K-IOVs.46 For comparison, control K-IOVs were derivatized by incubation with 10 or 100 mmol/L N-ethylmaleimide, or iodoacetamide before digestion with NTCB. The degree of protein 3 cross-linking was estimated by PAGE using a modification of the Steck method,47 omitting dithiothreitol and using 5% acrylamide. For comparison, control K-IOVs were oxidatively cross-linked by incubation with 1 mmol/L o-phenanthroline and 0.2 mmol/L CuSO₄.48 Amino acid analysis (Beckman 6300 Amino Acid analyzer) of purified 43 kD (ankyrin-binding) domain of protein 3 and 62 kD (spectrin-binding) domain of ankyrin were performed with β mercaptoethanol (0.1%) included in the hydrolysis reaction. The degree of protein 3 tyrosine phosphorylation was estimated using a modification of the method described by Harrison.48 Briefly, cells were resuspended to an hematocrit of 50% and incubated at 37°C for 20 minutes with varying combinations of 1 mmol/L Na₂VO₃, and 3 mmol/L H₂O₂. They were then subjected to PAGE using the Laemmli system49 with 9% acrylamide and transferred to nitrocellulose.50 The transferred proteins were stained with amido black and/or were blocked and immuno stained after having been exposed to monoclonal (ICN, Costa Mesa, CA) or polyclonal (GIBCO, Gaithersburg, MD) antibody to phosphotyrosine. Antiphosphotyrosine antibody on the blots was detected using the ECL detection system (Amersham, Arlington Heights, IL).

RESULTS

Sickle spectrin is normal. We have previously reported that sickle spectrin binding to ankyrin, as measured by binding to control IOVs, is normal.38 For this report, we examined spectrin self-association by extracting it from sickle RBC membranes at 37°C and 4°C, and measuring the degree of oligomerization using nondenaturing PAGE. As shown in Fig 1A, there is no difference in the pattern of oligomerization, and no abnormal bands in sickle spectrin.

The binding of sickle spectrin to F-actin was measured by incubating increasing amounts of 125I-sickle spectrin with F-actin with and without protein 4.1 and measuring 125I-spectrin bound to the actin pellet. The results in Fig 2A indicate that sickle spectrin functions normally.

Sickle ankyrin is abnormal in binding to spectrin, but normal in binding to protein 3. We have previously reported that there is a functional defect in sickle ankyrin—normal spectrin binds poorly to sickle IOVs.38 This observation is consistent with data presented in Fig 1B showing that while there is little difference in the composition of normal and sickle RBC ghosts, or hypotonic, or isotonic skeletons, there are differences between the hypertonic skeletons. At 300 mmol/L, fewer stable sickle skeletons are formed. Those that are, are devoid of essentially all ankyrin and protein 3, while these proteins are maintained in normal skeletons. At 600 mmol/L, both the normal and sickle skeletons have lost ankyrin and protein 3. Because the composition of the shells changes at different ionic strengths, the best internal comparison is the ankyrin/actin ratio. For normal RBCs, the ratio was 1.3 ± 0.17 in ghosts; 1.6 ± 0.3 in 150 mmol/L shells; and 1.3 ± 0.6 in 300 mmol/L shells. For sickle RBCs, the
Fig 1. (A) Nondenaturing PAGE of 37°C and 4°C control (C) and sickle (S) spectrin extract. (B) SDS-PAGE of sickle and control RBC membrane ghosts (gh) and skeletons at various ionic strengths (mmol/L). (C) SDS PAGE of control (C), sickle reticulocyte-rich fraction (S-top), sickle ISC-rich fraction (S-bot) stripped inside-out vesicles digested with NTCB. (D) Immunoblot of SDS-PAGE of control and sickle erythrocytes incubated with vanadate and/or peroxide as indicated. The blot is stained with antibody to phospho-tyrosine.

The ability of sickle ankyrin to bind to protein 3 was tested by incubating normal K-IOVs, with increasing concentrations of sickle 125I-ankyrin and measuring binding by sedimentation. As shown in Fig 3A, this function of ankyrin is normal.

Amino acid analysis of normal and sickle 62-kD (spectrin-binding) domain of ankyrin showed no significant difference in cysteic acid content (data not shown).

Sickle protein 3 is abnormal in its ability to bind ankyrin, but has no demonstrable differences in cysteine modification or phosphotyrosine content. We tested the ability of sickle protein 3 to bind to ankyrin by incubating sickle K-IOVs.
with increasing concentrations of $^{125I}$ ankyrin. As seen in Fig 3A, this assay indicates a defect in sickle protein 3. In a parallel experiment, we incubated sickle K-IOVs with protein 4.1, another protein that binds to the cytoplasmic domain of protein 3, although with lower affinity. As seen in Fig 3B, this assay was normal. The experiment indicates that the overall geometry (eg, sidedness) of the sickle K-IOV is normal, and that the ankyrin binding defect does not indicate a gross, global distortion of the cytoplasmic portion of protein 3. However, the high-affinity ligand for protein 4.1 on K-IOVs is glycophorin, and although this protein appears normal in sickle K-IOVs, we may be missing a subtle defect of the protein 4.1 binding region of protein 3, which appears to be near the ankyrin site.51

Amino acid analysis of normal and sickle 43 kD (ankyrin-binding) domain of protein 3 showed no significant difference in cysteic acid content (data not shown).

We took advantage of the system devised by Thevenin et al46 and used NTCB digestion of control and SS K-IOVs to determine if there was modification of Cys$^{201}$ and Cys$^{317}$. Because these are the only cysteines in the cytoplasmic portion of protein 3, NTCB cleavage gives a simple pattern and can demonstrate if either and/or both have been derivitized. Cleavage at 201 results in a peptide of ~23 kD, additional cleavage at 317 results in a ~13-kD peptide. Failure to cleave at 201, results in a ~36 kD peptide, while failure to cleave at all results in no released peptides. As shown in Fig 1C, when normal K-IOVs were digested and run on SDS PAGE (7.5% to 15% gradient Laemmli), three major bands (at ~36, 24, and 12 kD) representing the three major peptides were seen. In the presence of increasing amounts of the CYS-modifying agents N-ethylmaleimide and iodoacetamide, these bands were reduced (data not shown). The digestion pattern of normal and sickle (separated into light and dense fractions) NTCB-cleaved K-IOVs was the same. In addition, we have not found any evidence of sulfhydryl cross-linking of sickle protein 3, as judged by the appearance of high molecular weight aggregates in nonreduced SDS gels of sickle K-IOVs (data not shown).

We used the method designed by Harrison et al4* to compare the degree of tyrosine phosphorylation of the cytoplasmic domains of normal and sickle protein 3, Fig 1D. Using antibody to detect phosphotyrosine in the protein 3 region of untreated whole cells blotted onto nitrocellulose, we found no difference between normal and sickle cells. When these cells were incubated with vanadate and peroxide to inhibit phosphatase, an expected increase in phosphotyrosine was found in normal and sickle cells. The only difference between normal and sickle cells was seen when vanadate was included and peroxide excluded from the incubation mixture. Under these circumstances, the amount of phosphotyrosine on sickle cells was markedly increased. These data indicate that no difference in phosphorylation is detected in vivo, but that the inherent peroxide-generating capacity of sickle cells enhances the potency of vanadate as a phosphatase inhibitor in vitro.

Sickle protein 4.1 is normal. Normal protein 4.1 eluted from DEAE cellulose between 13.8 and 14.6 mmho/cm; sickle protein 4.1 eluted between 14.0 and 14.6 mmho/cm. We examined the ability of $^{125I}$-sickle protein 4.1 from one patient to bind to K-IOVs, and as shown in Fig 3C, the binding was normal. These findings are different from those
4.1. As shown in Fig 2B, there was no difference in binding of ankyrin to control IOVs; vesicles from control and sickle RBCs. (A) Binding stripped IOVs: increasing concentrations of either normal or sickle protein 4.1 to control (W) and sickle spectrin immobilized on sepharose beads and found no abnormalities (data not shown). We tested the function of sickle protein 4.1 in the spectrin-actin-protein 4.1 complex in several different assays. We incubated $^{125}$I-spectrin in the presence of F-actin, and increasing concentrations of either normal or sickle protein 4.1. As shown in Fig 2B, there was no difference in binding. Using the same basic assay, this time with unlabeled spectrin and normal and sickle $^{125}$I-proteins 4.1, Fig 2C, there was also no difference. To see whether a subtle defect of sickle protein 4.1 and/or sickle spectrin would be detected when both of these proteins were used together, we examined the binding of sickle $^{125}$I-spectrin to actin in the presence of increasing concentrations of normal or sickle protein 4.1. As shown in Fig 2D, even when both sickle spectrin and sickle protein 4.1 were used, the complex formation was normal. We examined the binary interaction of sickle protein 4.1 and spectrin immobilized on sepharose beads and found no abnormality (data not shown).

DISCUSSION

We examined the major protein-protein interactions in sickle RBC membranes. Instead of finding diffuse abnormalities involving the horizontal interactions (spectrin self-association, spectrin-actin–protein 4.1 complex), as well as the vertical interactions (spectrin-ankyrin–protein 3), we found that only vertical interactions were affected. As we have previously described, sickle ankyrin binds poorly to spectrin, and as we show here, sickle protein 3 binds poorly to ankyrin. Sickle spectrin and protein 4.1 are spared.

The unimpaired ability of sickle spectrin to bind to ankyrin, self-associate, and form a complex with protein 4.1 and actin is characteristic of spectrin from $\alpha$ and $\beta$ thalassemia RBCs, but different from what we found in spectrin purified from $\beta$ unstable hemoglobinopathy cells. In those cells we discovered that spectrin bound poorly to actin in the presence of protein 4.1, and also noted an abnormal "fast-mobility" band on nondenaturing PAGE. These abnormalities resembled injuries that could be induced by dimide oxidation of normal spectrin. Because the effects of dimide on spectrin are dose-dependent we hypothesize that unstable hemoglobinopathy spectrin is sufficiently oxidized that it functions abnormally, but that the oxidant injury of sickle, and thalassemia spectrin is not severe enough to cause a measurable defect. This differing degree of oxidation is also likely to explain why the ankyrin defect seen in unstable hemoglobinopathy cells is more severe than that seen in sickle cells.

We did not show any abnormality of sickle protein 4.1. In our experience, this protein eluted at the same ionic strength as normal protein 4.1, bound normally to K-IOVs, bound normally to spectrin, and participated normally in the formation of the spectrin-actin–protein 4.1 complex. To stress the analysis of the spectrin-actin-protein 4.1 complex formation to show a more subtle abnormality, we also performed our assays using both sickle spectrin and sickle protein 4.1 in the same system. Even under these circumstances we found no abnormalities of complex formation. Interestingly, normal protein 4.1 function is maintained in $\alpha$ thalassemia, a disorder of denatured $\beta$-globin chains (as in sickle cell disease), but is abnormal in complex formation in $\beta$ thalassemia.

We were not surprised to find that sickle protein 3 binds poorly to ankyrin. Protein 3 is the only membrane protein known to have a binding site for hemoglobin, especially avid for denatured hemoglobin. In sickle cells, the binding of denatured hemoglobin in the form of "micro-Heinz bodies" causes protein 3 to aggregate with abnormally clustered ankyrin, glycoporphin, and autologous IgG, associating free iron with the aggregates, and limiting protein 3 mobility. It is quite possible that the ankyrin binding site of protein 3 becomes damaged and/or inaccessible in the process. In this context, it is intriguing that protein 4.1 binds normally to sickle protein 3 despite the fact that these proteins both bind near the N-terminus and compete when studied at very high concentration. In contrast to what is known about the relatively simple protein 3 binding sites for hemoglobin, much less is known about the ankyrin binding site(s).
The amino acid structure of the cytoplasmic domain suggests that approximately 36% is helical and that there is extensive folding. Cross-linking studies indicate that Cys201 and Cys317 are close enough to be cross-linked in the native molecule despite their distance in linear terms. The region between residues 175 and 190 is hinge-like, proline-rich, and able to exist in several pH-dependent conformations that influence ankyrin binding. Cross-linking studies indicate that Cys201 and Cys317 are cross-linkable and must be in their native, unmodified state in order to bind ankyrin.

We examined the phosphorylation of Tyr8 and Tyr21, and the status of the of the 201 and 317 cysteines in sickle protein 3 to see whether modification of these residues is related to the abnormal ankyrin binding. Despite what we expected, we found no evidence of either cysteine modification or enhanced tyrosine phosphorylation in sickle protein 3. At present, we do not know if this represents an issue of the sensitivities of the assays we employed. We are continuing to explore the cytoplasmic domain of sickle protein 3 for potential alterations, and the possibility that its state of dispersion/oligomerization in the membrane, confirmation, or physical association with hemoglobin plays a role in its diminished capacity to bind ankyrin.

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