Differing Erythrocyte Membrane Skeletal Protein Defects in Alpha and Beta Thalassemia

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Abstract

Thalassemic red cells show irregular morphology and maldistribution of glycoproteins and sialic acids. These changes are compatible with damage to the red cell membrane skeleton. To test this possibility, we systematically studied the interconnections of skeletal proteins in patients with a form of alpha thalassemia (HbH disease), in patients with beta thalassemia intermedia, and in normal individuals. Alpha- and beta-thalassemic spectrin functions normally in spectrin self-association, binding to normal inside-out vesicles (IOVs), and binding to actin in the presence and absence of normal protein 4.1. Binding of normal spectrin to beta:thalassemic IOVs is normal but alpha-thalassemic IOVs are defective and bind only half the normal amount of spectrin (66 ± 5 vs. 120 ± 16 μg spectrin dimer/mg IOV protein, respectively). A different defect is detected in beta thalassemia, in which protein 4.1 shows markedly reduced ability ($48 \pm 7\%$ of normal) to enhance the binding of normal spectrin to actin and a decreased ability to bind normal spectrin in a binary interaction, compared with normal protein 4.1 (24 ± 1 and 43 ± 1 μg protein 4.1/mg spectrin, respectively). As no quantitative deficiency of beta-thalassemic protein 4.1 is detected, we assume an acquired lesion is present, which affects about half of the protein 4.1 molecules. These findings indicate that specific, localized, yet different defects exist in the skeletal proteins of alpha- and beta-thalassemic red cells. The different molecular lesions imply that the mechanism of hemolysis and probably the interaction of unpaired globin chains with the membrane differs in the two diseases.

Introduction

Studies of the thalassemic syndromes have concentrated intensively on the primary genetic defects in hemoglobin synthesis (1). The mechanisms leading to the premature destruction of the abnormal red blood cells (RBCs) in the bone marrow sinusoids and the peripheral circulation have not been clearly defined. It is generally assumed that the extra, unmatched globin chains precipitate in the erythrocyte, resulting in widespread membrane dysfunction (see reference 2 for review). Indeed, alterations in the phospholipid and cholesterol content, increased intracellular calcium concentration, and changes in

the membrane sialic acid residues have been documented in beta thalassemia (2). Thalassemic red cells are more sensitive to oxidant stress, as reflected by an increase in malonyldialdehyde formation, a decrease in polyunsaturated fatty acids (3), an increase in protein cross-linking (4), and a decrease in titratable membrane thiols (5). This oxidant susceptibility may be due to excessive oxidation of the unstable globin chains and the subsequent release of oxygen radicals that theoretically might damage any component of the red cell membrane.

The abnormal morphology and irregular shape of thalassemic red cells suggest that the erythrocyte membrane skeleton must be damaged. The skeletal protein network is thought to be important in regulating different functions, including the deformability and stability of red cell membranes (6). Analyses of the latter parameters in thalassemic cells show that whereas both alpha- (HbH)[1] and beta-thalassemic membranes are unusually rigid, beta-thalassemic membranes are also fragile and fragment twice as rapidly as normal at high shear stress (7). We have also recently examined the membrane protein skeletons of beta-thalassemic red cells by scanning electron microscopy and found abnormalities in their shape and protein composition (8).

The goal of this study was to characterize the defects in the membrane skeletons of alpha- and beta-thalassemic red cells, and to analyze the similarities and differences between the two syndromes. We therefore studied systematically the major skeletal proteins interactions in patients with alpha and beta thalassemia. We found a qualitative defect in the binding of normal spectrin to inside-out vesicles (IOVs) in alpha thalassemia, and a different defect in the interaction of spectrin, actin, and protein 4.1 in beta thalassemia. The latter is caused by the presence of abnormal protein 4.1 molecules, with reduced ability to bind normal spectrin. These findings indicate that specific, localized skeletal protein damage occurs in thalassemia, and the damage differs in alpha and beta thalassemia.

Methods

Subjects. Venous blood samples in citrate-phosphate-dextrose were collected from splenectomized and nonsplenectomized normal individuals ($n = 20$) and from patients with alpha thalassemia (HbH disease; $n = 3$) and beta thalassemia intermedia ($n = 11$). Informed

1. *Abbreviations used in this paper:* Buffer A, 130 mM KCl, 20 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, 1 mM DTT, 1 mM MgCl$_2$, and 0.5 mM sodium azide, pH 7.5; buffer B, 150 mM NaCl, 4 mM sodium phosphate, 10 mM Tris, 0.5 mM DTT, 0.1 mM ATP, 0.01 mM CaCl$_2$, 4 mg/ml BSA, pH 7.4; buffer C, 10 mM sodium phosphate, 130 mM KCl, 20 mM NaCl, 1 mM EDTA, 0.5 mM sodium azide (pH 7.6), 1 mg/ml BSA, and 0.1% NP-40; buffer D, 130 mM KCl, 20 mM NaCl, 10 mM Tris Cl, 1 mM EDTA, 1 mM NaN$_3$, and 0.1 mM DTT, pH 7.6; HbH, hemoglobin H disease; IOV, inside-out vesicle; RBC, red blood cell.

consent was obtained from all participants. The thalassemic samples were obtained from patients who rarely require blood transfusions. None of the patients had been transfused in the 4 mo preceding the study. The detailed hemoglobin analysis and globin chain synthesis ratios of many of the patients have been previously described (9). Some samples were obtained from Israeli patients, and were shipped on ice by air express, accompanied by a normal control. All blood samples were received and studied within 24–36 h after blood collection. No differences were observed between samples shipped from Jerusalem and samples obtained locally.

Preparation of red cell membranes and membrane proteins. RBC membranes were prepared by hypotonic lysis in 5 mM sodium phosphate, pH 8 (10) with the addition of 1 mM EDTA and protease inhibitors (2 mM diisopropyl fluourophosphate, 5 μg/ml leupeptin) to the lysis buffer. Ratios of various membrane proteins were determined by running RBC membranes on a 3.5–17% exponential SDS-polyacrylamide gel (11). In most experiments, the Coomassie Blue-stained bands of spectrin, ankyrin, protein 3, protein 4.1, and globin were cut from four to six lanes, the dye was eluted with 25% pyridine, and the relative protein content was estimated by absorbance at 605 nm (12). In some experiments, the ratios were determined by densitometry of Coomassie Blue-stained gels.

Spectrin-depleted IOVs were separated from low ionic strength extracts by sedimentation and layered on a Dextran barrier (13). The sidedness of the IOVs was determined by measuring the glyceraldehyde-3-phosphate dehydrogenase activity before and after the addition of 0.2% Triton X-100, as described by Steck and Kant (14). Spectrin was isolated by low ionic strength extraction with 0.1 mM sodium phosphate, pH 8, at 37°C, and purified by gel filtration chromatography on Agarose A15M 200-400 mesh (Bio-Rad Laboratories, Richmond, CA) as described (15).

Protein 4.1 was purified from fresh red cell membranes by high salt extraction with 1 M KCl, as described by Tyler (16) and modified by Cohen and Foley (17). Actin was prepared from rabbit skeletal muscle by the method of Spudich and Watt (18). It has been previously shown that this actin is equivalent to erythrocyte actin in assays of spectrin-actin-protein 4.1 interactions (19).

The spectrin and protein 4.1 were iodinated with ^{125}I-Bolton Hunter reagent (New England Nuclear, Boston, MA) (20). Analysis of protein concentration was performed by the method of Lowry (21) using a BSA standard.

Membrane skeletal protein interactions. Spectrin self-association was analyzed by nondenaturing gel electrophoresis at 0°C, as described (22). The proportions of spectrin dimer, tetramers, and oligomers in low temperature (4°C), low ionic strength extracts were studied directly. The interconversion of spectrin tetramers to dimer was also determined during incubation of these extracts for 6 h at 30°C (22). Interactions of spectrin and ankyrin on IOVs were studied by rebinding ^{125}I-spectrin dimer to spectrin depleted IOVs in 130 mM KCl, 20 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, 1 mM DTT, 1 mM MgCl$_2$, and 0.5 mM sodium azide, pH 7.5 (buffer A) for 90 min at 0°C. Free and membrane-bound radioactivity was determined by pelleting the IOVs through 20% sucrose in the binding buffer (wt/vol), as described by Goodman and Weidner (23). Samples of ^{125}I-spectrin were heat denatured (60°C, 10 min) and tested at various spectrin concentrations in each experiment to estimate the amount of nonspecific binding. Such binding was < 15% of the values of untreated spectrin and was routinely subtracted.

Interaction of thalassemic spectrin with F actin and protein 4.1 was studied in an assay designed by Wolfe et al. (24), as modified by Becker et al. (25), where increasing concentrations of ^{125}I-spectrin dimer (10–30 μg) were incubated with 24 μg F actin in the presence or absence of protein 4.1 (0 or 6 μg) in 150 mM NaCl, 4 mM sodium phosphate, 10 mM Tris, 0.5 mM DTT, 0.1 mM ATP, 0.01 mM CaCl$_2$, 4 mg/ml BSA, pH 7.4 (buffer B) for 60 min at 23°C. In a few experiments, designed to test the function of protein 4.1, increasing concentrations of 4.1 (0–3 μg) were incubated with fixed amounts of ^{125}I-normal spectrin dimer (12 μg) and F actin (21 μg) for 1 h at 24°C in 130

mM KCl, 20 mM NaCl, 10 mM Tris Cl, 1 mM EDTA, 1 mM NaN3, and 0.1 mM DTT, pH 7.6 (buffer D). In both cases the F actin was then collected by centrifugation (48,000 g for 120 min) through a 10% (wt/vol) sucrose cushion in the binding buffer, and the pellet was counted to determine the amount of bound ^{125}I-spectrin. Control samples containing ^{125}I-spectrin alone were included in each assay and the nonspecific counts, which did not exceed 5%, were subtracted from the binding data. The assay was also performed in the presence and absence of DTT to test whether thalassemic skeletal proteins had incurred reversible oxidative damage.

In another set of experiments, a fixed amount of normal ^{125}I-spectrin (10 μg) was incubated with 21 μg F actin, in the presence of increasing concentrations of thalassemic or normal protein 4.1 (0–3 μg), under the same experimental conditions.

Interaction of spectrin and purified protein 4.1 was studied by binding ^{125}I-protein 4.1 to spectrin-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) in a modification of the method described by Becker et al. (26). Briefly, normal spectrin dimer (1–2 mg/ml) was attached to CNBr-Sepharose 4B (5 mg spectrin/g dry beads) in 0.1 M sodium bicarbonate and 0.15 M NaCl, pH 8 at 4°C for 16 h mixing end over end. The coupling efficiency was generally > 90%. Control beads were prepared by coupling Sepharose to BSA at the same concentration. These were used to assess nonspecific binding. A fixed amount of spectrin-Sepharose (40 μg spectrin) or an equivalent amount of BSA-Sepharose was incubated with ^{125}I-protein 4.1 (0–18 μg) in 10 mM sodium phosphate, 130 mM KCl, 20 mM NaCl, 1 mM EDTA, 0.5 mM sodium azide, (pH 7.6), 1 mg/ml BSA, and 0.1% NP-40 (Calbiochem-Behring Corp., La Jolla, CA) (buffer C) for 90 min at 0°C. The spectrin-Sepharose or albumin-Sepharose and protein 4.1 mixtures were layered over 20% sucrose in the same buffer, centrifuged at 5,000 rpm for 15 min, and the pellets were assayed for radioactivity. The nonspecific binding on the BSA-Sepharose was < 10% of the binding values to the spectrin-Sepharose and was subtracted. The purified spectrin and protein 4.1 were clarified by centrifugation at 100,000 g for 45 min before binding in these experiments.

PAGE. PAGE in the presence of SDS was performed using the continuous method of Fairbanks (10) as modified by Steck (27), and the discontinuous method of Laemmli (28). PAGE in the absence of detergents was performed on 3% acrylamide tube gels.

Results

Composition of ghosts and vesicles. SDS-PAGE of red cell membranes and spectrin-depleted IOVs from thalassemic patients and normal individuals are shown in Fig. 1 *A* and *B* and quantified in Table I. There is a mild to moderate decrease in the spectrin content of both alpha-thalassemic (13% decrease) and beta-thalassemic (23% decrease) membranes. Similar spectrin deficiency has been observed previously in beta thalassemia by Shinar et al. (18). There is also an increase in the hemoglobin content of the alpha- and beta-thalassemic membranes, reaching 10±2 and 13±4%, respectively, of the total membrane protein, as calculated by densitometry or pyridine elution. An increase of cytoplasmic proteins, like catalase, is also detected, which has been described for other hemolytic anemias (29). The protein composition of spectrin-depleted thalassemic and normal IOVs is comparable (Fig. 1 *B,* Table I), except for the presence of hemoglobin, which is normally undetectable, but reaches 0.5–5% of the total thalassemic membrane protein content. No quantitative deficiency of ankyrin or protein 4.1 is detected when thalassemic ghosts and IOVs are studied using pyridine elution of Coomassie Blue-stained gel bands (Table I). There is no difference in sidedness between thalassemic and normal IOVs.

Protein 4.1 purified from thalassemic membranes has the same mobility on SDS-PAGE as normal 4.1 (Fig. 1 *C*), but has
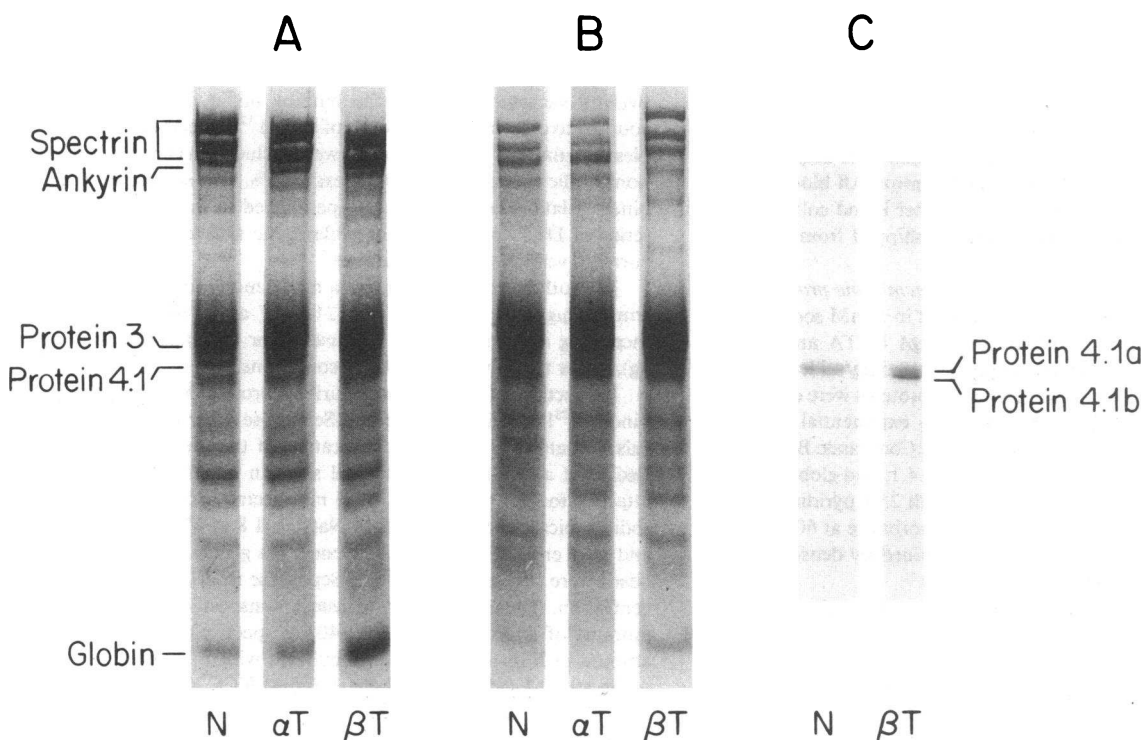
Figure 1. Gel electrophoresis of red cell membranes and isolated protein 4.1 from thalassemic patients and normal individuals. (*A*) Normal, alpha-thalassemic, and beta-thalassemic red cell membranes. (*B*) Normal, alpha-thalassemic, and beta-thalassemic spectrin-depleted IOVs. Electrophoresis was performed using a continuous system (11) and 30 μg of membranes and IOVs were applied to the gel. Both types of thalassemic membranes contain increased amounts of globin and other cytoplasmic proteins. (*C*) Normal and beta thalassemic protein 4.1. Electrophoresis was performed using a discontinuous system (28). There is an increased amount of the 4.1b subfraction in the thalassemic protein 4.1, presumably due to the young age of the thalassemic red cells (30).

a more prominent 4.1b component, presumably due to the young age of the thalassemic erythrocytes (30).

Low ionic strength spectrin extracts (at 4 and 37°C) from thalassemic patients are normal in SDS-PAGE mobility, gel filtration elution pattern, and yield (data not shown).

Spectrin self-association. Thalassemic spectrin-spectrin interactions are normal. Both alpha- and beta-thalassemic spectrin extracts show normal ratios of spectrin dimer/spectrin tetramer in both low (4°C) and high (37°C) temperature extracts.

There is a small increase in the percentage of spectrin oligomers in the thalassemic extracts (thalassemic 38%; normal 29%) as evaluated by densitometry of low temperature extracts on 3% nondenaturing gels (Fig. 2 *A;* Table II). We also detect a small amount (∼ 4% of the total spectrin) of a fast mobility band migrating approximately in the position of spectrin monomer (26) in splenectomized patients with alpha ($n = 1$) and beta thalassemia ($n = 4$) (Fig. 2 *B,* Table II). This was not observed in nonsplenectomized thalassemic patients or in either splenectomized or nonsplenectomized individuals. This band may represent oxidized spectrin (26) or a complex between spectrin and globin (31).

In solution, equivalent proportions of alpha thalassemic, beta thalassemic, and normal spectrin tetramer convert to dimer (alpha thalassemic 92%; beta thalassemic 94%; normal 93%) during 6-h incubations at 30°C (data not shown).

Spectrin ankyrin interactions. The ankyrin binding sites of thalassemic spectrins are normal as assessed by rebinding alpha- or beta-thalassemic ^{125}I-spectrin dimer (0–80 μg/ml) to normal spectrin-depleted IOVs (Fig. 3 *A*).

The binding of normal ^{125}I-spectrin to beta-thalassemic IOVs is also normal (Fig. 3 *B*) and comparable to that reported by Bennett and Branton (13); however, alpha-thalassemic IOVs bind less spectrin than normal IOVs (Fig. 3 *B*). Scatchard analyses (Fig. 3 *B, inset*) indicate that the binding capacity of

Table I. Quantitation of Membrane Proteins in Normal and Thalassemic Ghosts and IOVs

Ratio	Normal	Alpha thalassemia	Beta thalassemia
Membranes			
Spectrin/protein 3	1.51	1.32	1.16
Ankyrin/protein 3	0.39	0.39	0.44
4.1/protein 3	0.37	0.46	0.42
Globin/protein 3	0.24	0.35	0.49
IOVs			
Spectrin/protein 3	0.31	0.25	0.42
Ankyrin/protein 3	0.24	0.27	0.22
4.1/protein 3	0.38	0.36	0.36
Globin/protein 3	ND*	0.05	0.10

Ratios of dye eluted from Coomassie blue-stained gel bands by 25% pyridine (12). Average of experiments on two unrelated individuals; each in triplicate.
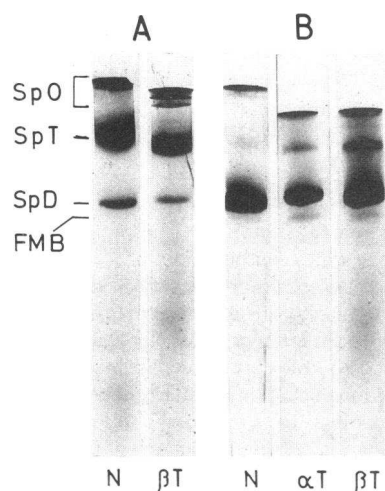* ND, none detected.

Figure 2. Nondenaturing gel electrophoresis of normal (N) and thalassemic (α T, β T) spectrin extracts. Samples were obtained by incubating red cell membranes at either (*A*) 4°C for 16 h or (*B*) at 37°C for 30 min in low ionic strength buffer. Note the increased amount of spectrin oligomer (SpO) in the beta-thalassemic but not the normal spectrin extracts prepared at 4°C. Also note the presence of a fast migrating band (FMB) in the 37°C spectrin extracts. SpT represents spectrin tetramer; SpD, spectrin dimer.

alpha-thalassemic IOVs is 66±5 μg spectrin dimer/mg IOV protein, whereas normal IOVs bind approximately twice as much spectrin: 120±16 μg spectrin dimer/mg IOV protein. The binding affinities are similar: the K_d for normal and alpha-thalassemic IOVs are 45 and 53 μg/ml, respectively.

Spectrin-actin-protein 4.1 interactions. The interaction of thalassemic spectrins with F actin and protein 4.1 is normal. Alpha- and beta-thalassemic spectrin dimers show normal binding to F actin in the presence or absence of normal protein 4.1 (Fig. 4 *A*). This normal binding ability is maintained when the assay is done in the absence of DTT (data not shown). This rules out the possibility that thalassemic spectrins undergo reversible oxidative damage that might have been corrected under the usual experimental conditions.

In contrast, the ability of beta-thalassemic protein 4.1 to enhance spectrin-actin interaction is abnormal. As shown in Fig. 4 *B*, beta-thalassemic protein 4.1 has markedly impaired ability to enhance the binding of normal spectrin dimer to actin (48±7% of normal), when compared with normal protein 4.1. The ability of alpha thalassemic protein 4.1 to interact with normal spectrin and actin is comparable to normal.

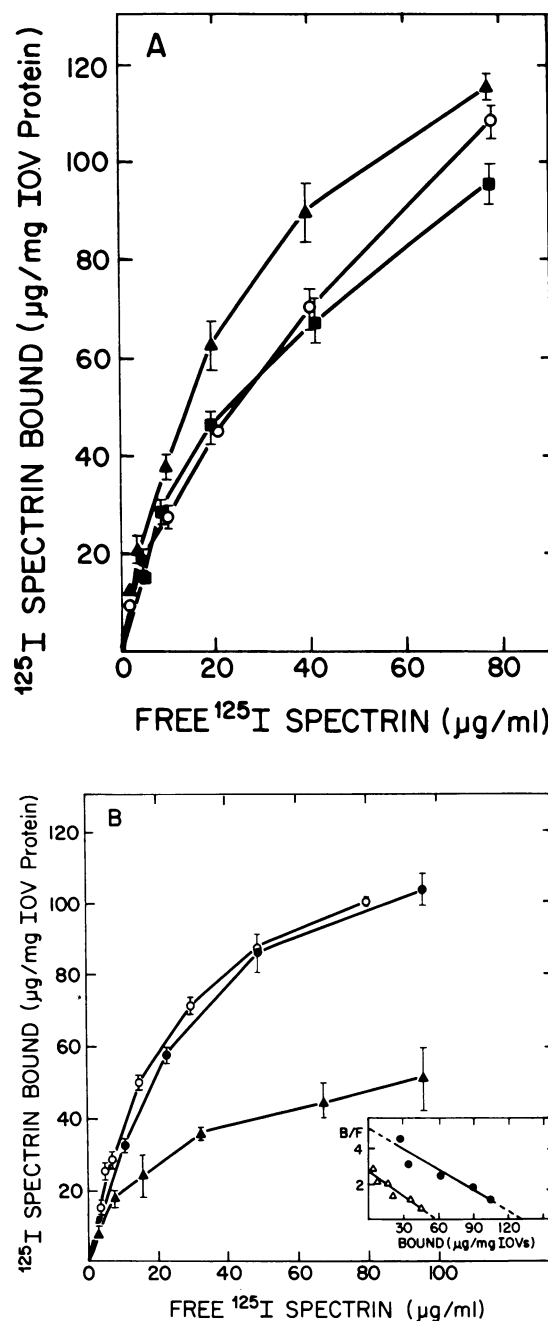The binding of beta-thalassemic 4.1 to spectrin is also ab-

Figure 3. (*A*) Binding of normal (o), alpha-thalassemic (▲), and beta-thalassemic (■) spectrin dimer to normal spectrin-depleted IOVs in two different experiments. Various concentrations of ^{125}I-spectrin dimer (0–100 μg/ml) were incubated with 16.7 μg IOVs for 90 min at 0°C in buffer A (see Methods). Error bars depict the SD. Note that thalassemic spectrin functions normally in this assay. (*B*) Binding of normal spectrin dimer to normal (o), alpha-thalassemic (▲), and beta-thalassemic (■) spectrin-depleted IOVs. The results of 10 experiments are shown. The data plotted according to the Scatchard equation (*inset*) show that the maximal capacity of normal and alpha-thalassemic IOVs was 120±14 and 66±5 μg spectrin dimer/mg IOVs protein, respectively.

Table II. Percent of Spectrin Oligomers, Tetramers, Dimers and Fast Migrating Band in 4 and 37°C Spectrin Extracts of Alpha- and Beta-Thalassemic Membranes

	4°C			37°C		
	SpO*	SpT	SpD	SpT	SpD	FMB
Normal	29±1	55±1	16±1	6±3	94±4	ND[‡]
Alpha thalassemia	38±2	49±1	15±1	10±2	86±2	5±1
Beta thalassemia	38±3	50±1	12±2	12±2	84±3	4±2

* SpO, Spectrin oligomers; SpT, spectrin tetramers; SpD, spectrin dimers; FMB, fast migrating band.
[‡] ND, none detected.

normal. When this binary interaction is studied by binding protein 4.1 to normal spectrin attached to Sepharose beads, a reduction of 45±2% is detected in the ability of beta-thalassemic protein 4.1 to bind normal spectrin (Fig. 5). Scatchard
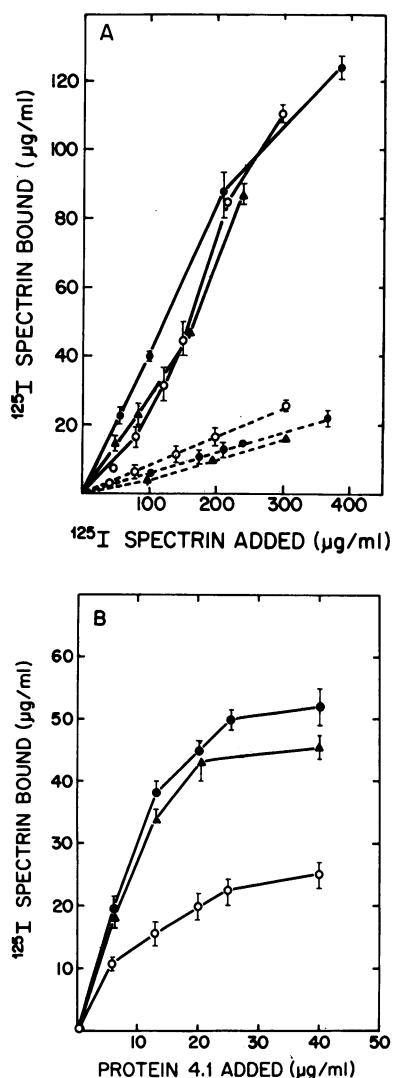
Figure 4. (A) Binding of normal (o), alpha- (▲), and beta-thalassemic (■) spectrin dimer to actin in the presence (—) and absence (--) of normal protein 4.1. The results of four experiments are presented. Increasing concentrations of normal and thalassemic spectrin dimer (0–30 μg) were incubated with 24 μg actin, with or without normal protein 4.1 (6 μg) as described in Methods. Error bars depict the SD. Note that both alpha- and beta-thalassemic spectrin dimer functioned normally in these interactions. (B) Binding of ^{125}I-normal spectrin dimer to actin in the presence of normal (o), alpha-thalassemic (▲), and beta-thalassemic (■) protein 4.1 in four experiments. Increasing concentrations of protein 4.1 (0–3 μg) were incubated with fixed amounts of ^{125}I-normal spectrin dimer (12 μg) and 21 μg F actin for 1 h at 24°C in buffer D (see Methods). Note there is a substantial decrease in the ability of beta thalassemia protein 4.1 to enhance the binding of normal spectrin to F actin, whereas alpha-thalassemic protein 4.1 functions normally in this interaction.

Figure 5. Interaction of purified protein 4.1 with spectrin. Binding of normal (o, △) and beta-thalassemic (●, ▲) protein 4.1 to spectrin-Sepharose in two experiments. Various concentrations of ^{125}I-protein 4.1 (0–20 μg) and a fixed amount of spectrin (40 μg) attached to Sepharose 4B were incubated in buffer C for 90 min at 0°C (see Methods). The curves were corrected for nonspecific binding by subtracting the binding of protein 4.1 to BSA-Sepharose. Error bars depict the SD. There was a 45±2% reduction in the ability of beta-thalassemic protein 4.1 to bind to normal spectrin dimer in this system. Scatchard analysis (insert) shows that the binding affinity of beta-thalassemic protein 4.1 is diminished compared with normal (K_d [thal] = 1.2 μM, K_d [normal] = 0.26 μM).

analyses shows that the binding affinity of beta-thalassemic protein 4.1 for spectrin is diminished (K_d = 1.2 μM), compared with normal (K_d = 0.26 μM). Note that the binding capacity of the immobilized spectrin in these experiments is only a portion of the expected, assuming equimolar amounts of spectrin and protein 4.1. This may indicate that some of the spectrin dimers are not properly oriented to bind protein 4.1 when they are coupled to the CNBr-Sepharose beads, or it may mean that some of the spectrin denatures during coupling.

The described alteration in beta-thalassemic protein 4.1 function is not caused by a quantitative deficiency, as measured by pyridine elution of the 4.1 band from SDS gels of thalassemic membranes (Table I). There is also no evidence suggesting increased aggregation of the thalassemic protein 4.1 in the membranes or in solution (data not shown), such as that recently described for protein 4.1 from sickle erythrocytes (32). The abnormal beta-thalassemic 4.1 presumably results from an acquired lesion or lesions that affect about half the 4.1 molecules. This assumption is supported by the abnormal elution profile of beta-thalassemic protein 4.1 from DEAE TSK-G50S anion exchange columns, which differs from that
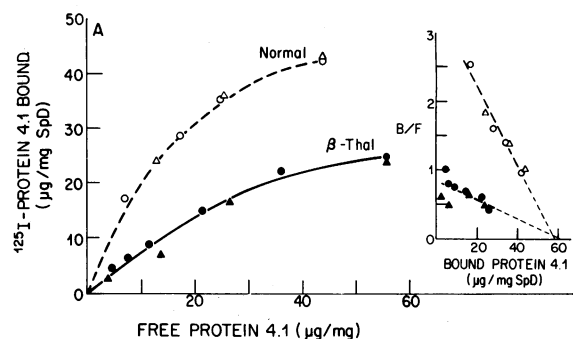
of alpha-thalassemic and normal protein 4.1 (5 different experiments). Alpha-thalassemic and normal protein 4.1 elute at 142±10 mM NaCl, whereas beta-thalassemic 4.1 elutes at 116±8 mM NaCl. This observation indicates that both structural and functional alterations are present in protein 4.1 purified from beta-thalassemic red cells.

Discussion

Previous indirect evidence suggests that skeletal membrane proteins of thalassemic erythrocytes are abnormal in shape, composition, and function (7, 8). We, therefore, analyzed the different skeletal protein interactions in thalassemic red cells in search of specific defects. We found that such defects are relatively few in number and differ in alpha and beta thalassemia (Table III).

Spectrin, the major skeletal protein, incurred some slight damage in both alpha and beta thalassemia as shown by the presence of a trace amount of a fast mobility band on nondenaturing gels. Despite this limited structural alteration, thalassemic spectrin functions normally in spectrin self-association and in its interactions with other membrane skeletal proteins.

Table III. Skeletal Protein Functions in Thalassemic Erythrocytes

Interaction	Alpha thalassemia	Beta thalassemia
Thalassemia spectrin self-association	Normal	Normal
Thalassemia spectrin-normal IOVs	Normal	Normal
Normal spectrin-thalassemia IOVs	Abnormal (50±6%)	Normal
Thalassemia spectrin-actin-normal 4.1	Normal	Normal
Normal spectrin-actin-thalassemia 4.1	Normal	Abnormal (48±7%)
Normal spectrin-thalassemia 4.1	Normal	Abnormal (45±2%)

A major defect is detected in the interaction of normal spectrin with its binding sites on membranes from alpha-, but not beta-, thalassemic red cells. The decreased ability of alpha-thalassemic IOVs to bind spectrin cannot be attributed to a quantitative deficiency of ankyrin, to unusual contamination of binding assays with outside-out membrane vesicles, or to significant proteolysis, as judged by quantitative analyses of electrophoresis gels and by sidedness assays. Alpha-thalassemic IOVs have increased hemoglobin attached to them, which reaches ~ 5% of the total IOVs protein, or one to two molecules of hemoglobin for every molecule of ankyrin. Theoretically, this could account for the reduction in spectrin binding of alpha-thalassemic IOVs; however, beta-thalassemic IOVs, with a similar or even greater amount of attached hemoglobin, bind spectrin normally (Fig. 3 *B*). The binding defect may, therefore, result from interference of the easily denatured, unpaired beta globin chains with the spectrin binding sites on the membrane by either steric hindrance or direct oxidative damage to the thalassemic ankyrin. The abnormal spectrin binding is similar to the defects described in other syndromes that involve the beta globin chain such as sickle cell disease (33) and severe unstable beta-hemoglobinopathies like Hb Nottingham or Hb Brockton (34). The excess beta chains in HbH disease are unstable and readily precipitate to form hemichromes (35), which bind to protein 3 with high affinity (36). X ray studies show that the cytoplasmic domain of protein 3 binds normal hemoglobin by inserting its NH-terminal end into the 2,3-diphosphoglycerate cavity between the beta globin chains (37). The alpha globin chains do not participate in this interaction. If hemichromes of beta globin tetramers (HbH) bind in a similar fashion, this might explain why ankyrin, which binds nearby on protein 3, is apparently damaged in alpha thalassemia but not beta thalassemia.

The defect in beta-thalassemic RBCs involves the skeletal protein interaction between the core skeletal components spectrin, actin, and protein 4.1. Beta-thalassemic protein 4.1 binds poorly to normal spectrin and only partially amplifies the binding of this spectrin to actin. Alpha-thalassemic protein 4.1 behaves normally in these interactions. As no quantitative deficiency of protein 4.1 is detected in beta-thalassemic membranes, we have to assume that an acquired lesion is present, which affects ~ 50% of the beta-thalassemic protein 4.1 molecules. This assumption is supported to some extent by the abnormal elution profile of beta-thalassemic protein 4.1 from DEAE anion exchange columns. It might be caused by changes in the charge of beta-thalassemic protein 4.1, perhaps due to oxidation of its amino acid side chains.

Protein 4.1 interacts with a variety of different membrane components. It participates in spectrin-actin-4.1 interactions (19, 38–40), but it also binds to the red cell membrane via the glycophorins (41–43) and possibly protein 3 (44), and phosphatidylserine (45–47). Recent data show that it has a crucial role in regulating red cell membrane stability (48). As previously mentioned, beta-thalassemic membranes show increased membrane fragility (7). Similar fragility is observed in patients with total or partial RBC protein 4.1 deficiency (48, 49) as well as in defects involving spectrin self-association (50, 51), spectrin-actin-4.1 interactions (47, 49), and severe spectrin deficiency (6). Beta-thalassemic red cells are moderately spectrin deficient (23% less spectrin than normal) and this deficiency may contribute to the observed membrane fragility (7); it is difficult to apportion the relative contributions of spectrin deficiency and the functional defect present in beta-

thalassemic protein 4.1. The possibility that spectrin deficiency in thalassemic membranes may be secondary to the defects in spectrin-IOV binding (alpha thal) or spectrin-4.1 interactions (beta thal) must also be considered.

That different molecular defects exist in the skeletal proteins of alpha- and beta-thalassemic red cells is of great interest and supports our hypothesis that unpaired alpha and beta globin chains may bind to and damage the membrane in different ways. Indeed, previous electron microscopic data suggest that this might be the case, as the morphological appearance of nucleated, as well as mature RBC is different in alpha (51) and beta thalassemia (52).

The loss of critical interactions between the core skeletal proteins (more evident in beta thalassemia), as well as interactions between the skeletal proteins and the overlying lipid bilayer (more evident in alpha thalassemia), might contribute to abnormal clustering of transmembrane proteins. This, in turn, could stimulate binding of autologous immunoglobulins (53, 54), and early recognition and removal of those erythrocytes from the circulation.

Further functional and structural studies are required for better understanding of the mechanism(s) responsible for such defects, and their role in the pathophysiology of the severe hemolysis in thalassemia.

Acknowledgments

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