

The Instability of the Membrane Skeleton in Thalassemic Red Blood Cells

By J. Yuan, A. Bunyaratvej, S. Fucharoen, C. Fung, E. Shinar, and S.L. Schrier

The thalassemias are a heterogeneous group of disorders characterized by accumulation either of unmatched α or β globin chains. These in turn cause the intramedullary and peripheral hemolysis that leads to varying anemia. A partial explanation for the hemolysis came out of our studies on material properties that showed that β -thalassemia (β -thal) intermedia ghosts were very rigid but unstable. A clue to this instability came from the observation that the spectrin/band 3 ratio was low in red blood cells (RBCs) of splenectomized β -thal intermedia patients. The possible explanations for the apparent decrease in spectrin content included deficient or defective spectrin synthesis in thalassemic erythroid precursors or globin chain-induced membrane changes that lead to spectrin dissociation from the membrane during ghost preparation. To explore the latter alternative, samples from different thalassemic variants were obtained, ie, β -thal intermedia, HbE/ β -thal, HbH (α -thal-1/ α -thal-2), HbH/Constant Spring (CS), and homozygous HbCS/CS. We searched for the presence of spectrin in the first lysate of the standard ghost preparation. Normal individuals and patients with autoimmune hemolytic anemia, sickle cell anemia, and anemia due to chemotherapy served as controls. Using gradient so-

dium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, no spectrin was detected in identical aliquots of the supernatants of normals and these control samples. Varying amounts of spectrin were detected in the first lysate supernatants of almost all thalassemic patients. The identification of spectrin was confirmed by Western blotting using an affinity-purified, monospecific, rabbit polyclonal antispectrin antibody. Relative amounts of spectrin detected were as follows in decreasing order: splenectomized β -thal intermedia including HbE/ β -thal; HbCS/CS; nonsplenectomized β -thal intermedia, HbH/CS; and, lastly, HbH. These findings were generally confirmed when we used an enzyme-linked immunosorbent assay technique to measure spectrin in the first lysate. Subsequent analyses showed that small amounts of actin and band 4.1 also appeared in lysates of thalassemic RBCs. Therefore, the three major membrane skeletal proteins are, to a varying degree, unstably attached in severe thalassemia. From these studies we would postulate that membrane association of abnormal or partially oxidized α -globin chains has a more deleterious effect on the membrane skeleton than do β -globin chains.

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EKTACYTOMETRIC studies have shown abnormalities of the material properties of red blood cells (RBCs) and their membranes in the several forms of thalassemia.^{1,2} The membranes of both α -thalassemia (α -thal) and β -thalassemia (β -thal) variants are quite rigid, but the membrane instabilities are different.^{1,2} In severe α -thal (hemoglobin-H disease) the membranes are hyperstable, whereas in severe β -thal (β -thal intermedia) the membranes are unstable, particularly in samples from patients who have been splenectomized.¹

Membrane stability is thought to be controlled by the content and function of its skeleton, which is principally composed of ternary complexes of spectrin, actin, and band 4.1 arranged in apparent hexagonal arrays.³⁻⁶ Spectrin dimer self association is defective in severe forms of both α -thal and β -thal.⁷ In severe β -thal intermedia, the spectrin/band 3 ratio is low; this observation pointed to a deficiency of spectrin in the skeleton⁸ accounting for the instability. There are several possible causes for such a deficiency, including deficient synthesis of spectrin in thalassemic erythroid precursors, abnormal spectrin function in forming the ternary complex, and abnormal function of those proteins that bind the spectrin-rich skeleton to the membrane.⁹

In these experiments, we explored the tightness of spectrin binding to the membrane. Ordinarily during ghost preparation by standard stepwise hypotonic hemolysis,¹⁰ virtually no spectrin is detectable in the first hemolysate. Therefore, we looked for the abnormal appearance of spectrin in the first hemolysate of the standard ghost membrane preparation of several sorts of thalassemic RBCs.

MATERIALS AND METHODS

Materials. High-quality sodium dodecyl sulfate (SDS) was purchased from BDH (Poole, UK). Rabbit polyclonal anti-band 4.1 and antispectrin were generated as described previously.^{8,11,12} As previously shown,^{8,12} the monospecific antispectrin used reacts 1.7 times more against α spectrin than against β spectrin, as determined

by Western blotting and quantitative laser densitometry (data not shown)

In some experiments, we used an affinity-purified anti-band 4.1 antibody kindly provided by Dr Joel Chasis (Lawrence Berkeley Laboratory, Berkeley, CA). Rabbit polyclonal antibody against band 3 was a generous gift from Dr Philip Low (Purdue University, Lafayette, IN). Mouse monoclonal antiactin antibodies were purchased from Amersham (Arlington Heights, IL). The secondary antibodies used in Western blotting were a horseradish peroxidase-linked goat antirabbit IgG or goat antimouse IgG obtained from DAKO (Carpinteria, CA). Reagents used in the determination of spectrin by enzyme-linked immunosorbent assay (ELISA) are described below. All other reagents were the best analytical grade available.

Collection of blood. Venous blood was obtained from thalassemic patients and normal controls and shipped on ice in citrate phosphate dextrose or acid citrate dextrose from Bangkok, Thailand under protocols approved by the Thalassemia Center, Siriraj Hospital

From the Thalassemia Center, Siriraj Hospital, Bangkok, Thailand; the Department of Pathology, Ramathibodi Hospital, Bangkok, Thailand; Magen David Adom, Tel Hashomer, Israel; and the Division of Hematology, Stanford University School of Medicine, Stanford, CA.

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Address reprint requests to Stanley Schrier, MD, Division of Hematology, Stanford University School of Medicine, Stanford, CA 94305.

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(Bangkok, Thailand) and the Department of Pathology, Ramathibodi Hospital (Bangkok, Thailand). These samples were compared with samples from controls and patients with autoimmune hemolytic anemia, sickle cell anemia, and chemotherapy-induced anemia at Stanford University Medical Center (Stanford, CA). This latter group of patients consisted of patients entering complete remission in acute myeloid leukemia (AML) who no longer require RBC transfusion. Blood was drawn under protocols approved by Stanford University Committee on Human Subjects in Research.

Preparation of hemolysate. The RBCs were washed three times in phosphate-buffered saline (PBS) to remove plasma and buffy coat. The cells were then incubated at 37°C for 1 hour with the protease inhibitors 2 mmol/L diisopropylfluorophosphate (DFP), 10 μ g/mL leupeptin, and 10 μ g/mL pepstatin and again washed twice in PBS and packed. The hematocrit (Hct) of the cells was measured and a volume of RBC suspension was added that approximated 1 mL of RBCs with an Hct of 100. These RBCs were lysed in 20 mL of lysis buffer consisting of 5 mmol/L sodium phosphate, 1 mmol/L EDTA, pH 8.0, and 20 μ g/mL phenylmethyl sulfonyl fluoride (PMSF) to inhibit proteolysis. The lysed cells were centrifuged at 12,000 RPM, 17,400g for 20 minutes. The first lysate was quantitatively collected for analysis.

SDS-polyacrylamide gel analysis (SDS-PAGE). Ten milliliters of first lysates was centrifuged at 12,000 RPM, 17,400g for 15 minutes. One hundred fifty microliters of clear supernatant was added to standard solubilizer solution and loaded on 6% to 18% gradient SDS-PAGE gels and electrophoresed. The gel was stained with Coomassie brilliant blue and destained in 7% acetic acid solution.^{8,12}

Western blot analysis. Samples were electrophoresed on SDS-PAGE, transferred onto nitrocellulose, and then reacted with rabbit polyclonal antibodies to spectrin, band 4.1, and band 3 (all three at 1:500 dilution in PBS) and mouse monoclonal antibodies to actin (1:500 dilution in PBS). The reaction was developed by adding either horseradish peroxidase-linked goat antirabbit IgG or antimouse IgG (1:1,000 dilution in PBS), followed by the addition of the substrate 4-chloronaphthol.^{12,13}

Quantification of spectrin in the first hemolysate. The amounts of spectrin appearing in first lysate were determined as follows. The supernatant from the first lysate underwent SDS-PAGE as described above and a fresh normal ghost preparation was included in each gel to establish the region where α - and β -spectrin migrate. From each supernatant sample, strips of the gel that contained these regions were carefully cut and placed in separate 1-mL Eppendorf tubes. A piece of gel equal in area to the strips containing spectrin served as a control. One milliliter of 25% pyridine solution was added to each tube. The tubes were capped and agitated overnight. The next day, spectrophotometer readings were taken at 605 nm and recorded. The absolute amount of spectrin in the first hemolysate was estimated as follows. The protein content and concentration of a fresh ghost sample was determined by the Lowry method standardized using bovine serum albumin. The sample was then subjected to SDS-PAGE exactly as described above, stained with Coomassie brilliant blue, and then analyzed by laser densitometry. The densitometer data provided the absolute concentration of spectrin present in the ghost sample. A spectrin standard curve was then established by adding a serial dilution of this ghost preparation to sequential wells and performing SDS-PAGE. After staining, the spectrin containing areas were eluted into pyridine (as above) and the absorption at 605 nm was plotted versus the known content of spectrin on the gel. The values of spectrin used for this standard curve were based on our prior analyses of thalassemic hemolysates and were chosen to contain and overlap the values initially observed. Using interpolation, the absolute values for spectrin in the patients' first hemolysates were determined. We had added a precisely measured volume of packed RBCs to each lysate. Each patient's resulting ghosts were

also subjected to SDS-PAGE and the proportion of spectrin was calculated by laser densitometry. Therefore, we could determine the proportion of spectrin that appeared in each first hemolysate. This calculation was based on the following presumptions: 1 mL of packed RBCs (Hct at a theoretical 100) consists of 10^{10} normal RBCs and, when converted to ghosts, contains 7 mg of membrane protein of which α - and β -spectrin (bands 1 and 2) comprise 30% or 2.1 mg of spectrin.

ELISA. To confirm more definitively the amount and proportion of spectrin that dissociates from RBC membranes during hypotonic lysis, an ELISA method was used. For this experiment an entirely new shipment was obtained from Bangkok consisting of samples from patients not previously analyzed and including two normal shipment controls. Several preliminary experiments were performed to establish a standard curve spanning the anticipated spectrin concentration. Standards were run in duplicate, whereas patient samples were performed in triplicate. One hundred microliters of lysates and spectrin standard (Sigma Chemicals, St Louis, MO) was coated directly onto a 96-well ELISA plate (ICN Biochemicals, Horsham, PA) and incubated overnight at 4°C. After blocking with 200 μ L of 5% bovine serum albumin (BSA) in PBS per well for 2 hours at 37°C, the plate was washed four times with 0.05% Tween 20 (Sigma Chemicals) in PBS. To each well we added 100 μ L of the Ig fraction of our monospecific polyclonal antispectrin antibody (diluted 1:250 with 5% BSA in PBS), which was followed by incubation at 37°C for 2 hours. The plate was again washed and 100 μ L of peroxidase-linked donkey antirabbit Ig antibody (1:5,000 dilution with 1% BSA in PBS; Pierce, Rockford, IL) was added and incubated at 37°C for 1 hour. After a final fourfold wash with 0.05% Tween 20 in PBS, the plate was developed using the o-phenylenediamine (OPD) ELISA detection kit (Pierce). The plate was read at 490 nm using an ELISA plate reader (Flow Laboratories, McLean, VA). Patient hemolysates were prepared and analyzed when the samples arrived; these results are presented in Table 2. Three days later, another set of hemolysates was prepared from the same RBCs and run again with substantial agreement (data not shown).

RESULTS

Spectrin content of first lysate. After lysing a precise volume of packed RBCs, the first supernatant was collected and analyzed on a gradient SDS-PAGE, as shown in Fig 1A and B. Normal controls do not have any visible protein in the α - or β -spectrin region of the gel. In contrast, all of the thalassemic samples show varying amounts of visible spectrin in the first lysate, indicating that some spectrin was dissociated from the membrane cytoskeletal network during the first hypotonic lysis. The number of patients with each thalassemic variant studied is listed in Table 1. Other patient controls studied included autoimmune hemolytic anemia (n = 3), sickle cell anemia (n = 2), and chemotherapy-induced anemia (n = 3; Table 1); the results are shown in Fig 2. No protein bands in the spectrin region were visible in these patient's lysates.

Confirmation of the presence of spectrin. To be sure that the bands seen on the gels were indeed spectrin, Western blotting analyses were performed (Fig 3). The bands seen in our thalassemic lysates were, in fact, immunoreactive spectrin and none was detected in our normals or patient controls.

Quantification of the spectrin in the hemolysate. To estimate the amount of the spectrin that was dissociated from the membrane, the protein bands in the spectrin region were

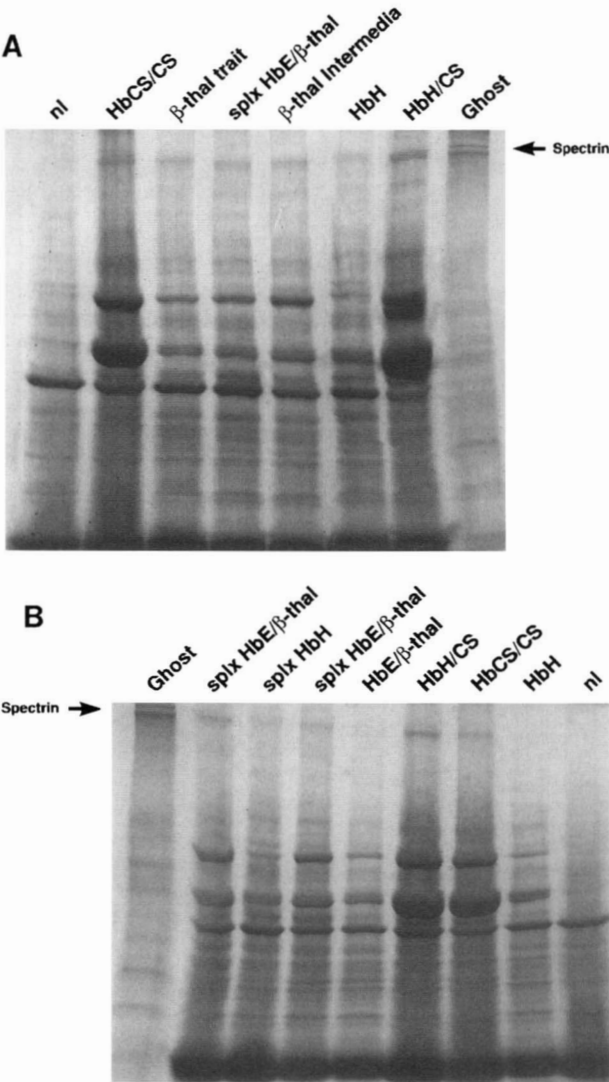


Fig 1. SDS-PAGE of first lysate of thalassemic samples from Bangkok (A and B). The RBCs were washed and lysed as described. One hundred fifty microliters of the first lysate was collected and solubilized in SDS, separated electrophoretically on 6% to 18% nonlinear gradient polyacrylamide gels, and stained with Coomassie blue.

cut out and eluted into pyridine. The results are shown in Fig 4, in which the proportion of spectrin dissociated from the membrane by the first hypotonic lysis step is indicated along with disease severity as manifested by the patient's hemoglobin concentration. Four different thalassemic groups are shown and contrasted with several normal individuals and 2 patients with α -thal trait. The splenectomized β -thal patients have the most severe anemia while having the highest proportion of spectrin dissociation. Homozygous hemoglobin Constant Spring (CS) RBCs show considerable spectrin dissociation, but the anemia is quite modest.

Because these results were critical for our observation, an ELISA method was also used to detect spectrin in the first lysate. The results are shown in Table 2 and are consistent with the results reported above for the semiquantitative

Table 1. Diseases Studied

	No.
NI	8
AHA	3
Chemotherapy-induced anemia (AML)	3
SS	2
HbE/ β -thal (S)	3
HbE/ β -thal (NS)	7
Hb CS/CS	7
Hemoglobin H (α -thal-1/ α -thal-2)	5
Hb H/CS	5
α -thal-1 trait	3

Abbreviations: NI, normal individuals; AHA, autoimmune hemolytic anemia; SS, sickle cell anemia; HbE/ β -thal (S), splenectomized β -thal intermedia; HbE/ β -thal (NS), nonsplenectomized β -thal intermedia; Hb CS/CS, homozygous Hb Constant Spring; Hb H/CS, hemoglobin H/Constant Spring.

method. However, the patients with homozygous Hb CS/CS in this shipment did not have as much spectrin dissociation as seen previously. The generally high values of spectrin dissociation seen in β -thal intermedia were confirmed as were the low values for classical HbH disease.

Detection of other membrane proteins in the first lysate. With the evidence that some spectrin dissociates from the membrane in the several forms of severe thalassemia tested, it became important to determine if spectrin was unique in this regard. We therefore tested the first lysate for the presence of actin and band 4.1 using Western blotting and found both proteins in virtually all of our thalassemic patient samples and little if any in normal patients or patient controls

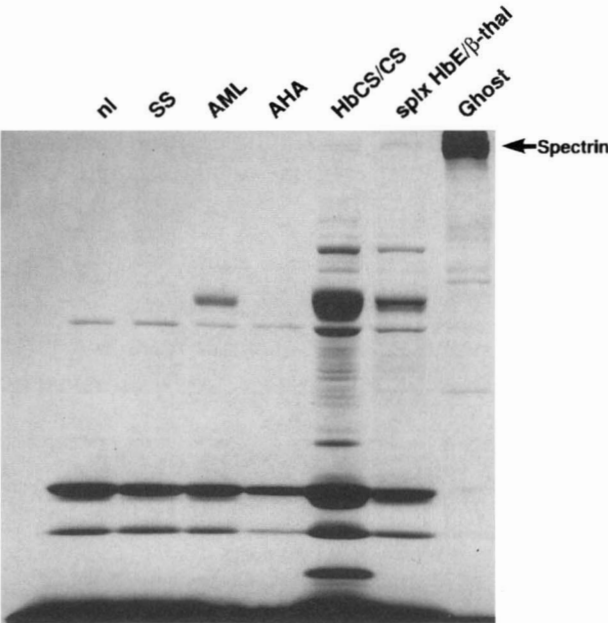


Fig 2. SDS-PAGE of samples from local and shipment controls. Patients samples included sickle cell anemia, autoimmunhemolytic anemia, and a patient with AML recovering from induction with an elevated reticulate count with no longer transfusion dependent.

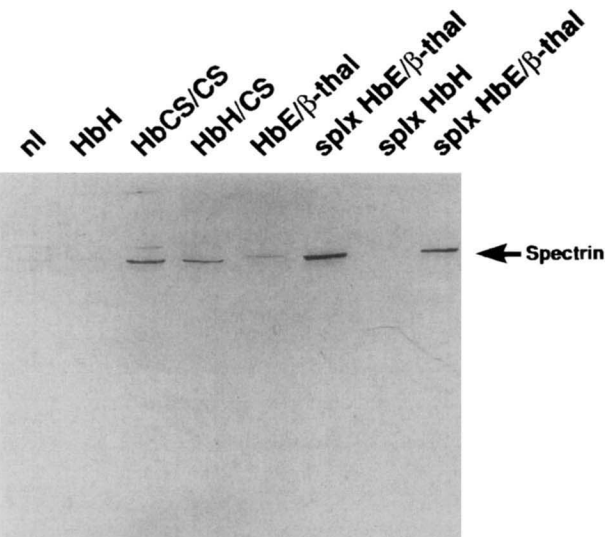


Fig 3. Western blotting studies of first lysates. After SDS-PAGE separations, the samples were transferred to nitrocellulose and reacted first with rabbit antispectrin antibodies and then with horseradish peroxidase-conjugated goat antirabbit antibodies. The immunoblots were developed using 4-chloronaphthol as substrate.

(Figs 5 and 6). To determine if there was a general membrane instability, we analyzed the first lysate in Western blotting using an anti-band 3 antibody and found none in patients, normals, or patient controls (data not shown).

DISCUSSION

In normal RBCs and in RBCs from patients with sickle cell anemia, autoimmune hemolytic anemia, and chemotherapy-induced anemia, spectrin is tightly bound to the membrane skeleton and to transmembrane proteins and very little, if any, appears in the first hemolysate.¹⁴ In both α -thal and β -thal, spectrin appears in the first hemolysate (Fig 1A and B), indicating a degree of instability of spectrin binding to the skeleton or to the transmembrane proteins. Western blot-

ting confirms the presence of spectrin (Fig 3). Spectrin in the first hemolysate of thalassemic RBCs was measured and the amounts in decreasing order were as follows: splenectomized β -thal intermedia including HbE/ β -thal; Hb CS/CS; nonsplenectomized β -thal intermedia; HbH/CS; and, lastly, HbH disease (Fig 4). ELISA measurements generally confirmed these findings. The fact that the β -thal variants and some of the CS variants produce relatively more spectrin dissociation than did classical HbH disease (α -thal-1/ α -thal-2) suggests that the binding of an α -globin chain to the membrane (the excess α globin in β -thal and the α -Constant Spring in HbH/CS disease and in some patients HbCS/CS)¹⁵ produces relatively more spectrin dissociation than does β -globin chain binding.

It would be convenient to speculate that the spectrin dissociation occurring in splenectomized β -thal intermedia RBCs accounts for the membrane instability seen in the ghosts of these RBCs. However, we cannot make that obvious linkage because some Hb CS variants show similarly large amounts of spectrin dissociation and their membranes are known to be hyperstable.¹⁵

Our initial observations were confined to an analysis of spectrin in the first hemolysate, because spectrin bands could be seen on SDS-PAGE. No bands migrating in the position of band 4.1 or actin could be visualized on standard SDS-PAGE. However, because both of these membrane skeletal proteins are present of approximately 15% of the content of spectrin, their presence might be below the detectability of SDS-PAGE analysis with Commassie blue staining. Therefore, we searched for the presence of these membrane skeletal proteins in Western blotting and found small amounts of both band 4.1 and actin in the first lysates of virtually all of our severely affected thalassemic patients. No band 4.1 or actin was found in the lysates of normals or our patient controls. Furthermore, no band 3 was found in any of our patients or controls, and the band 3 content of membranes is equal to or greater than that of spectrin. Therefore, we concluded that the major transmembrane protein, band 3, is stably inserted into the membrane but that the membrane skeletal proteins represented by spectrin, actin, and band 4.1

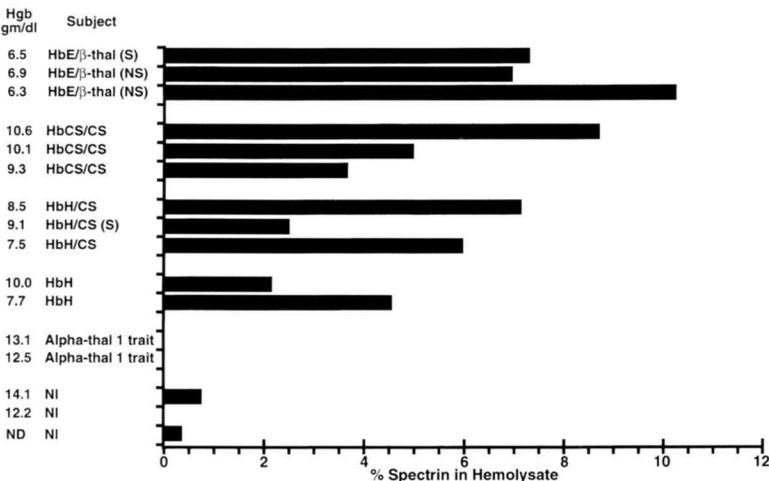


Fig 4. The amount of spectrin that dissociates from the membrane is indicated for five specific thalassemic variants along with the patients' hemoglobin content.

Table 2. Percentage of Spectrin in Hemolysate Determined by ELISA

Disease Type	Hgb (g/dL)	Spectrin (%)
HbE/ β -thal (NS)	10.1	3.9
HbE/ β -thal (NS)	7.9	6.2
HbE/ β -thal (NS)	7.9	11.8
HbE/ β -thal (NS)	6.4	4.6
HbCS/CS	9.8	2.3
HbCS/CS	10.6	2.6
HbH/CS	10.9	2.4
HbH	6.8	1.9
HbH	9.7	2.0
NI	16.6	1.3
NI	17.0	1.3

are unstably attached to the membrane in all of the thalassemic variants tested.

An alternative and unlikely explanation for our findings is that thalassemic RBCs contain cytosolic spectrin, band 4.1, and actin. This possibility is highly unlikely given the recent studies of membrane assembly.¹⁶ Furthermore, if cytosolic spectrin were present, it would be more likely to be α -spectrin that is synthesized at three times the rate as β -spectrin. However, inspection of Fig 1A and B shows that, if anything, β -spectrin appears to be present in somewhat larger amounts than α -spectrin. The observation is further buttressed by the fact that our antispectrin antibody reacts

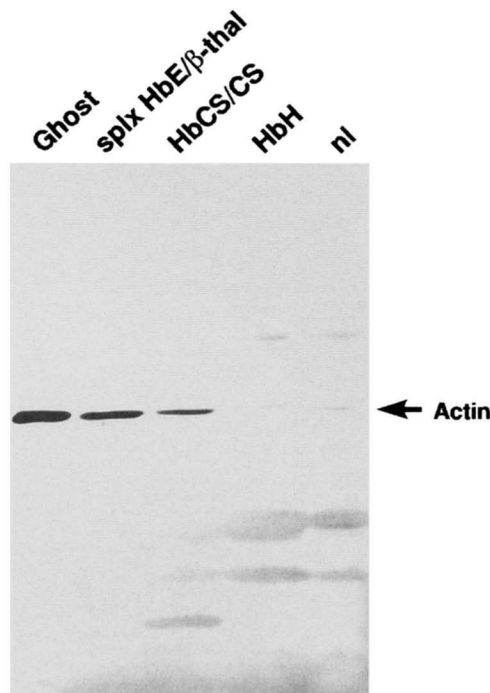


Fig 5. Western blotting studies of patients and control lysates. After SDS-PAGE separations, the samples were transferred to nitrocellulose and reacted first with antiactin monoclonal antibodies and then with peroxidase-conjugated goat antimouse antibodies. The immunoblots were developed using 4-chloronaphthol as substrate.

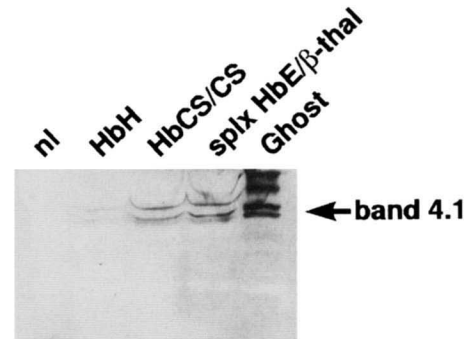


Fig 6. Western blotting studies of patients and control lysates. After SDS-PAGE separations, the samples were transferred to nitrocellulose and reacted first with affinity-purified anti-band 4.1 and then with peroxidase-conjugated goat antirabbit antibodies. The immunoblots were developed using 4-chloronaphthol as substrate.

1.7 times more with α -spectrin than with β -spectrin (see Materials and Methods).

It is not clear why β -spectrin may be present in somewhat greater concentration than α -spectrin in our thalassemic lysates. It may be that the proteases that degrade α -spectrin are very active in thalassemic RBCs in vivo, leaving relatively larger amounts of β -spectrin behind.

Therefore, three membrane skeletal proteins are dissociated from thalassemic RBCs when challenged by hypotonic lysis. The degree of spectrin instability does not account for the membrane instability detected by ektacytometry. The reported defective spectrin dimer self association could also contribute to the membrane instability.⁷ The accumulation of α -globin chains at the membrane (either excess α in severe β -thal or α CS in some of the HbCS variants) presumably destabilizes the membrane skeleton more than the β -globin chain accumulation that occurs in classical Hb-H disease. At this point, it is not clear how this membrane instability could contribute to the peripheral hemolysis seen in thalassemia. It is likely that the accumulation of the globin chains at the membrane or their oxidation products produces derangement sufficient to account for as much as 10% of spectrin dissociation during hypotonic lysis.

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