Molecular Basis of Spectrin and Ankyrin Deficiencies in Severe Hereditary Spherocytosis: Evidence Implicating a Primary Defect of Ankyrin

By Manjit Hanspal, Sa-Hyun Yoon, Huilan Yu, Jatinder S. Hanspal, Stephen Lambert, Jiri Palek, and Josef T. Prchal

While varying degrees of spectrin deficiency have been found in the majority of patients with hereditary spherocytosis (HS), a combined severe deficiency of both spectrin and the spectrin-binding protein, ankyrin, has been reported only in two patients with severe HS. To elucidate the molecular basis of these protein deficiencies, we have studied the synthesis, assembly, and the mRNA levels of spectrin and ankyrin in peripheral blood reticulocytes in one of the previously reported probands. Pulse-labeling studies showed that in HS reticulocytes, the synthesis of α-spectrin was comparable with control reticulocytes while that of β-spectrin was increased about fourfold, presumably reflecting increased erythropoietic drive. On the HS reticulocyte membrane, the amount of newly assembled spectrin was reduced to about half of the control values, presumably reflecting a decrease in the synthesis of α- and β-spectrin.

The primary defect is thought to reside in the red blood cell (RBC) membrane skeleton. This submembranous protein network is organized as a hexagonal lattice of fibers of spectrin tetramers that are interconnected by junctional complexes of oligomeric actin, with the aid of proteins 4.1, adducin, and possibly other proteins including myosin, tropomyosin, and band 4.9. Each of the spectrin tetramers is composed of the αβ-spectrin heterodimers that self-assemble at their head region into tetramers. The skeleton is attached to the plasma membrane via the association of β-spectrin with ankyrin (protein 2.1) and protein 4.1 which, in turn, binds to several integral membrane proteins.

The assembly of the membrane skeleton that occurs during erythroid development has been previously studied in rat and chicken erythroid cells and, to some extent, in erythroblastic cell lines. In both rat and chicken erythroblasts, α-spectrin is synthesized in a twofold to threefold excess over β-spectrin. The two polypeptides assemble in equimolar amounts on the membrane forming a stable skeletal network, while the excess cytosolic α- and β-spectrins are rapidly degraded. In the final stages of erythropoiesis, the synthesis of ankyrin and protein 4.1 and the incorporation of these proteins into the membrane continue well after the synthesis of other components of the membrane skeleton has been downregulated.

In the majority of patients with HS, a partial deficiency of spectrin has been observed. In two subsets of HS patients, this spectrin deficiency may represent a primary lesion: (1) in rare HS kindreds with an autosomal recessive form of the disease, where HS has been linked to an abnormality involving α-II domain of spectrin, and (2) in a small subset of patients with dominantly inherited HS, where the primary defect involves abnormal β-spectrin that binds poorly to the protein 4.1. In contrast, in many HS patients, particularly those with the autosomal dominant form of the disease, spectrin deficiency does not seem to represent the primary molecular defect as shown by recent preliminary studies failing to link HS with polymorphism of the synthesis of the spectrin binding protein, ankyrin: the ankyrin synthesis was nearly absent in the cytosol and the amounts of membrane-associated ankyrin were reduced to about half of the normal values. The changes in the amounts of spectrin and ankyrin mRNAs quantitated by slot blot and Northern blot analyses were comparable with changes in the synthesis of these proteins: The α-spectrin mRNA was within a control range and the β-spectrin mRNA was slightly increased, while the amounts of ankyrin mRNA were reduced to about 50% of control values. We conclude that the primary defect underlying the combined spectrin and ankyrin deficiency is a deficiency of ankyrin mRNA leading to a reduced synthesis of ankyrin which, in turn, underlies the decreased assembly of spectrin on the membrane.

© 1991 by The American Society of Hematology.
reticulocytes (α-spectrin) or even increased (β-spectrin) as were the amounts of reticulocyte mRNA for both spectrin polypeptides. In a striking contrast, the assembly of spectrin on the membrane was markedly reduced. We further show that the underlying molecular defect involves a reduced synthesis of ankyrin, as indicated by marked decrease in ankyrin synthesis in the cytosol and its reduced assembly on the membrane together with a marked decrease in the amount of the reticulocyte mRNA. Furthermore, the residual ankyrin was unstable as evidenced by the presence of ankyrin degradation products in the cytosol that were not detected in control reticulocytes. Thus, the primary defect involves a reduced synthesis of ankyrin resulting in a reduced incorporation of ankyrin into the RBC membrane which, in turn, leads to a reduced assembly of spectrin on the membrane.

MATERIALS AND METHODS

Subjects. Normal reticulocytes were isolated from the peripheral blood of patients with sickle cell anemia or other hemolytic disorders and from patients with hemochromatosis undergoing therapeutic phlebotomy.

Clinical data on subject CC has been summarized by Coetzee et al. In brief, the subject is a 41-year-old white man with chronic hemolytic anemia since infancy, who underwent a splenectomy at 3 months of age. The peripheral blood smear showed marked microspherocytosis, poikilocytosis, and polychromasia. The proband's deceased father suffered from alcoholic hepatic cirrhosis and anemia; however, he was recorded to have normal hemoglobin concentration and erythrocyte indices at the time of military training, whereas his deceased mother was hematologically normal as are two living siblings.

Venous blood was collected in tubes containing acid citrate dextrose as anticoagulant at the University of Alabama, Birmingham. Blood was immediately processed for reticulocyte RNA preparation. For synthetic studies, the samples were cooled and transported to Boston, MA overnight on ice in an insulated container and analyzed immediately.

Fractionation of reticulocytes and nucleated RBC precursors. Reticulocytes of sickle cell and pyruvate kinase deficiency patients were used as control reticulocytes in the present study. Reticulocytes were isolated from the peripheral blood of control and HS individuals. Blood was centrifuged at 1,000g for 5 minutes and the cells were washed three times with Hank's balanced salt solution (HBSS) without Ca2+ and Mg2+ (M.A. Bioproducts, Walkersville, MD). Four milliliters of this cell suspension was then mixed with 18 mL 75% Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) in HBSS resulting in a starting density of 1.09 g/mL, and centrifuged in a fixed angle rotor at 45,000g for 30 minutes at 4°C. After the run, 1-mL fractions were collected from the top of the gradient and diluted with HBSS. The cells were harvested by centrifugation and reticulocytes were isolated by staining with New Methyline Blue (J.T. Baker Chemical Co, Phillipsburg, NJ). RNA content of reticulocytes was determined by means of the orcinol technique. Reticulocytes in fraction numbers 9 to 12 corresponding to Percoll density of 1.07 g/mL contained 40 ± 5 μg of RNA per 107 cells. These fractions were pooled and used in the present studies.

Human nucleated RBC precursors were isolated from the sternal or rib marrow using a combination of Ficoll-Paque and a discontinuous Percoll gradient, as described earlier. The sternal and rib marrow represented a discarded marrow during sternotomy and rib resection, respectively.

Pulse labeling of cells with (35S) methionine. Human reticulocytes or nucleated RBC precursors, 2 to 3 × 107, were washed twice in minimum essential medium (MEM) without methionine (Flow Laboratories, McLean, VA), resuspended in 10 mL of the same medium containing 10% fetal calf serum prewarmed to 37°C, and incubated at 37°C for 15 minutes. They were then labeled for different lengths of time with 300 μCi (35S) methionine (1,000 Ci/mmol; New England Nuclear, Boston, MA). For the pulse-chase experiment, further incorporation of (35S) methionine was stopped by the addition of unlabeled methionine (0.4 mM/L) and the incubation was then continued for different time periods. At the end of the labeling period, 10 vol of 155 mM/L choline chloride, 5 mM/L HEPES, pH 7.1, were added and the cells were harvested by centrifugation.

Isolation of plasma membranes. (35S) methionine-labeled cells were treated with Diisopropyl fluorophosphate (Sigma Chemical Co, St Louis, MO) before isolating plasma membranes. Plasma membranes were isolated by using the procedure of Chan. The cells were suspended in hypotonic buffer (10 mM/L Tris HCl, pH 7.5 10 mM/L KCl, and 1.5 mM/L MgCl2) and then disrupted with 40 strokes of a tight-fitting Dounce homogenizer. An appropriate volume of 2 mol/L sucrose was added immediately to the homogenate to restore isotonicity. The homogenate was then layered over a sucrose step gradient (28%/50% sucrose, 1 mL 75% Percoll, Pharmacia Fine Chemicals, Uppsala, Sweden) in HBSS. The fractionation of the resulting gradient was monitored by the absorbance at 260 nm and the fractions containing the plasma membranes were collected. The membranes were washed four times with buffer A. The final pellet was resuspended in HBSS and centrifuged at 100,000g for 1 hour.

Immunoprecipitation of spectrin and ankyrin. Membranes and cell lysates isolated from (35S) methionine-labeled cells were used for immunoprecipitation of spectrin and ankyrin. Spectrin immunoprecipitation was performed as described earlier, whereas for ankyrin immunoprecipitation, the method used was a modification of Marcantonio and Hynes. The samples in 0.1 mol/L Tris-HCl, pH 8.5, 0.15 mol/L NaCl, 2.4 mM/L MgCl2, and 5 mM/L Tris-HCl, pH 7.4) and centrifuged in an SW 41 rotor (Beckman Instruments, Inc, Fullerton, CA) at 117,000g for 40 minutes. The membrane fraction, at the 28%/50% sucrose interface, and the soluble fraction on top of the sucrose gradient were collected. The membrane fraction was diluted with 20 mM/L Tris-HCl, pH 7.4, and centrifuged at 22,000g for 10 minutes. The resulting membrane pellet was washed once more before it was solubilized in sodium dodecyl sulfate (SDS) sample buffer.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were separated by SDS-PAGE according to the buffer systems of Fairbanks et al and Laemmli. The gels were processed for fluorography with En'Hance (New England Nuclear), dried, and exposed to Kodak XAR-5 x-ray film (Eastman Kodak, Rochester, NY). The autoradiograms were scanned at 570 nm with a Quick-Scan (Helena Laboratories, Beaumont, TX), and the area under each peak was integrated.
Preparation of reticulocyte RNA. Blood from the spectrin- and ankyrin-deficient patient and from a patient with reticulocytosis undergoing therapeutic phlebotomies, were used as a source of human reticulocyte RNA. Total reticulocyte RNA was isolated by the method of Groossens and Kan, and modified by additional purification of the acid RNA precipitate by the guanidine HCl-phenol method. Poly (A) RNA was isolated by oligo(dT)-cellulose chromatography, and by one to three passages through oligo-(dT) resin. All human tissues were obtained from the Tissue Procurement Facility of the University of Alabama at Birmingham (UAB) using protocols previously approved by the UAB Human Use Committee.

Preparation of β-spectrin, α-spectrin, ankyrin, and α-globin cDNA probes. The human β-spectrin cDNA probe was isolated from the plasmid pHaSp5.7 previously characterized in our laboratory. The human α-spectrin cDNA probe was prepared from the plasmid pHaSp5 (kindly provided by Dr Peter Curtis, The Wistar Institute of Anatomy and Biology, Philadelphia, PA) by polymerase chain reaction with two primers flanking the PstI site of pBR322. The human ankyrin cDNA probe was prepared from the plasmid pHank19A that contains a 2-kb insert spanning part of the spectrin binding domain of ankyrin. The human α-globin cDNA probe (kindly provided by Dr Steven Liebhaber, School of Medicine, University of Pennsylvania) was obtained from plasmid pHo2A, and α-globin cDNA was separated from the plasmid DNA by PstI digestion using standard techniques.

Slot blotting and quantitation of spectrin and ankyrin transcripts. Poly (A) RNA samples were applied on nitrocellulose paper by using a slot blot system (Schleicher & Schuell, Keene, NH). The quantity of each poly (A) RNA sample was assayed spectrophotometrically. The filter was hybridized with an appropriate cDNA probe labeled with 32P by nick translation. In addition, each nitrocellulose filter was analyzed by α-globin cDNA probe. Thus, the amount of globin mRNA in each reticulocyte mRNA sample served as an internal standard of poly (A) RNA purity and quantity. The hybridization and washing was performed under stringent conditions as described elsewhere. Autoradiograms were scanned using an LKB 2400 Ultrascan XL laser densitometer (LKB Produkter, Broma, Sweden) and the amount of message was calculated by comparison of the relative intensity of hybridization of the sample with that of an α-globin message in the same sample. In these studies, the control was a subject of normal globin chain synthesis. The Northern blot hybridization. Poly (A) RNAs were fractionated on 1.4% agarose formaldehyde gels followed by transfer to nitrocellulose filters and hybridization to cDNA probe. Conditions for hybridization and washing were performed as previously described. The Northern blot membrane was subsequently rehybridized with α-globin cDNA probe; thus, the globin mRNA in each sample served as an internal standard of poly (A) RNA quantity and purity.

Chromosomal analysis and Southern blotting of ankyrin DNA. Cytogenetic analysis of the spectrin- and ankyrin-deficient patient was performed from a bone marrow aspirate. The chromosomal spreads were examined using a modification of Hozier and Lindquist’s technique. All metaphases were GTO banded. Southern blot analyses were performed using standard techniques. Two different cDNA human ankyrin probes were used. Probe 13A is a 1.5-kb probe spanning both spectrin and band 3 binding domains, while probe 25A is a 1.58-kb cDNA probe covering the 5’ coding region of ankyrin molecule and extending downstream to the band 3 binding domain. In each Southern blot analysis, at least five control subjects were used. These were patients with hereditary spherocytosis and their unaffected relatives. The enzymes used with probe 13A were HindIII, PvuII, EcoRI, BamHI, PstI, KpnI, Sal I, Xba I, TaqI, BglII, and BglI; those used with probe 25A were KpnI, BglI, and BamHI. Similar studies were performed using the α-spectrin genomic and cDNA probes and two β-spectrin cDNA probes covering β7 to β14 repetitive domains.

RESULTS

Synthesis, assembly, and turnover of spectrin in reticulocytes. The synthesis of spectrin was studied in reticulocytes after a pulse-label with (35S) methionine. In the cytosol of control reticulocytes, the synthesis of α- and β-spectrins was comparable with that previously reported in nucleated RBCs from the rat and human. In contrast, on the membrane the two chains of spectrin assembled in equimolar amounts as shown previously for rat, human, and chicken erythroid cells. In the cytosol of HS reticulocytes, the synthesis of α-spectrin was identical to control while the synthesis of β-spectrin was increased about fourfold (Fig 1A). On the HS membrane, the two polypeptides were assembled stoichiometrically but their amounts were markedly reduced to about half the control values. We have obtained identical results by two independent methods: (1) direct quantitation of spectrin synthesis and assembly from the autoradiogram of SDS-PAGE of plasma membranes and cell lysates, and (2) spectrin immunoprecipitation (data not shown). As suggested earlier in our previous report, the extent of spectrin synthesis in reticulocytes is dependent on the age and fragility of these cells as determined by their RNA contents. Early reticulocytes synthesize significant amounts of both α- and β-spectrins, while at the late reticulocyte stage spectrin synthesis is markedly diminished (data not shown). Therefore, early reticulocytes were used throughout in the present study for both the control and patient samples.

To ascertain that the synthesis of spectrin in reticulocytes is comparable with that in their nucleated RBC precursors, we have compared the synthesis and assembly of spectrin in nucleated RBC precursors and reticulocytes. The synthetic ratios of spectrin α- and β-subunits in reticulocytes and in their nucleated RBC precursors were similar, implying that reticulocytes represent a useful system to study the synthesis and assembly of spectrin in patients with hereditary hemolytic anemias associated with deficiencies of membrane proteins (data not shown).

The stability of the newly synthesized α- and β-spectrins in the membrane and cytosol was examined by a pulse-chase of control and HS reticulocytes labeled with (35S) methionine and chased in a medium containing unlabeled methionine. Both in control and HS reticulocytes (Fig 1B), approximately equimolar proportions of labeled α- and β-spectrin entered the membrane fraction and reached a plateau by 1 hour following the commencement of the chase. As previously reported by us for the rat erythroid precursors, there was no detectable turnover of either control or HS spectrin in the membrane during the 3-hour chase period. However, in the soluble fraction of both
The synthesis of α-spectrin in the cytosol of HS reticulocytes is similar following a 15-minute labeling, the cells were incubated in a medium fourfold. On the control while the synthesis of β-spectrin is increased about (S).

The synthesis of α-spectrin in the cytosol of HS reticulocytes is similar to control while the synthesis of β-spectrin is increased about fourfold. On the HS membrane, the amount of the two polypeptides is reduced to about half the control values. In membranes of both the control and HS reticulocytes, there is no detectable turnover during the 3-hour chase period. In the cytosols, however, α- and β-spectrins are gradually turning over with similar half-lives in control and HS reticulocytes. These experiments were performed four times with similar results.

Fig 1. Representative synthesis, assembly, and turnover of spectrin in HS and control reticulocytes. An equal number (2 × 10⁷) of age-matched HS (C.C) and control (N) reticulocytes were labeled with (35S) methionine for different time periods. For pulse-chase in (B), following a 15-minute labeling, the cells were incubated in a medium containing cold methionine for 0, 1, 2, and 3 hours. At the end of pulse-label or pulse-chase incubations, the cells were lysed to obtain their plasma membranes and cell lysates, which were then examined by SDS-PAGE. The autoradiograms generated from these gels were scanned at 570 nm and the area under each peak was integrated. (A) The synthesis of α-spectrin in the cytosol of HS reticulocytes is similar to control while the synthesis of β-spectrin is increased about fourfold. On the HS membrane, the amount of the two polypeptides is reduced to about half the control values. (B) In the membranes of both control and HS reticulocytes, there is no detectable turnover during the 3-hour chase period. In the cytosols, however, α- and β-spectrins are gradually turning over with similar half-lives in HS and control reticulocytes. These experiments were performed four times with similar results.

control and HS reticulocytes, the amount of labeling increased during the first hour after the onset of the chase and subsequently declined. The slopes of the curves for soluble HS and control α- and β-spectrins were identical, suggesting that they turn over with similar half-lives. The initial increase in radioactivity is probably due to the completion of the synthesis of polypeptides labeled during the pulse period.

Synthesis, assembly, and turnover of ankyrin. Our findings of normal synthesis and turnover of spectrin in HS reticulocytes were in a striking contrast to a reduced assembly of spectrin on the HS RBC membrane. Therefore, we have asked whether or not the reduced assembly of spectrin on the HS membrane is caused by a defective synthesis of the spectrin-binding protein ankyrin. As in the studies of spectrin synthesis, an equal number of (35S) methionine-labeled reticulocytes matched for their RNA content were used, and their plasma membranes and cell lysates isolated as described in Materials and Methods. Ankyrin was immunoprecipitated from both the plasma membranes and cell lysates, and the resulting immunoprecipitates were analyzed on SDS-polyacrylamide gels (Fig 2A). In control reticulocytes, the amounts of ankyrin synthesized in the cytosol exceed those assembled on the membrane, as has also been shown in avian erythroid cells.⁴⁶ In contrast, no intact ankyrin was detectable in the cytosol of HS reticulocytes and only immunoreactive degradation products (indicated by arrows in Fig 2A) were seen that were absent in the nonimmune serum controls (data not shown). While we were unable to detect newly synthesized intact ankyrin in the HS cytosol, some newly synthesized ankyrin was found in the membrane preparations of HS reticulocytes; however, it was reduced to about half the control values (Fig 2B).

To determine the turnover of the newly synthesized ankyrin, (35S) methionine-labeled control and HS reticulocytes were chased for various lengths of time in a medium containing unlabeled methionine. Ankyrin was then immunoprecipitated from both the plasma membranes and cell lysates isolated from an equal number of cells for each time point. In membranes of both the control and HS reticulocytes (Fig 2C), the total amount of radioactivity increased for some time after the beginning of the chase and then reached a plateau that remained constant for up to 120 minutes of the chase, indicating that the membrane-associated ankyrin was stable. The turnover of membrane-associated HS ankyrin was the same as in the control samples. In contrast to membrane-associated ankyrin, the amount of radiolabeled ankyrin in the soluble fraction of control reticulocytes declines gradually, implying that the soluble ankyrin is gradually degraded. Because there was no detectable intact ankyrin in the HS reticulocyte cytosol, the pulse-chase could not be performed.

Quantitation of α-, β-spectrin, and ankyrin mRNAs in reticulocytes. We have previously shown that human reticulocytes have a low abundance of both α and β spectrin mRNA.⁴⁸,⁵⁰ The relative abundance of the spectrin and ankyrin mRNAs in control and HS reticulocytes was quantitated by using a slot-blot analysis (Fig 3), and the amounts were expressed as a ratio to the amount in α-globin mRNA used as an internal standard (Table 1). While the amount of α-spectrin mRNA was identical to the control sample, the amount of β-spectrin appeared slightly greater as judged by relative intensity of β-spectrin signal. This increase in β-spectrin mRNA was less apparent when the results were adjusted to the relative amounts of α-globin mRNA, used as an internal standard for each sample. However, the difference was relatively minor, with the mean ratio of relative values of β-spectrin and α-globin mRNA signal .705 as compared with control value of .64. This apparent mild increase of β-spectrin message of HS patients was not statistically significant with the P value of .805. In a striking contrast, the amount of ankyrin mRNA was markedly reduced as compared with a control sample. While the data presented in Fig 3A suggest that the propositus had less than 50% of ankyrin mRNA as compared with the control, the correction for the amount and purity of poly(A)⁺ RNA by α-globin internal standard showed that the deficiency of ankyrin mRNA is about 50% of normal (Table 1).
Northern blot analysis of reticulocyte poly (A)⁺ RNA hybridized with a human ankyrin cDNA probe is shown in Fig 4. As previously shown, ankyrin mRNA was present as two distinct species of 7.2 and 9.0 kb. The amounts of both the species was dramatically reduced in the HS reticulocytes as compared to a control sample, consistent with our slot-blot analysis.

To more accurately quantitate the ankyrin message in reticulocytes, the filter from Fig 4 was rehybridized with human α-globin cDNA probe. Because of the marked differences in abundance of ankyrin and human α-globin message in reticulocytes, the rehybridized membrane was exposed for only 30 minutes at room temperature, and the globin signal and the major ankyrin 7.2-kb signal were quantitated by laser densitometry. The ratio of globin to ankyrin signal was 10.5 as compared with the same measure-
Quantitation of spectrin and ankyrin mRNAs in HS and control reticulocytes. Poly (A)⁺ RNA samples were applied on nitrocellulose paper by using a slot-blot apparatus. The filter was hybridized with (A) α-spectrin, (B) β-spectrin, and (C) ankyrin cDNA probes. Hybridization and washing conditions were the same as described previously. While there are no significant differences in the amounts of control and HS reticulocyte α- and β-spectrin mRNAs (see text), ankyrin mRNA is markedly reduced in HS reticulocytes relative to a control sample.

A detailed chromosomal analysis of the HS subject was normal as were the major ankyrin fragments produced by multiple restriction enzymes using two ankyrin cDNA probes, coding for 5' and mid to 3' regions of human ankyrin gene. These data make the possibility of chromosomal rearrangement or deletion at the site of ankyrin gene unlikely. Likewise, no rearrangement or deletion of α- and β-spectrin genes were found on Southern blot analyses with α- and β-spectrin probes. Thus, the partial decrease of the ankyrin mRNA is likely to be the result of a point mutation resulting in either a reduced mRNA transcription or a reduced mRNA stability, rather than a partial deletion of the ankyrin gene.

**DISCUSSION**

The recently reported severe HS with a combined severe deficiency of both spectrin and the spectrin-binding protein ankyrin gave us an opportunity to investigate the molecular basis of these combined deficiencies and the role of ankyrin in the assembly of spectrin on the normal and HS mem-

**Table 1. α-, β-Spectrin and Ankyrin mRNA Content of Reticulocytes**

<table>
<thead>
<tr>
<th>Subject</th>
<th>α-Spectrin/α₂-Globin</th>
<th>α-Spectrin/α₂</th>
<th>Ankyrin/α₂-Globin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.54 ± 0.14 (4)</td>
<td>0.64 ± 0.417 (8)</td>
<td>0.172 (2)</td>
</tr>
<tr>
<td>HS</td>
<td>0.52 ± 0.12 (4)</td>
<td>0.705 ± 0.353 (4)</td>
<td>0.095 (2)</td>
</tr>
</tbody>
</table>

The amounts of spectrin and ankyrin mRNA are expressed as mean (± SD) ratios to the amounts of α₂-globin mRNA in the same sample. The numbers in parentheses indicate the number of determinations.
brane. As previously reported, the amounts of spectrin and ankyrin in the RBC membranes of the HS proband were about half the normal amounts. Furthermore, previously reported structural and functional studies of the residual spectrin and ankyrin, including the binding of ankyrin to ankyrin-depleted inside-out vesicles, showed no abnormalities, raising a possibility that the underlying defect involves a reduced synthesis or increased turnover of either spectrin or ankyrin. Because the synthesis and assembly of spectrin α- and β-subunits in human reticulocytes and the nucleated RBC precursors are very similar, we have used the former system to evaluate the molecular basis of a combined spectrin and ankyrin deficiency in this patient.

As previously reported for nucleated RBC precursors, we have found that in control reticulocytes, α-spectrin is synthesized in excess of β-spectrin. We further found that in the HS reticulocytes the α-spectrin synthesis in the cytosol was normal while β-spectrin synthesis was increased about fourfold. In contrast, the incorporation of newly synthesized α- and β-spectrins into the HS membrane was reduced to about half the control values while the turnover of both cytosolic and membrane-associated spectrin was normal. Thus, despite normal (α-spectrin) or even increased (β-spectrin) synthesis, the assembly of the newly synthesized spectrin chains into the HS membrane was markedly reduced. The reason for the increased β-spectrin synthesis is likely related to a highly stimulated erythropoiesis: this is suggested by recent data showing that in erythroblasts isolated from the spleens of mice infected with the anemia-inducing strain of Friend virus (FVA cells) induced with erythropoietin or murine erythroleukemia (MEL) cells induced with dimethyl sulfoxide (DMSO), the synthesis of β-spectrin is selectively increased. Therefore, it is possible that the HS reticulocytes represent a similar system of stress erythropoiesis with increased synthesis of β-spectrin. The finding of normal α- and increased β-spectrin synthesis is consistent with our findings of normal amounts of α-spectrin mRNA, although the increase of β-spectrin synthesis was more striking than the increase in mRNA level, presumably due to a more efficient translational process.

We further found that the marked decrease of the assembly of the newly synthesized spectrin on the membrane appears to be related to abnormalities in the synthesis of ankyrin. In control reticulocytes, the amounts of ankyrin synthesized in the cytosol are in excess of those assembled on the membrane (Fig 2). In contrast, no intact ankyrin was detected in the cytosol of HS reticulocytes, and only immunoreactive ankyrin degradation products were present. Furthermore, the amounts of newly synthesized ankyrin which were stably assembled on the HS membrane were markedly reduced to about half the control value. Immunoprecipitations of other proteins like 4.1 or band 3 fragments of the skeleton of the human erythrocyte membrane were not performed because of the limited amounts of samples available. However, plasma membranes derived from (35S) methionine-labeled HS and control reticulocytes, as examined by SDS-PAGE followed by autoradiography, showed that in HS reticulocyte membranes the incorporation of all other newly synthesized proteins except spectrin and ankyrin was similar to control reticulocyte membrane, suggesting that the synthesis of other proteins was normal.

Our findings of reduced ankyrin synthesis were consistent with a marked decrease in ankyrin mRNA. The normal chromosomal studies as well as an absence of abnormalities on Southern blots using multiple restriction enzymes and two ankyrin probes excluded a deletion of a major segment of the ankyrin gene. Thus, it is likely, but remains to be proven, that the partial ankyrin mRNA deficiency is a result of a point mutation leading to a reduced expression or a reduced stability of the ankyrin mRNA.

Although the above data indicate that a deficiency of ankyrin mRNA and, consequently, a reduced synthesis of ankyrin constitutes the primary molecular defect, the exact inheritance pattern cannot be unequivocally established in this particular proband because of unavailability of his parents for study. However, several points suggest that the patient is either a homozygote or a compound heterozygote for a defect involving reduced synthesis of ankyrin: (1) All relatives of this patient were hematologically normal with the exception of the father whose anemia was nonhemolytic and likely acquired. (2) In our studies of ankyrin synthesis in normal reticulocytes, we detected large amounts of ankyrin synthesized in the cytosol with only a fraction of this newly synthesized ankyrin being assembled on the membrane. In contrast, there was no detectable ankyrin synthesis in the cytosol of the patient's reticulocytes, although some ankyrin (about 50% of normal) was found assembled on the membrane. (3) Furthermore, the deficiency of both spectrin and ankyrin in our proband as originally reported in reference 30 appears more profound than in the recently studied cases of HS associated with a deletion of the ankyrin gene in one of the two chromosome pairs.

Our results further show that the decrease in spectrin assembly on the HS reticulocyte membrane is of a similar magnitude as the decrease in ankyrin incorporation into the membrane, suggesting that the assembly of spectrin on the membrane is limited by the amount of membrane-associated ankyrin. Hence, the underlying defect seems to be similar to that of the nb/nb spectrin-deficient mutant mice involving a reduced synthesis of ankyrin, which in part is also partially degraded before its attachment on the membrane. We conclude that the resulting partial deficiency of membrane-associated ankyrin leads to a secondary deficiency of spectrin, that, in turn, leads to a weakened interaction of the membrane skeleton with the lipid bilayer and integral membrane proteins, facilitating loss of surface area and spherocyte formation.

REFERENCES
3. Byers TJ, Branton D: Visualization of the protein associations
in the erythrocyte membrane skeleton. Proc Natl Acad Sci USA 82:6153, 1985


44. Thomas PS: Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc Natl Acad Sci USA 77:5201, 1980


55. White RA, Biokenmeier CS, Lux SE, Barker JE: Ankyrin and the hemolytic anemia mutation, nb, map to mouse chromosome 8: Presence of the nb allele is associated with a truncated erythrocyte ankyrin. Proc Natl Acad Sci USA 87:3117, 1990

Molecular basis of spectrin and ankyrin deficiencies in severe hereditary spherocytosis: evidence implicating a primary defect of ankyrin

M Hanspal, SH Yoon, H Yu, JS Hanspal, S Lambert, J Palek and JT Prchal