Hereditary Spherocytosis With Spectrin Deficiency Due to an Unstable Truncated β Spectrin

By Hani Hassoun, John N. Vassilikis, James Murray, Scott J. Yi, Manjit Hanspal, Christine A. Johnson, and Jiri Palek

Red cell membrane protein analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and direct quantitation by radioimmunoassay or cytofluorometry defines four distinct subsets of patients with hereditary spherocytosis: Patients with isolated spectrin deficiency, combined spectrin and ankyrin deficiency, band 3 deficiency, and protein 4.2 deficiency. In regard to the first group, only one mutation of β spectrin has been reported in the literature. We describe a spectrin variant characterized by a truncated β chain, and associated with hereditary spherocytosis and isolated spectrin deficiency. The clinical phenotype consists of a moderate hemolytic anemia with spherocytosis and frequent spiculation of the red cells. We present the biochemical characteristics of this mutant protein and show that it constitutes only 12% of the total spectrin on the membrane. We show that the truncation of the protein is the result of a single point mutation at position +1 (G → A) of the donor consensus splice site of intron 17 leading to an aberrant β spectrin transcriptional message lacking exons 16 and 17. To elucidate the basis for the decreased amount of the truncated protein on the membrane and the overall spectrin deficiency, we provide evidence that the mutated gene is transcribed but its mRNA is less abundant than its normal counterpart in reticulocytes; we also show that the mutant protein is synthesized in decreased amounts in the cytoplasm of erythroid progenitor cells, and appears to be susceptible to proteolytic degradation. This mutant spectrin underscores the importance of the regulatory role played by the β spectrin molecule in the assembly of αβ spectrin heterodimers on the membrane.

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Hereditary Spherocytosis (HS) is a common hemolytic anemia characterized by a chronic hemolysis with a broad spectrum of clinical severity ranging from an asymptomatic condition to life-threatening anemia requiring splenectomy. The red cells present a varying degree of surface area deficiency resulting in a spherocytic phenotype and increased osmotic fragility. In addition to the more common autosomal dominant form, a more severe autosomal recessive one has been described.1-3 It is now recognized that this disorder is associated with defects of the erythrocyte membrane skeleton.

This membrane skeleton is a protein lattice that laminates the inner side of the red cell membrane and that is responsible for the shape and deformability of the cell.3,4 Spectrin, the major structural component of this skeleton, is formed by the association of two subunits, α and β, both consisting of contiguous homologous repeats of approximately 106 amino acids folded into a triple helical structure.3-5 α and β spectrin are intertwined in an antiparallel manner to form heterodimers.3,5 This side-to-side association involves an initial attachment of the two subunits at a specific nucleation site, which on β spectrin comprises the four first repeated segments and on α spectrin the last four repeated segments.6,7

The skeleton is attached to the membrane mainly by ankyrin, which links spectrin to the transmembrane protein, band 3. The ankyrin binding domain of spectrin has been mapped to the 15th repeated segment of the β spectrin subunit.8

In patients with HS, the biochemical analysis of the red cell membrane proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), or by direct quantitation using radioimmunoassay or fluorocytometry has identified four distinct abnormalities corresponding to four different subsets of patients: (1) isolated spectrin deficiency,1,2,9 (2) combined spectrin and ankyrin deficiency,1,2,9 (3) band 3 deficiency,10-12 and (4) defects of protein 4.2,13-15 The correlation between the biochemical defects and the severity of the hemolysis remains poorly defined.

At the molecular level, point mutations and deletions involving the genes of band 3, ankyrin, protein 4.2 and spectrin are being uncovered, further defining the four subsets of patients described above. Point mutations and small deletions involving the band 3 gene have been described in patients with HS and band 3 deficiency.12,23 Likewise point mutations of the ankyrin gene24,25 and the protein 4.2 gene have been disclosed in the subsets of patients with ankyrin deficiency and protein 4.2 deficiency, respectively. In the patients with isolated spectrin deficiency, no mutations of α spectrin have been reported so far. This is most likely due to the fact that α spectrin is synthesized in approximately threefold excess of β spectrin14,15 with the redundant, unassembled α chains undergoing degradation in the lysosomal compartment.26 Consequently, the β spectrin synthesis appears to be the rate limiting factor for the assembly of spectrin αβ heterodimers on the membrane. Therefore, in contrast to α spectrin, defects of β spectrin are more likely to be expressed in the heterozygous state. A point mutation occurring in the N-terminal region of β spectrin has been described, presumably leading to an unstable protein and subsequent spectrin deficiency.16,17 This β spectrin mutation also resulted in the defective binding of spectrin to protein 4.1. We have also previously reported, in a preliminary form, a truncated β spectrin due to a large genomic deletion resulting in an in-frame skipping of exons 23 and 24.18 The
corresponding portion of the protein represents most of repeated segment 12 and part of repeated segment 13. We also showed that, although the synthesis and stability of the truncated protein were unaffected by the deletion, its incorporation into the membrane skeleton was defective in erythroblasts. We postulated that the proximity of the deleted segment also showed that, although the synthesis and stability of spectrin characterized by a truncated \( \beta \) spectrin and associated with hereditary spherocytosis and overall spectrin deficiency. We show that the truncation results from a single point mutation at position +1 (G \( \rightarrow \) A) of the donor consensus splice site of intron 17 leading to an unstable \( \beta \) spectrin transcriptional message, which lacks exons 16 and 17. We also show that this mutant spectrin may be subject to rapid proteolytic degradation in the cytoplasm of erythroblasts resulting ultimately in overall spectrin deficiency on the membrane.

MATERIALS AND METHODS

Patient

The family under study consists of three members: The patient was incidentally found to be mildly anemic at age 4 although no diagnosis was reached at that time. At age 5, he presented with marked fatigue following an episode of streptococcal pharyngitis and was found to have a hemoglobin of 8 g/dL. After recovery from this acute event, his hemoglobin stabilized between 10 and 11 g/dL, with a reticulocyte count around 7, an MCV of 80, and an undetectable haptoglobin. His peripheral blood smear showed marked spherocytosis (Fig 1) and the red cell osmotic fragility was markedly increased. No splenomegaly was found by physical examination. The parents of the patient are asymptomatic and present no detectable hematologic abnormalities.

Analysis of the Erythrocyte Membrane Protein

These methods have been described previously and include (1) erythrocyte membrane preparation\(^{15,14}\); (2) analysis of the erythrocyte membrane proteins by SDS-PAGE (3.5% to 17% gradient Fairbanks gels and 10% Laemmli polyacrylamide gels\(^{15}\); (3) immunoblotting using polyclonal antibodies raised against \( \alpha \) spectrin, \( \beta \) spectrin, and ankyrin\(^{15}\), and (4) spectrin extraction at 4\(^{\circ}\)C\(^{15}\) and relative determination of spectrin dimers (SpD) and spectrin tetramers (SpT) by nondenaturing gel electrophoresis.\(^{16}\)

PCR Amplification of Reticulocyte \( \beta \) Spectrin cDNA

Total RNA isolated from reticulocytes\(^{17}\) was reverse transcribed using random primers. Overlapping segments of the \( \beta \) spectrin cDNA were amplified by the polymerase chain reaction (PCR). The segment encompassing exons 16 and 17 was amplified using two sets of primers independently: sense primer BS1 with antisense primer BS54 and sense primer 339 with antisense primer BS62. Each 50-\( \mu \)L reaction mixture contained 50 mmol/L KCl, 10 mmol/L Tris-HCl, 2 mmol/L MgCl\(_2\), 0.1% gelatin, 100 pmol of each primer, 0.2 mmol/dL dNTP, and 2 U of Taq polymerase. Each cycle consisted of 1 minute at 95\(^{\circ}\)C, 1 minute at 57\(^{\circ}\)C, and 3 minutes at 72\(^{\circ}\)C. The amplified DNA was fractionated on a 1% agarose gel and stained with ethidium bromide.

Southern Blot Analysis of Genomic DNA

Ten micrograms of genomic DNA isolated from peripheral blood mononuclear cells,\(^{18}\) were digested with two restriction enzymes independently (\( XbaI \) and \( EcoRI \)). The DNA fragments resulting from this digestion were fractionated on a 0.7% agarose gel, transferred to a nylon membrane (Zetaprobe, Bio-Rad Laboratories, Richmond, CA) by nonalkaline Southern blotting. Probes were labeled with \(^{32}\)P dATP by random priming (Megaprime DNA labeling system, Amersham, UK) and hybridized to the nylon membrane as described.\(^{19}\)

PCR Amplification of Intron-Exon Boundaries

Genomic DNA was isolated from peripheral blood leukocytes as described previously.\(^{20}\) Amplification of introns 15, 16, and 17 was carried out in order to amplify the donor and acceptor splice sites flanking exons 16 and 17. For the small introns 16 and 17, the amplifications were performed in a 50-\( \mu \)L reaction mixture containing 50 mmol/L KCl, 10 mmol/L Tris-HCl, 2 mmol/L MgCl\(_2\), 0.1% gelatin, and 100 pmol of each primer. The primers used were sense primer 304 with antisense primer BS44 for intron 16, and sense primer BS 43 with antisense primer BS42 for intron 17. A 40 cycle amplification was carried out, each cycle consisting of 1 minute at 95\(^{\circ}\)C, 1 minute at 50\(^{\circ}\)C, and 3 minutes at 72\(^{\circ}\)C. For intron 15, which exceeds 4 kb, the amplification was performed using the conditions described previously for amplification of large DNA fragments\(^{18}\) and using sense primer BS1 with antisense primer 351. All primers used to amplify individual introns are designed within the adjacent coding exon sequences.

Direct Sequencing of Amplified DNA

DNA fragments generated by PCR amplification were purified using the geneclean system (BIO 101, Natick, MA) and sequenced using the Sequenase kit, version 2.0 (USB, Amersham, Cleveland, OH) as recommended by the manufacturer with the following minor modification: the initial alkaline denaturation of the template is replaced by simple heat denaturation in presence of the sequencing primer (95\(^{\circ}\)C for 5 minutes), followed by quick chilling on ice for 10 minutes.

Subcloning and Sequencing of Amplified DNA

DNA fragments generated by PCR amplification were purified using the geneclean system (BIO 101) and subcloned into the pCRII cloning vector (Invitrogen Corp, San Diego, CA). After transformation in competent Escherichia coli cells, the recombinant DNA clones were sequenced by the deoxyxynucleotide sequencing method of Sanger, Nicklen, and Coulson.\(^{21}\)

*Fig 1. Peripheral blood smear. Note the significant spherocytosis as well as the presence of dense spinculated cells.*
cDNA Quantitation by PCR

mRNA was prepared from peripheral blood reticulocytes and reverse transcribed to cDNA using random primers. To quantify the relative amounts of the normal and abnormal transcripts, we amplified by PCR a cDNA segment overlapping the truncated fragment using primers BS1 and BS42 located on exons 15 and 18, respectively. This amplification resulted in a 1,100 bp DNA fragment from the normal allele, and a 140 bp fragment from the truncated allele. A 500-μL reaction mixture containing 50 mmol/L KCl, 10 mmol/L Tris-HCl, 2 mmol/L MgCl2, 0.1% gelatin, 100 pmol of each primer, 0.2 mmol/L radioactive dATP, 0.2 mmol/L of the other dNTP, and 2 U of Taq polymerase, was aliquoted in 15 PCR tubes. A 30 cycle PCR amplification was initiated, each cycle consisting of 1 minute at 95°C, 1 minute at 55°C, and 2 minutes at 72°C. The tubes were removed successively at the end of each cycle between cycle 15 and cycle 30. The DNA fragments resulting from this amplification were fractionated on a 5% polyacrylamide gel, for 10 hours, at 180V and their amount quantified by exposure of the gel to autoradiography followed by densitometric analysis of the film. Also, after localization by autoradiography, the bands were cut off the gel and the radioactive signal quantified by beta spectrometer. The relative abundance of the truncated band to the normal band was determined for each one of the 15 samples.

In Vitro Assay of Spectrin Synthesis, Stability, and Incorporation Into the Membrane

The methods used for this assay have been extensively described elsewhere. Briefly, they include (1) a two-phase liquid culture system for the maturation of erythroid progenitors using the system described by Fibach et al and (2) pulse labeling and pulse chase experiments of late erythroblasts isolated from liquid culture.

Two-phase liquid culture system for the maturation of erythroblast progenitors. Peripheral blood was obtained from healthy donors and patients, and mononuclear cells were separated by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) gradient density centrifugation. The mononuclear cells were collected and cultured in a two-phase liquid culture system as described earlier. Briefly, in the first phase, mononuclear cells (10^6/mL) were cultured in alpha minimum essential medium (MEM) (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) (HyClone, Logan, UT) and 10% conditioned medium collected from cultures of the 5637 bladder carcinoma cell line. After a 1-week incubation at 37°C in an atmosphere of 5% CO2, the nonadherent, nonphagocytic cells were harvested and cultured in the second phase in alpha medium containing 30% FCS, 1% deionized BSA, 0.1 mmol/L 2-mercaptoethanol, 1.5 mmol/L glutamine, 0.1 mmol/L dexamethasone, 100 μg/mL phenylmethyl sulphonyl fluoride, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 μg/mL dithiothreitol, 1 mmol/L phenylmethyl sulphonyl fluoride, 1 mmol/L leupeptin, and 10% Laemmlili gel (data not shown). The extra band reacted with a β spectrin polyclonal antibody, when analyzed by Western blotting (Fig 2B). This truncated protein constituted approximately 12% of the total β spectrin present on the membrane.

Biochemical Characterization of the Mutant β Spectrin

An additional band migrating between band 2.1 and 2.2, with an estimated molecular weight of 190 kDa was revealed by 3.5% to 17% gradient SDS-PAGE analysis of the patient’s red cell membrane proteins. (Fig 2A). This band could also be visualized on 10% Laemmlili gel (data not shown). The extra band reacted with a β spectrin polyclonal antibody, when analyzed by Western blotting (Fig 2B). This truncated protein constituted approximately 12% of the total β spectrin present on the membrane. Using densitometric analysis of SDS-PAGE gels, the spectrin to band 3 ratio (including the truncated protein) was 0.84 (Normal: 0.97 ± 0.10) indicative of an overall moderate spectrin deficiency on the membrane. Nondenaturing gel electrophoresis of spectrin extracts performed at 4°C showed a normal spectrin dimers to tetramers ratio, suggesting that the deleted portion of β spectrin does not affect the spectrin heterodimer contact site (data not shown). The red cells of both parents had no detectable membrane abnormality as assessed by these methods.

Characterization of the Molecular Defect Underlying the Truncated β Spectrin

PCR amplification of overlapping cDNA fragments spanning the entire β spectrin cDNA was carried out. Amplification of the sequence derived from exons 15 to 18, was carried out using sense primer BS1 with antisense primer BS42, and revealed two size DNA products in the patient (Fig 3A), one of expected size (1,100 bp) and a truncated fragment (140 bp). This finding was confirmed using a second set of primers (sense primers 339 with antisense primer BS42 shown in Fig 3A) to amplify the sequence spanning exon 14 to 18. Sequencing of the truncated 140 bp abnormal PCR product revealed a 960 bp in-frame deletion of the sequence normally contributed by exons 16 and 17 (Fig 3B). These exons encode the C-terminal part of repeated segment 6, the entire repeated segments 7 and 8, as well as most of repeated segment 9 (Fig 3C).

In order to elucidate the underlying molecular defect, we first focused our efforts on detecting a large genomic deletion as it appeared to be the most likely event that would be responsible for more than one exon skipping. Genomic DNA, prepared from the three family members, was analyzed by Southern blot hybridization using two restriction enzymes independently (EcoRI and Xba 1), and a cDNA...
Fig 2. Biochemical analysis of the red cell membrane proteins. (A) SDS-PAGE analysis by 3.5% to 17% gradient Fairbanks gel of the red cell membrane proteins of the patient (P) and both parents (F and M) revealing an extra band ($\beta'$ spectrin) in the patient's sample, migrating between bands 2.1 and 2.2. (B) Western blot analysis of red cell membrane proteins separated on a 10% Laemmli gel, using an antibody directed against $\beta$ spectrin. Note the truncated band ($\beta'$ spectrin) in the patient (P) absent from the normal sample (C).

probe extending from exon 15 to exon 18. No abnormalities could be detected (data not shown), suggesting that a large genomic deletion was unlikely to be the underlying molecular defect. We next addressed the possibility of a point mutation within the consensus splice site sequences flanking exons 16 and 17. Using primers designed within exons 15, 16, 17, and 18 (Fig 4), we amplified by PCR introns 15, 16, and 17 as described in the Methods section. The direct sequencing of intron 17 revealed a point mutation at position +1 (G → A) of the donor splice site of that intron. No other

Fig 3. PCR amplification of reticulocyte $\beta$ spectrin cDNA. (A) The sequences corresponding to exons 14 to 18 and exons 15 to 18 of the $\beta$ spectrin cDNA were amplified in the three family members using two sets of primers independently: primers 339 with BS42 and primers BS1 with BS42, respectively, shown in the diagram. The first lane is the molecular weight marker. Note in both series of amplifications the fragment that is truncated by 960 bp in the patient only (P). (B) Subcloning and sequencing of the 1,100 bp fragment in the patient revealed the normal sequence corresponding to exon 15 to exon 18 of the $\beta$ spectrin cDNA (data not shown). However, the sequence corresponding to exons 16 and 17 were deleted from the 140 bp fragment as shown. This deletion of 960 bp did not alter the reading frame. (C) Schematic representation of $\beta$ spectrin showing the region deleted from the protein (shaded area). This corresponds to the C-terminal end of repeated segment 6, the entire repeated segments 7 and 8, as well as most of repeated segment 9.
mutations were detected at any other intron-exon boundaries. Likewise, no mutations were found when exons 16 and 17 were amplified by PCR and sequenced (data not shown). Sequencing of the donor splice site of intron 17 using genomic DNA from both parents did not show the same mutation, suggesting a de novo mutation in the proband (data not shown).

**Molecular Basis for the Spectrin Deficiency**

To understand the mechanism underlying the imbalance between the amounts of the normal and the truncated β spectrin present on the membrane, we have examined the stability of the transcriptional message, as well as the synthesis and stability of the truncated protein.

We first investigated the stability of the mRNA by relative quantitation of cDNA as described in the Methods section (Fig 5). The PCR amplification of the patient’s cDNA, using primers BS1 and BS42 flanking the deleted segment, yields two fragments, one of 1,100 bp corresponding to the normal allele and a 140 bp fragment corresponding to the mutant allele. The relative amount of these two products was examined after each PCR cycle between cycles 20 and 30 as described in the Methods section. Despite the smaller size of the mutant band (which one would therefore expect to be preferentially amplified), we find that the ratio of the truncated to the normal band remains low at 0.34 ± 0.08. This result suggests that the mRNA generated by the transcription of the mutant β spectrin gene is less abundant than its normal counterpart in the cytoplasm of reticulocytes and may therefore be unstable.

We next examined the synthesis and stability of the newly synthesized β spectrin using an in vitro liquid culture system allowing the isolation of late erythroblasts from peripheral blood burst-forming unit-erythroids (BFU-Es) Pulse label and chase experiments described in the Methods section reveal that the truncated β spectrin can be immunoprecipitated from the erythroblast cytosolic fraction (Fig 6). However, after 90 minutes of labeling (ie, at time 0), it is much less abundant than the normal protein and represents only a small fraction of the total β spectrin in the cytoplasm. This result is in agreement with the finding of a decreased amount of the mutant mRNA in reticulocytes. Furthermore, the presence in the patient’s sample of several degradation products of β spectrin (absent in control samples) suggests an increased susceptibility to proteolytic degradation of the truncated protein. The expected high ratio of α spectrin to β spectrin which reflects the excessive synthesis of α spectrin is further increased in the patient compared with the control (Fig 6), confirming the overall deficiency in normal β spectrin in the cytoplasm of the patient’s erythroblasts.

We can conclude from these experiments that the decrease in the spectrin incorporation into the membrane is probably the consequence of the concerted occurrence of an unstable transcriptional message and an increased susceptibility to proteolytic degradation of the mutant protein.

**DISCUSSION**

Several mutations of the red cell skeletal proteins have been described in association with hereditary spherocytosis. However, within the subset of patients with isolated spectrin deficiency, a single report describing a point mutation of a conserved aminoacid of the β spectrin gene has been published. Based on bacterial expression of β spectrin peptides, it was presumed that the spectrin deficiency was the conse-
The cytosolic fraction was isolated and spectrin immunoprecipitated after incubation for different time intervals. The cytoplasm and degraded normally over the ensuing 120 minutes. In contrast, the truncated protein is very faintly seen at 0 minutes. Furthermore, erythroblasts were labeled for 90 minutes with [35S] methionine. Further incorporation was stopped by the addition of unlabeled methionine. Synthesis of spectrin was determined by densitometric analysis of the autoradiogram. The expected high ratio of degraded spectrin in the cytoplasm of erythroblasts. (B) α spectrin to β spectrin ratio over time, indicating the truncated protein is rapidly degraded in the cytoplasm of erythroblasts. (B) α spectrin to β spectrin ratio over time (0, 60, and 120 minutes) in the cytoplasm of erythroblasts as determined by densitometric analysis of the autoradiogram. The expected high ratio of α spectrin to β spectrin, which reflects the excessive synthesis of α spectrin, is further increased in the patient compared with the control, confirming the overall deficiency in normal β spectrin in the cytoplasm of the patient's erythroblasts.

Fig 6. Synthesis, stability, and membrane incorporation of spectrin in the patient's erythroblasts. (A) In this pulse chase experiment, erythroblasts were labeled for 90 minutes with [35S] methionine. Further incorporation was stopped by the addition of unlabeled methionine. The cytosolic fraction was isolated and spectrin immunoprecipitated after incubation for different time intervals (0, 60, and 120 minutes). The autoradiogram from the patient's sample shows that at 0 minutes (i.e., after 90 minutes of labeling), the normal β spectrin is present in the cytoplasm and degrades normally over the ensuing 120 minutes. In contrast, the truncated protein is very faintly seen at 0 minutes. Furthermore, several degradation products of β spectrin are seen on the patient's autoradiogram, which may suggest that the truncated protein is rapidly degraded in the cytoplasm of erythroblasts. (B) α spectrin to β spectrin ratio over time (0, 60, and 120 minutes) in the cytoplasm of erythroblasts as determined by densitometric analysis of the autoradiogram. The expected high ratio of α spectrin to β spectrin, which reflects the excessive synthesis of α spectrin, is further increased in the patient compared with the control, confirming the overall deficiency in normal β spectrin in the cytoplasm of the patient's erythroblasts.

sequence of an increased protein degradation in vivo, as mutant peptides were unstable and highly susceptible to oxidative injury and proteolytic degradation. We have also previously reported, in a preliminary form, a truncated β spectrin mutant lacking exons 23 and 24 (corresponding to most of repeated segments 12 and the first helix of repeated segment 13) associated with an HS phenotype. We were able to show that despite normal synthesis and stability of the protein in the cytoplasm of erythroblasts, the mutant protein was not incorporated into the membrane skeleton. We postulated that conformational changes of the protein affecting the nearby ankyrin binding domain or, less likely, the heterodimer nucleation site was responsible for the decrease in the skeletal incorporation of the defective spectrin. In this report, we have described another truncated protein associated with an HS phenotype and show that it constitutes only 12% of the total spectrin on the membrane. We show that the deletion of exons 16 and 17 corresponding to the C-terminal end of repeated segment 6, the entire repeated segments 7 and 8, as well as most of repeated segment 9, is due to a single point mutation at position +1 (G → A) of the donor consensus splice site of intron 17 leading to an aberrantly spliced β spectrin transcriptional message. This mutation has occurred de novo as it is absent in both parents. Unlike spectrin Durham, the resulting mRNA appears to be unstable. Furthermore, the pulse chase studies of newly synthesized spectrin provide evidence that the mutant protein may also be highly susceptible to proteolytic degradation.

Previous studies have revealed that the α and β subunits of spectrin, like other subunits of multimeric complexes are synthesized in excess of the amounts assembled on the membrane, and that excess unassembled subunits, unlike their membrane bound counterparts, are rapidly degraded in the cytoplasm. However, the amount of α spectrin synthesized exceeds by several-fold the amount assembled on the membrane, and by threefold the amount of β subunit synthesized. Furthermore, the two subunits of spectrin are degraded by two distinct intracellular pathways: while α spectrin is degraded slowly by a lysosomal-type pathway, with a half-life of 2 hours, the unassembled β subunit is degraded much faster by a soluble cytoplasmic factor with a half-life of about 15 minutes. These observations suggested that the extent of assembly of newly synthesized subunits may be regulated at the posttranscriptional level. Although availability of and accessibility to the ultimate stabilizing membrane receptor may play an important role in this regulation, it is also believed that selective proteolysis of key components may provide a means of control of multimeric subunit assembly. More specifically, in the case of spectrin, the limiting factor for its assembly on the membrane, may be the rate of turnover of its most unstable component, the β subunit. This is supported by the fact that the rate of degradation of β spectrin closely matches its rate of synthesis and rate of assembly on the membrane. This model would readily accommodate the hypothesis proposed in this report, which attributes the spectrin deficiency to a decreased incorporation of the mutant spectrin into the membrane, resulting from a marked shortage in β spectrin, the limiting factor of spectrin assembly.

Although we cannot exclude the possibility that another mutation in conjunction with the one described here may contribute to the phenotype, the previous finding of cosegregation of the HS phenotype with a mutant β spectrin supports the concept that a defect in β spectrin could be manifest in the heterozygous state. It is worth noting that in the mouse model of β spectrin mutant (the jaundiced mouse ju/ju), the homozygous state of the mutation results in a severe hemolytic anemia while the heterozygous mouse, which has
a 50% decrease in β spectrin synthesis, would appear normal.49 Several hypotheses could be proposed to reconcile this mouse model with the one described here: it is not unlikely that the heterozygous mouse has a mild, compensated hemolytic picture with no clinical significance as is the case with our patient. Alternatively, the presence of the truncated protein on the membrane may have some other deleterious effect that is not clearly understood.

It is interesting to note that this truncated protein is the result of the simultaneous deletion of exons 16 and 17, due to a single point mutation at position +1 of the donor consensus splice site of intron 17. A recent survey of mammalian mutations available in the Genbank database identified over 100 fully characterized splice site mutations associated with defective genes.50 These were classified in four types: exon skipping, activation of a cryptic splice site, creation of a pseudo-exon within an intron, and intron retention. The first pattern, exon skipping, is the most frequently encountered (51%), and mutations of the donor splice site are more common than those of the acceptor splice site. Among these, there is no single description of mutation of the donor splice site resulting in the deletion of the involved exon as well as the further upstream exon as reported here in the case of spectrin Winston-Salem. However, mutations in which the further downstream exon is also skipped have been described by others.51 This double exon skipping phenomenon supports the concept that parameters other than the splice site sequences play a significant role in the exon definition model. This model invokes pairing between the splice sites across an exon, as opposed to pairing across an intron. In this exon definition model, the pre-mRNA is scanned by the splicing machinery searching for a pair of closely spaced splice sites.52 However, it appears that, aside from the appropriate splice sequences, other constraints are imposed: a minimum as well as a maximum size seem to be necessary for the exon definition. Only 3.5% of exons are longer than 300 nucleotides, and only 1% are longer than 400 nucleotides. Furthermore, it is also clear from the observations gathered on the differential recognition of exons (alternative splicing), as well as mutually exclusive exons, that factors other than splice site sequences may be crucial.53 Steric hindrance, secondary structures of the mRNA, and enhancer sequences, among other mechanisms have been proposed as plausible explanations. The double exon skipping observed in spectrin Winston-Salem, lends credence to the exon definition model and its observed constraints. Despite having a high scoring splice signal,54 the size of exon 16 (757 nucleotides) is unsuitably large, a common finding in alternatively spliced exons.55 We propose that the double exon skipping described in spectrin Winston-Salem represents an example of mutually inclusive exons, in which the definition of exon 16, due to its unusually large size, is contingent to the recognition of exon 17 by the splicing mechanism. This may occur by means of specific secondary structure of the mRNA, or other poorly understood mechanisms.

It should finally be pointed out that the phenotype presented by the patient is very similar to the one described in spectrin Durham,56 consisting of a mild to moderate hemolytic anemia, associated with a significant spherocytosis and frequent spiculated red cells on the peripheral blood smear. Acanthocytes were also reported in spectrin Kissimmee.57 The significance of this observation remains, at this point, uncertain, however, the presence of acanthocytes may be a feature of β spectrin mutations in HS.

In summary, we have described a new truncated β spectrin mutant, caused by a point mutation involving the donor splice site of intron 17, and resulting in a double exon skipping. We have provided evidence that the instability of the transcriptional message and the susceptibility of the truncated spectrin to proteolytic degradation concomitantly led to the scarcity of the aberrant protein and ultimately to spectrin deficiency on the membrane. This mutant spectrin underscores the importance of the regulating role played by the β spectrin molecule in the assembly of the αβ spectrin heterodimers on the membrane.

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H Hassoun, JN Vassiliadis, J Murray, SJ Yi, M Hanspal, CA Johnson and J Palek