Spectrin $\beta^{\text{Tandí}}$, a Novel Shortened $\beta$-Chain Variant Associated With Hereditary Elliptocytosis Is Due to a Deletional Frameshift Mutation in the $\beta$-Spectrin Gene

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An Argentinian family with hereditary elliptocytosis (HE) associated with a shortened spectrin (Sp) chain was studied. As with most of the other shortened $\beta$-Sp-chains that have been described, this variant, called $\beta$Spp$t^{\text{Tandí}}$, has impaired ability to participate in Sp dimer self-association, has lost its ability to become phosphorylated, and is associated with the presence of increased amounts of the $\alpha$ 74-Kd fragment after partial tryptic digestion of Sp. The $3^{'\prime}$ ends of the $\beta$-Sp gene of affected patients were analyzed. cDNA was prepared by reverse transcription of peripheral blood mRNA and amplified by the polymerase chain reaction (PCR) using primers corresponding to sequences 400 bp apart on the cDNA, spanning the last three exons ($X$, $Y$, $Z$) of the $\beta$-Sp gene. Gel electrophoresis of the cDNA amplification products using electrophoresis on polyacrylamide gel showed the presence of one band, the size of which was apparently the same as the band amplified from mRNA of a normal control. cDNA from one HE patient was subcloned and sequenced. Several clones showed the presence of a 7-bp deletion at codon 2041 in exon X. Genomic DNA of all the affected members of the family were amplified by PCR using primers flanking the deletion and corresponding to sequences 128 bp apart on exon X. Analysis of the PCR products using electrophoresis on polyacrylamide gel showed the presence of 121- and 128-bp bands in all HE subjects, and an additional doublet migrating more slowly than the two bands, which corresponded to the presence of heteroduplexes. The mutation results in a shift of the normal reading frame and leads to a new amino acid sequence at the C-terminus of the mutant $\beta$-Sp chain. A new in-frame stop codon is encountered downstream, leading to premature chain termination. The identification of the molecular defect in Sp$t^{\text{Tandí}}$ provides information regarding the region of the $\beta$-Sp chain that is important for both Sp dimer self-association and an indication of potential sites of phosphorylation of the chain.

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**HEREDITARY** elliptocytosis (HE) is a heterogeneous group of disorders characterized by the presence of elliptical erythrocytes.$^{1,2}$ Its clinical presentation ranges from an asymptomatic condition to severe hemolysis, also referred to as hereditary pyropoikilocytosis (HPP).$^{3}$ In all cases, the underlying alterations involve the cytochrome membrane skeleton.$^{1,2}$ The membrane skeleton is a network of proteins that laminates the inner surface of the lipid bilayer and is responsible for the strength and flexibility of the membrane.$^{4}$ Its main components include spectrin (Sp), ankyrin, protein 4.1, and actin. Sp is composed of an $\alpha$- and a $\beta$-chain that intertwine to form a rodlike dimer. In turn, $\alpha$ $\beta$ dimers self-associate head to head to form tetramers and higher order oligomers. Using partial trypsin digestion, Sp chains have been dissected into domains ($\alpha$ to $\alpha$V and $\beta$1 to $\beta$IV domains).$^{5}$ The N-terminal $\alpha$ domain and the C-terminal $\beta$ domain participate in Sp dimer self-association.$^{6}$ Each chain is comprised mainly of multiple homologous repeats that are 106 amino acids long.$^{7,9}$ In a large number of patients with HE and HPP, Sp is defective in its ability to self-associate into tetramers and higher order oligomers.$^{10,11}$ This functional defect has been related to structural alterations of either the $\alpha$ or the $\beta$ domain. Most of the mutations of the $\alpha$ domain that have been characterized thus far are point mutations.$^{12,17}$

In a different fashion, structural alterations of the $\beta$-Sp chain often consist of truncated chains at their C-termini ($\beta'$ chains).$^{18-22}$ Identification and characterization of the cDNA encoding the $\beta$-Sp gene$^{9}$ have allowed the identification of the molecular basis of these shortened $\beta$-Sp chains. Splice site mutations are responsible for skipping, either of the antepenultimate exon X in both variants Spp$t^{\text{le Puy}}$ and Spp$t^{\text{Rouen}}$, whereas an insertional frameshift mutation in exon X is responsible for the variant Spp$t^{\text{Nice}}$.$^{26}$

An Argentinian family with HE and truncated Sp$\beta$-chain was studied in this report. This new variant Sp$\beta^{\text{Tandí}}$ has impaired ability to participate in Sp dimer self-association, has lost its ability to become phosphorylated, and is associated with the presence of increased amounts of the $\alpha$ 74-Kd fragment after partial tryptic digestion of Sp. Analysis of the $3^{'\prime}$ end of the $\beta$-Sp gene of the affected individuals showed a 7-bp deletion in the antepenultimate exon X of the gene that causes a shift in reading frame with premature chain termination. As a result, the Sp$\beta$-chain that is synthesized is 64 residues shorter than the normal chain and includes a variant C-terminus containing 32 novel amino acid residues.

**MATERIALS AND METHODS**

**Clinical material.** Six affected members of the family P. were examined. The pedigree of the family is shown in Fig 1. This family was of South American Indian ancestry and affected members presented a hemolytic form of HE. A summary of clinical and biochemical data is shown in Table 1. Blood was collected by venipuncture into sterile tubes containing...
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ACD anticoagulant; informed consent was obtained from each member of the family. Specimens were put on ice and transported in an insulated container from Tandil, Argentina to Paris, France. The delay from the time blood was drawn to the time of analysis was less than 36 hours. A control sample was sent each time.

Erythrocyte morphology and deformability. Cells were examined by light phase-contrast microscopy after fixation in 1% (vol/vol) glutaraldehyde in 5 mmol/L phosphate buffer, 150 mmol/L NaCl, pH 7.4. Cell deformability was studied using an ektacytometer and was followed as a function of the osmolality of the suspending medium, as previously described.27

Erythrocyte membrane protein analysis. The methods used have been previously described. They include: erythrocyte membrane preparation,28 analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the procedure of Laemmli29 or Fairbanks et al.,30 estimation of the amount of Sp in the membrane,13 extraction of Sp and analysis of Sp dimer and tetramer by nondenaturing gel electrophoresis,31 and limited tryptic digestion of Sp and Sp dimer separation by one-dimensional electrophoresis.32 To determine the presence of the Sp<sup>Trmd</sup> chain in Sp dimers and tetramers extracted at 4°C, the 4°C extract was first fractionated by nondenaturing gel electrophoresis. The lane was then cut out, incubated in SDS-containing Laemmli sample buffer, and loaded on top of SDS Laemmli gel for the second electrophoresis. Proteins were stained with silver.32 For Sp phosphorylation and purification of the truncated Sp<sup>Trmd</sup> chain, 32P-labeled Sp was extracted from membranes of red blood cells (RBCs) incubated with 32P,33 and the truncated β-chain was isolated by anion-exchange chromatography.28

Synthesis and amplification of reticulocyte β-Sp cDNA. Human reticulocyte RNA was prepared from peripheral blood as described.32 Total RNA, 0.5 μg, was transcribed into single-stranded cDNA by incubation for 30 minutes at 42°C in a 20-μL reaction mixture containing 2 μL of a 10x polymerase chain reaction (PCR) buffer (500 mmol/L KCl, 100 mmol/L Tris-HCl pH 8.3, 15 mmol/L MgCl<sub>2</sub>, 1 mg/mL gelatin), 1 μL of 50 mmol/L MgCl<sub>2</sub>, 1 mmol/L dNTPs, 20 U RNA (Promega Corp, Madison, WI) in 100 pmol/L of hexanucleotides, and 200 U of M-MLV reverse transcriptase (GIBCO-BRL, Cergy-Pontoise, France). The sample was then heated for 2 minutes at 100°C and quickly cooled on ice. The cDNA was then amplified by PCR in a 100-μL mixture containing 80 μL of 1x PCR buffer, 50 pmol/L of each primer, and 2 U of Taq polymerase. Thirty cycles of amplification were performed in a programmable heat block (Hybaid Thermal Reactor; Hybaid Limited, Teddington, Middlesex, UK). The cycles consisted of 10 seconds at 92°C, 30 seconds at 55°C, and 20 seconds at 72°C. The first cycle was initiated with a 4-minute denaturation step at 94°C. After the last cycle, the samples were incubated at 72°C for 7 minutes to ensure the completion of the last extension step. Amplified DNA products were analyzed by electrophoresis in 2% agarose gels. The sequence of the upstream and downstream primers used are: 5' - CGT (GAATTC) CTGCGCAGC-3' (primer 1) and 5' - CGT (AAGCTT) CTGCCAGC-3' (primer 2), respectively. Their 5' extremities contain recognition sequences (shown in parentheses) for the restriction enzymes BamHI and HindIII, respectively. They are designed to anneal to target sequences that span the last three exons and are located approximately 400 bp apart at the 3' end of the coding region of β-Sp cDNA.

A primer (primer 3) was designed to anneal to a target sequence located on the antepenultimate exon of the β-Sp gene 81 bp downstream to the 3' extremity of primer 1 (see Fig 6). This primer was used together with primer 1 in a single-step protocol using Taq polymerase to directly synthesize and amplify cDNA of RNAs isolated from reticulocytes.

Subcloning and sequencing of amplified cDNAs. The method used has been previously described in detail.35 Briefly, after purification by extraction with phenol, amplified cDNA was digested with BamHI and HindIII, fractionated by electrophoresis in low-melting agarose, excised from the gel, and recovered by phenol extraction at 65°C. The fragment was then subcloned in plasmid pGEM3Z (Promega) that first had been digested with BamHI and HindIII. Sequencing of positive subclones was performed by the dideoxy sequencing method with T7 DNA polymerase (Sequenase; US Biochemical Corp, Cleveland OH) using Sp6 and T7 promoter primers.

Genomic DNA analysis. Genomic DNA was prepared from peripheral blood leukocytes by standard methods. One microgram of DNA was used in each PCR amplification reaction. Primers 1 and 2, which flank a 128-bp segment on the antepenultimate exon X of the β-Sp gene, were used (see Fig 6). The amplification scheme was the same as used above for amplification of the β-Sp cDNA. Amplified products (5 to 10 μL from each PCR sample) were electrophoresed through vertical 10% polyacrylamide gel, stained in ethidium bromide, visualized under UV illumination, and then stained with silver.32

RESULTS

Erythrocyte morphology and deformability. Erythrocyte morphology of HE subject II<sub>1</sub> is shown on Fig 2. Elliptocytosis was prominent (90% of the cells); however, cells were more often smooth and bulky ovalocytes than elongated elliptocytes. All affected members of the family exhibited the same RBC morphologic picture. Ektacytometric curves showed a decrease in the index of deformability at isotonicity in all HE subjects, and a shift to higher osmolality of the index at hypoosmoticity, indicating spherocytic behavior of the cells (not shown).

Erythrocyte membrane protein analysis showed the presence of a truncated β-chain variant. SDS-PAGE of the erythro-

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Table 1. Clinical and Biochemical Data on the HE Family With the Truncated β-Chain Variant Sp<sup>Trmd</sup>

<table>
<thead>
<tr>
<th>Subject</th>
<th>Splenomegaly</th>
<th>Hematocrit (%)</th>
<th>SpD&lt;sup&gt;*&lt;/sup&gt;</th>
<th>Clinical Phenotype</th>
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<tr>
<td>I&lt;sub&gt;1&lt;/sub&gt;</td>
<td>No</td>
<td>34</td>
<td>8</td>
<td>Hemolytic HE</td>
</tr>
<tr>
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<td>Yes</td>
<td>32</td>
<td>30</td>
<td>17.5</td>
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<td>5</td>
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<td>I&lt;sub&gt;6&lt;/sub&gt;</td>
<td>Yes</td>
<td>36</td>
<td>25</td>
<td>18</td>
</tr>
</tbody>
</table>

*Normal values for percentage of Sp dimer (SpD) 3.6% ± 1.6%.

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Fig 1. Pedigree of the HE family with the truncated β-chain variant Sp<sup>Trmd</sup>. (Empty symbols) normal not studied; (half-filled symbols) HE Sp<sup>Trmd</sup>.
The band was observed between Spβ-chain and ankyrin, accounted for approximately 25% of the total β-chain, and was unequivocally at the expense of the normal β-chain (Fig 3). The variant β-chain appeared with fixed proportions in the six HE subjects of the family. This variant β-Sp was designated SpβTandii, which refers to the town where the HE subjects in this study are living. The Sp amount (including α, β, and truncated β) was quantified by densitometry in the six HE subjects, and its content expressed as a ratio to the amount in band 3 ranged from 0.81 to 0.92 (Sp/band 3 ratio in normal controls: 1.04 ± 0.13; n = 21). The other proteins appeared to be quantitatively normal in all subjects.

**SpβTandi** has impaired ability to participate in Sp dimer self-association. Sp dimer self-association was defective in HE subjects, as shown in Table 1. Two-dimensional electrophoresis of Sp crude extracts showed that the β' chain was recovered in dimer species (Fig 4).

**SpβTandii** is associated with the presence of increased amounts of the α/74-Kd fragment after partial tryptic digestion of Sp. In HE subjects studied, Sp tryptic digests were analyzed using one-dimensional gel electrophoresis and showed a slightly increased amount of the 74-Kd peptide (not shown).

The SpβTandii chain is not phosphorylated. To determine the ability of the SpβTandii truncated chain to undergo phosphorylation in the cell, the truncated chain had to be separated from the β-chain. Under these conditions, no
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Fig 4. Two-dimensional electrophoresis of spectrin crude extracts. O, Sp oligomers; T, Sp tetramers; D, Sp dimers; C, Sp crude extract from HE subject.

figerating appeared in the place of the truncated \( \beta \)-chain (Fig 5).

PCR amplification of reticulocyte \( \beta \)-Sp cDNA from HE subjects showed a single band product. To identify the molecular defect that causes the shortening of the \( \beta \)-Sp chain in \( \beta^{\text{Tandil}} \), the structures of \( \beta \)-Sp gene transcripts were analyzed. cDNA corresponding to the 3' end of \( \beta \)-Sp mRNA was transcribed from peripheral blood reticulocyte RNA of the affected HE subjects and amplified by PCR using primers 1 and 2 corresponding to sequences spanning the last three exons (X, Y, and Z) of the \( \beta \)-Sp gene (Fig 6). Agarose gel electrophoresis of the PCR products showed in all affected HE subjects the presence of a single cDNA product, the size of which (409 bp) was apparently identical to that of the PCR band obtained after amplification of transcripts synthesized from RNA of a normal control (not shown). This pattern suggested that the molecular defect responsible for the shortening of \( \beta^{\text{Tandil}} \) was not a splicing mutation leading to skipping of an exon, as has been reported recently for truncated \( \text{Sp} \) le Puy, \( \text{Sp} \) Gottingen, and \( \text{Sp} \) Rouen.23-25

Nucleotide sequencing of cDNAs products showed a 7-bp deletion in the antepenultimate exon X of the \( \beta \)-Sp gene. The amplified PCR product from HE subject 11 was subcloned into a plasmid vector. DNA sequencing of subcloned DNA of this subject showed in 8 of 13 separate subclones a 7-bp deletion in the sequence of exon X, at position 6219 of the \( \beta \)-Sp gene (Fig 7), whereas sequencing of five subclones showed a normal sequence from positions 6153 to 6543 (see also Fig 6).

Genomic DNA analysis confirmed the presence of the 7-bp deletion in other affected HE subjects of the family. Genomic DNAs from subjects I1, II1, II2, and II4 were amplified by PCR using primers 1 and 3, which correspond to sequences 128 bp apart on exon X that flank the position 6219 (see Fig 6). Electrophoresis of amplified products through vertical polyacrylamide gel showed the presence of a 121- and a 128-bp band in all HE subjects, and an additional doublet that was migrating more slowly than the two bands and that corresponded to the presence of heteroduplexes (Fig 8). The four fragments were isolated from the gel and analyzed by denaturing-gel electrophoresis followed by silver staining: the results obtained confirmed that each band of the slower migrating “doublet” was a hybrid DNA molecule formed by a normal strand and a 7-bp deleted strand (not shown).
Fig 7. DNA sequence of subcloned β-Sp cDNA from HE subject II. Nucleotide sequence of the normal (right) and the mutant (left) gene of HE subject II. The 7-bp deletion is boxed in the normal sequence.

Fig 8. PAGE of genomic DNA fragments amplified by PCR. Genomic DNA was amplified by PCR using primers 1 and 3 and electrophoresed through a 10% polyacrylamide gel. Bands were stained with silver. Lane 1, DNA molecular weight marker (PBR 322 digested with HaeIII); lane 2, HE subject I; lane 3, HE subject II; lane 4, normal control. The 121-bp mutant product, the 128-bp normal allele product, and the heteroduplexes (hd) are indicated by arrows.

PCR DNA products amplified from either RNA transcripts or genomic DNA showed the same electrophoretic pattern. The electrophoretic pattern of DNA fragments that had been amplified by PCR using primers 1 and 3 from either reticulocyte RNA or from genomic DNA of HE subjects were then compared. The results are shown in Fig 9. PCR DNA products amplified from either RNA transcripts or genomic DNA showed the same electrophoretic pattern in agarose gel, i.e., a 121- and a 128-bp band and slower bands corresponding to the presence of heteroduplexes.

The predicted sequence of the truncated β-Sp chain of SpβΤαndl DNA sequence and predicted amino acid sequence of the 3' end of β-Sp cDNA in normal Sp (N) and in SpβΤαndl (M). The 7-bp deletion starting at position 6219 after the normal codon 2041 creates a frameshift mutation in the translation of the sequence encoded by the antepenultimate exon of the β-Sp gene. A termination codon is encountered 97 bp downstream in the new reading frame. As a result, the C-terminal 96 amino acid residues of the normal β-Sp gene beginning at codon 2042 are replaced by a variant C-terminus 32 residues in length. Thus, there is a net loss of 64 amino acid residues in the variant β-chain Tandil, which corresponds to a size difference of approximately 8 Kd (Fig 10).

DISCUSSION

HE subjects with the SpβΤαndl exhibit similar clinical, morphologic, and rheologic features to those reported for other truncated β-chain variants associated with HE. All of these cases exhibited hemolytic HE, and ektacytometric studies have shown that a population of cells behaved as spherocytes (and D.Dhermy, unpublished data, June 1987). Interestingly, HE subjects with SpβΤαndl had normal
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amounts of Sp in the membrane. The identification of the molecular defects associated with truncated Spβ-chains in HE helps shed light on several questions about the structure-function relationships of Sp. Specifically, it provides information on both the region of the β-Sp chain that is important for Sp dimer self-association and an indication of potential new in-frame stop codon is encountered 97 bp downstream, leading to premature chain termination.

In the HE subjects with the Spo11/65 variant who are heterozygous for mutations in exon 2 of the Spa gene, the presence of the truncated variant Spβ-Rouen is normal up to codon 2073. If we assume that the novel C-terminus segment of the β-chain in Spβ-Rouen is normal up to codon 2073, it would appear to be located between residues 2041 and 2073. The location of the potential phosphorylation site can be further narrowed down because the truncated variant Spβ-Nice has a normal translated amino acid sequence up to codon 2045 (see Fig 11).

Fig 10. DNA sequence and predicted amino acid sequence of the 3' end of β-Sp CDNA in normal Sp (N) and in Spβ<sub>Tandil</sub> (M). In Spβ<sub>Tandil</sub>, the 7-bp deletion at position 6291 results in a shift of the normal reading frame and leads to a new amino acid sequence at the C-terminus. A new in-frame stop codon is encountered 97 bp downstream, leading to premature chain termination.

Fig 11. Comparative schematic representation of the C-terminus ends in Spβ le Puy,25 Spβ Gottingen,24 Spβ<sub>Tandil</sub>, Spβ Nice,26 and Spβ Rouen.26 Solid lines represent normal β-Sp sequence; dashed lines represent the variant C-terminal sequences. Splice site mutations are responsible for skipping, either of the antepenultimate exon X in both variants β-Sp le Puy and β-Sp Gottingen, or of the penultimate exon Y of the β-spectrin gene in variant β-Sp Rouen, whereas an insertional frameshift mutation in exon X is responsible for the variant β-Sp Nice. (Top) Schematic representation of the 3' end of the normal β-Sp cDNA. W, X, Y and Z: Last four exons of the β-Sp gene.
gous for the 154 Leu duplication and are clinically asymptomatic.\textsuperscript{39,40} In subjects with Spp\textsuperscript{Tanddil}, as well as in HE subjects with the other truncated \(\beta\)-Sp chain variants previously reported, existence of another yet unidentified factor that would add to the defect in Sp dimer association and worsen the instability of the membrane skeleton is possible.

Spp\textsuperscript{Tanddil} is the first documented example of an HE-associated \(\beta\)-Sp truncated variant that is caused by a microdeletion in the coding sequence of the \(\beta\)-Sp gene. The possible mechanisms that could give rise to this mutation are unclear. Interestingly, the deleted sequence is part of a tandem 6-bp direct-repeat sequence (see Fig 6). It would seem that repeat sequences are often involved in DNA rearrangements,\textsuperscript{41} which could be a result of either “slipped mispairing” or unequal chromosome crossing-over.

In this report we described a deletional frameshift mutation that causes the truncation of Spp\textsuperscript{Tanddil}. The elucidation of this defect adds to the diversity of mutations responsible for truncated \(\beta\)-Sp chains and increases understanding of the structure-function relationships of Sp, one of the critical RBC membrane proteins.

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Spectrin beta Tandil, a novel shortened beta-chain variant associated with hereditary elliptocytosis is due to a deletional frameshift mutation in the beta-spectrin gene

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