Hereditary Pyropoikilocytosis and Elliptocytosis in a White French Family With the Spectrin αI/74 Variant Related to a CGT to CAT Codon Change (Arg to His) at Position 22 of the Spectrin αI Domain

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We describe a white French family in which 12 subjects presented with hereditary elliptocytosis (HE) or hereditary pyropoikilocytosis (HPP). Eight of these subjects were shown to be heterozygous for a spectrin (Sp) αI/74 variant, as demonstrated by analysis of partial tryptic digestion fragments of spectrin. This abnormal peptide pattern was associated with a decreased ability of Sp dimers to self-associate. In this kindred, in which four generations were available for study, the clinical expression varied from mild HE to HPP with an intermediate status of hemolytic HE. The severity of the disease appeared to be correlated both with the estimated amount of variant Sp (42% to 65%) and the excess of Sp dimers found in the membrane (30% to 51%, with a normal value of 3.7% ± 1.6%). Reassociation studies using isolated Spα and β chains from an affected patient and an unaffected control subject showed that the Spα/74Kd abnormal tryptic peptide resulted from a defect in the Spα chain. Partial amino acid sequencing showed that the Spα/74Kd peptide resulted from cleavage at lysine residue 42 of the Spα/80Kd domain. Knowledge of the exon/intron organization of the human aSp gene allowed us to amplify by the polymerase chain reaction the second exon of the aSp gene in total cellular DNA of the HPP proposita. The amplified fragment was subcloned and sequenced. We found a G to A base substitution in the 22nd codon (CAT for CGT), which changes the normal arginine to a histidine. Hybridization of amplified DNAs with allele-specific oligonucleotides corresponding to the normal and mutant sequences confirmed the presence of the mutation in six other HE and HPP members of the family. The identification of this mutation at the DNA level confirmed the transmission of the same molecular defect in Sp through four generations but with different patterns of clinical expression.

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Spα and β chains from an affected patient and unaffected control subject showed that the Spα174 abnormal tryptic peptide pattern in this family resulted from a defect in the Spα chain. Study of DNA from an HPP proband showed a CGT to CAT codon change at position 22 of the αl domain, which changed the normal arginine to a histidine. Hybridization of amplified DNAs with allele-specific oligonucleotides corresponding to the normal and mutant sequences confirmed the presence of the mutation in six other HE and HPP members of the family.

MATERIALS AND METHODS

Case report. The pedigree of the family is shown in Fig 1. Five members of this family were previously reported. Briefly, the proposita V, and her two maternal uncles, IV, and IV, had a history of neonatal hemolytic anemia. Repeated blood transfusions were required before splenectomy, which was performed at 9 months of age for the proposita and at 11 and 7 years of age for patients IV, and IV, respectively.

The proposita's maternal grandmother III, as well as the proposita's mother IV, (Table 1), have compensated hemolytic anemia. The proposita's daughter VI, presented with hemolytic anemia when she was 2 months old. Her hemoglobin (Hb) level was 7.8 g/dL, with reticulocytes of 250,000/µL, and anisocytosis and poikilocytosis were noted on blood smears. At the age of 9 months, the hematologic picture evolved into typical mild HE with a normal Hb level and reticulocyte count. Subject VI, was splenectomized during childhood after a history of severe hemolytic anemia; at present he exhibits a normal Hb level with a high reticulocyte count. Subject VI, was splenectomized when he was 9 months old. At present, he exhibits a normal Hb level, high reticulocyte count (400,000/µL), anisocytosis, poikilocytosis, and microcytosis. Subject V, had a history of severe neonatal anemia; splenectomy was performed when he was 8 months old. At present, he exhibits a normal Hb level with a high reticulocyte count (500,000/µL), marked anisocytosis, poikilocytosis, and microcytosis (Table 1).

Subjects IV, and VI, exhibit typical mild HE with normal Hb levels and reticulocyte counts, and presence of elliptocytes on blood smear. Subject VI, has typical mild HE (Table 1); however, just after her daughter's birth, she experienced anemia with poikilocytosis (Hb of 9.6 g/dL and reticulocyte count of 280,000/µL), which regressed rapidly to the hematological picture of asymptomatic HE. Her daughter VII, did not have neonatal anemia; however, she exhibited a high reticulocyte count (430,000/µL), anisocytosis, and poikilocytosis with approximately 20% elliptocytes.

Thermal sensitivity and morphology of erythrocytes. The thermal sensitivity of erythrocytes was studied as previously described. Normal and heated cells were examined by light phase-contrast microscopy after fixation in 1% (vol/vol) glutaraldehyde in 5 mmol phosphate buffer and 150 mmol NaCl, pH 7.4.

Erythrocyte deformability and membrane stability. These studies were performed using an etkacytometer. Whole cell deformability was followed as a function of the osmolality of the suspending medium, as previously described. The membrane resistance to shear-induced fragmentation was measured as described.

Preparation and analysis of RBC membranes. Red cell membranes were prepared as described. Membrane proteins were analyzed by electrophoresis in sodium dodecyl sulfate polyacrylamide slab gels (SDS-PAGE), either with a 5% to 15% polyacrylamide gradient as described by Laemmli or with a 3.5% to 15% polyacrylamide gradient as described by Fairbanks et al. To estimate Sp-band-3 ratios, SDS polyacrylamide slab gels were scanned after Coomassie blue staining using a DU8 Beckman spectrophotometer at 550 nm.

Study of Sp dimer-tetramer equilibrium. Sp was extracted as described, by incubating ghosts overnight at 4°C in low ionic strength buffer. The content of spectrin dimers and tetramers was determined by non-denaturing gel electrophoresis as described.

Limited tryptic digestion of Sp. Limited tryptic digests of spectrin extracts were prepared as described. Sp peptides were separated by SDS-PAGE in a 7% to 15% polyacrylamide gradient and by two-dimensional electrophoresis as described by O'Farrell and modified by Speicher et al. One-dimensional SDS-PAGE of Sp tryptic digests were scanned at 550 nm after Coomassie blue staining.

Partial amino acid sequencing of Spα174 peptide. After separation by SDS-PAGE, tryptic peptides were transferred onto polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, MA); after staining with Coomassie blue, the band corresponding to the 74 Kd peptide was cut out, destained in methanol/acetic acid (50:5%), and stored at -20°C, before microsequencing analysis was performed with an Applied Biosystems Model 740A gas-phase sequencer (Cheshire, England). Reassociation studies of isolated Sp α and β chains. Separation of Sp α and β chains from an affected patient and a normal control

Fig 1. Pedigree of the family with the Spα174 variant. Arrow indicates HPP proposita V.
subject, reconstitution and subsequent tryptic digestion of the Sp αβ complexes were performed as described; briefly, Spα and Spβ chains were separated by anionic exchange chromatography on a Mono Q column (Pharmacia, Uppsala, Sweden), in the presence of 3 mol/L urea. Equal amounts of α and β chains were mixed and dialyzed against phosphate-buffered saline (PBS). Reassociation was verified using electrophoresis in non-denaturing polyacrylamide gels. Reassociated α and β chains were then digested by trypsin at 0°C for 5 hours with an enzyme to substrate ratio of 1:50 (wt:wt). Tryptic peptides were separated by SDS-PAGE using a 7% to 12% gradient gel.

**Amplification of DNA by the polymerase chain reaction.** DNA was prepared from nucleated blood cells as previously described. The two oligonucleotide primers 5’ CGT(GAATTC)TGAGAACTAGAATAAAG 3’ and 5’ CGT(GGATCC)GACATAACATATACATAAAG 3’ were synthesized using a Biosearch DNA synthesizer; their 5’ extremities contain recognition sequences (shown in parentheses) for the restriction enzymes EcoRI and BamHI, respectively; they were designed to anneal to target sequences 364 bases apart in genomic DNA, allowing the amplification of a segment of 3 mol/L urea. Equal amounts of α and β chains were mixed and dialyzed against phosphate-buffered saline (PBS). Reassociation was verified using electrophoresis in non-denaturing polyacrylamide gels. Reassociated α and β chains were then digested by trypsin at 0°C for 5 hours with an enzyme to substrate ratio of 1:50 (wt:wt). Tryptic peptides were separated by SDS-PAGE using a 7% to 12% gradient gel.

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**Subcloning and sequencing of the amplified DNA.** Amplified DNA from the HPP proband was purified by sequential extractions with phenol:chloroform, then chloroform, and precipitated with ethanol. Amplified DNA was then resuspended in 20 μL of the concentrated appropriate buffer recommended by the manufacturer and digested sequentially with restriction enzymes EcoRI and BamHI (Boehringer Mannheim, France). DNA was electrophoresed in a 2% NuSieve GTG agarose gel (FMC Bioproducts, Rockland, ME), the amplified band was cut out of the gel, and the DNA was recovered by phenol extraction at 65°C. The amplified fragment was then subcloned in plasmid pGEM 3Z (Promega Corp, Madison, WI), which had been first digested with restriction enzymes EcoRI and BamHI. After transformation with E. coli XL1BLUE competent cells (Stratagene, La Jolla, CA), recombinant clones were isolated and their DNA was purified; clones containing the amplified DNA were identified by digestion of the plasmid DNA with HindIII and EcoRI (Boehringer Mannheim) and electrophoresis in a 2% agarose gel. Sequencing of both strands of positive clones was done by the dideoxy sequencing method and T7 DNA polymerase using Sp6 and T7 promoter primers.

**Hybridization of amplified DNA with allele-specific oligonucleotides.** For hybridization analysis, 10 μL samples of amplified DNA were processed as described using a Gelman BiTrace membrane (Gelman Sciences Inc, Ann Arbor, MI). The membrane was prehybridized in 5× saline solution phosphate EDTA (SSPE), 5× Denhardt’s solution, and 0.5% SDS, for 1 hour at 50°C then hybridized at 62°C for 2 hours with 5× 10⁶ cpm/mL of radiolabeled oligonucleotide. The probe was 5′ end-labeled with (γ-32P) adenosine triphosphate (ATP) using T4 polynucleotide kinase. The membrane was washed twice for 15 minutes at room temperature in 2× SSPE, then twice for 4 minutes in 5× SSPE, 0.1% SDS at 62°C and subjected to autoradiography at −70°C using Dupont Kronex intensifying screens (Orsay, France).

**RESULTS**

**RBC stability and deformability.** Red cell thermal sensitivity, mechanical membrane stability, and RBC deformability studies were extended to other affected members of the family who were not available at the time of the first study.

In subject V, whose RBCs demonstrated marked anisocytosis and poikilocytosis, fragmentation occurred at 45°C. In HE subjects II, and IV, the mechanical stability was reduced to 15% of the control value. In subject V, RBCs were subjected to a constant shear stress, and their mechanical resistance was monitored as a function of time with an ektacytometer. Ghosts from HPP subject V, were very unstable; the mechanical resistance was reduced to 6% of the control value. In HE subjects II and IV, (subject III's daughter), the mechanical stability was reduced to 15% of the control value. In HE subject VI, and in her daughter, VII, the mechanical resistance was reduced to 10% of the control value.

Deformability of RBCs was measured as a function of the osmolarity of the suspending medium and expressed as the ektacytometric index (EI). In HPP subject V, RBC deformability was markedly decreased, with an EI at 0.20; the EI in hypotonicity was shifted to a higher osmolarity of 183 mosm (normal control values, 133 to 153 mosm), which is characteristic of the presence of spherocytic cells. Erythrocytes from HE subjects II, VI, and VII, generated trapezoidal shaped curves with a decreased EI in isotonicity (0.25, 0.24, and 0.27, respectively, with normal control values ranging between 0.41 and 0.57); as we reported...
previously, these trapezoidal curves are typical of HE with spectrin defects.

Analysis of membrane protein content. Densitometric scanning of RBC membrane proteins separated by SDS-PAGE showed no significant decrease in Sp content in the membranes of HE subjects III, IV, and VI; Sp:band 3 ratios were 0.91, 0.86, and 1.1, respectively, with normal control values of 1.05 ± 0.13 (n = 21). In HPP proband V, as well as in both of her HPP uncles, IV, and IV, Sp content was decreased in membranes, with an Sp:band 3 ratio of 0.70, 0.68, and 0.54, respectively. The values in subjects V, and IV, were similar to those found in 1986 (0.67 and 0.89, respectively) when these subjects were first studied. In RBC membranes of HPP subject V,, Sp content was decreased, with an Sp:band 3 ratio of 0.70 (Table I). HE subjects VI,, V,, and HPP subject VI, were not available for this study.

Analysis of Sp dimer self-association and limited tryptic digestion. In all seven subjects studied (HPP proband V,, HPP subjects IV, and IV, and HE subjects III, IV, and VI,), the relative amounts of Sp dimers in 4°C Sp extracts were increased, indicating a defective ability of Sp dimers to self-associate into tetramers. The proportion of Sp dimers (as a percentage of the total dimer plus tetramer pool) ranged between 30% and 51%, with normal control values of 3.6% ± 1.6% (Table I).

In all seven individuals studied, peptide patterns obtained after limited tryptic digestion of crude Sp and separation by electrophoresis showed a decrease in the α1/80Kd peptide with a concomitant increase in the 74Kd peptide (Fig 2). The amount of 74Kd peptide was evaluated as the proportion of 74Kd peptide in the 74Kd + 80Kd peptide pool. The area under the corresponding peaks was calculated by densitometric scanning of the peptides produced after 20 hours of incubation with trypsin and separation by one-dimensional gel electrophoresis. The percentage of 74Kd peptide varied from 42% to 65%, with the highest levels in HPP subjects (Table 1). In normal controls, the percentage of 74Kd peptide in Sp tryptic digests was 15 ± 4% (n = 18). The amount of the 74Kd peptide measured in subjects IV, V, and the proband V, was similar to that found in 1986 (Table 1) when these subjects were first studied.

Partial amino acid sequence of the α174 Kd peptide. Figure 3 shows a segment of the sequence of the normal α1/80Kd peptide compared with the N-terminal sequence of the α1/74Kd peptide from HE subject VI,. The N terminus is leucine, tryptic cleavage having occurred after lysine residue 42.

Reassociation studies of isolated Spα and Spβ chains. Spα and Spβ chains were isolated from a normal control subject and HE subject VI, using anionic exchange chromatography (Mono Q column) in the presence of 3 mol/L urea. The isolated chains were then allowed to reassociate and form hybrid dimers, which were then subjected to limited tryptic digestion. As shown in Fig 4, the tryptic peptide pattern obtained with the Sp hybrid dimer composed of control Spα chain and HE subject Spβ chain showed a normal content of the 80Kd peptide; in contrast, the peptide pattern obtained with the Sp hybrid dimer composed of HE Spα chain and control Spβ chain showed a decrease in the 80Kd peptide and a concomitant increase in the 74Kd peptide, demonstrating the involvement of the Spα chain from the HE subject in the pathologic process.

DNA amplification and sequencing. We used oligonucleotide primers complementary to intronic sequences flanking either side of exon 2 of the Spα gene to amplify total cellular DNA of HPP proband V,. Exon 2 encodes amino acids 3 to 82 of the Spα1/80Kd domain. The amplified fragment was subcloned, and 17 independent clones were isolated and subjected to DNA sequence analysis. In 11 clones, a G to A base substitution was found in codon 22 (CGT to CAT), which changes the normal arginine to a histidine (Fig 5); in the six other clones, the DNA sequence of exon 2 was normal.

Hybridization of amplified DNA with allele-specific oligonucleotides. DNA from six HPP or HE subjects of the family (HE subjects III, IV, V, and HPP subjects IV, IV, and V, and V,) was amplified and hybridized to radiolabeled allele-specific oligonucleotides corresponding to the normal and mutant sequences. As shown in Fig 6, the amplified DNAs

![Image](https://via.placeholder.com/150)

**Fig 2.** SDS-PAGE of partial tryptic digests of spectrin from different family members: HE subjects VI,, III, and IV,; HPP subjects IV, IV, V, (proposita), and V,; normal controls (C).
**Fig 3.** Partial amino acid sequence of the 74Kd peptide from HE subject VI. Tentatively identified residues are shown in parentheses. Numbering of amino acid residues is according to Speicher et al.7

from the six subjects with HE or HPP Spα1/74 (as well as DNA of the HPP proband) hybridized to both probes consistent with the fact that they are heterozygous for the mutation. In contrast, amplified DNA from two normal control subjects hybridized only to the oligonucleotide corresponding to the normal sequence.

**DISCUSSION**

HE and HPP with the Spα1/74 variant are characterized by increased susceptibility of the Spα1/80 domain to limited tryptic digestion with the production of abnormally high levels of the 74Kd peptide. Spα1/74 was the first variant described in HE and HPP. In the family reported here, the expression of mutant Sp was estimated by the percentage of the 74Kd peptide in tryptic digests, which is actually proportional but not necessarily equal to the amount of the mutant Sp. The percentage of the 74Kd peptide varied from one patient to another, but it was identical in the same patient on two separate occasions separated by a 4-year

**Fig 4.** Reassociation studies of isolated Spα and β chains. SDS-PAGE was performed after limited tryptic digestion of reconstituted Sp complexes from: lane 1, normal control αSp subunit + normal control βSp subunit; lane 2, normal control αSp subunit + βSp subunit of HE subject VI; lane 3, αSp subunit of HE subject VI + normal control βSp subunit; and lane 4, α + β Sp subunits of HE subject VI. Arrows indicate 80 and 74 Kd peptides.

**Fig 5.** DNA sequences derived from the normal and mutant alleles of HPP proposita VI. The arrow marks the site of the mutation. Numbering of amino acid residues is according to Speicher et al.7

**Fig 6.** Hybridization of amplified DNAs from family members with allele-specific oligonucleotides (ASO). One microgram of each genomic DNA was amplified as described in Materials and Methods. The amplified DNA from each individual was hybridized separately on duplicate filters to a probe corresponding to either the normal sequence (N) or the mutant sequence (M). The sequence of the ASO probe N is 5' CACTTCCTGACGCCTCTCCTG 3'; the sequence of the ASO probe M is 5' CAGGAGAGGCATCAGGAAAGTGTG 3'. N is complementary to the (+) strand of the normal allele; M is complementary to the (−) strand of the Spα1/74 allele.
interval. The amount of mutant Sp correlated with indicators of membrane skeleton dysfunction, such as erythrocyte thermal sensitivity, mechanical membrane instability, percentage of Sp dimers in 4°C Sp extracts, and clinical expression. The HPP patients who have the more severe clinical picture show the highest percentage of Sp variant and Sp dimers in 4°C Sp extracts. Thus, in this family, the severity of the disease appears to correlate in part with the estimated amount of mutant Sp in the erythrocyte membrane. This correlation suggests the existence of a threshold in the amount of variant Sp above which the RBC membrane becomes very unstable and the clinical picture very severe. However, this threshold depends not only on the amount of mutant Sp but also on the nature of the mutation. The Sp1/74 variant in this family, as well as the Sp1/78 variant (39 Arg to Ser) that we reported recently,47 seem to be associated with a more severe phenotype than, for instance, the Sp1/65-66 variant.47 We observed individuals with Sp1/65 HE in whom the percentage of variant Sp ranged up to 80% of the total Sp, without causing either a substantial defect in Sp self-association or RBC fragmentation and severe hemolysis.48 In the family described here, a 60% level of the Sp1/74 variant was associated with very unstable RBC membranes and a severe hemolytic disorder.

The mechanism(s) underlying the variable expression of the mutant Sp remains unknown: the amount of mutant Sp present in the erythrocyte membrane could be regulated transcriptionally or depend on a posttranscriptional event involving, for instance, the assembly of Sp in the membrane skeleton. In fact, at least two different defects of the membrane skeleton have been identified in HPP: the presence of an abnormal Spα chain with defective self-association properties and a partial deficiency of Sp.49 It has been suggested that HPP might result from double heterozygosity for two different defects: a structural mutation of the α1 Sp domain inherited from a parent with HE and a second unknown disorder that might affect Sp synthesis and is completely asymptomatic in the simple heterozygous state in the other parent.47 It must be mentioned that in the HPP proband, sequence of exon-2 from the other allele was normal. In the family with the Sp1/74 variant described here, no alterations were detected in erythrocytes from the proposita's father but with one parent was also free of any Sp abnormality.34 In the present family, the presumptive "silent factor" carried by the HPP proposita's unaffected parent must be widespread in the population, since it is expected to be present in the heterozygous state not only in the HPP proposita's father but also in the unrelated father of HPP subjects IVa and IVb, the mother of HPP subject V, and the father of HPP subject VI. It must also be noted that reassociation studies using isolated Spα and β chains from an HPP subject of the family (IVa) and an unaffected control subject gave results that were similar to those observed with HE patient VI.31

The cleavage site producing the 74Kd peptide in this kindred occurs after lysine residue 42, near the NH2-terminal end of the Spα domain. Identical results were obtained in other kindreds by Coetzer et al.50 This site is partially accessible in Sp from normal individuals, since low amounts of the 74Kd peptide are observed in digests of normal Sp47 and also result from cleavage after Lys 42.12,51 Studies by Speicher et al on the normal affinity purified α1/80Kd peptide showed that the cleavage site producing the 74Kd peptide was located after Arg 39.7 This discrepancy could be explained by the fact that the latter work was done using the isolated α1 peptide; indeed, we observed that the cleavage site at Arg 39 is more accessible than that at Lys 42 when tryptic digestion is performed on isolated normal α Sp chain.51 In the case of other Spα variants associated with HE and HPP, limited tryptic digestion of Sp results in a decrease in the Spα/80 peptide and the concomitant appearance of a "new" peptide of lower molecular weight that is not observed in normal control digests. These results indicate that the tryptic cleavage sites generating these variants, such as Lys 10 for Spα/78, Arg 131 for Spα/65, Arg 250 or Lys 252 for Spα/50b, and Arg 462 for Spα/50a13,14,25 are not readily accessible in normal Sp. Abnormally high levels of the 74Kd peptide observed in Sp tryptic digests from patients with HE or HPP Spα/74 can be due not only to a mutation of the Spα chain (this work and reference 52) but also to a defect of the Spβ chain, including a shortened variant β Sp chain.54 All of these mutations probably induce a conformational change in the Spα/80Kd domain leading to abnormal exposure of the same tryptic cleavage site.25 Among Sp mutations that have been thus far characterized at the DNA level, Spα/165-68, Spα/50b, and Spα/50b are located at the end of helix 3 of repeats 1, 2, and 4, respectively.25 Both Spα/78 mutations described lie at the end of the single helix of partial repeat 1', helix comparable with helix 3 of repeats 1 to 5.13 All of these mutations give rise to amino acid changes that expose tryptic cleavage sites lying upstream of the mutation. The comparable position of all of these mutations within the Sp repeat structure argues for the importance of the structural integrity of helix 3. The mutation responsible for the Spα/74 variant in this HE/HPP kindred is also located in the single helix of partial repeat 1'; however, the Spα/74 mutation in this case gives rise to an amino acid change that exposes a tryptic cleavage site downstream of the mutation. The diversity of mutations affecting the N terminus of SpαI domain should provide a better understanding of structure/function relationships of this part of the molecule.

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Hereditary pyropoikilocytosis and elliptocytosis in a white French family with the spectrin alpha I/74 variant related to a CGT to CAT codon change (Arg to His) at position 22 of the spectrin alpha I domain

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