Two Elliptocytogenic $\alpha^{I/74}$ Variants of the Spectrin αI domain

Spectrin Culoz (GGT→GTT; αI 40 Gly→Val) and Spectrin Lyon (CTT→TTT; αI 43 Leu→Phe)

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Abstract

Spectrin $\alpha^{I/74}$ elliptocytosis results from abnormalities involving the "head" region of spectrin dimer. Increased susceptibility to trypsin enhances cleavage of the α spectrin chain, yielding an increased amount of the αI 74-kD fragment at the expense of the αI 80-kD parent fragment. Recently we showed that the mutations causing the Spα$^{I/74}$ abnormality may lie in the α- or the β-chain, and that spectrin Culoz and spectrin Lyon were two ($\alpha^{I/74}$)α-variants, respectively. We now show that the spectrin Culoz αI domain undergoes prominent tryptic cleavage after Lys 42, whereas cleavage prevails after Arg 39 in spectrin Lyon. Applying the polymerase chain reaction (PCR) technique to exon 2 of the spectrin αI domain, we have established that the mutation responsible for spectrin Culoz is αI 40 Gly→Val; GGT→GTT. Applying the PCR technique to the cDNA derived from reticulocyte mRNA, we have shown that the mutation responsible for spectrin Lyon is αI 43 Leu→Phe; CTT→TTT. Studies of normal controls and of family members using dot blot hybridization with allele-specific oligonucleotide probes confirmed these results. Variants such as spectrin Culoz and spectrin Lyon should provide insight into a region that participates in spectrin dimer self-association and whose susceptibility to proteolysis must reflect subtle conformational changes. (*J. Clin. Invest.* 1990. 86:548–554.) Key words: Sp $\alpha^{I/74}$ elliptocytosis • spectrin mutants • spectrin α-gene

Introduction

The red cell membrane skeleton is a protein network that underlies the inner surface of the membrane (for review, see reference 1). It determines the unique shape and deformability of the erythrocyte. Spectrin represents its main component and occurs, in vitro, as an αβ dimer. Peptide mapping of spectrin partial tryptic digests allowed the division of the α- and β-chains into five and four domains, respectively (2). The αI and βI domains, which face each other, participate in dimer self-association. The αI domain appears in the form of an 80-kD fragment, which is further cleaved into a minor 78-kD fragment and a prominent 74-kD fragment. The amount of the 74-kD peptide varies depending on the samples and/or the experimental conditions. In addition, the precise cleavage point seems to be heterogeneous, occurring either after arginyl residue 39 (3) or lysyl residue 42 (4, 5). The αI domain is composed of five 106-amino acid repeats (6) designated α-1 to α-5. In turn, each repeat can be folded into three helices. Between repeat α-1 and the NH_2 terminus lies a partial repeat, termed α-1′ and containing part of helix 3 of the following repeats. At the gene level, the NH_2-terminal part of the αI domain is encoded by exon 1 (Met −6 to Thr 2) and exon 2 (Val 3 to Gln 82) (7).

Hereditary elliptocytosis (HE)[1] refers to a set of conditions in which the red cells have an elliptical shape. In recent years, HE has been shown to be associated with a number of abnormalities of spectrin or protein 4.1 (for review, see references 8 and 9). Many changes involve the αI and βI domains and as a result weaken spectrin dimer self-association. The spectrin $\alpha^{I/74}$ (Spα$^{I/74}$) abnormality is one of these changes. It is characterized by an increase of the αI 74-kD fragment at the expense of the parent αI 80-kD fragment (which encompasses nearly the entire αI domain). The Spα$^{I/74}$ alteration occurs sporadically among both black (10–13) and white populations (14–16). It generates highly variable clinical and morphological manifestations.

Initially it was assumed that the primary mutation would lie in the α-chain itself. However, several findings put this view in question: the Spα$^{I/74}$ abnormality was observed in association with β-chain mutants that carry deletions of variable lengths in their COOH-terminal region (17); in other families, haplotype studies (18) or DNA sequencing (19) ruled out defects of the α spectrin gene, or at least of exon 2 of this gene, as the cause of the alteration. Yet the possibility remained that the α-gene would be involved in some cases. Using in vitro spectrin dimer reconstitution, Pothier et al. (20) were able to assign the Spα$^{I/74}$ abnormality to either the α- or β-chain, making possible the distinction between ($\alpha^{I/74}$)α-variants and ($\alpha^{I/74}$)β-variants. Accordingly, spectrin Culoz and spectrin Lyon, which are elliptocytogenic variants found in two unrelated French Caucasian families, appeared as ($\alpha^{I/74}$)α-variants. Some differences in the protein phenotype indicated that these variants would not be identical. Using amplification of genomic DNA or of cDNA derived from reticulocyte mRNA, we

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1. *Abbreviations used in this paper:* ASO, allele-specific oligonucleotides; HE, hereditary elliptocytosis; PCR, polymerase chain reaction; Spα$^{I/74}$, spectrin $\alpha^{I/74}$.

found that spectrin Culoz is due to an αI 40 Gly→Val substitution (GGT→GTT) and that spectrin Lyon results from the αI 43 Leu→Phe (CTT→TTT) substitution.

Methods

Case reports

Case reports concerning spectrin Culoz and spectrin Lyon have been presented before (20). Briefly, spectrin Culoz was found in a Caucasian girl and her father (family CH). It was clinically asymptomatic and even associated with normal red cell morphology in the father. We noted several important features. (*a*) There was an unexpectedly high percentage of spectrin dimers in 4°C extracts: 67.3% in the proposita, presenting with clinically asymptomatic elliptocytosis, and 51.4% in the clinically asymptomatic father with normal morphology (controls: 6.4±1.9% [n = 12]) (20). We have no explanation for such high spectrin dimer percentage values, which also occur in spectrin Lyon (see below). We have discussed previously (20) that the Spα$^{I/74}$ mutation weakens spectrin self-association to the point that part of the tetramer may dissociate even at 4°C, leading to an overestimation of the dimer in vitro with respect to the percentage in situ. (*b*) The amount of the αI 74-kD fragment, after digestion in standard buffer (17) and expressed as the 74/(80+78+74) ratio (×100), was 51.0±1.2 (two determinations) in the father and 65.7±2.0 (two determinations) in the proposita, vs. 21.7±1.2 in six controls. These values are in good agreement with those reported before using a different buffer (20). (*c*) There was a duplication of the αI 74-kD fragment into an upper (74a) and a lower (74b) fragment, both yielding a positive reaction with anti-αI domain antibodies.

Spectrin Lyon was found in a Caucasian woman and her baby (family AU). In the mother, a picture of compensated hemolysis was observed. The baby was clinically unaffected with normal red cell morphology. The percentage of spectrin dimers in 4°C extracts was 40.7 and 34.4% in the proposita and her son, respectively (controls: 6.4±1.9 [n = 12]) (20). As in spectrin Culoz, high values were obtained. The 74/(80 + 78 + 74) ratio (×100), after digestion in standard buffer (17), was 56.5±1.2 (two determinations) in the proposita and 43.3±3.9 (two determinations) in her son (vs. 21.7±1.2 in six normal controls). These values are in good agreement with those reported before using a different buffer (20). The αI 74-kD fragment, using standard electrophoretic procedures, did not appear duplicated.

Analysis of the Spα$^{I/74}$ abnormality

The standard techniques that were used have been described or referred to previously (17). Tryptic digests of spectrin were analyzed on 7–15% linear polyacrylamide gels.

To best resolve the αI 74-kD fragment into its 74a and 74b subcomponents, digests were analyzed on 7–10% exponential polyacrylamide gels (21). In the control, the digestion times were 20 and 40 h (vs. 20 h in spectrin Culoz and spectrin Lyon). A 40-h digestion time in the control was carried out to give the 74-kD peak a height closer to that seen with the mutant spectrins. In spectrin Culoz, the αI 74-kD fragment was conspicuously duplicated as noted previously using a lower resolution electrophoretic system (20). In spectrin Lyon as well as in the normal controls, the above conditions allowed the demonstration of a duplicated αI 74-kD fragment that was not observed using the lower resolution electrophoretic system. In all cases, it became possible to determine the heights of 74a and 74b peaks and to deduce the 74a/74b ratio.

To carry out amino acid sequence analysis of the αI 74-kD fragment NH_2-terminal region (or of each of its 74a and 74b subcomponents), spectrin digests were run under special conditions. We used reagent- or electrophoresis-grade reagents purchased from Bio-Rad Laboratories (Richmond, CA) or Sigma Chemical Co. (St. Louis, MO) to prevent the artifactual blocking of the NH_2 terminus. Gels were left overnight at 4°C to ensure complete polymerization, sodium thioglycolate (0.1 mmol/liter) was added to the cathode reservoir, and a high protein/acrylamide ratio was used. The gels were blotted onto polyvinylidene fluoride (PVDF) membranes (Immobilon; Millipore/Continental Water Systems, Bedford, MA) as previously described (4). Because the gels had to be overloaded for the purposes of the present experiment (100 μg of digest), the separation of the 74a and 74b subcomponents was decreased. Whereas both components could be cut out separately in spectrin Culoz with limited cross-contamination, we had to cut out the αI 74-kD fragment as a single band in spectrin Lyon and in the control. All fragments were sequenced using a gas-phase sequenator (model 470A; Applied Biosystems Inc., Foster City, CA) coupled on-line with a 120A PTH–amino acid analyzer (Applied Biosystems Inc.). The determination of the NH_2-terminal segment of the mixture of 74a and 74b peptides (in spectrin Lyon and the normal control) will be discussed in Results.

DNA or cDNA amplification, cloning, and DNA sequencing

Spectrin Culoz. An Xba I restriction polymorphic site has been described in the 5′ end of the α spectrin gene (22–24). Genomic DNA from the proposita was digested with Xba I, transferred onto a nylon membrane, and hybridized using the genomic 3021-E1 probe obtained by Linnenbach et al. (25).

To elucidate the mutation responsible for spectrin Culoz, we chose to amplify exon 2 (amino acids 3–82) of the α spectrin gene. We synthesized two 28-mer oligonucleotide primers (a and b in Table I) that were complementary to intronic sequences 5′ and 3′ of exon 2, respectively. These oligonucleotides carried an Eco RI site at their 5′ end to facilitate subsequent cloning. The polymerase chain reaction (PCR) was performed as previously described (4) except that the hybridization step was done at 53°C instead of 42°C. The amplified products were digested with Eco RI and cloned into M13mp18 (Appligene, Strasbourg, France). Positive clones were revealed using the 3021-E1 probe and sequenced by the dideoxynucleotide method of Sanger et al. (26) using the sequenase kit (United States Biochemical Corp., Cleveland, OH).

Spectrin Lyon. To elucidate the mutation responsible for spectrin Lyon, we chose to amplify the cDNA derived from reticulocyte mRNA, following the demonstration by Tse, B., and B. G. Forget (unpublished data) that amplified α spectrin cDNA can be obtained from reticulocyte RNAs. PCR primers c and d (Table I) were synthesized to cover a region extending from (and including) codon −6 to codon 171, e.g., a region encompassing exons 1–4 (7).

Total RNA was extracted from peripheral blood as described by Itoh et al. (27). Reverse transcription was performed according to the methods of Frohman et al. (28) and Goblet et al. (29), with some modifications. 2 μg of total RNA in H_2O was heated at 65°C for 10 min, chilled in ice, and mixed with 5 μl of a buffer containing 67 mM Tris HCl (pH 8.80), 6.7 mM $MgCl_2$, 16.7 mM $(NH_4)_2SO_4$, 1.5 mM of each dNTP, 0.5 μg of primer d (Table I), and 200 U of Moloney Murine Leukemia Virus reverse transcriptase (Bethesda Research Laboratories, Cergy-Pontoise, France). The reaction (final volume, 50 μl) was incubated at 42°C for 90 min. To amplify the cDNA, the reaction mixture was supplemented with 0.5 μg of primer c (Table I) and 2.5 U of the Taq polymerase (Perkin Elmer Cetus, Norwalk, CT). The amplification procedure was performed as first described by Saiki et al. (30): the first denaturation set at 93°C for 5 min; then 30 cycles of 1 min 30 s annealing at 53°C, 2 min of extension at 72°C, 1 min of denaturation at 93°C; the last cycle was set for 5 min of extension. A pattern of several bands was obtained. The right sized band was purified by squeeze-freeze (31) and reamplified as follows: cDNA (0.1 μg) was added to 5 μl of a buffer containing 67 mM Tris HCl (pH 8.80), 4.7 mM $MgCl_2$, 16.7 mM $(NH_4)_2SO_4$, 1 mM of each dNTP, 0.5 μg of primers c and d (Table I), and 2.5 U of Taq polymerase. The amplification reaction (final volume, 50 μl) was performed as above.

Amplified cDNA was digested with Eco RI (Boehringer Mannheim GmbH, Mannheim, FRG) to regenerate the Eco RI sites on the 5′ end of each primer. The right sized band was purified by squeeze-freeze. Cloning and sequencing were done as described for spectrin Culoz.

Table I. PCR Primers and ASO Probes

	Spectrin Culoz		Spectrin Lyon
	PCR primers		
a	5′GCGAATTCTGAGAACTAGCAATTAACAG3′ Eco RI	c	5′GCGAATTCATGGAGCAATTTCCAAAGGA3′ Eco RI
b*	5′GCGAATTCCCATTAACATTAACATAAAG3′ Eco RI	d*	5′GCGAATTCTTGTCTCCAATCCACTCTA3′ Eco RI
	ASO probes		
	Codon 40		Codon 43
N_1	5′TGAGAGGGGTCAGAAGCTT3′	N_2	5′GGTCAGAAGCTTGAGGATT3′ Hind III
	↓		↓
M_1	5′TGAGAGGGTTCAGAAGCTT3′	M_2	5′GGTCAGAAGTTTGAGGATT3′

The oligonucleotides are complementary to the antisense strand or the sense strand (*). PCR primers a and b correspond to sequences found in the introns flanking exon 2. PCR primers c and d correspond to sequences present in exon 1 and exon 4, respectively. An Eco RI site was incorporated into all primers. In mutant ASO probes M_1 and M_2, the arrow indicates the substituted bases. The Hind III restriction site, underlined in ASO probe N_2, is lost in M_2.

Dot blot analysis of spectrin Culoz and spectrin Lyon DNA

We synthesized four 19-mer allele-specific oligonucleotides (ASOs) complementary to the antisense strand. Their sequences are presented in Table I. ASO N_1 (normal) and ASO M_1 (mutant) were used for the detection of the spectrin Culoz mutation. ASO N_2 (normal) and M_2 (mutant) were used for the spectrin Lyon mutation. ASO probes were 5′-labeled with [^{32}P]γ-ATP using the T4 polynucleotide kinase (Boehringer Mannheim GmbH). Fresh samples of amplified DNA or cDNA were used for dot blot analysis. Additional controls consisted of cloned mutant DNA from both propositi (positive controls) and cloned normal DNA (negative controls). 4% of the reaction product was applied to a nylon membrane (Zetabind; Amersham [France], Les Ulis, France) using a dot blot system (Bethesda Research Laboratories). For both spectrin Culoz and Lyon, replicate samples were analyzed. Prehybridization was carried out in 5× SSPE, 5× Denhardt's, and 0.5% SDS for 1 h at 52°C for spectrin Culoz and at 50°C for spectrin Lyon. Hybridization was carried out by adding 140 ng of ^{32}P-labeled ASO probes for 16 h at 52°C for spectrin Culoz and 4 h at 50°C for spectrin Lyon. Membranes were rinsed twice in 2× SSC, 0.1% SDS at room temperature for 5 min, and then in 5× SSC, 0.1% SDS at 57°C for 10 min for spectrin Culoz. For spectrin Lyon, washes were carried out at 54°C for ASO N_2 and at 56°C for ASO M_2. Autoradiography was performed for 1 h using Kodak XAR5 film.

Results

Protein studies

In addition to the features previously reported (20) and briefly reviewed in Methods, the carriers of either spectrin Culoz or spectrin Lyon displayed no apparent increase of the minor αI 78-kD fragment and showed minor changes in the 17–22-kD region. Although the latter may be of some relevance, they were not analyzed in detail. Attention was focused on the αI 74-kD fragment.

Using high resolution electrophoretic conditions, it became apparent that the duplication of the αI 74-kD fragment into 74a (upper) and 74b (lower) subcomponents was a general rule (Fig. 1). Only the ratio between both subcomponents varied in different samples. In controls, the 74a/74b ratio was 0.88±0.14 (n = 8) (digestion time, 20 h), or 0.80±0.11 (n = 7) (digestion time, 40 h), indicating the predominance of the lower αI 74-kD subcomponent (Fig. 1). Based on the proportions of amino acid PTH derivatives obtained at each cycle, amino acid sequence analysis (Table II) of the 74a + 74b mixture was consistent with an overlap between a peptide

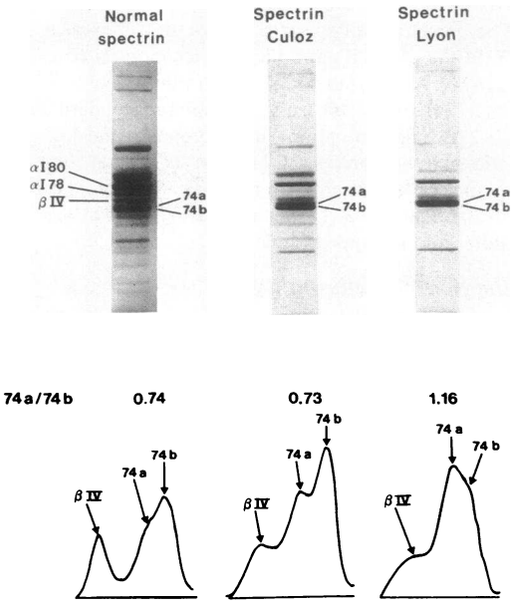

Figure 1. One-dimensional analysis of spectrin partial digests using high resolution electrophoretic conditions. Electrophoresis was carried out using a 7–10% exponential polyacrylamide gel according to Laemmli (21). The duplication of the αI 74-kD fragment is obvious in spectrin Culoz but poorly resolved in normal control spectrin and in spectrin Lyon. However, scanning at 570 nm reveals duplication in all cases, as shown by the profiles below each gel. The indicated 74a/74b ratios are indicated for the particular profiles shown; the reproducibility of these values is discussed in the text. To increase the amount of the αI 74-kD fragment in the normal control, a digestion for 40 h was used vs. 20 h for the mutant spectrins.

Table II. Amino Acid Sequence Analysis of the NH_2-terminal Regions of the 74a and 74b Fragments

Normal spectrin					
	40	41	42	43	44
74a	X	Gln	Lys	Leu	Glu
+					
74b	X	Glu	Asp	Ser	Tyr
	43	44	45	46	47
Spectrin Culoz					
74a	40	41	42	43	44
	Gly	Gln	Lys	Leu	Glu
	Val		(Asp)		
74b	Leu	Glu	Asp	Ser	Tyr
	43	44	45	46	47
Spectrin Lyon					
	40	41	42	43	44
74a	X	Gln	Lys	Leu	Glu
				Phe	
+					
	X	Glu	X	Ser	Tyr
74b	Phe				
	43	44	45	46	47

Amino acid sequence analysis was carried out on mixed 74a and 74b subcomponents in control spectrin and in spectrin Lyon, due to the lack of separation of the two subcomponents. On the other hand, the subcomponents were analyzed separately in spectrin Culoz. From the 74a and 74b mixtures, it was possible to decipher overlapping sequences starting at positions 40 and 43, respectively. The NH_2-terminal residue was often difficult to identify (see text). In particular, Gly 40 and Leu 43 were not detected in the first cycle using the 74a + 74b mixture from spectrin Lyon, whereas Phe 43 (the substituted residue at position 43) was recognized in this cycle. In spectrin Culoz, Asp 45 (position 3 of subcomponent 74b) contaminated Lys 42 (position 3 of subcomponent 74a). The mutations are underlined.

starting at position 40 (peptide 74a) and a peptide starting at position 43 (peptide 74b). For example, cycle 3 yielded 4.1 pmol of Asp (peptide 74b, position 45) and 0.3 pmol of Lys (position 42, peptide 74a), respectively. Due to the small amounts of peptides that were used, however, some cycles could not be interpreted because of unavoidable contamination by other free amino acids and/or other minor peptides.

In the proposita for spectrin Culoz, the 74a/74b ratio was 0.78±0.16 (two digestions, 20 h; one and four determinations, respectively). In her father, the ratio was 0.88±0.04 (one digestion, 20 h; two determinations). Such values indicated the prevalence of the lower αI 74-kD fragment. Amino acid sequence analysis, carried out in separated 74a and 74b subcomponents, ascertained that the peptides starting at positions 40 and 43 corresponded to subcomponents 74a and 74b, respectively. The NH_2-terminal residue was often difficult to identify. Presumably this results from free amino acids that contaminate the sample and/or the reaction mixture, and also from the lack of a preceding cycle to be used as a reference point, as in the case for subsequent cycles. Incidentally, the presence of valine in position 40 was an indication of the mutation involved in spectrin Culoz.

In the proposita for spectrin Lyon, the 74a/74b ratio was 1.22±0.14 (one digestion, 20 h; four determinations). In her son (one digestion, 20 h; two determinations), the ratio was 1.33±0.07. Such values indicated the prominence of the upper αI 74-kD fragment. Amino acid sequence analysis of the 74a + 74b mixture was, again, consistent with the above ratio. For example, cycle 5 yielded 1.4 pmol of Glu (position 44, peptide 74a) and 0.6 pmol of Tyr (position 47, peptide 74b), respectively; as before, unavoidable contaminations made some cycles uninterpretable. Incidentally, the presence of phenylalanine in position 43 was an indication of the mutation involved in spectrin Lyon.

Gene studies

Spectrin Culoz. The proposita and her father and mother were homozygous for the absence of the Xba I polymorphic site in the 5′ end of the α spectrin gene (22–24). Therefore, this site could not serve as a marker for one of the two chromosomes. Consequently, exon 2 was sequenced in seven clones from the proposita. In two clones, the G→T substitution was found in position 2 of codon 40 (GGT→GTT; Gly→Val) (Fig. 2). The rest of the sequence of exon 2 was normal. Using dot blot analysis (Fig. 3), the normal ASO probe (N_1) hybridized with amplified DNA from the father, mother, proposita, and control. The mutant probe (M_1) gave a signal with amplified genomic or cloned DNA from the proposita and also with amplified genomic DNA from her father (who carries the $Sp\alpha^{I/74}$ abnormality without elliptocytosis). On the contrary, the mutant probe did not react with amplified genomic DNA from the mother (who does not carry the $Sp\alpha^{I/74}$ abnormality), or with amplified genomic or cloned normal control DNA.

Spectrin Lyon. Exon 2 was sequenced in five clones from the proposita. In two clones, a C→T substitution was found in position 1 of codon 43 (CTT→TTT; Leu→Phe) (Fig. 4). Using dot blot analysis (Fig. 5), the normal ASO probe (N_2)

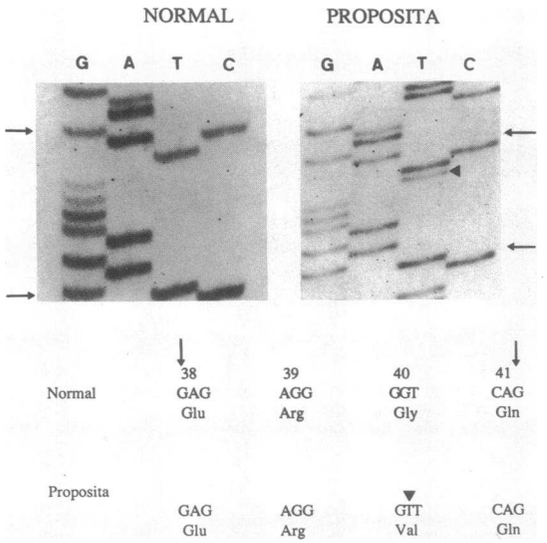

Figure 2. DNA sequence from the spectrin gene of the proposita with spectrin Culoz. Individual subclones from PCR-amplified genomic DNA were sequenced using the dideoxynucleotide method of Sanger et al. (26). The normal DNA subclone was from the proposita's mother. The arrows bracket the sequence surrounding the mutation. The numbers refer to the α spectrin amino acid sequence, according to Speicher et al. (3). ▶, New T at position 2 of codon 40.

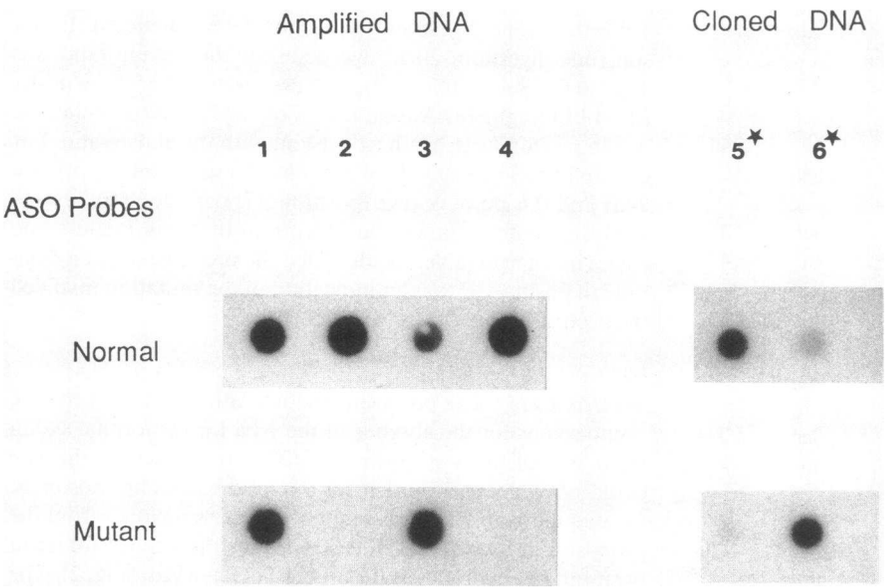

Figure 3. Dot blot analysis of DNA from the family with spectrin Culoz. Amplified genomic DNA or cloned DNA was hybridized with the normal or the mutant ASO probes N_1 and M_1, respectively (Table I), using long or short (★) exposure times. Lane *1*, father (heterozygous for spectrin Culoz); lane *2*, mother (normal); lane *3*, proposita (heterozygous for spectrin Culoz); lane *4*, normal control; lane *5*, cloned DNA from a normal control (exon 2 sequenced); lane *6*, DNA of mutant clone from the proposita. There were no other family members available for testing.

gave a positive hybridization signal with: (*a*) amplified cDNA and amplified genomic DNA from the proposita, (*b*) amplified genomic DNA from the carriers of spectrin Culoz (see above) and of spectrin Tunis ($\alpha^{I/78}$; CGG→TGG [αI 35; Arg→Trp]) (4), and (*c*) cloned DNA from a normal control. The mutant probe (M_2) gave a positive signal with amplified cDNA, amplified genomic DNA, and cloned DNA from the proposita. Due to the G-T stable mismatch, a more stringent wash (56°C instead of 54°C) was necessary to extinguish the signal obtained with the ASO probe in individuals not carrying spectrin Lyon, while it left unaltered the signal obtained with the mutant DNA (Fig. 5). Finally, Hind III digestion of the amplified genomic DNA of the carrier of spectrin Lyon yielded a fragment of ~ 540 bp (as visualized by ethidium bromide fluorescence) in addition to smaller fragments of ~ 150 and 400 bp, confirming the loss of the Hind III restriction site in exon 2 caused by the mutation in codon 43 (not shown).

Discussion

The $Sp\alpha^{I/74}$ abnormality was the first change to be recognized at the protein level in the spectrin αI domain. It was subsequently shown to occur in sporadic fashion among blacks as well as Caucasians, and to be associated with a highly variable clinical phenotype. Thus, the characteristics of the $Sp\alpha^{I/74}$ abnormality contrast, for example, with those of the $Sp\alpha^{I/65}$ ab-

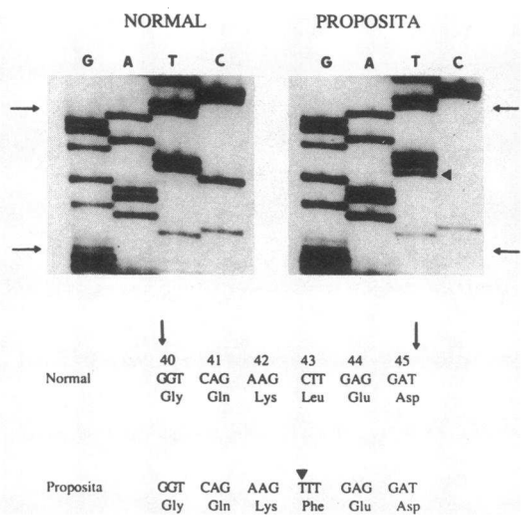

Figure 4. DNA sequence from α spectrin cDNA of the proposita with spectrin Lyon. Individual subclones from PCR-amplified cDNA were sequenced as in Fig. 2. The normal sequence is from a normal subclone of the proposita. The arrows bracket the sequence surrounding the mutation. The numbers refer to the α spectrin amino acid sequence (3). ▶, New T in position 1 of codon 43. There were no other family members available for testing.

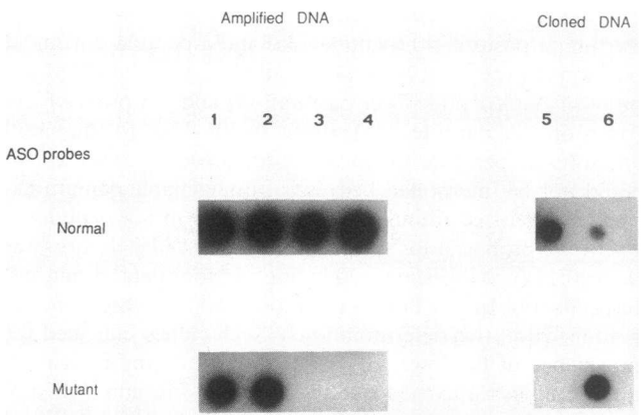

Figure 5. Dot blot analysis of DNA from the proposita with spectrin Lyon. Amplified genomic DNA, amplified cDNA, and cloned cDNA were hybridized with the normal or the mutant ASO probes (M_2 and N_2, Table I). Lane *1*, amplified cDNA from the proposita (heterozygous for spectrin Lyon); lane *2*, amplified genomic DNA from the proposita (amplification was carried out using primers a and b of Table I); lane *3*, amplified genomic DNA from a spectrin Culoz heterozygote; lane *4*, amplified genomic DNA from a spectrin Tunis ($Sp\alpha^{I/78}$) heterozygote (4); lane *5*, DNA of normal cDNA clone from the proposita; lane *6*, DNA of mutant cDNA clone from the proposita.

normality (32, 33). The latter is found exclusively, and sometimes at a relatively high frequency (34), among people residing in, or originating from Africa; its phenotype is more homogeneous, although some variability still occurs at the morphological and biochemical levels. In all cases investigated thus far, the Sp$\alpha^{I/65}$ abnormality appears to result from duplication of α spectrin codon 148 (TTG = Leu) (7, 35, 36). To a certain extent, the Sp$\alpha^{I/74}$ abnormality more closely resembles the Sp$\alpha^{I/50}$ abnormality, although the latter is also restricted to people living in, or originating from Africa. It is associated with a clinical picture of variable severity and results from several different mutations (7, 35). On the whole, recent information makes it likely that the Sp$\alpha^{I/74}$ abnormality would result from a number of different mutations.

Consistent with this view, but somewhat unexpectedly, the Sp$\alpha^{I/74}$ abnormality was found to be associated with alterations in both the α- and β-chains of spectrin. That abnormal β-chains could generate the Sp$\alpha^{I/74}$ abnormality was initially suspected in light of the following results: (*a*) the Sp$\alpha^{I/74}$ alteration was observed in patients carrying β-chain variants with different deletions in their COOH-terminal region (17), (*b*) it failed to appear in linkage with the α spectrin gene in some other Sp$\alpha^{I/74}$ families (18), and (*c*) it was associated with no abnormality of exon 2 of the latter gene in still another Sp$\alpha^{I/74}$ family (19). Using in vitro spectrin dimer reconstitution experiments, Pothier et al. (20) recently assigned the ultimate mutation to either the α- or β-chain, in a variety of cases of Sp$\alpha^{I/74}$ HE. Spectrin Culoz and spectrin Lyon appeared as ($\alpha^{I/74}$)α-variants because their α-chains, but not their β-chains, were associated with the generation of the Sp$\alpha^{I/74}$ abnormality after partial tryptic digestion of reconstituted spectrin. Recent findings strengthened the view of a heterogeneous molecular basis for the Sp$\alpha^{I/74}$ abnormality. In one family, an Ala→Pro substitution in the last repeat of the β spectrin chain was observed (37). In another family the $\alpha^{I/74}$ phenotype was related to a point mutation at position 22 of spectrin α-chain: Arg→His; CGT→CAT (38).

Cleavages after both Arg 39 and Lys 42 yield the αI 74-kD fragment, or more precisely, its 74a and 74b subcomponents. It is not known whether the cleavage after Lys 42 implies an initial cleavage after Arg 39 or if it can occur independently. In any event, the final results vary according to the mutant. Spectrin Culoz favors cleavage after Lys 42 and spectrin Lyon after Arg 39. Such differences in behavior imply differences in the conformational changes induced by each mutation. Along with the 74/αI ratio, e.g., the amount of the αI 74-kD fragment as the percentage of the whole αI domain, the 74a/74b ratio is another useful parameter for the characterization of Sp$\alpha^{I/74}$ variants.

It is noteworthy that all of the αI domain mutations causing elliptocytosis elucidated thus far occur in the same area of the polypeptide chain repeats: (*a*) helix 3 overlapping repeats α-2 and α-3 or α-4 and α-5 for several Sp$\alpha^{I/50}$ variants (7, 35); (*b*) helix 3 overlapping repeats α-1 and α-2 for the Sp$\alpha^{I/65}$ variant (7, 35, 36); (*c*) single helix overlapping repeats α-1′ and α-1 for the Sp$\alpha^{I/78}$ variants (4, 23); (*d*) single helix overlapping repeats α-1′ and α-1, again, for the two present Sp$\alpha^{I/74}$ variants and the case of Garbarz et al. (38). Helix 3 must play a critical role in achieving the appropriate rigidity over the whole length of the dimer.

From the results available at this time, however, it is likely that the relationship between the specific mutation and the local conformational change represents a complex issue. For example, the αI 35 Arg→Trp substitution (spectrin Tunis [$\alpha^{I/78}$]) results in a dramatic increase in the cleavage after Lys 10 but little cleavage after Arg 39 and/or Lys 42 (4). The αI 39 Arg→Ser substitution (23) also results in increased cleavage after Lys 10 (yielding the Sp$\alpha^{I/78}$ phenotype) without causing an increase in the αI 74-kD fragment. On the other hand, mutations located downstream at positions 40 and 43 (spectrin Culoz and spectrin Lyon, respectively), or a mutation located upstream (αI 22 Arg→His) (38), produce the Sp$\alpha^{I/74}$ phenotype. In the latter cases, the αI 78-kD fragment is normal or decreased; potential increased cleavage after Lys 10 would be masked by the truncation resulting from cleavage after Arg 39 and/or Lys 42. Therefore, much remains to be learned about how a given mutation will produce a given phenotype.

It should be pointed out that the amino acid substitutions responsible for spectrin Culoz and spectrin Lyon bring about little modification in charge or steric hindrance, unlike the appearance of helix-breaking prolyl residues in several Sp$\alpha^{I/50}$ variants (7, 35). However, this conclusion does not allow an accurate prediction of the actual conformational changes that develop. Pothier et al. (20) have shown that the Sp$\alpha^{I/74}$ abnormality was still present in α^{Culoz}-chains separated from β-chains but not in isolated α^{Lyon}-chains. The presence of the β-chain seems to be needed for the Sp$\alpha^{I/74}$ abnormality in α^{Lyon}-chains to become apparent. It is also unclear why the 74a/74b ratio has a different value in spectrin Culoz vs. spectrin Lyon.

In this work, we have reported the nature of the mutations that are responsible for two elliptocytogenic Sp$\alpha^{I/74}$ spectrin α-chain variants: spectrin Culoz (GGT→GTT; αI 40 Val→Gly) and spectrin Lyon (CTT→TTT; αI 43 Leu→Phe). The availability of such variants should help to define the structural conformation around positions 39–42 of spectrin αI domain that is involved in the $\alpha^{I/74}$ cleavage.

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