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Mutation of a highly conserved isoleucine disrupts hydrophobic interactions in the αβ spectrin self-association binding site

Patrick G Gallagher[1], Zhushan Zhang[2], Jon S Morrow[2] and Bernard G Forget[3,4]

[1]*Department of Pediatrics;* [2]*Department of Pathology;* [3]*Department of Internal Medicine and* [4]*Department of Genetics, Yale University School of Medicine, New Haven, CT, USA*

We studied an infant with severe neonatal hemolytic anemia and hyperbilirubinemia that evolved into a partially compensated ellipto-poikilocytic anemia. His father had typical elliptocytosis. Their erythrocyte membranes demonstrated structural and functional defects in spectrin. Genetic studies revealed that the proband and his father were heterozygous for an α-spectrin mutation, Ile24Thr, in the αβ spectrin self-association binding site. The proband also carried the low expression allele α^{LELY} *in trans*, influencing the clinical phenotype. The importance of isoleucine in this position of the proposed triple helical model of spectrin repeats is highlighted by its evolutionary conservation in all α spectrins from *Drosophila* to humans. Molecular modeling demonstrated that replacement of a hydrophobic isoleucine with a hydrophilic threonine disrupts highly conserved hydrophobic interactions in the interior of the spectrin triple helix critical for spectrin function.
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Spectrin, the principal structural component of the erythrocyte membrane, is also the most abundant protein, comprising 25–30% of membrane protein. In the red cell, spectrin functions include maintenance of cellular shape, regulation of lateral movement of integral membrane proteins, and provision of structural support for the lipid bilayer.[1] Spectrin is composed of two subunits, α and β spectrin, that despite some similarities are distinct proteins encoded by separate genes.[2–6] α and β spectrin chains intertwine in an antiparallel manner to form αβ heterodimers. The αβ heterodimers self-associate to form tetramers and oligomers, the primary functional unit of spectrin in the red cell.[1]

Detailed study of α and β spectrin, including cDNA cloning, has yielded a better understanding of these important membrane proteins. Knowledge of the primary structure of spectrin has allowed determination of the precise genetics defects in cases of hereditary elliptocytosis (HE) and hereditary pyropoikilocytosis (HPP).[6] Analyses of these mutations have provided important information on the structure and function of spectrin in the red cell and shed additional insight into the pathogenesis of these disorders.

Of particular importance to our understanding of spectrin structure and function has been the identification of mutations in the regions of α and β spectrin critical for self-association.[7,8] This report describes a novel α-spectrin mutation, Ile24Thr, discovered in an elliptocytosis/poikilocytosis kindred that is located in the region of the αβ-spectrin self-association binding site. This mutation disrupts highly conserved hydrophobic interactions in the interior of the spectrin triple helix that are critical for αβ spectrin self-association, highlighting the importance of isoleucine 24 in spectrin structure and function.

Materials and methods

Erythrocyte Membrane Preparation And Quantitation of Spectrin Content

Erythrocyte membranes were prepared from peripheral blood as previously described.[9,10] Membrane proteins were analyzed by electrophoresis in SDS–PAGE either with a 5–15% polyacrylamide gradient as described by Laemmli[11] or using a 3.5% polyacrylamide gel as described by Fairbanks *et al*.[9] To estimate spectrin/band–3 ratios, SDS polyacrylamide slab gels were scanned after Coomassie blue

Correspondence: PG Gallagher, Department of Pediatrics, Yale University School of Medicine, 333 Cedar Street, PO Box 208064, New Haven, CT 06520-8064, USA.
E-mail: patrick.gallagher@yale.edu
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Table 1 Oligonucleotide primers

αI-spectrin–flanking exon 2
5′-CGTGAATTCTGAGAACTAGCAATTAACAG-3′ (sense)
5′-CGTGGATCCCCATTAACATTAACATAAAG-3′ (antisense)
βI-Spectrin–flanking exons 30 and 31
5′-GCGAATTCAGGAGTGAACGATTGGGTGCT-3′ (sense)
5′-CGAAGCTTGGTCCCAATGTCAGGT-3′ (antisense)

staining using a DU8 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA, USA at 550 nm.

Study of Spectrin Dimer–Tetramer Equilibrium

Spectrin was extracted by incubating ghosts overnight at 4°C in low ionic strength buffer.[12] The content of spectrin dimers and tetramers was determined by nondenaturing gel electrophoresis as described.[13]

Limited Trypic Digestion of Spectrin

Limited tryptic digests of spectrin extracts were prepared as described.[10] Spectrin peptides were separated by two–dimensional gel electrophoresis with isoelectric focusing (IEF) as described by O'Farrell[14] modified by Speicher *et al*.[15]

Amplification, Subcloning, and Nucleotide Sequencing of Amplified Genomic DNA

Genomic DNA was amplified, subcloned, and sequenced as described.[16] Oligonucleotide primers used to amplify exon 2 of the human α-spectrin gene and exons 30 and 31 of the β-spectrin gene are listed in Table 1.

Identification of the α^{LELY} Allele

The α^{LELY} allele was determined using a PCR-amplification techniques as described.[17]

Dynamic Molecular Modeling

Dynamic molecular modeling of the spectrin self-association domain was performed as described.[18] These computations place the starting structure of the spectrin self-association domain in a 6-Å water shell that contains ~11 1000 water molecules. The application of this approach and its fidelity has been described.[16,18]

Results

Patients

The proband, an African–American male, had hemolytic anemia and extreme jaundice in the neonatal period requiring intensive phototherapy. His hematocrit decreased from 42 to 22% in the first 2 weeks of life. Blood smear showed hemolysis and elliptocytosis. At 9 month-of-age, the spleen tip was palpable and the liver edge was 1 cm below the costal margin. The hematocrit was 30%, and the reticulocyte count 3%. The blood smear showed numerous elliptocytes and significant numbers of poikilocytes and schistocytes. The father's hematocrit was 43%, and the reticulocyte count 3.9%. His blood smear showed occasional elliptocytes. Blood smear and hematologic indices of the mother were normal.

Studies of Erythrocyte Membrane Proteins

Qualitative and quantitative analyses of erythrocyte membranes

One-dimensional SDS-PAGE analyses of erythrocyte membranes from the proband and his parents were qualitatively normal (not shown). Quantitative analyses of spectrin content, measured by the ratio of spectrin to band 3 was normal in the mother (1.1) and father (1.04), and decreased in the proband (0.91), compared to that of a normal control (1.0).

Limited tryptic digestion of spectrin

Limited tryptic digestion of normal spectrin followed by two-dimensional gel electrophoresis yields a pattern of five major proteolytically resistant domains of α spectrin and four proteolytically resistant domains of β spectrin.[15,19,20] The 80 kDa αI domain encodes the NH_2-terminus of α spectrin that interacts with sequences from the 17th repeat of β spectrin to form the binding site for spectrin self-association.[21,22]

Limited tryptic digestion of spectrin followed by two-dimensional gel electrophoresis with IEF (Figure 1) revealed new peptides of 74 kDa in samples from the proband as well as in his father. Increased

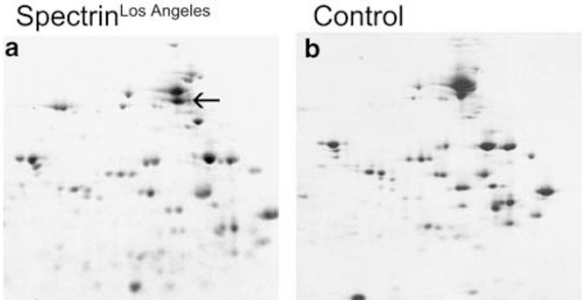

Figure 1 Limited tryptic digestion of spectrin. Red cell spectrin from members of the spectrin$^{Los\ Angeles}$ kindred was partially digested with trypsin and fractionated by two-dimensional gel electrophoresis with IEF (SDS/PAGE with IEF). Spectrin digests from the father (**a**) demonstrate increased amounts of a 74 kDa spectrin peptide (arrow), located just below the 80 kDa αI domain peptide, compared with normal (**b**). Digests from the proband demonstrated increased amounts of the 74 kDa αI domain peptide (not shown). In tryptic digests from the mother, the αI domain peptide was normal.

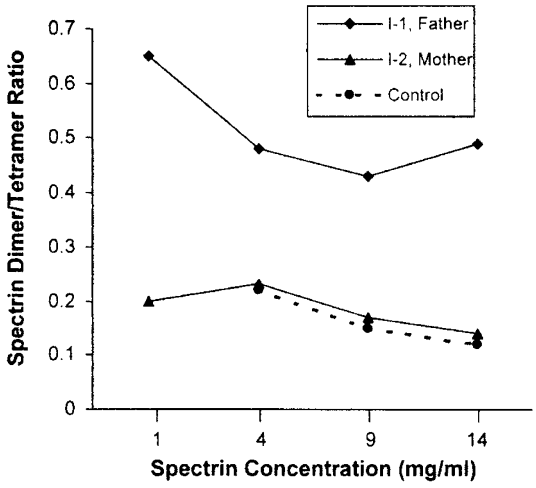

Figure 2 Spectrin self-association assays. Studies of spectrin self-association were performed using spectrin extracted from erythrocyte membrane ghosts at low ionic strength at 4°C and then equilibrated at various concentrations. The increased amounts of spectrin dimers compared with tetramers at higher concentrations of the father's spectrin indicate a moderate degree of impairment of spectrin self-association.

amounts of the αI/74 kDa peptide have been associated with structural defects of spectrin in individuals with HE and HPP.

Digests also revealed polymorphisms of the αII domain of spectrin.[23,24] The mother had polymorphisms of type 2 and type 4; the father, types 1 and 4; and the proband, type 4 only. Therefore, in this family, the αI/74 kDa abnormality is associated with an α-spectrin gene that also carries the type 4 polymorphism of the αII domain.

Spectrin self–association
Studies of spectrin's ability to self-associate were performed using spectrin extracted from erythrocyte ghosts under low ionic strength. The impaired formation of tetramers as a function of spectrin concentration is apparent in the elliptocytic father compared to the hematologically normal mother and a control (Figure 2). These studies indicate a moderate degree of impairment in spectrin self-association. In general, the degree of impairment of spectrin self-association parallels clinical severity in patients with HE or HPP.[25,26] Insufficient spectrin was available from the proband to perform these studies.

Molecular Genetic Analyses

Nucleotide sequencing
Mutations associated with the variant 74 kDa peptide of the αI domain of spectrin have been identified in the regions of the αI and βI spectrin genes encoding the binding site of spectrin self-association. This corresponds to exon 2 of αI-spectrin gene and exons 30–31 of the βI-spectrin gene.[27] Genomic DNA from the proband corresponding to exon 2 of the αI-spectrin gene and exons 30–31 of the βI-spectrin gene was amplified by PCR. Nucleotide sequence analysis of these amplification products revealed heterozygosity for a single nucleotide substitution (ATC to ACC) that changes an isoleucine (aliphatic, hydrophobic) to a threonine (hydroxyl, hydrophilic) at codon 24 of the αI-spectrin chain. We have named this mutation spectrin$^{\text{Los Angeles}}$.

PCR-based mutation detection
The authenticity of this mutation was confirmed by restriction enzyme digestion of amplified genomic DNA. The spectrin$^{\text{Los Angeles}}$ mutation disrupts an *Xho*II restriction enzyme site, PuGATCPy, allowing for rapid PCR-based detection in genomic DNA of each family member. Oligonucleotide primers (Table 1) flanking exon 2 of the α-spectrin gene amplify a fragment of 372 bp. Amplified genomic DNA fragment from a normal individual and the mother digested into fragments of 56, 81, and 235 bp. Amplified genomic DNA fragments from the proband and his father yielded fragments of 56, 81, 235, and 316 bp, indicating heterozygosity for the spectrin$^{\text{Los Angeles}}$ mutation.

α^{LELY} allele
A PCR-based detection method was utilized to determine the status of the low expression allele α^{LELY}.[17] The proband and his mother are heterozygous for the α^{LELY} allele (not shown). The father lacks the α^{LELY} allele. Thus, compared to the elliptocytic father without the α^{LELY} allele, in the proband, inheritance of the α^{LELY} allele *in trans* is likely to increase the relative expression of the mutant spectrin$^{\text{Los Angeles}}$ allele, thereby worsening the clinical phenotype.

Dynamic Molecular Modeling

The spectrin$^{\text{Los Angeles}}$ mutation occurs at position 79 of partial repeat 1 within helix 3 of the proposed triple helical conformational model of spectrin repeats, corresponding to position C4 of the crystallographic structure of the spectrin repeat.[2,28] Most mutations of the 80 kDa αI domain of spectrin occur in helix 3 of the proposed model and are often at or adjacent to highly conserved residues of the homologous 106-amino-acid repeats of spectrin.[27] The importance of isoleucine in this residue is highlighted by its evolutionary conservation in all reported α spectrins from *Drosophila* to humans (Table 2). A previous mutation in codon 24, Ile24Ser spectrin$^{\text{Lograno}}$, was described in a French kindred,[34] further indicating the importance of codon 24 in spectrin function.

Molecular modeling
Development of dynamic molecular modeling allows prediction of the conformational rearrangements in the predicted structure induced by point

Table 2 Evolutionary conservation of isoleucine in position 79 of partial repeat 1 of the proposed triple helical model of α-spectrin

Sequence	*Species and Isoform*	*References*
TAEE**I**QERR	Human αI, wild type	Sahr *et al*[3]
TAEE**T**QERR	Human αI, spectrin$^{\text{Los Angeles}}$	This report
TAEE**I**QHRR	Murine αI	Wandersee *et al*[29]
TAED**I**QERR	Human αII	Moon and McMahon[30]
TAED**I**QERR	Rat αII	Hong and Doyle[31]
TAED**I**QERR	Chicken	Wasenius *et al*[32]
TVED**I**QERR	*Drosophila*	Dubreuil *et al*[33]

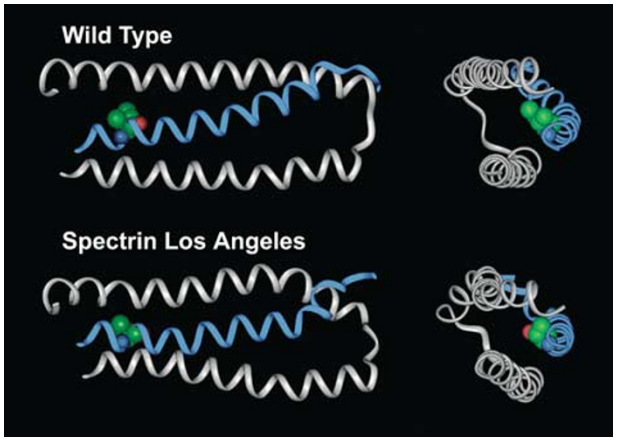

Figure 3 Dynamic molecular modeling of the structural effects of the spectrin$^{\text{Los Angeles}}$ mutation. A triple helical repeat representing the αβ spectrin self-association contact site is shown. The A and B helices of βI spectrin are shown in white and the C helix of αI spectrin is shown in blue. Amino acid 24, the location of the spectrin$^{\text{LosAngeles}}$ mutation is shown as a space-filling representation. Dynamic molecular modeling indicates that replacement of a hydrophobic isoleucine with a hydrophilic threonine disrupts conserved hydrophobic interactions in the interior of the triple helix and disrupts the conformation of the normal αβ spectrin self-association domain. Lateral (left) and end-on (right) views are shown.

mutations. Modeling of the Ile24Thr mutation is shown in Figure 3. This mutation disrupts critical hydrophobic interactions between helix C and helix A (F2014 of β spectrin).[28] In general, interactions of helix C with helices A and B provide stability to the spectrin self-association unit.[18]

In previous studies, comparison of the degree of predicted structural disruption in the self-association site with clinical severity revealed a strong correlationr.[18] The degree of structural deviation, as derived by the root-mean-square deviation from the predicted spectrin backbone, for the Ile24Thr spectrin$^{\text{Los Angeles}}$ mutation was 3.296 Angstroms (normal 2.846 Å). Thus, the predicted structural disruption of the spectrin$^{\text{Los Angeles}}$ mutation correlates with the clinical severity observed.

Discussion

The model of spectrin as a protein-containing multiple α-helical, coiled coil, triple helical repeats was first predicted by direct protein sequencing.[2] Refinements and phasing of the model have been made as determination of the primary structure,[3,4] X-ray, nuclear magnetic resonance, and spectroscopic studies of spectrin have been completed.[28,35–43] These studies predict that the first and third helices are parallel and the second helix is antiparallel. The amphipathic repeats are stabilized by hydrophobic interactions of each repeat at the interior core of each repeat.

Heptad periodicity, detectable from inspection of the primary sequence and in the three-dimensional structure, has been suggested to promote stable folding of a single spectrin repeat.[44] The majority of conserved amino acids in spectrin repeats are amino acids that occupy heptad positions *a* and *d* that make up the hydrophobic core of the coiled coil. This is true for isoleucine 24, located at position C4 in heptad *d*, which is one of the most highly conserved hydrophobic residues in all spectrin repeats.[38] In addition to the hydrophobic packing in the core, electrostatic interactions between charged side chains of various amino acids, primarily at heptad positions *e* and *g*, help stabilize the repeat fold[45] and contribute to the formation of stable dimers.[46]

Molecular modeling permits analysis of the key residues involved in spectrin inter- and intrachain interactions. Interestingly, the majority of α-spectrin mutations associated with HE and HPP are predicted to disrupt hydrogen bond and salt bridge interactions. In only one other case, spectrin$^{\text{Lyon}}$,[47] L49F, is there replacement of an amino acid directly involved in hydrophobic interactions.

Spectrin$^{\text{Los Angeles}}$ was detected only in the heterozygous state, and when *in trans*, to the α^{LELY} allele as occurs in the proband, is associated with a severe clinical phenotype. As noted by Zhang *et al*,[18] even though heterozygous α-spectrin self-association binding site mutations are among those most commonly found in HE and HPP patients, homozygous mutations of the α-spectrin self-association site have not been found, perhaps because complete disruption of spectrin self-association is incompatible with life. The significant disruption of hydrophobic interactions in the core of the αβ spectrin repeat caused by the spectrin$^{\text{Los Angeles}}$ mutation

suggests that homozygosity for this mutation would also be incompatible with life.

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