SUPPLEMENTARY MATERIALS AND METHODS

Genomic DNA

Genomic DNA was isolated from blood leukocytes using the Puregene DNA purification kit (Gentra, MN, USA). Exon 2 of the SPTA gene was amplified by PCR and sequenced using a 3130 Genetic Analyzer (Applied Biosystems, CA, USA). Primer sequences were described previously.[22]

Reticulocyte mRNA

Total RNA was isolated from reticulocytes as previously described. The RNA precipitate was further purified using Tri-Reagent (Molecular Research Center, OH, USA) and reverse transcribed using Superscript III First-Strand Synthesis SuperMix including random primers (Invitrogen, CA, USA).

Quantitation of the SPTA mRNA transcript

Reticulocyte SPTA mRNA levels were determined using TaqMan mRNA Expression Assays and normalized to an endogenous HPRT control (Applied Biosystems, CA, USA). Quantitative allele-specific PCR (qRT-ASPCR) for αSp reticulocyte mRNA was performed on a 7500-Sequence Detection-System (Applied Biosystems, CA, USA) according to a modification of a previously described method utilizing complementary oligonucleotides containing a locked nucleic acid and a mismatch primer. Screening for SPTAαLELY was performed as previously described. Primer sequences are listed in Supplementary Table 2.

Erythrocyte membrane protein analysis

Red cell membranes were prepared as described[5]. Briefly, erythrocytes were lysed with a hypotonic buffer (3mM sodium phosphate, pH 8, 0.1mM EDTA, 0.1mM phenylmethylsulphonyl fluoride) and the ghosts were pelleted by centrifugation at 30,000*g* for 15 minutes at 4ºC and washed until clear of hemoglobin. The protein concentration was determined using the Coomassie Plus™ Protein Assay Reagent (Pierce Biotechnology Incorporated, USA). Aliquots were solubilized by boiling for 1 minute in

10mM Tris-HCl pH 8, 1mM EDTA, 1% SDS, 5% sucrose, 2% β-mercaptoethanol and 20-25µg protein resolved on a 4-17% Fairbanks exponential gradient SDS-polyacrylamide gel.[15] Coomassie Blue stained gels were scanned on a Hoefer GS300 transmittance/reflectance scanning densitometer and the accompanying GS365W software (Hoefer Scientific Instruments, USA) and the ratio of spectrin to band 3 was quantified for each individual.

Spectrin was analysed as previously described[5, 6]. Briefly, spectrin was extracted from the membranes in a low ionic strength buffer of 0.1mM sodium phosphate, pH 8, 0.1mM EDTA, 0.1mM 1,4-dithiothreitol (Roche Diagnostics GMBH, Germany) at 4ºC overnight. Aliquots of 10µg spectrin were resolved on 3% acrylamide/0.3% agarose non-denaturing gels and stained with Coomassie blue. The percentage spectrin dimers (SpD) out of total SpD and spectrin tetramers (SpT) was determined by densitometry. Fifty micrograms spectrin was subjected to limited tryptic digestion with 0.5µg trypsin (Sigma-Aldrich Corporation, USA) for 18 hours at 4ºC and the peptides were resolved on a 10% SDS-PAGE gel.[16] Relative amounts of the 80 and 74kDa peptides were determined by densitometry of the Coomassie-stained gels.

Comparative modeling of the spectrin tetramerization site

The crystal structure of the erythrocyte Sp tetramerization complex[19] is available in the Protein Data Bank (http://www.rcsb.org; accession number 3LBX). A putative model of the partial repeat 0 of helix C of αSp with the R34P mutation was generated using the Protein homology/analogy recognition engine V2.0 (Phyre2) algorithm (http://www.sbg.bio.ic.ac.uk/phyre2/) and the crystallized erythrocyte Sp tetramerization complex as a template. The model quality was assessed using the Qmean server (http://swissmodel.expasy.org/qmean/cgi/index.cgi). The model of the mutant Sp tetramerisation site was superimposed on the crystallized erythrocyte Sp tetramerization complex structure using Swiss PDB viewer (http://spdbv.vital-it.ch/refs.html). Images were modified using Pymol Molecular Graphics System, version 1.3, Schrödinger, LLC. The helical propensity of normal and mutant Sp was analysed using Agadir (http://agadir.crg.es/).

Supplementary Table 1. Clinical data of Families A and B

Pedigree number	Age	Sex	Hb (g/L)	MCV (fl)	Hct (fraction of 1)	WBC (10^9/L)	Platelets (10^9/L)	Erythrocyte morphology
Range			120-180	82-98	0.43-0.52	3.6-11.2	140-440	
Family A								
A-I-1	83	M	127	88	0.378	9.2	317	**HE**
A-I-3	79	M	96-125	84-87	0. 26-0.34	5.1	400-614	**Atypical HPP**
A-II-1	56	F	151	82	0.434	5.49	311	**N**
A-II-2	52	F	120	81	0.348	7.57	265	**HE**
A-II-3	47	M	152	84	0.422	5.4	260	**N**
A-II-4	48	M	153	95	0.477	6.3	218	**N**
A-II-5	42	M	181	98	0.563	6.4	177	**N**
A-II-6	41	F	130	98	0.41	7.4	173	**HE**
A-III-1	20	M	ND	86	ND	ND	ND	**HE**
A-III-2	11	M	133	83.1	0.371	8.4	311	**HE**
Family B								
B-I-1	39	F	140	82.3	0.409	8.69	553	**Atypical HPP**
B-I-2	38	F	114	86	0.329	6.23	262	**N**
B-I-3	35	F	130	85	0.383	5.76	214	**HE**
B-II-1	17	M	147	87	0.433	5.71	374	**N**
B-II-2	12	F	135	89	0.39	7.05	315	**N**
B-II-3	9	F	137	87	0.403	4.98	244	**N**
B-II-4	8	F	147	88	0.416	8.67	395	**N**
B-II-5	8	M	128	81	0.359	5.32	327	**N**
B-II-6	10	F	151	88	0.429	5.64	273	**N**
B-II-7	7	M	141	87	40.5	7.63	282	**HE**
B-II-8	4	F	127	80	0.359	7.64	354	**N**

Abbreviations:
N: Normal; HE: Hereditary Elliptocytosis; HPP: Hereditary Pyropoikilocytosis
ND: No data

Supplementary Table 2. Primer and probe sequences to quantitate SPTA mRNA

Primer/Probe	Sequence 5' to 3'
FAM-AS-SP-MGB	6FAM-CACTTCCTGACGCCTC-MGBNFQ
SP-LNA-F	5'-GAAACCGTTGTGGAGAGCAGT-3'
SP-LNA-G-R	5'-CGCTCCTTGAAACTTTGAT*A*gG-3'*
SP-LNA-C-R	5'-CGCTCCTTGAAACTTTGAT*A*gC-3'*

*The mismatched nucleotide is depicted in lowercase and the locked nucleic acid base is underlined and italicized.

Supplementary Table 3. Helical propensity of αSp at the tetramerization site is reduced in the P34 mutant

Amino acid	Amino acid position	Helical propensity*	
		Wild type (R34)	Mutant (P34)
A	21	4.4	3.5
E	22	4.6	3.7
E	23	5.4	4.3
I	24	5.9	4.6
Q	25	6.1	4.7
E	26	6.1	4.6
R	27	6.3	4.4
R	28	5.0	2.9
Q	29	3.6	1.2
E	30	3.0	0.5
V	31	3.0	0.4
L	32	2.8	0.1
T	33	2.8	0.0
R / P	34	2.4	0.1
Y	35	0.8	0.1
Q	36	0.6	0.1
S	37	0.5	0.2

*Helical propensity was predicted using Agadir (http://agadir.crg.es/) at pH = 7.4, temperature = 37ºC and ionic strength = 134mM.