Four different mutations in codon 28 of alpha spectrin are associated with structurally and functionally abnormal spectrin alpha I/74 in hereditary elliptocytosis.

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Research Article

Hereditary elliptocytosis (HE) Sp alpha I/74 is a disorder associated with defective spectrin (Sp) heterodimer self-association and an abnormal tryptic cleavage of the 80-kD alpha I domain of Sp resulting in increased amounts of a 74-kD peptide. The molecular basis of this disorder is heterogeneous and mutations in codons 28, 46, 48, and 49 (codons 22, 40, 42, and 43 in the previous nomenclature which did not include the six NH2-terminal amino acids) have been reported. In this study we present data on seven unrelated HE Sp alpha I/74 kindred from diverse racial backgrounds in whom we identified four different mutations all occurring in exon 2 of alpha Sp at codon 28. Utilizing the polymerase chain reaction we established a CGT----CTT; Arg----Leu 28 mutation in one kindred of Arab/Druze origin. In two unrelated white kindred of English/European origin the substitution is CGT----AGT; Arg----Ser 28 and in two apparently unrelated white kindred from New Zealand, the mutation is CGT----TGT; Arg----Cys 28. Finally, in one American black kindred and in a black kindred from Ghana the mutation involves CGT----CAT; Arg----His 28. Allele specific oligonucleotide hybridization confirmed that the probands are heterozygous for the respective mutant alleles. All four point mutations abolished an Aha II restriction enzyme site which allowed verification of linkage of the mutation with HE Sp alpha I/74. Our results […]

Four Different Mutations in Codon 28 of α Spectrin Are Associated with Structurally and Functionally Abnormal Spectrin $\alpha^{I/74}$ in Hereditary Elliptocytosis

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Abstract

Hereditary elliptocytosis (HE) Sp $\alpha^{I/74}$ is a disorder associated with defective spectrin (Sp) heterodimer self-association and an abnormal tryptic cleavage of the 80-kD αI domain of Sp resulting in increased amounts of a 74-kD peptide. The molecular basis of this disorder is heterogeneous and mutations in codons 28, 46, 48, and 49 (codons 22, 40, 42, and 43 in the previous nomenclature which did not include the six NH_2-terminal amino acids) have been reported. In this study we present data on seven unrelated HE Sp $\alpha^{I/74}$ kindred from diverse racial backgrounds in whom we identified four different mutations all occurring in exon 2 of α Sp at codon 28. Utilizing the polymerase chain reaction we established a CGT → CTT; Arg → Leu 28 mutation in one kindred of Arab/Druze origin. In two unrelated white kindred of English/European origin the substitution is CGT → AGT; Arg → Ser 28 and in two apparently unrelated white kindred from New Zealand, the mutation is CGT → TGT; Arg → Cys 28. Finally, in one American black kindred and in a black kindred from Ghana the mutation involves CGT → CAT; Arg → His 28. Allele specific oligonucleotide hybridization confirmed that the probands are heterozygous for the respective mutant alleles. All four point mutations abolished an Aha II restriction enzyme site which allowed verification of linkage of the mutation with HE Sp $\alpha^{I/74}$. Our results imply that codon 28 of α Sp is a "hot spot" for mutations and also indicate that Arg 28 is critical for the conformational stability and functional self association of Sp heterodimers. (*J. Clin. Invest.* 1991. 88:743–749.) Key words: α spectrin gene • "hot spot" mutations • hereditary pyropoikilocytosis • polymerase chain reaction • clinical heterogeneity

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Introduction

Hereditary elliptocytosis (HE)[1] is heterogeneous in terms of clinical presentation, inheritance, and the underlying molecular defect (1, 2). The most common abnormality involves a defective spectrin (Sp) self association (3–6). Sp, the major component of the red cell membrane skeleton, consists of two elongated chains which are arranged in an antiparallel fashion to form $\alpha\beta$ heterodimers (SpD); the SpD, in turn, interact in the head region of the two chains forming tetramers (SpT) and oligomers (3, 7). Limited tryptic digestion of Sp has allowed the identification of five α Sp (αI–αV) and four β Sp (βI–βIV) structural domains (8). The self association of SpD into SpT involves the NH_2-terminal αI and the COOH-terminal βI domains (7). α and β Sp are composed of multiple homologous repeat units, each consisting of ~ 106 amino acids (9), except for the NH_2- and COOH-terminal residues of both α and β Sp, as well as segment 10 of α Sp, which are atypical and unrelated to the conserved repeat motif (10, 11).

Seven distinct structural defects of the αI domain, associated with impaired SpD-SpD interaction, have been defined on the basis of abnormal limited tryptic digest maps in which there is a decrease in the normal 80-kD αI domain and a concomitant increase in one or more lower molecular weight peptides (2). These defects of Sp are designated according to the molecular weight of the abnormal peptide (1). HE subjects who carry one or more of these abnormal spectrins exhibit marked variation in clinical severity as well as the degree of functional impairment of SpD self association (12). Recent studies have indicated that Sp $\alpha^{I/74}$ is the most severe defect, whereas Sp $\alpha^{I/65}$ results in only mild dysfunction (13). Partial amino acid sequencing of the NH_2-terminal portion of the abnormal peptides has revealed amino acid changes in close proximity to the cleavage site in subjects with Sp $\alpha^{I/65}$, Sp $\alpha^{I/46}$, and Sp $\alpha^{I/50b}$ (14). Knowledge of the genomic exon/intron arrangement of the αI domain of Sp (15) has allowed the verification of these changes at the DNA level and has also facilitated the study of defects located either upstream or further away from the cleavage site. Sp $\alpha^{I/65}$ appears to be a homogeneous defect due to a single mutation because all cases thus far investigated involve a duplication of the leucine codon (TTG) at position 154 (formerly

1. *Abbreviations used in this paper:* ASO, allele-specific oligonucleotide; HE, hereditary elliptocytosis; HPP, hereditary pyropoikilocytosis; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; Sp, spectrin; SpD, spectrin heterodimer; SpT, spectrin tetramer.

148, see abstract and below) of α Sp (16–18). In contrast, recent studies on Sp $\alpha^{I/74}$ have revealed that this defect is heterogeneous in origin and that the primary mutation may occur in either the α Sp or β Sp genes (18–20). Thus far, four α Sp and one β Sp point mutations have been described in Sp $\alpha^{I/74}$ subjects involving changes of α Sp amino acids 22 (21), 40 (22), 42 (23), and 43 (22) and amino acid 2053 in β Sp (20). In these reports, the amino acids of α Sp have been numbered according to the amino acid sequence of limited tryptic digest peptides of α Sp (9). However, the full-length cDNA sequence of α Sp predicts an additional six NH_2-terminal amino acids (10). Therefore, by agreement with other researchers in the field (Alloisio, N., J. Delauney, D. Dhermy, and B. Forget), the numbering used in this report has now been modified to include the additional six residues. The previously reported mutations (20–23) are thus located in codons 28, 46, 48, and 47.

In this study, we describe four mutations in codon 28 (new nomenclature; this corresponds to codon 22 of previous nomenclature) of α Sp in seven unrelated kindred with HE from diverse racial backgrounds. Allele specific oligonucleotide hybridization confirmed that the probands were heterozygous for the defects. All four point mutations abolished an Aha II restriction enzyme site and this allowed screening of family members to verify linkage of the mutation with HE.

Methods

Subjects. The pedigrees of the seven unrelated Sp $\alpha^{I/74}$ kindred are shown in Fig. 1. Family 6 is one of the two original black American kindred in whom the Sp $\alpha^{I/74}$ defect was first detected (24) and clinical and protein data on family 4 have been reported previously (25). Data on the remaining five kindred have not been published. Clinical and biochemical data on affected individuals are given in Table I. Criteria for the diagnosis of the clinical phenotype have been outlined (2) and are based mainly on (*a*) review of the peripheral blood film, (*b*) presence or absence of hemolysis, and (*c*) the inheritance pattern.

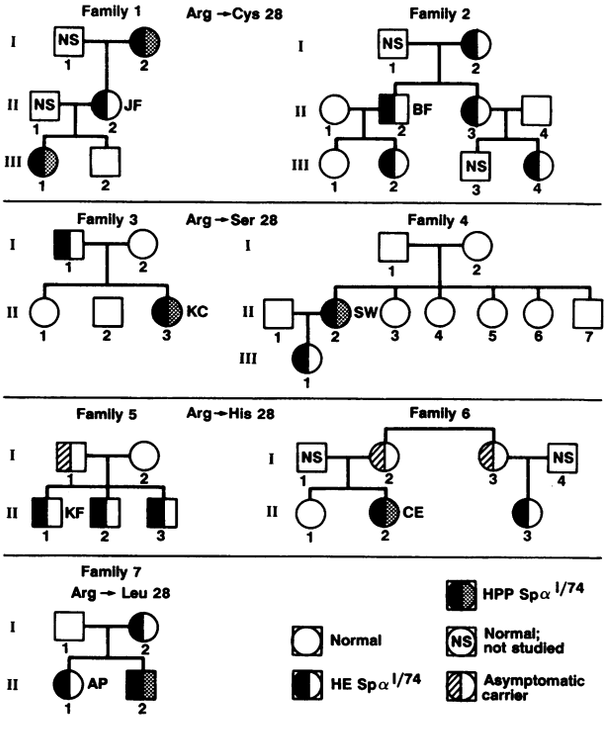

Figure 1. Pedigrees of the seven unrelated HE Sp $\alpha^{I/74}$ kindred with four different codon 28 mutations. Families 1–4 are Caucasian, families 5 and 6 are Black, and family 7 is of Arab/Druze origin.

In all seven kindred, the clinical severity varies markedly between family members and ranges from asymptomatic carrier to HPP (Fig. 1 and Table I). Families 1 and 2 are apparently unrelated white kindred from New Zealand and families 3 and 4 are white kindred of English/European origin. Family 5 is a black kindred from Ghana, whereas family 7 is of Arab/Druze origin.

In all these individuals, there is an increase in the percentage SpD extracted from the membrane at 4°C compared to control values of 5.0±3.6% (n = 35). Asymptomatic carriers have 25–31% SpD; HE subjects range from 36–55% SpD and HPP individuals have 60–70% SpD in their membrane extracts. This functional Sp self association defect is due to the presence of abnormal Sp $\alpha^{I/74}$ in all cases as evidenced by limited tryptic digestion of Sp (data not shown). The membrane Sp content, expressed as a Sp/b3 ratio, is decreased in all HPP subjects (normal Sp/b3 1.0±0.1, n = 60), as well as in some of the HE individuals with severe hemolysis (Table I). The remaining HE subjects and asymptomatic carriers have a normal Sp content.

Erythrocyte membrane protein analysis. The methods used have been previously referred to or described in detail (24, 26, 27). They include erythrocyte membrane protein preparation (26, 28) and analysis by SDS-PAGE (3.5–17% acrylamide gradient gels) (26, 29); spectrin extraction (26) and analysis of SpD and SpT by nondenaturing gel electrophoresis (4); limited tryptic digestion of Sp (24) followed by SDS-PAGE (10% acrylamide) (30) and immunoblotting with a polyclonal anti-α I Sp antibody (26). On some probands, two-dimensional isoelectric-focusing/SDS-PAGE of limited tryptic digests of Sp was performed (24). Amino acid sequence analysis (31) of the abnormal 74-kD α I peptide was carried out in the laboratory of Dr. P. Matsudaira at Massachusetts Institute of Technology, Cambridge, MA.

Restriction fragment length polymorphism (RFLP) analysis. Genomic DNA was isolated from peripheral leukocytes of ACD anticoagulated blood (32) and 10 μg DNA were digested with the restriction endonucleases Xba I, Msp I, Pvu II, and Taq I (Boehringer Mannheim GmbH, Mannheim, FRG). DNA fragments were fractionated on a 0.7% agarose gel (33) and transferred to a nylon membrane (Zetaprobe, Bio-Rad Laboratories, Inc., Richmond, CA) by alkaline Southern blotting (34) in 0.4 M NaOH. A 13-kb genomic α I Sp probe (clone 3021 E1) (35) and a 738 bp β Sp cDNA probe (36) were labeled with ^{32}P dCTP (New England Nuclear, Boston, MA) by random priming (37) and hybridized to the filters as described by the Zetaprobe manufacturer.

Genomic DNA amplification and sequencing. Exons 1 and 2 of α Sp were amplified by the polymerase chain reaction (PCR) (38) utilizing oligonucleotide primers complementary to the flanking intron sequences (Fig. 2). P1 (5'GAATTCGACTGGACAGTTCCAT3') and P2 (5'TTAGGGTCTGCTCTGAGGCAAT3') amplified a ±1,500-bp fragment containing both exons, whereas P2 and P3 (5'CACATATAAGCGGGGCAACAT3') amplified a 348-bp fragment containing only exon 2. 1 μg of genomic DNA was amplified for 30 cycles using Taq polymerase (Perkin Elmer Corp., Norwalk, CT) in a Perkin Elmer Cetus DNA thermal cycler. Each cycle consisted of 1 min denaturation at 94°C, 1 min annealing at 55°C and 2 min extension at 72°C.

Subcloning and sequencing of amplified DNA. The PCR reaction mixes were analyzed on 1% agarose gels and the amplified DNA was purified by elution from the gel using Geneclean (Bio 101, La Jolla, CA). The PCR products were blunt-end ligated into Hinc II digested pGEM 3Z vector (Promega Biotec, Madison, WI) (33). Positive subclones were sequenced on both strands by dideoxy sequencing (39) using T7 DNA polymerase (Sequenase; United States, Biochemical Corp., Cleveland, OH) and plasmid Sp6 and T7 promoter primers.

Direct sequencing of amplified DNA. Genomic DNA was amplified as above using primers P2 and P3. 5 μl of the PCR reaction mix were reamplified as described, using only P2. This resulted in accumulation of single stranded DNA which was purified either on Centricon 30

Table I. Clinical and Biochemical Data on HE Sp $\alpha^{I/74}$ Kindred

Subject	Splenectomy	Hct	Retics	%SpD*	Sp/b3*	Clinical phenotype	Mutation
		%	%				
Family 1							
I.2	Yes	30	2.1	52	0.74	HPP	Arg → Cys 28
II.2′	No	41	1.5	49	0.91	Mild HE	Arg → Cys 28
III.1	Yes	34	6.3	68	0.67	HPP	Arg → Cys 28
Family 2							
I.2	Yes	34	2	53	0.86	Severe hemolytic HE	Arg → Cys 28
II.2	Yes	36	1.5	53	0.83	Severe hemolytic HE	Arg → Cys 28
II.3	Yes	32	1.8	55	0.81	Severe hemolytic HE	Arg → Cys 28
III.2	No	36	1.9	48	0.90	Mild HE	Arg → Cys 28
III.4	Yes	15‡	17‡	60	0.71	HPP	Arg → Cys 28
Family 3							
I.1	No	45	2	36	1.00	Mild HE	Arg → Ser 28
II.3	No	12–18	5–14	50	0.77	Severe hemolytic HE	Arg → Ser 28
Family 4							
II.2	Yes	35	4–5	61	0.69	HPP	Arg → Ser 28
III.1	No	35	1.9	44	0.96	Mild HE	Arg → Ser 28
Family 5							
I.1	No	NA	N	26	0.99	Asymptomatic carrier	Arg → His 28
II.1	No	NA	↑	48	0.97	Hemolytic HE	Arg → His 28
II.2	No	NA	↑	51	0.96	Hemolytic HE	Arg → His 28
II.3	No	NA	↑	Transfused		Hemolytic HE	Arg → His 28
Family 6							
I.2	No	40	0.9	25	1.02	Asymptomatic carrier	Arg → His 28
I.3	No	47	1.8	31	0.97	Asymptomatic carrier	Arg → His 28
II.2	No	21–25	8–16	76	0.80	HPP	Arg → His 28
II.3	No	34.5	5.2	32	0.98	Mild HE	Arg → His 28
Family 7							
I.2	No	NA	1.4	39	0.91	Mild HE	Arg → Leu 28
II.1	No	NA	12	73	1.00	Severe hemolytic HE	Arg → Leu 28
III.2	No	NA	20	66	0.82	HPP	Arg → Leu 28

NA, not available.
* Normal values for % of SpD, 5.0±3.6%; Sp/b3, 1.0±0.1. ‡ Presplenectomy values.

(Amersham, UK) or model 30,000 filters (Millipore Corp., Bedford, MA). The supernatant was concentrated by ethanol precipitation and sequenced using T7 DNA polymerase and P3 as sequencing primer.

Allele-specific oligonucleotide hybridization. Normal and mutant allele-specific oligonucleotides (ASO) were synthesized on an Applied Biosystems oligonucleotide synthesizer (Applied Biosystems Inc., Foster City, CA) and purified. The normal ASO sequence was 5′CAGGAGAGGCGTCAGGAAGTG3′ (codon 28 is underlined) and the mutant ASO sequences were identical except for the relevant nucleotide substitution at codon 28.

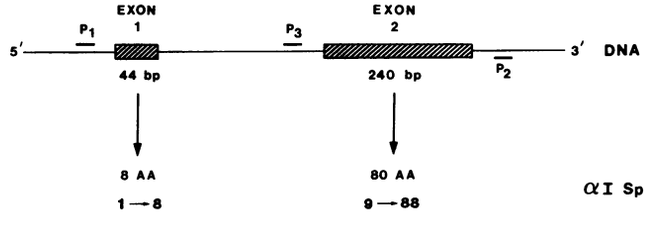

Figure 2. Exon/intron arrangement of the NH_2-terminal portion of αSp. P1–P3 are oligonucleotide primers used for PCR amplification. AA denotes amino acids.

Genomic DNA was amplified as described and 10 μl of the PCR reaction mix were transferred to a nylon membrane (Zetaprobe) using a slot blot apparatus (40). Normal and mutant ASO probes were end labeled with gamma ^{32}P ATP using T4 polynucleotide kinase (33). The slot blots were hybridized with either mutant or normal ASO probes for 2 h at 62°C in 5× SSPE, 5× Denhardts solution, 0.5% SDS. Membranes were washed twice for 15 min at room temperature in 2× SSPE, followed by two to three washes for 5–10 min at 62°C in 2× SSPE, 0.1% SDS and subjected to autoradiography at −70°C using intensifying screens (New England Nuclear).

Restriction endonuclease analysis of amplified DNA. DNA from normal and affected family members was amplified as described using P2 and P3. The amplified product was purified by phenol chloroform extraction and ethanol precipitation; digested with Aha II (New England Biolabs, Beverly, MA) and analyzed on 2% agarose gels.

Results

Coinheritance of mutant Sp $\alpha^{I/74}$ with HE and HPP. In all seven kindred, the clinically affected individuals exhibited a striking increase in the amount of SpD in the red cell membrane extracts (Table I), ranging from 25–76% SpD out of the total SpD and SpT pool as compared with control values of 5.0±3.6% (*n*

= 35). Limited tryptic digestion of Sp revealed the presence of mutant Sp $\alpha^{I/74}$ as evidenced by a marked increase in the amount of the 74-kD α I peptide and a concomitant decrease in the normal 80-kD α I domain of Sp (data not shown). The clinical presentation was variable, including asymptomatic carrier state, mild HE, HE with hemolysis and HPP. The latter was characterized by microspherocytosis and micropoikilocytosis with only occasional elliptocytes, which correlated with a decreased membrane spectrin content as well as a more severe clinical and biochemical presentation (Table I). In hematologically normal family members, SpD self association and limited tryptic digests, as well as Sp content, were all normal.

Assignment of the defect to α Sp. The primary defect in Sp $\alpha^{I/74}$ can reside either on the α Sp or the β Sp chain as indicated by RFLP studies (18) or limited tryptic digestion of α β Sp mixed hybrids (19). In the present study, either RFLP analysis or limited tryptic digestion of isolated α and β chains was employed in some kindred in an attempt to assign the defect to one of the two Sp chains. For RFLP studies, DNA from affected and nonaffected individuals were analyzed with a genomic α I Sp probe which is polymorphic for the restriction enzymes Xba I, Pvu II, and Msp I (41). The Xba I polymorphism, for example, distinguishes two alleles corresponding to restriction fragment lengths of 4.05 and 2.20 + 1.85 kb, respectively. In three pedigrees, one of the 4.05-kb alleles segregated with the inheritance of HE Sp $\alpha^{I/74}$ which would be compatible with linkage to α Sp indicating that the primary defect could be in the α I domain of Sp (data not shown). RFLP studies using a Taq I polymorphism for β Sp were not informative.

Elucidation of the α Sp gene mutation. Amino acid sequencing of the 74-kD αI peptide in four probands revealed that the cleavage site was at Lys 48 (residue 42 in previous nomenclature) compared with the normal cleavage site at Arg 45 (previously residue 39) (42). We therefore analyzed exons 1 and 2 of α Sp, which code for the first 88 amino acids (six NH_2-terminal amino acids as predicted by the DNA sequence [15] plus 82 amino acids according to Speicher's nomenclature [42]). Genomic DNA was amplified by PCR and sequenced either directly or after subcloning. DNA sequencing of subcloned DNA from probands JF (family 1) and BF (family 2) revealed a single base change of C → T in exon 2 of both probands as exemplified in Fig. 3 for JF. To verify the mutation, several subclones from separate PCR reactions were sequenced to ensure that both alleles were analyzed. In subject JF, four out of 10 subclones of PCR 1 contained the mutation, as well as three out of four subclones of PCR 2. In subject BF, the mutation was present in four out of 12 subclones of PCR 1 and, two out of 11 subclones of PCR 2 (Fig. 3). This changed the normal CGT codon to TGT resulting in an Arg → Cys substitution at amino acid 28 (previously residue 22). All remaining codons of exon 2, as well as exon 1, were normal.

In the remaining five kindred, direct sequencing of PCR-amplified DNA was carried out and three different mutations at codon 28 were identified (Fig. 4). In this type of sequencing both normal and mutant alleles are sequenced simultaneously resulting in the presence of the normal and mutant nucleotides at the same position in the sequencing ladder. In two unrelated white families (kindred 3 and 4), the point mutation was CGT → AGT which resulted in an Arg → Ser substitution (Fig. 4, *left*). In the Arab Druze kindred (family 7) (Fig. 4, *center*), the point mutation was CGT → CTT causing substitution of Arg by Leu and in two unrelated black kindred (families 5 and 6), a CGT → CAT mutation changed Arg to His (Fig. 4, *right*).

Allele-specific oligonucleotide hybridization. To verify the various different nucleotide substitutions, PCR-amplified DNA of the probands were hybridized with either a normal or a mutant ASO probe and the results are shown in Figs. 5 and 6. CE (II.2, family 6) and KF (II.1, family 5) are heterozygous for the CAT (His 28) defect because their DNA hybridized to both the normal and the mutant probe (Fig. 5). Likewise, SW (II.2, family 4) and KC (II.3, family 3) are heterozygous for the AGT (Ser 28) mutation and AP (II.1, family 7) is a heterozygote for the CTT (Leu 28) abnormality (Fig. 6).

Linkage of the codon 28 mutations to HE Sp $\alpha^{I/74}$. The normal DNA sequence has an Aha II restriction cleavage site at codon 28 which is eliminated by the base changes and therefore restriction enzyme analysis could be used to test for the presence of the mutations. Exon 2 of α Sp was amplified in affected and normal individuals from all kindred and after digestion with Aha II, the samples were analyzed on 2% agarose gels. An example of one kindred (family 1) is shown in Fig. 7. In the normal individual from this kindred, both normal alleles contain the restriction site and are therefore cleaved into two fragments of 222 and 126 bp, respectively. In contrast, the HE individuals from this family are heterozygous for Sp $\alpha^{I/74}$ and contain a normal allele which is digested by Aha II and a mu-

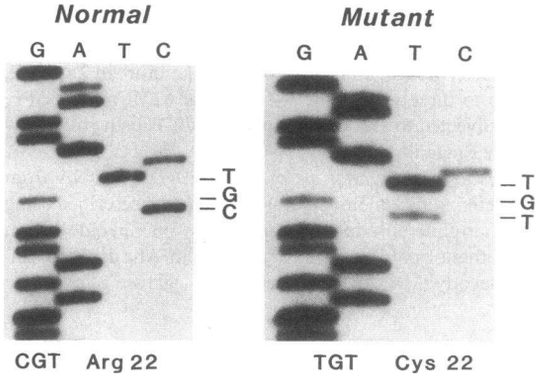

Figure 3. Normal and mutant DNA sequence of part of the α Sp gene in proband II.2 (JF) of family 1. Genomic DNA was amplified by PCR, subcloned, and sequenced. A point mutation in codon 28 that changes the normal subclone sequence of CGT (Arg) to TGT (Cys) in the mutant subclones was detected.

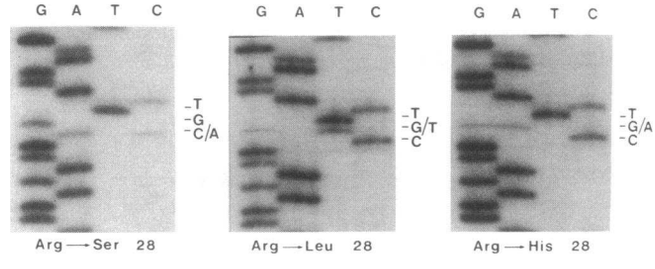

Figure 4. Normal and mutant DNA sequence of part of the α Sp gene in proband II.3 of family 3 (*left*, CGT → AGT); proband II.1 of family 7 (*center*, CGT → CTT) and proband II.1 of family 5 (*right*, CGT → CAT). Genomic DNA was amplified by PCR and sequenced directly resulting in the presence of the normal and mutant nucleotide at the same position in the sequencing ladder.

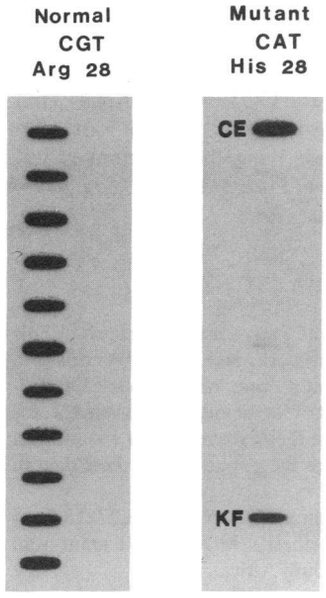

Figure 5. Allele specific oligonucleotide (ASO) hybridization. DNA from various affected HE Sp $\alpha^{I/74}$ and normal family members were amplified by PCR and hybridized to either a normal ASO or a mutant ASO. Note that CE (II.2, family 6) and KF (II.1, family 5) are both heterozygous for the mutation.

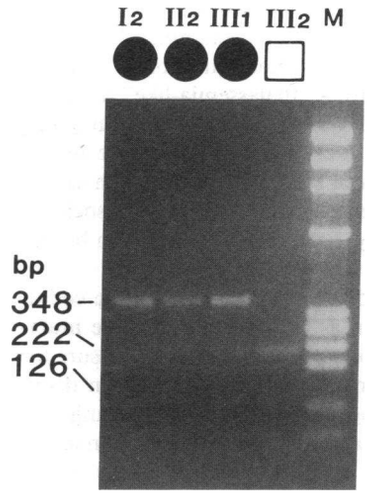

Figure 7. Aha II restriction enzyme analysis. Genomic DNA from family 1 was amplified by PCR producing a 348-bp fragment which was digested with Aha II and analyzed on 2% agarose gels. Affected HE/HPP Sp $\alpha^{I/74}$ individuals are heterozygous for the mutant allele which has lost the Aha II site and hence remains intact (348-bp fragment), and the normal allele which is cleaved by Aha II (222- and 126-bp fragments). The normal family member does not contain the mutant allele. M denotes size markers.

tant allele which lacks the cleavage site and remains intact, resulting in the presence of three fragments on the gel (348 bp and 222 + 126 bp). The codon 28 mutation is therefore linked to the inheritance of HE Sp $\alpha^{I/74}$ in this kindred. Aha II digests of amplified DNA from the remaining six kindred gave identical results (data not shown).

Discussion

In this study, we have described four different mutations in codon 28 (previously referred to as codon 22) of α Sp in seven unrelated HE Sp $\alpha^{I/74}$ kindred from diverse racial backgrounds. The point mutations resulted in the substitution of Arg 28 by either Cys, Ser, Leu, or His. The latter mutation is identical to that previously described in one white French kindred (21). In an independent simultaneous study the Arg → Leu mutation was also found in one black individual and the Arg → Ser substitution in one white subject (23).

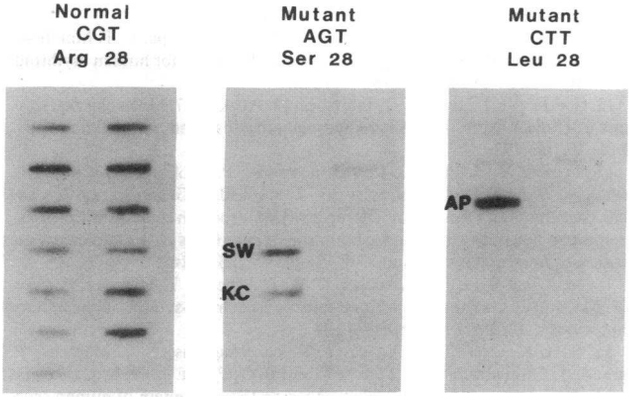

Figure 6. Allele specific oligonucleotide (ASO) hybridization. DNA from various affected HE Sp $\alpha^{I/74}$ and normal family members were amplified by PCR and hybridized to either a normal ASO or a mutant ASO. Note that SW (II.2, family 4), KC (II.3, family 3) and AP (II.1 family 7) are heterozygous for the respective mutations.

The Arg 28 mutations only occurred in individuals whose red cells contained the structurally and functionally defective Sp $\alpha^{I/74}$ and were absent in normal family members and control individuals. Furthermore, because Arg 28 is located near the 74-kD tryptic cleavage site and is part of the self-association site, these data imply that the codon 28 mutations are involved in the observed functional and structural Sp $\alpha^{I/74}$ abnormalities. In the triple-helical model proposed for Sp (9), Arg 28 is located in repeat 1 of α Sp, at the NH_2-terminal end of helix 3, adjacent to the turn region. Helix 3 thus appears to be critical in maintaining the structural and functional integrity of the Sp molecule. The importance of helix 3 is further evidenced by the presence of mutations in this helix in various other repeat units of α Sp. In these cases the mutations occurred at the COOH-terminal end of the helix, adjacent to the connecting region: in Sp $\alpha^{I/65}$ (repeat 2), Sp $\alpha^{I/46}$ (repeat 3), Sp $\alpha^{I/50b}$ (repeat 5), Sp Lyon (repeat 1), and Sp Culoz (repeat 1) (14–16, 22).

Our studies further indicate that Arg 28 is a vital residue for the normal function and conformational stability of α Sp. Substitution of Arg 28 with an amino acid residue of different size and/or charge alters the conformation of the head region of α Sp which in turn influences the function and structural integrity of the molecule. This is manifested by a decreased SpD self-association interaction and an enhanced susceptibility of the $\alpha^{I/74}$ tryptic cleavage site at Arg 45 or Lys 48 (previously residues 39 and 42), resulting in an increased production of the 74-kD peptide after digestion with trypsin. Interestingly, the type of amino acid substitution at position 28 does not appear to affect the stability of the molecule in a differential manner, because all four mutations (Arg 28 → Cys/Ser/His/Leu) produce a similar range of functional (%SpD) and structural (74-kD peptide) abnormalities (Table I).

Within each family investigated, there is a marked variation in the clinical expression ranging from an asymptomatic carrier state to HPP. This heterogeneity has previously been shown to correlate with the amounts of both SpD and the 74-kD peptide, as well as with the absolute Sp content of the membrane (12). Because all affected individuals from one kindred are heterozygotes for the identical mutation, which is present in only one of the two alleles, it is clear that additional factors modulate the expression of the defective gene and the abnormal protein product. These may be *cis*-acting factors such as a

linked polymorphism or mutation occurring elsewhere in the α Sp gene or alternatively, *trans*-acting factors involving linkage with another, as yet unknown, defect. An example of the latter possibility would be the concurrent inheritance of an α Sp codon 28 mutation together with a "thalassemia-like" defect of Sp synthesis (1), which would cause a partial Sp deficiency and result in an altered red cell morphology and a more severe clinical presentation, as seen in some individuals with severe hemolytic HE and HPP. Identical point mutations, associated with variable clinical severity, have also been noted in hemophilia A (43).

The occurrence of four different mutations in one codon allows interesting speculation as to the origin of these mutations. Firstly, the existence of a positive selection pressure for individuals heterozygous for a mutant allele has been documented in the case of hemoglobin S, E, and thalassemic disorders, where resistance to malaria caused by a mutation in one of the globin genes confers a selective survival advantage (44). In our study of α Sp mutations, this would probably be an unlikely event because the families are of different ethnic and geographic origins unrelated to malarial endemic regions. Secondly, Arg 28 may be a crucial amino acid in the Sp molecule so that any change in this residue would alter the function and structural conformation of Sp and hence manifest as a disease state, whereas changes in the surrounding residues would not have a deleterious influence and hence only result in clinically silent polymorphisms which would not be detected. This likewise seems an unlikely explanation because the molecular basis of Sp $\alpha^{I/74}$ is heterogeneous and mutations at codons 46, 48, and 49 (previously referred to as codons 40, 42, 43) have been reported (22, 23). An equally unlikely possibility is that the Arg 28 mutations are tolerated because the resulting structural and functional alterations could be assimilated by the red cell membrane, whereas mutations in other possibly more critical residues maybe potentially lethal and thus selected against. A strong case against this possibility is a recent study of a family with a severely dysfunctional Sp mutation (13). The homozygote proband carrying this mutant Sp had a life threatening hemolysis and her mutant Sp was completely devoid of the propensity to form SpT. In contrast, heterozygote family members had a relatively mild hemolytic HE only, because the adverse consequences of this mutant Sp were diminished by the product of the normal Sp allele.

We believe that the most likely conclusion is that the codon 28 may represent a "hot spot" for mutations because it is encoded by the nucleotides CGT, and CG dinucleotides have been implicated as mutation "hot spots" for several reasons: (*a*) CG dinucleotides are observed at a much lower rate than expected in exon sequences, implying negative selection or repression (45); (*b*) evolutionary studies on vertebrate genes indicated a high rate of CG substitution (46); (*c*) restriction enzymes that contain CG dinucleotides in their recognition sequence (e.g., Taq I, Msp I) detect a high frequency of polymorphisms (47); and (*d*) a large number of recurrent point mutations involving CG → TG and CG → CA transitions have been found in human genetic disease, e.g., hemophilia A (48, 49) and adenosine deaminase deficiency (50).

The mechanism underlying this frequent generation of mutations is presumably due to preferred methylation of C residues in this position in the heavily methylated human genome (51) which can subsequently undergo spontaneous deamination to T (52, 53). This would result in a C → T transition or alternatively in a G → A transition if 5 methyl-C deamination occurred on the antisense strand. Two of the mutations noted in our study, in four unrelated kindred, involve such transitions (CGT → TGT; Arg → Cys and CGT → CAT; Arg → His) and thus codon 28 may be regarded as a "hot spot" for α Sp mutations. The mechanism underlying the recurrent mutations involving C → A and G → T transversions (Arg 28 → Ser/Leu) remains to be resolved.

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