Sp$\alpha^{V/41}$: a Common Spectrin Polymorphism at the αIV–αV Domain Junction

Relevance to the Expression Level of Hereditary Elliptocytosis Due to α-Spectrin Variants Located in *trans*

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Abstract

Spectrin α-chain mutants associated with hereditary elliptocytosis are highly variable in their level of expression. It has been assumed that the degree of elliptocytosis can be increased when the spectrin α chain, encoded by the α gene in *trans* to the variant, is expressed at a low level. We now provide strong evidence for the existence of low-level expression of spectrin α chains. This condition is referred to as the $\alpha^{V/41}$ polymorphism. It has been observed in 15 different families or individuals of French, North African, and African ancestry in which seven distinct elliptocytogenic α-spectrin variants were co-inherited. Whenever the $\alpha^{V/41}$ polymorphism was present, the severity of the biochemical, morphological, and, sometimes, the clinical phenotype of elliptocytosis was increased. The $\alpha^{V/41}$ polymorphism was also frequently encountered among 36 unrelated control subjects in the heterozygous or homozygous states, and was entirely asymptomatic in both cases. The main biochemical feature was an increased susceptibility to proteolysis of the αIV–αV domain junction. Alteration of the facing βIV domain of spectrin was demonstrated by in vitro spectrin dimer reconstitution experiments. It appears that the $\alpha^{V/41}$ polymorphism is often required for α-spectrin elliptocytogenic variants to become manifest in the heterozygous state. Thus, α-spectrin–related elliptocytosis may be viewed as a bifactorial condition. (*J. Clin. Invest.* 1991. 87:2169–2177.) Key words: hemolytic anemia • skeleton • spectrin

Introduction

The red cell membrane skeleton is a protein network beneath the inner surface of the lipid bilayer of the red cell (for reviews, see references 1–3). It determines the unique mechanical properties of the erythrocyte. The major proteins are spectrin, an $\alpha\beta$ dimer, actin, and protein 4.1. Using partial tryptic digestion, the spectrin α chain has been divided into five domains (αI–αV) and the β chain into four domains (βI–βIV) (4). The αI and βI domains play a critical role in spectrin dimer self-association. The αV and βIV domains participate in the interaction with actin and protein 4.1. Elliptocytogenic mutations have been found mainly in the αI and the βI domains (for reviews, see references 2 and 3). More rarely, they affect the αII domain. Under certain circumstances, the most deleterious mutations may result in hereditary pyropoikilocytosis (HPP).

An intriguing feature of elliptocytogenic α-spectrin mutants is their variable expression level with regard to the proportion of variant, the percentage of elliptocytes and/or poikilocytes, and the degree of hemolysis and anemia. This variability has been repeatedly observed with the αI domain mutants designated Sp$\alpha^{I/78}$, Sp$\alpha^{I/74}$, Sp$\alpha^{I/65}$, Sp$\alpha^{I/50}$, and Sp$\alpha^{I/46}$, and also with an αII mutant referred to as Sp$\alpha^{II/21}$ (for reviews, see references 2 and 3). Similarly, two shortened α-chain variants have been observed that show a great variability in their level of expression (5, 6). The variability is so pronounced that, in some kindreds, the same mutation can be associated with either normal red cell shape, elliptocytosis, or pyropoikilocytosis, depending on the family member. For a given mutant, a good correlation usually exists among the biochemical, morphological, and clinical parameters (2, 3). These features may be influenced by a factor that modulates the effect of the elliptocytogenic spectrin α allele (7, 8). Because spectrin α chains are normally synthesized in large excess relative to β chains (9, 10), it has been assumed that if the level of α chains produced by the α allele in *trans* to the elliptocytogenic α allele is decreased, the proportion of the variant α chain incorporated into the membrane skeleton would increase, enhancing the morphological and clinical manifestations of the disorder. The variability of these manifestations is so common in families with α-spectrin–related hereditary elliptocytosis (HE) that the modulating factor must be very common. Using α-spectrin gene haplotype analysis (11) in a large family (family TI) with $\alpha^{I/65}$ HE, we recently obtained evidence that the factor is linked to the spectrin α allele that lies in *trans* to the elliptocytogenic allele (12).

In 15 families or individuals with seven different elliptocytogenic spectrin α-chain mutants, we now show that the increase in the level of expression of the HE phenotype is associated with protein alterations affecting the αIV–αV domain junction of the α chain encoded by the α allele in *trans* to the elliptocytogenic α allele. This protein phenotype has been designated the $\alpha^{V/41}$ polymorphism. It is very common in the normal population. It is clinically and morphologically silent in the heterozygous and the homozygous states.

This article is dedicated by the authors to the memory of Dr. Claude Féo, Institut National de la Santé et de la Recherche Médicale U299, Le Kremlin-Bicêtre, France.

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1. *Abbreviations used in this paper:* HE, hereditary elliptocytosis; HMM, high molecular mass; HPP, hereditary pyropoikilocytosis.

Case reports

The study encompassed 15 different families or individuals with HE. Of these 15 families, 9 have been previously reported (8, 13–17) (Table I). A good correlation exists between the clinical and morphological features and various biochemical alterations: percentage of spectrin dimers, association constant (K_a) of the spectrin tetramers, and percentage(s) of the abnormal spectrin fragment(s) after partial tryptic digestion. The most relevant features of the six other families or individuals are summarized below.

Family NO (Tunisia; $\alpha^{I/74}$HE). A severe $\alpha^{I/74}$ elliptocytosis with a marked poikilocytic component was found in an anemic boy. His father, carrying the $\alpha^{I/74}$ mutation, was clinically normal and morphologically nearly normal. His mother and his sister were normal. We found that the $\alpha^{I/74}$ elliptocytogenic mutation was the αI 22 Arg→His (CGT→CAT) substitution (Baklouti et al., manuscript submitted for publication), first reported by Garbarz et al. (18).

Family HD (Tunisia; $\alpha^{I/65}$HE). HE was discovered by chance in a healthy man (80–90% elliptocytes) and in his mother (50–60% elliptocytes). The father had normal erythrocytes. The Spα$^{I/65}$ abnormality was found. The relative amount of the $\alpha^{I/65}$ variant (65/αI, see Methods) was (×100) 64% in the propositus ($K_a = 0.8\pm0.1 \times 10^5\ M^{-1}$, two determinations) and 48% in his mother ($K_a = 2.5\pm0.3 \times 10^5\ M^{-1}$, two determinations), K_a in controls was $4.7\pm0.6 \times 10^5\ M^{-1}$ ($n = 33$). The mutation, determined by the polymerase chain reaction and dot blot hybridization (17), was the duplication of TTG codon 148 (TTG: Leu), as in all cases of Spα$^{I/65}$ HE previously studied among North Africans and African and American blacks (17, 19, 20).

Individual SE (Tunisia; $\alpha^{I/65}$HE). This woman had 85% bulky elliptocytes. The Spα$^{I/65}$ variant was found (65/αI, 38%; K_a, not done). In one brother of the proposita, elliptocytosis was also present, confirming the hereditary nature of the condition. No study at the gene level was performed.

Individual TA (Tunisia; $\alpha^{I/65}$HE). This woman had 100% elliptocytes, including 30% rod-shaped erythrocytes. The Spα$^{I/65}$ abnormality was found (65/αI, 58%; K_a, not done). Family studies and studies at the gene level were not performed.

Family BA (Guinea, Atlantic West Africa; $\alpha^{I/65}$HE). Elliptocytosis (30%) was discovered in a girl with normal hemoglobin level. The mother was normal. The father was not studied. The Spα$^{I/65}$ abnormality was found in the proposita (65/αI, 46%; $K_a = 1.8\pm0.3 \times 10^5\ M^{-1}$, two determinations). Using PCR and dot blot hybridization (17), we found the duplication of codon 148 (TTG: Leu).

Family FE (Tunisia; $\alpha^{II/31}$). Elliptocytosis (100%) was found in an asymptomatic boy. His father was clinically and morphologically normal but his mother displayed mild elliptocytosis (20%). Three other children of the sibship displayed asymptomatic elliptocytosis (100%). The twin sister of the propositus had normal red cells. Partial tryptic digestion disclosed an apparently new alteration of the spectrin αII domain, the causal mutation of which is under study.

Methods

General procedures. Most of the methods used in the present study have been described or referred to elsewhere (8, 13). Digestion of crude spectrin 37°C extracts was performed at 4°C for 20 h in a buffer containing 10 mM phosphate, 40 mM NaCl, pH 8.0. The trypsin/spectrin

Table I. Families Investigated

Families	Country of origin	Number of individuals examined	Primary mutation	References
Spα$^{I/78}$ abnormality				
TR	Tunisia	3 (2)	αI 35 Arg → Trp	14, 15
Spα$^{I/74}$ abnormalities				
CH	France	3* (1)	αI 40 Gly → Val	16
AU	France	2* (1)	αI 43 Leu → Phe	16
NO	Tunisia	4* (1)	αI 22 Arg → His	See *Case reports*
Spα$^{I/65}$ abnormality				
AK	Algeria	3 (2)	αI 148 + Leu	8, 12 (family TI only), 17
AZ	Morocco	3 (3)		
BE	Algeria	4* (1)		
TI	Algeria	10 (8)		
RA	Algeria	4 (3)	αI 148 + Leu	17
HD	Tunisia	4 (2)	αI 148 + Leu	See *Case reports*
SE	Tunisia	1 (1)	ND	
TA	Tunisia	1 (1)	ND	
BA	Guinea	2 (1)	αI 148 + Leu	
Spα$^{II/31}$ abnormality				
FE	Tunisia	7 (5)	Work in progress	See *Case reports*
Spα$^{II/21}$ abnormality				
HA	Algeria	4* (2)‡	Work in progress	13

The number of elliptocytic subjects is shown in parentheses. * One of these persons carries the elliptocytogenic mutation but has red cells with normal shape. ‡ The elliptocytic child is an $\alpha^{II/21}/\alpha^{II/21}$ homozygote. ND, not determined.

ratio was 1:100 (wt/wt). Polyclonal anti-αIII, anti-αIV, and anti-αV domain antibodies were prepared as previously described for raising antibodies against the αII domain (13). Anti-βIV domain antibodies (nonreactive with the βIV 30- and 28-kD fragments) were obtained as reported elsewhere (21). In vitro spectrin dimer reconstitution experiments were performed as described by Pothier et al. (22).

Quantitation of αI domain abnormalities. After Coomassie Blue staining, one-dimensional gels were scanned (570 nm). The $\alpha^{I/78}$ abnormality was expressed as the 78/(80 + 78) percentage (14). The $\alpha^{I/74}$ abnormality was expressed as the 74/(80 + 78 + 74) percentage, or the 74/αI percentage (16). The $\alpha^{I/65}$ abnormality was expressed as the 65/(80 + 78 + 74 + 65) percentage, or the 65/αI percentage (8). The quantitation of the above abnormalities was based on the relative amount of a given peptide. Since the latter (except for the αI 65-kD fragment) is also generated by normal spectrin and since its relative amount after a 20-h digestion is an arbitrary value, the calculated percentages represent an estimate of the actual proportion of the mutant spectrin.

Quantitation of αII domain variants. Quantitation of the $\alpha^{II/21}$-spectrin variant (family HA in Table I) was obtained after two-dimensional electrophoresis and Coomassie Blue staining. The αII type 1 spots (46, 35, 30, and 25 kD), or the αII type 2 spots (52, 39, 34, and 29 kD) (23) were cut out, pooled and eluted. The optical density (590 nm) of the eluate was determined (13). The amount of the spots was expressed relative to that of the rather constant βIII 33-kD spot. The amount of the $\alpha^{II/21}$-spectrin was determined by subtracting the value obtained as described above from that found in the normal control subjects (13). The $\alpha^{II/31}$-spectrin was quantitated in a similar fashion, starting from the unique abnormal αII spots (manuscript in preparation). The quantitation of αII spots, corresponding to the normal haploid complement of spectrin, allowed us to accurately determine (by subtraction) the actual proportion of both $\alpha^{II/21}$ and $\alpha^{II/31}$ variants.

Quantitation of the αV 41-kD fragment and of other fragments. The $\alpha^{V/41}$ polymorphism was defined by the percentage of the αV 41-kD fragment (αV 41,%), e.g., the ratio (×100) of the optical density of the αV 41-kD peptide to the overall optical density of α and β chain-derived peptides after one-dimensional polyacrylamide gel electrophoresis (7–15% linear gradient of acrylamide). Replicate determinations of some samples demonstrated the high reproducibility of the $\alpha^{V/41}$ polymorphism assay (not shown). The amounts of the αIV 52-kD and the αV 25-kD bands, respectively, and that of high molecular mass (HMM) peptides (> 80 kD) were expressed in a similar fashion. Two-dimensional electrophoresis was also used to determine the amounts of the following spots: αIV 52 kD; αV 41 and 25 kD; βIV 74, 70, 52, 46, 41, 30, and 28 kD. The amounts of the spots derived from two-dimensional gels were expressed relative to the rather constant amount of the βIII 33-kD spot.

Statistical analysis. Values are expressed as mean ± SD. The significance of the difference between two means was determined with the *t* test (using mean ± 2 SD). In figures, the mean ± 2 SD is also shown.

Results

Genetic analysis. We first present the genetic analysis of the $\alpha^{V/41}$ polymorphism interacting with a variety of elliptocytogenic α alleles. For this analysis, the $\alpha^{V/41}$ polymorphism is defined solely on the basis of an increase in the αV 41-kD fragment.

Families HD and BE, with $\alpha^{I/65}$ HE, illustrate the phenomenon (Fig. 1). In each family, the 65/αI percentage is either baseline (48% and 37%), correlating with few or no elliptocytes, or high (64% and 63%), correlating with a 100% elliptocytosis. High values of the 65/αI percentage are associated with intermediate levels of the αV 41-kD peptide (~ 1.5%). Baseline values of the 65/αI percentage are associated with low levels of the αV 41-kD peptide (~ 0.9%). Family members without elliptocytosis had increased amounts of the αV 41-kD fragment, but to varying degrees. In the father of family HD, the amount was intermediate (1.5%), e.g., comparable to that seen in his elliptocytic child. In the mother of family BE, the amount was high (3.2%). Further analysis indicates that intermediate and high levels of the αV 41-kD fragment correspond to heterozygosity (genotype $\alpha/\alpha^{V/41}$ or $\alpha^{HE}/\alpha^{V/41}$) and homozygosity (genotype $\alpha^{V/41}/\alpha^{V/41}$), respectively, for the $\alpha^{V/41}$ polymorphism.

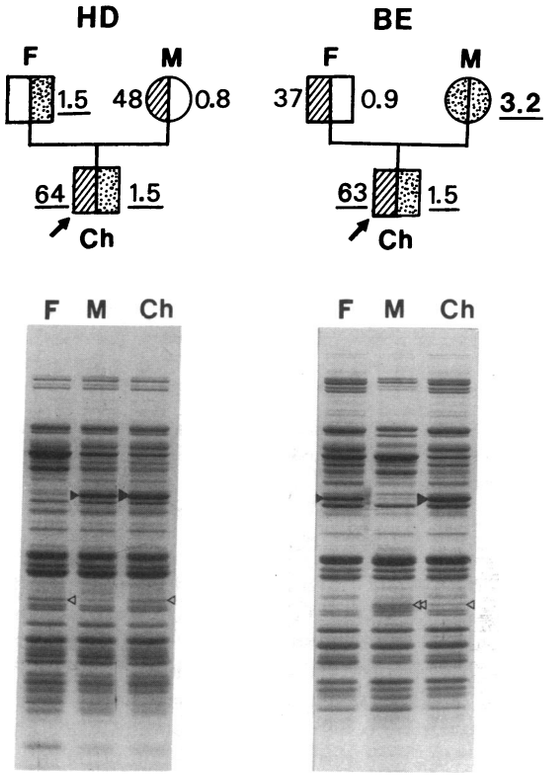

Figure 1. Pedigrees of families HD and BE and their respective protein phenotypes. (*Top*) Genetic trees. (▨) $\alpha^{I/65}$ allele; (▩) $\alpha^{V/41}$ polymorphism. Abbreviations: F, father; M, mother; Ch, child. (↗) propositus. The 65/αI percentage and the percentage of the αV 41-kD fragment are indicated on the left and right of each family member, respectively. (*Underlined values*) High expression level of the $\alpha^{I/65}$ abnormality or intermediate level of the $\alpha^{V/41}$ polymorphism. (*Underlined boldface value*) High level of the $\alpha^{V/41}$ polymorphism. (*Bottom*) Peptide maps. (*Small solid arrowheads*) Baseline value of the αI 65 kD fragment; (*large solid arrowheads*) high value of this fragment; (◁) intermediate amount of the αV 41-kD fragment; (◁◁) high amount of this fragment.

Similar results were obtained in all cases of $\alpha^{I/65}$ HE (seven families and two individuals) (Fig. 2). The 65/αI percentage displayed a bimodal distribution, including baseline values of 44.3±3.5 (n = 14) and high values of 62.3±2.2 (n = 9). The difference between the means (t test) was highly significant ($P < 0.001$). The levels of the αV 41-kD fragment also displayed a statistically significant bimodal distribution ($P < 0.001$), including mode A (low levels, assumed to reflect the absence of the $\alpha^{V/41}$ allele) of 0.79±0.14% (n = 14) and mode B (intermediate levels, assumed to reflect heterozygozity for the $\alpha^{V/41}$ allele) of 1.44±0.19% (n = 9). Whenever the 65/αI percentage was baseline values, the level of the αV 41-kD fragment was low. On the contrary, whenever the 65/αI percentage was high,

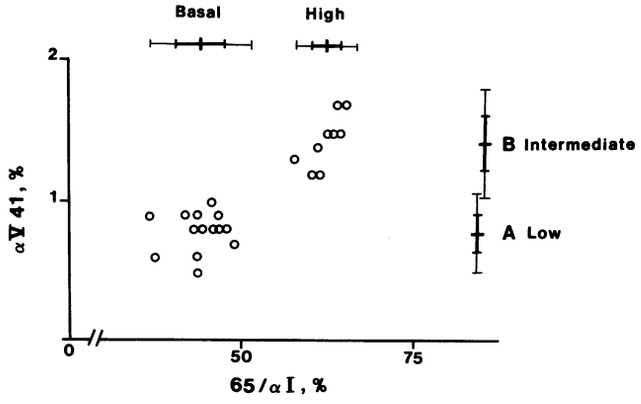

Figure 2. Expression of the $\alpha^{I/65}$ and the $\alpha^{V/41}$ alleles. Mode A, low level of the αV 41-kD fragment; mode B, intermediate level of the αV 41-kD fragment.

the level of the αV 41-kD fragment was intermediate. In the previously reported family TI (12), the fact that all combinations were achieved in the sibship (α/α, $\alpha^{I/65}/\alpha$, $\alpha/\alpha^{V/41}$, and $\alpha^{I/65}/\alpha^{V/41}$; not shown) provided further evidence that both the $\alpha^{I/65}$ and the $\alpha^{V/41}$ alleles were in *trans* to each other.

Detailed studies were not feasible in families with other types of HE, because each kindred had a different variant interacting with the $\alpha^{V/41}$ polymorphism. Nevertheless, in every family, it was possible to distinguish between a baseline and a high level of expression of the elliptocytogenic variant (Table II). Whenever the elliptocytogenic variant displayed a baseline level of expression, the amount of the αV 41-kD fragment was low ($\alpha^{V/41}$ polymorphism was absent). On the contrary, whenever the elliptocytogenic variant displayed a high level of expression, the amount of the αV 41-kD fragment was intermediate ($\alpha^{V/41}$ polymorphism was present in the heterozygous state).

When all of the quantitative results of the αV 41-kD fragment were combined (Fig. 2 and Table II), a statistically significant bimodal distribution was obtained ($P < 0.001$): mode A was 0.75±0.16% ($n = 20$) and mode B was 1.52±0.28% ($n = 18$). Finally, the amount of the αV 41-kD fragment had a trimodal distribution in 36 normal control individuals (Fig. 3): mode A was 0.78±0.15% ($n = 17$), mode B was 1.65±0.17% ($n = 17$), and mode C (high levels; homozygosity for the $\alpha^{V/41}$ allele) was 3.45±0.21% ($n = 2$). The differences between the means of modes A and B, and the means of modes B and C were highly significant ($P < 0.001$), (even though mode C was limited to two values). In the normal (non-HE) parents of $\alpha^{HE}/\alpha^{V/41}$ compound heterozygous children, the distribution of the expression level of the $\alpha^{V/41}$ allele was significantly bimodal ($P < 0.001$): mode B was 1.52±0.08% ($n = 5$), and mode C was 3.23±0.25% ($n = 3$) (Fig. 3). Because all of the children of these parents were compound heterozygotes for an elliptocytogenic spectrin α allele and the $\alpha^{V/41}$ allele, the parent not carrying HE carried the $\alpha^{V/41}$ allele, as expected. It is noteworthy that all four elliptocytic children of the homozygous ($\alpha^{V/41}/\alpha^{V/41}$) father (I.1) in family FE were compound heterozygotes ($\alpha^{II/31}/\alpha^{V/41}$) (Table II).

Protein phenotype. The complete protein phenotype defining the $\alpha^{V/41}$ polymorphism is illustrated in Figs. 4 and 5. It was established by testing all of the carriers in the present study at least once, and sometimes on multiple occasions. The main feature was enhanced tryptic cleavage at the αIV–αV domain junction, resulting in increased amounts of the αV 41-kD and of the αIV 52-kD fragments. The facing βIV domain displayed an enhanced susceptibility to proteolysis. No detectable modifications were noted in the αI, αII, βI, βII, and βIII domains.

The increase in production of the αV 41-kD fragment was confirmed by varying the time of tryptic digestion (data not shown) and was accompanied by an increase in the αV 25-kD subfragment. The total increase in both fragments (αV [41 + 25]/βIII 33, ×100) was assessed in a homozygote after two-dimensional electrophoresis: 138 compared with 17 in normals. An intermediate value of 35 was obtained in a heterozy-

Table II. Interaction between Elliptocygenic α Alleles (α^{HE}) Other Than the $\alpha^{I/65}$ Allele and the $\alpha^{V/41}$ Allele

Family	α^{HE}	$\alpha^{V/41}$
TR		
I.1	—	1.6
I.2	31.3	1.0
↗ II.1	39.3	1.9
CH		
I.1	51.0	0.7
I.2	19.6 (normal)	1.6
↗ II.1	65.7	1.5
AU		
↗ I.2	56.4	1.6
II.1	45.3	0.5
NO		
I.1	30.0	0.6
I.2	24.0 (normal)	**3.0**
II.1	(normal)	1.7
↗ II.2	44.8	1.8
FE		
I.1	—	**3.5**
I.2	27	0.5
II.1	60	1.4
II.2	59	1.3
II.3	73	1.3
↗ II.4	68	1.3
II.5	—	1.1
HA		
I.1	10	0.6
I.2*	39	2.3§
II.1*	—	1.7
↗ II.2	100‡	0.8

Family members are designated as previously published (see references in Table I) or, in the case of families NO and FE, ↗ is the propositus, I.1 is the father, I.2 is the mother, II.1, etc. are the children. The amount of the α^{HE} variant is that listed in the references cited in Table I. (*Underlined values*) High expression level of the α^{HE} alleles and intermediate level of the αV 41-kD fragment. (*Underlined boldface values*) High level of the αV 41-kD fragment. Member NO II.1 was not studied in the present series; however, she was found to be normal in another series (unpublished data).

* αII type 2 polymorphism in *cis* to the $\alpha^{V/41}$ polymorphism.

‡ $\alpha^{II/21}/\alpha^{II/21}$ homozygote.

§ This unusually elevated value for an $\alpha^{V/41}$ heterozygote is addressed in the Discussion.

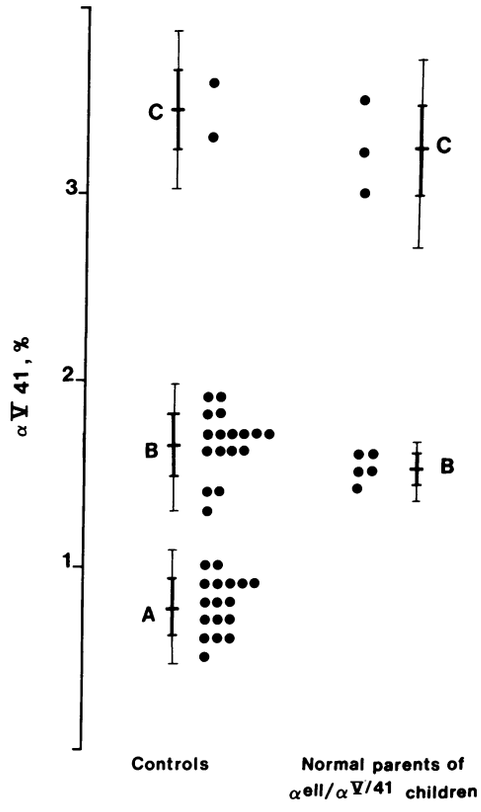

Figure 3. Expression of the $\alpha^{V/41}$ polymorphism in persons without an elliptocytogenic variant (in *trans*). Modes A and B are as defined in Fig. 2. Mode C, high level of the αV 41-kD fragment. (*Left*) Trimodal distribution in 36 unrelated control subjects. (*Right*) Bimodal distribution in normal parents of subjects who are compound heterozygotes for one elliptocytogenic α allele and the $\alpha^{V/41}$ allele.

gote. The αV 41-kD fragment appeared as a doublet in the five homozygotes examined, with a less intense lower band. This lower band was not always visible in heterozygotes. The $\alpha^{V/41}$ abnormality may provide two alternative sites for proteolytic cleavage, leading to two αV 41-kD fragments instead of one.

The αIV 52-kD fragment (αIV52/βIII33, ×100) was also increased: 70 in an $\alpha^{V/41}/\alpha^{V/41}$ individual and 47 in an $\alpha/\alpha^{V/41}$ individual compared with 19 in a normal α/α individual. The redistribution of the αIV 52-kD spots at the benefit of the most cathodic spots seems to represent an independent polymorphism, also encountered in the absence of the $\alpha^{V/41}$ polymorphism (unpublished data). We also noted an increase in the αIV 17-kD spot (Fig. 4) and, to a lesser degree, an increase in the αIV 46-, 41-, and 25-kD spots (not indicated in Fig. 4).

A dramatic difference in the pattern of the HMM peptides (> 80 kD) was observed (Fig. 4). These peptides were termed a, b, c, d, and e. Bands a and b were themselves composed of doublets. Band c included bands c_1 and c_2, as shown in the two-dimensional separations and immunoblots (Fig. 5). Immunoblots allowed the determination of the domain composition of those peptides and to propose a model (Fig. 5). In normal α chains, the αIV–αV domain junction was highly resistant to hydrolysis as shown by the predominance of HMM peptides containing this junction: peptides a and b, representing the αIII–αIV–αV block; and peptides c_2 and e, representing the αIV–αV block. In $\alpha^{V/41}$ chains, the αIV–αV junction became highly susceptible to hydrolysis: peptides a, b, c_2, and e decreased or disappeared, giving rise to peptides c_1 and d (αIII–αIV block). Quantitation of HMM peptides and of the αIV 52-kD and αV 41- and 25-kD fragments (after one-dimensional separation) showed that the increase in the latter accounted for the decrease in the former: in an $\alpha^{V/41}/\alpha^{V/41}$ homozygote, the amount of the αIV 52-kD + αV (41- + 25-kD) fragments was 18.3% and that of the HMM peptides 8.9% compared with levels of 14.3% and 13.5%, respectively, in a normal (α/α) individual. Thus, it appears that the responsible mutation would accelerate proteolysis of the αV domain. The increase in the αIV 52-kD fragment was not as pronounced as the αV (41- + 25-kD) fragments because some αIII–αIV fragment remained as bands c_1 and d among HMM peptides (Figs. 4 and 5). The αIII 52-kD fragment was not noticeably modified.

Changes were also noted in the βIV domain (Figs. 4 and 5). In $\alpha^{V/41}/\alpha^{V/41}$ homozygotes, the βIV 74- and 70-kD fragments were decreased. In the amino-terminal portion of the βIV domain, there was a decrease in the βIV 52-kD fragment and in the βIV 41-kD fragment (upper fraction U) and an increase in the βIV 46-kD fragment and in the βIV 41-kD fragment (lower fraction L). In the carboxy-terminal portion, there was a decrease in the βIV 30-kD fragment and an increase in the βIV 28-kD fragment. In $\alpha/\alpha^{V/41}$ heterozygotes, changes involving the βIV domain were intermediate and more difficult to interpret. In all cases, the amounts of the involved fragments ([total βIV]/βIII33, ×100) remained nearly unchanged: 208 ($\alpha^{V/41}/\alpha^{V/41}$ individual), 188 ($\alpha/\alpha^{V/41}$), and 183 (α/α).

There was a parallel between the changes observed in the αIV and αV domains, and those seen in the βIV domain. The genetic analysis supports the presence of a mutation altering primarily the spectrin α chain and inducing secondary changes in the partner β chain. The opposite situation was formally ruled out by in vitro spectrin dimer reconstitution experiments. The αV 41-kD peptide was increased after digestion of reconstituted dimers containing the α chain but not the β chain of an $\alpha^{V/41}/\alpha^{V/41}$ homozygote. The amount of the $\alpha^{V/41}$ fragment obtained after digestion of different reconstituted dimers for 20 h and electrophoresis in one dimension was as follows: 2.2% (αβ), 2.6% (αβ), 2.1% (αβ), and 2.7% (αβ) (underlined chains were isolated from an $\alpha^{V/41}/\alpha^{V/41}$ homozygote and non-underlined chains from a normal (α/α) individual). These results were confirmed using two other digestion times: 7 and 16 h (data not shown). The βIV domain conformational change of the βIV domain may occur in native $\alpha^{V/41}$-β dimer; however, the possibility also exists that the change develops during the digestion process, once the αV 41-kD domain has been prematurely removed.

It has not yet been possible to measure directly the absolute amount of the $\alpha^{V/41}$-β dimers. The total amount of spectrin being constant, it seems reasonable to assume that the increased amount of any elliptocytogenic α chain reflects a reduced assembly of the $\alpha^{V/41}$ chain, encoded by the α allele in *trans* to the elliptocytogenic α-allele. In one case however (HA, II.1 [13]), the actual amount of $\alpha^{V/41}$-β dimers could be tentatively measured. This person is a compound heterozygote for normal α chains and α chains with both the $\alpha^{V/41}$ polymorphism and the αII type 2 polymorphism (23) ($\alpha/\alpha^{II\ type\ 2\text{-}V/41}$). The spots representing the αII type 2 domain accounted for ~ 25% of all αII domain spots. In parallel quantitative studies, we observed that the αII type 2 peptides (from member II.1 of

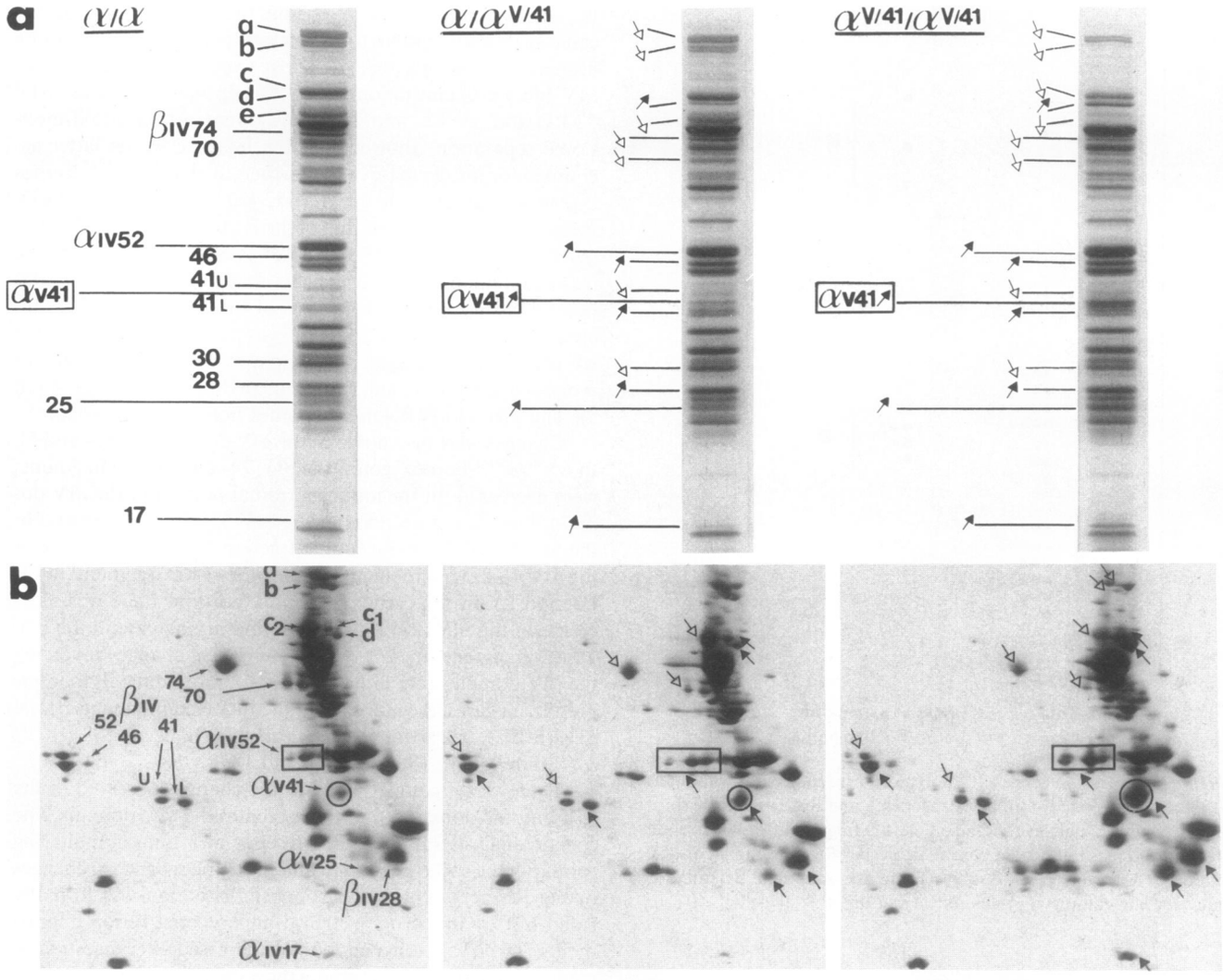

Figure 4. The protein phenotype defining the $\alpha^{V/41}$ polymorphism, using (*a*) one-dimensional and (*b*) two-dimensional electrophoretic separation. Bands a, b, c (c_1 and c_2), d, and e: HMM peptides (> 80 kD). βIV 41 U and L bands: upper and lower components of the β IV 41-kD fragment, respectively. In an $\alpha/\alpha^{V/41}$ heterozygote and an $\alpha^{V/41}/\alpha^{V/41}$ homozygote, the variations of the fragment intensities are indicated by upward (↗) or downward (↘) arrows. (*Vertical arrow* [↓]) Disappearance of band e in the $\alpha^{V/41}/\alpha^{V/41}$ homozygote; the remaining material corresponds to a faint, undesignated band lying immediately above band e in α/α and $\alpha/\alpha^{V/41}$ individuals (see Fig. 5).

family HA) were produced at the same rate and with the same yield as αII (type 1) peptides from normal individuals (not shown). Assuming (*a*) that the αII type 2 mutation did not influence by itself the expression level of its α chain and (*b*) that the $\alpha^{V/41}$ mutation, if distinct, behaved nevertheless in a similar fashion as encountered in other families, it is tentatively suggested that the $\alpha^{V/41}$ polymorphism decreases by ~ 50% the amount of $\alpha^{V/41}$-β dimers incorporated into the membrane.

Frequency of the $\alpha^{V/41}$ polymorphism. One striking finding of our study was the frequency of the $\alpha^{V/41}$ alteration which we therefore refer to as a polymorphism. In 36 normal, unrelated controls (28 of French origin, 5 of North African origin, and 3 of unknown origin), there were 17 $\alpha/\alpha^{V/41}$ heterozygotes and 2 $\alpha^{V/41}/\alpha^{V/41}$ homozygotes. Thus, we estimate that 29% of α-spectrin genes on chromosome 1 (24) carry the $\alpha^{V/41}$ allele. These results pertain essentially to people of French origin. The small number of control subjects of North African ancestry does not allow us to draw conclusions on the frequency of the $\alpha^{V/41}$ polymorphism in the North African population, although three out of five control subjects displayed a $\alpha^{V/41}$ chromosome. 12 of the HE families (from the total of 15) were of North African ancestry. The fact that 9 of the 12 propositi in these North African families were compound heterozygotes, carrying the $\alpha^{V/41}$ allele in addition to an elliptocytogenic allele, suggests that the $\alpha^{V/41}$ allele must also be common in North Africa.

Discussion

The present study describes the characterization at the protein level of a common α-spectrin chain polymorphism. This polymorphism, designated $\alpha^{V/41}$, may explain why elliptocytosis due to α-spectrin chain variants manifest such a variable degree of severity even in a given family. One long-standing hypothesis (7, 8) proposes that the nonelliptocytogenic α chain encoded by the α gene in *trans* to the mutant α gene might be incorporated into the membrane in varying amounts. As a result, more

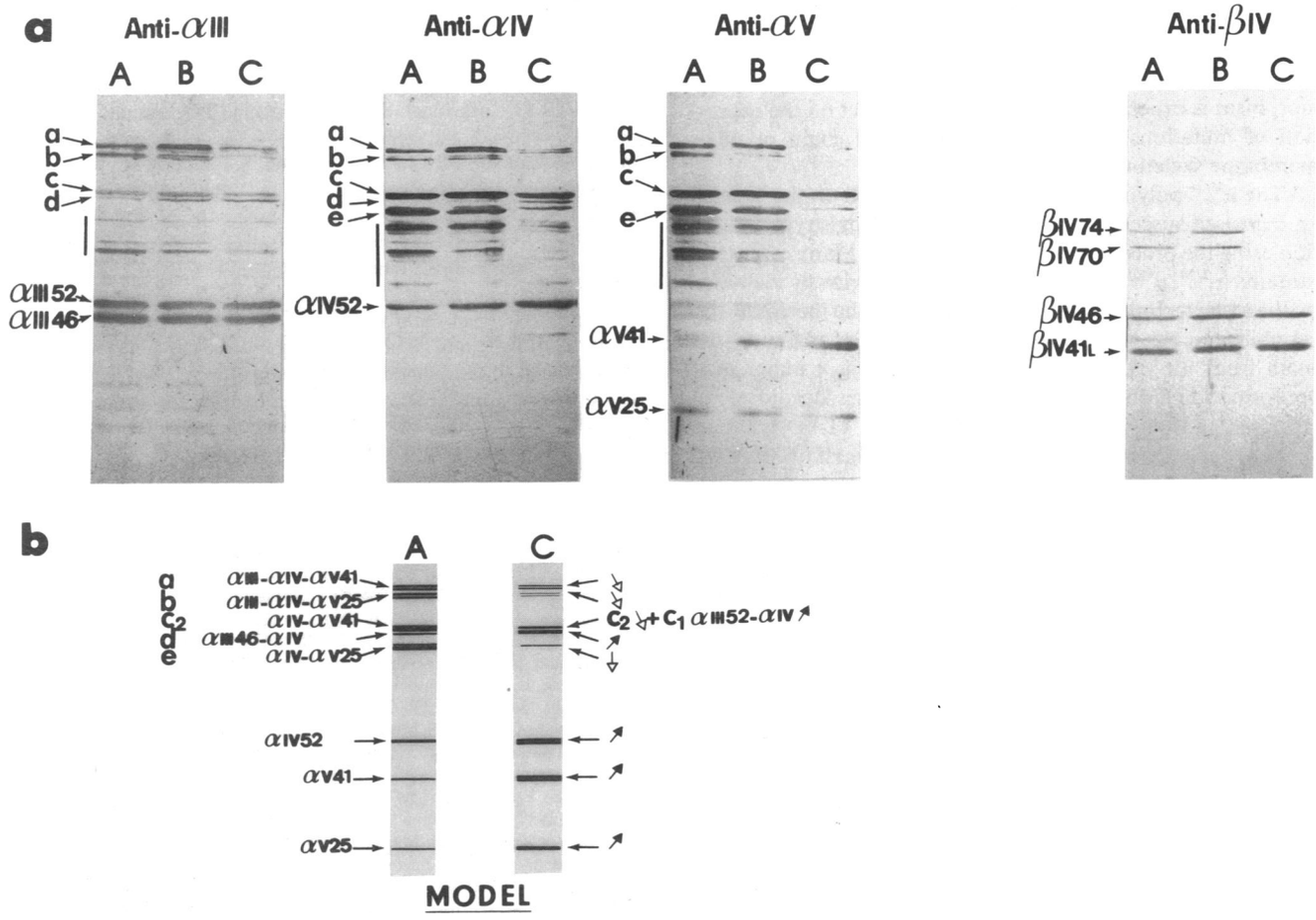

Figure 5. Immunoblots of one-dimensional electrophoretic separations of spectrin digests (*a*) Anti–αIII, anti–αIV, anti–αV, and anti–βIV domain polyclonal antibodies were used. (*A*) α/α; (*B*) $\alpha/\alpha^{V/41}$; (*C*) $\alpha^{V/41}/\alpha^{V/41}$. (*Vertical lines*) Peptides that are poorly visualized using Coomassie Blue staining because they are hidden by other peptides: they correspond to partially digested fragments of the αIII–αIV–αV block. (*b*) Model of peptide pattern in (*A*) normal and (*C*) homozygous $\alpha^{V/41}$ individuals. (*Short downward arrows in C* [↘]) Decrease of all HMM peptides (a, b, and c_2) containing the αIV–αV domain junction. (*Short vertical arrow in C* [↓]) Disappearance of band e (the remaining material corresponds to a faint band (presumably αIV41–αV41) above band e in *A;* see also Fig. 4). (*Short upward arrows in C* [↗]) Reciprocal increase of peptides of the αIII–αIV block (c_1 and d) and of the αIV and αV domains. The duplication of bands a and b must be due to the same difference as that appearing between the αIII 52-kD and the αIII 46-kD fragments during proteolysis.

or less of the variant elliptocytogenic α chains would be incorporated into the membrane. Haplotype analysis in a large, informative Algerian family (family TI) with $\alpha^{I/65}$ HE supports the view that the compounding factor responsible for this variable phenotype is associated with the spectrin α gene located in *trans* to the mutant α gene (12). In the current study, careful measurements in a number of families clearly indicated that, whenever the expression level of an elliptocytogenic spectrin α chain mutant was increased in terms of biochemical amounts, morphology, and, sometimes, clinical severity, an increase in the αV 41-kD fragment was present. When the parents of individuals with such a phenotype were available for study, it was invariably demonstrated that one had transmitted the elliptocytogenic variant whereas the other was either heterozygous or homozygous for the $\alpha^{V/41}$ polymorphism and had transmitted it to the affected child.

The unusually high level of the αV 41-kD fragment in member I.2 ($\alpha^{II/21}/\alpha^{II\ type\ 2\text{-}V/41}$) of family HA suggested that the $\alpha^{II/21}$ HE allele might itself be expressed at a low level (it is nearly undetectable in the heterozygous state [$\alpha^{II/21}/\alpha$], as in family member HA I.1 of Table II). The low level of expression of the $\alpha^{II/21}$ chain was not related to the presence of the $\alpha^{V/41}$ polymorphism in *cis,* as indicated by the finding of $\alpha^{II/21}/\alpha^{II/21}$ family member II.2. If an $\alpha^{V/41}$ mutation were associated in *cis* with an elliptocytogenic mutation, the latter would probably be expressed at an extremely low level. It is not known whether the $\alpha^{V/41}$ polymorphism results from a unique mutation or from an array of mutations.

In family NO, member II.2 displayed a clinically severe elliptopoikilocytosis and was a $\alpha^{I/74}/\alpha^{V/41}$ compound heterozygote. In contrast, his father, who was free of clinical and nearly free of morphological abnormalities, was an $\alpha^{I/74}/\alpha$ simple heterozygote. This example illustrates how dramatic the sensitizing effect of the $\alpha^{V/41}$ allele may be when the latter is placed in *trans* to the elliptocytogenic α alleles that are very deleterious. Therefore, we suggest that in cases other than homozygosity and compound heterozygosity for variant α-spectrin alleles (25–27), HPP may involve the co-inheritance of the $\alpha^{V/41}$ allele or another low output α-spectrin allele. The possibility exists that the αII domain mutation or polymorphism that is asso-

ciated with some cases of recessively inherited hereditary spherocytosis in Caucasians (28) may be enhanced by the $\alpha^{V/41}$ polymorphism or a related disorder. In contrast, the $\alpha^{V/41}$ polymorphism is expected to have little or no effect on the expression of mutations that affect the β-spectrin chain or other membrane skeleton proteins.

The $\alpha^{V/41}$ polymorphism was characterized on the basis of an increased susceptibility of spectrin to partial tryptic digestion using the procedure of Speicher et al. (4). Many α-spectrin mutants ($\alpha^{I/74}$, $\alpha^{I/65}$, $\alpha^{I/46}$, . . .) have been previously identified by the same technique. It is noteworthy that the increased susceptibility to cleavage leads to increase release of the αV domain from the αIII–αIV–αV block, implying a major local conformational change. In spectrin molecules devoid of the $\alpha^{V/41}$ polymorphism, the amount of the αV 41-kD fragment was only slightly increased even when the digestion time was increased to 68 h (not shown). Therefore, it may be anticipated that the causal mutation lies in the vicinity of the αIV–αV domain junction.

The basis for the association between the structural change defining the $\alpha^{V/41}$ polymorphism and the low amount of α-spectrin remains to be determined. A reduction of the $\alpha^{V/41}$ spectrin mRNA would likely indicate a pretranslational process: reduced transcription or abnormal splicing. The latter could result from the nucleotide change that causes the protein alteration. Alternatively, a normal amount of the $\alpha^{V/41}$ spectrin mRNA would indicate a posttranslational phenomenon. For example, the spectrin $\alpha^{V/41}$ chain may be less efficiently recruited by the β chain. Incidently, it seemed present in a patient described by Becker et al. (29).

The high frequency of the $\alpha^{V/41}$ polymorphism was unexpected. This polymorphism was present in 29% of the chromosomes tested, resulting in 47% heterozygotes and 6% homozygotes in a control group composed mainly of persons of French ancestry. Taking 0.29 as the theoretical frequency of $\alpha^{V/41}$ chromosome, one would expect 8% homozygotes: the actual value of 6% fits with this value. The $\alpha^{V/41}$ polymorphism also appears to be frequent in persons of North African ancestry who constituted most of our cases of HE. The actual frequency of the $\alpha^{V/41}$ polymorphism in the black population remains to be determined.

The homozygous state for the $\alpha^{V/41}$ polymorphism is asymptomatic presumably because of the normally large excess of α chains synthesized (9, 10). This fact may have allowed the high frequency of the $\alpha^{V/41}$ allele. However, some deleterious effects are observed in the case of compound heterozygosity for the $\alpha^{V/41}$ polymorphism and various elliptocytogenic spectrin α-chain mutations. It is noteworthy that many of our HE propositi were compound heterozygotes. A clinically severe HE/HPP phenotype can result from homozygosity or compound heterozygosity for elliptocytogenic α-chain variants. Alternatively, many of these variants are likely to be detected clinically (or morphologically) when they are associated with the $\alpha^{V/41}$ polymorphism. Therefore, α-spectrin–related HE should be viewed as a bifactorial condition.

In conclusion, we have characterized a very common spectrin polymorphism that (*a*) lies at the αIV–αV domain junction, (*b*) results in a low percentage of the corresponding α chain in the membrane, and (*c*) probably represents the modulating factor that has been sought to explain the variation in the expression of spectrin α-chain mutants.

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