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Spectrin self-association site: characterization and study of β-spectrin mutations associated with hereditary elliptocytosis

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Most of hereditary elliptocytosis (HE) cases are related to a spectrin dimer (SpD) self-association defect. The severity of haemolysis is correlated with the extent of the SpD self-association defect, which itself depends on the location of the mutation regarding the tetramerization site. This site is presumed to involve the first C helix of the α chain and the last two helices, A and B, of the β chain to reconstitute a triple helical structure (A, B and C), as observed along spectrin. Using recombinant peptides, we demonstrated that the first C helix of the α chain and the last two helices of the β chain alone are not sufficient to establish interactions, which only occurred when a complete triple-helical repeat was added to each partner. One adjacent repeat is necessary to stabilize the conformation of both N- and C-terminal structures directly involved in the interaction site and is sufficient to generate a binding affinity similar to that observed in the native molecule. Producing peptides carrying a β^{HE} mutation, we reproduced the tetramerization defect as observed in patients. Therefore, the βW2024R and βW2061R mutations, which replace the invariant tryptophan and a residue located in the hydrophobic core, respectively, affect α–β interactions considerably. In contrast, the βA2013V mutation, which modifies a residue located outside any presumed interacting regions, has a minor effect on the interaction.

INTRODUCTION

Hereditary elliptocytosis (HE) is a haemolytic disease related to defects within the erythrocyte membrane skeleton. HE is a heterogeneous disorder in terms of clinical severity, red-cell-morphology abnormalities and underlying molecular defects. Its clinical presentation ranges from an asymptomatic condition to fatal hydrops fetalis, with intermediate phenotypes such as mild HE showing compensated haemolysis and severe haemolytic anaemia, characterized by red-cell fragmentation (hereditary pyropoikilocytosis) [1,2]. HE results from mutations in the genes encoding the spectrin α and β chains and protein 4.1. These proteins (together with actin) are the major components of the erythrocyte skeleton, the complex protein network responsible for the characteristic shape and the unique physical properties of red cells, such as deformability and remarkable stability to shear stresses.

Spectrin is composed of two elongated subunits, an α and a β chain (280000 and 246000 Da, respectively), which are non-covalently associated side-by-side in an antiparallel orientation to form heterodimers (spectrin dimers, SpDs). Involving each α-chain N-terminus and each β-chain C-terminus, SpDs self-associate head-to-head to form tetramers that constitute the long flexible filaments of the network. SpD self-association is crucial in maintaining the stability and the integrity of the cell, as clearly pointed out by studies on HE. In almost all cases of HE caused by a spectrin molecular defect, there is a more-or-less pronounced deficiency in SpD self-association. Under such conditions, the severity of haemolysis depends on the extent of the spectrin self-association defect [2]. The same interaction has also been demonstrated to be critical for normal development in *Drosophila* [3].

Each spectrin chain is mainly made up of a succession of repeating segments flanked by non-consensus structures [4–6]: 22 repeats in the α chain and 17 in the β chain. The repeating units, which are about 106 amino acids long, show low sequence identity (roughly 20%), but some residues are highly conserved, notably leucine and tryptophan at positions 26 and 45 of the repeats, respectively. As first proposed by Speicher and Marchesi [4], and recently confirmed by crystallographic and NMR data on a repeating unit of *Drosophila* α spectrin [7] and chicken brain α spectrin [8], the repeating motifs are folded in a triple-helical coiled-coil structure made up of helices A, B and C. Previous CD studies and analyses of protease sensitivity of *Drosophila* and human α-spectrin recombinant peptides have led to determination of the phasing of the native folded structure: the beginning of the stable conformational unit is shifted 20–30 residues toward the C-terminal end relative to the originally suggested alignment of the repeat-unit sequences [9,10]. Thus the conformational unit begins around the invariant leucine at position 26 in the repeat sequence and continues up to the same residue of the following repeat, involving helices A and B of one repeat unit and helix C of the following repeat. The crystallographic studies suggest the presence of a long helix C–A rather than two separate helices C and A, this long helix making the link between two triple-helical bundles.

According to the phasing and the triple-helical structure of the motif, the N-terminal end of the α chain begins with an isolated C helix (α1), whereas the β chain ends in an incomplete structure (β17), consisting of helices A and B, followed by an unfolded segment called domain III. It has been proposed that the two A and B helices of the last repeat β17 interact with the first C helix of the α chain to constitute a triple-helical structure similar to the repeats along the remainder of spectrin molecule (Figure 1) [11,12].

This model was first inferred from the relationship between the severity of the spectrin self-association defect and the location of the underlying mutations found in HE. Most amino acid substitutions located in the helices that were presumed to be involved in the self-association site are related to more severe

Abbreviations used: HE, hereditary elliptocytosis; SpD, spectrin dimer; GST, glutathione S-transferase; IPTG, isopropyl β-D-thiogalactoside.

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spectrin self-association defects than those located outside these helices. Shortened β-chain variants truncated at the C-terminal end with partial or complete deletion of the final helix B cannot interact with the N-terminus of the α chain [13,14]. This model was reinforced by studies involving partial or complete deletions of these helices from large recombinant peptides corresponding to the ends of the α and β chains. Loss of the first 16 residues of the α chain decreased its binding affinity to the β chain by about 50% [12]. Further truncations of recombinant peptides encompassing the αI domain, by deleting the 27, 30 or 49 residues, totally abolished detectable head-to-head association with the complementary β subunit [10]. In a similar way, deletions of the two last helices of the β chain led to a loss of binding to the α partner [15].

These data demonstrated the involvement of these three helices in the dimer–dimer contact site but did not indicate whether they alone are sufficient for binding. To determine whether the three helices compose the complete self-association site and to better define the bounds of this site, we have chosen to express several recombinant peptides encompassing different parts of the β-chain C-terminal domain and the α-chain N-terminal domain. We have tested their ability to associate with their respective partners and with SpDs. We have defined the minimum lengths of peptides required to generate the strong interaction observed in the native molecule and we have also determined the dissociation constant for the interaction of univalent peptides.

It is now well established that the severity of HE related to α^{HE} mutations depends on two parameters: (i) the location of the HE mutation in relation to the tetramerization site; and (ii) the proportion of mutated spectrin α chain present in the membrane, because the expression of the α^{HE} allele can be modulated by the presence *in trans* of a low-expression α allele, such as the α^{LELY} allele [16].

In HE arising from mis-sense β mutations, the clinical heterogeneity is also related to the severity of the spectrin self-association defect. Although all known mutations are located within the two helices in the self-association site, their effect on this function is variable. Two parameters, such as the nature of the substitution and the location of the mutation, could affect this function. To address this question, we have reproduced three previously defined β^{HE} mutations using site-directed mutagenesis on the recombinant peptides and we have analysed their effect on the interaction between the β-chain C-terminus and the α-chain N-terminus.

EXPERIMENTAL

Construction of the spectrin peptide expression plasmids

To identify the minimum length of peptides required in the tetramerization site, several recombinant peptides encompassing different parts of the C-terminal region of the β chain and of the N-terminal extremity of the α chain were expressed as glutathione S-transferase (GST) fusion proteins in *Escherichia coli* from the plasmid pGEX-2T (Pharmacia-Biotech) and the plasmid pGEX-KG [17].

The DNA fragments were obtained by PCR amplification of human spectrin cDNA clones (β29 and αB6 clones were generous gifts from B. Forget, Yale University, NH, U.S.A.), using either *Taq* polymerase (Gibco–BRL) or Vent polymerase (Biolabs) with the primers as shown in Table 1.

For the four α-spectrin constructs cloned into pGEX-2T plasmid, the upstream primer contained a *Bam*H1 restriction site, whereas the downstream primer contained a stop codon followed by an *Eco*R1 restriction site. The four α-spectrin constructs, α1–50 (containing the first C helix), α1–85 (helix C–A), α1–124 (helices C–A and B) and α1–154 (first C helix followed by a triple-helical repeat) encode the first 50 (6.7 kDa), 85 (10.6 kDa), 124 (15.2 kDa) and 154 (18.3 kDa) residues of the spectrin α chain, respectively (Figure 1).

Concerning the β-spectrin constructs cloned in pGEX-KG vector, the 5′-end primer has an *Eco*R1 restriction site, whereas the 3′-end primer contained a stop codon, immediately followed by a *Hin*dIII restriction site.

The β-spectrin construct β17C, from residue R2004 to Y2137 (15.9 kDa), consists of the last two A and B helices of the β chain, followed by the non-homologous C-terminal end; this corresponds to a partial fragment of repeat β17 and complete domain III (Figure 1). The β-spectrin construct β17, from residue R2004 to E2083 (10 kDa), contains only the last two A and B helices and corresponds to the β17C peptide without the domain III. The β-spectrin construct β16–17C, from residue Q1898 to Y2137 (28.7 kDa), and the β-spectrin construct β16–17, from residue Q1898 to E2083 (22.8 kDa), correspond to the β17C and β17 peptides, including the last complete triple-helical repeat, respectively.

The purified PCR-amplified cDNAs were sequentially or simultaneously cut with the appropriate enzymes (Gibco–BRL) and directionally cloned into the restricted vector using standard techniques. *E. coli* strain JM109 (Stratagène) and strain OMPT−, a protease deficient strain (a generous gift from J. M. Clément, Institut Pasteur, Paris, France) were transformed by pGEX-2T and pGEX-KG plasmids, respectively. Transformant colonies were screened by peptide expression after induction by isopropyl β-D-thiogalactoside (IPTG, Appligene; 0.5 mM). Then the appropriate candidates were expanded for sequencing using Sequenase 2 (United States Biochemical Corp.) to confirm the fidelity of the construct. Because of residual polylinker sequences in the plasmid, the α-spectrin peptides have two additional residues (G and S) at the N-terminal end after thrombin cleavage and the β-spectrin peptides have the additional residues GSPGISGGGGGI at the N-terminal end, as verified by microsequencing.

Site-directed mutagenesis

Mutations were introduced in the β-spectrin cDNA cloned in the pGEX-KG plasmids according to the site-directed mutagenesis method known as long primer–unique-site elimination mutagenesis [18] and modified as previously described [19]. The template target plasmid containing the cloned insert corresponding to the β16–17C construct was amplified using two kinds of mutant primers: (i) the selection primer (sense primer) eliminates the unique *Bam*HI site by converting it to a *Kpn*I site (see bold sequence in Table 1); and (ii) the other primers (reversed primers) contain the desired mutations (Table 1).

Expression and purification of recombinant peptides

Cells were grown at 37 °C in Terrific Broth medium containing ampicillin (100 μg/ml). Overnight cultures were diluted (1:10) in Terrific Broth and were grown to an attenuence of 0.8–0.9 at 600 nm, and diluted again (4:5) prior to induction with 0.5 mM IPTG. Growth was continued for 1.5–2.5 h according to the stability of the recombinant peptide. After centrifugation, the bacterial pellets were frozen and stored at −20 °C. Frozen cells from 1 litre of culture were resuspended in 25–50 ml of 20 mM phosphate buffer (pH 7.3)/150 mM NaCl/0.5% Triton X100 containing 1 mM EDTA, 0.5 mM AEBSF [4-(2-aminoethyl)-benzenesulphonyl fluoride] or PMSF, 2 μg/ml leupeptin, 2 μg/ml pepstatin, 100 Units/ml aprotinin and 1 mM β-mercaptoethanol, and lysed by sonication. The expressed GST–spectrin fusion

Table 1 Design of recombinant α- and β-spectrin tetramerization-site peptides

Bold text indicates mutations within the primers. See text for more details.

Peptides	Amino acids	5′ Primer	3′ Primer
β16–17C	1898–2137	5' GG**GAATTC**AGCTAGTGGACACGGCGGAT 3'	5' GG**AAGCTT**TTACTAGTAGGGGTGAGAGGG 3'
β16–17	1898–2083	5' GG**GAATTC**AGCTAGTGGACACGGCGGAT 3'	5' GG**AAGCTT**TTACTACTCTGCAATCTGGCG 3'
β17C	2005–2137	5' GG**GAATTC**GGCTCCGCATGTTGCTGGAG 3'	5' GG**AAGCTT**TTACTAGTAGGGGTGAGAGGG 3'
β17	2005–2083	5' GG**GAATTC**GGCTCCGCATGTTGCTGGAG 3'	5' GG**AAGCTT**TTACTACTCTGCAATCTGGCG 3'
α1–50	1–50	5' GG**GGATCC**GAGCAATTTCCAAAGGAAAC 3'	5' GG**GAATTC**ATCCTCAAGCTTCTGACCC 3'
α1–85	1–85	5' GG**GGATCC**GAGCAATTTCCAAAGGAAAC 3'	5' GG**GAATTC**TTATTAGTTGGGTCTTCATAGCTCT 3'
α1–124	1–124	5' GG**GGATCC**GAGCAATTTCCAAAGGAAAC 3'	5' GG**GAATTC**TTAGCAGAATGACCCATGGTAA 3'
α1–154	1–154	5' GG**GGATCC**GAGCAATTTCCAAAGGAAAC 3'	5' GG**GAATTC**TTACAACTGGTCACCCTTCTC 3'
α-UR2	152–259	5' GG**GGATCC**CAGTTGCTGCGGGCCCTGAA 3'	5' GG**GAATTC**TTAATTGGACAGAGCTTTCTG 3'
β16–17C	A2023V	5' GATCTGGTTCCGCGTGG**TA**CCCCGGGAATTTCCG 3'	5' GGGCAATCAGCCAC**A**CCTCAGCCACAGAG 3'
β16–17C	W2024R	5' GATCTGGTTCCGCGTGG**TA**CCCCGGGAATTTCCG 3'	5' CTGGGCAATCAGCC**T**CGCCTCAGCCACAG 3'
β16–17C	W2061R	5' GATCTGGTTCCGCGTGG**TA**CCCCGGGAATTTCCG 3'	5' CGCTCTGCCC**T**GCTGGCCGTG 3'

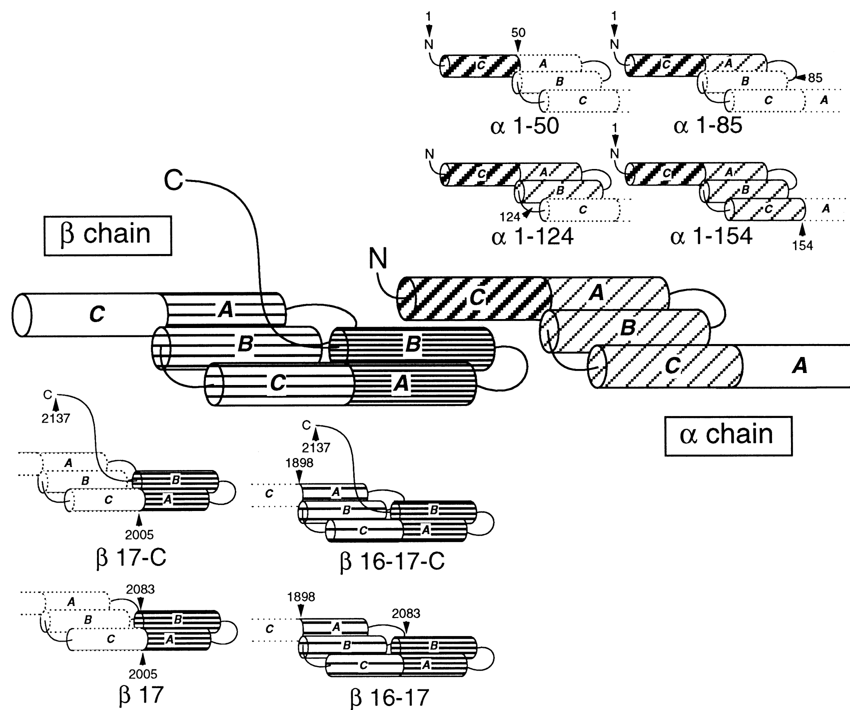


Figure 1 Models of the self-association site and of the recombinant peptides used in this study

The positions of residues corresponding to the beginning and end of each recombinant peptide are indicated by arrows.

proteins were affinity purified on a glutathione–Sepharose column (Pharmacia-Biotech). The spectrin peptides were cleaved from the carrier protein by thrombin digestion on the affinity column. Spectrin peptides were purified to homogeneity by anionic exchange chromatography either on Mono Q or on Resource Q columns (Pharmacia-Biotech).

All the peptides were highly expressed in bacteria, but they differed in their solubility and stability. The α peptides (α1–50, α1–85 and α1–124) and the β peptides (β17C and β17 peptides) were less soluble and stable than α1–154, β16–17C and β16–17 peptides, which contain a complete triple-helical repeat. The β17C and β16–17C fusion peptides, which contain the non-homologous part of the C-terminal end of the β chain, were susceptible to proteolysis. To reduce insolubility and proteolysis

of the less-stable peptides, expression was performed at 25–30 °C instead of 37 °C, and the β16–17C and β17C peptides were expressed in a protease-deficient stain, OMPT−.

After thrombin cleavage of fusion proteins immobilized on the glutathione–Sepharose Cl-4B affinity column, the α1–50, α1–85 and α1–154 recombinant peptides were highly purified and had an apparent molecular mass in gel electrophoresis that was in agreement with the calculated value. The α1–124 peptide was proteolysed during thrombin cleavage and consequently was not available for further investigation. Subsequent anion-exchange chromatography of the α1–154 peptide revealed elution of a predominant peak and other, minor, peaks. The α1–50 and α1–85 peptides were poorly eluted from the anion-exchange column, even at high salt concentration. Thus, both these peptides were used after the affinity column without any further purification steps.

The β17 and β17C peptides were highly unstable after thrombin cleavage. The β16–17 peptide, and particularly the β16–17C peptide containing domain III, required subsequent anion-exchange chromatography to eliminate proteolytic products.

As previously observed [17], the series of glycine residues near the thrombin cleavage site, introduced in the fusion protein by the plasmid pGEX-KG, allowed almost complete cleavage (95–100 %) compared with that achieved with fusion proteins expressed from the plasmid pGEX-2T (50–75 %).

Binding assays

As indicated in the Figure legends, various mixtures of recombinant peptides in the form of fusion proteins, isolated peptides or ^{125}I-labelled peptides were incubated in isotonic buffer (150 mM NaCl/20 mM Tris, pH 7.4) at 0 °C for at least 4 h. Free and bound species were analysed following three different procedures. (i) When α-spectrin peptides were mixed with β-spectrin peptides as GST-fusion proteins, bound and free α-spectrin species were separated by centrifugation after adding glutathione agarose beads. After three successive centrifugations and washes, pellets containing bound ligands were analysed by SDS/PAGE [20]. (ii) Interactions between α- and β-spectrin peptides, free of GST, were analysed by rapid non-denaturing electrophoresis (15 min) at 4 °C using a Phast System (Pharmacia-Biotech). When one partner was ^{125}I-labelled, Coomassie Blue-stained bands were excised from the gel and quantified by γ-radiation counting (Beckman 7000 γ counter). Peptides were radiolabelled with Na^{125}I (Amersham) by the chloramine T method.

CD spectroscopy

The far-UV CD spectra were recorded on a Jasco 700 spectropolarimeter in 0.1 or 0.2 cm quartz cells at 20 °C. The protein concentration was determined by UV absorption at 280 nm, using the known molar extinction coefficient of the Trp, Tyr and Cys residues in the sequence. The spectra were obtained as an average of 10 runs and were corrected for the contribution of the buffer. The final CD data are shown as mean residue molar ellipticity in units of degrees·cm^{-2}·dmol^{-1}.

Other procedures

Native spectrin dimers were extracted from human erythrocyte membrane by incubation at 37 °C in a low ionic-strength buffer and dimer–tetramer formation was followed by non-denaturing electrophoresis as described previously [21].

SDS/PAGE was performed either as described by Laemmli [20] or by using a Phast system. Gels were stained with Coomassie Brillant Blue.

Western blots were performed from Phast gels on PVDF membranes using diffusion blotting. Recombinant polypeptides were detected by rabbit polyclonal antibodies directed against either α- or β-spectrin chains. Blots were incubated with the peroxidase-conjugated anti-rabbit immunoglobulin antibodies from goat and developed using Nitro Blue Tetrazolium as substrate [22].

Protein concentrations were determined by absorbance at 280 nm using extinction coefficients calculated from sequences.

RESULTS

CD studies

A coherent analysis of specific intermolecular interactions between biomolecules requires minimal information on the

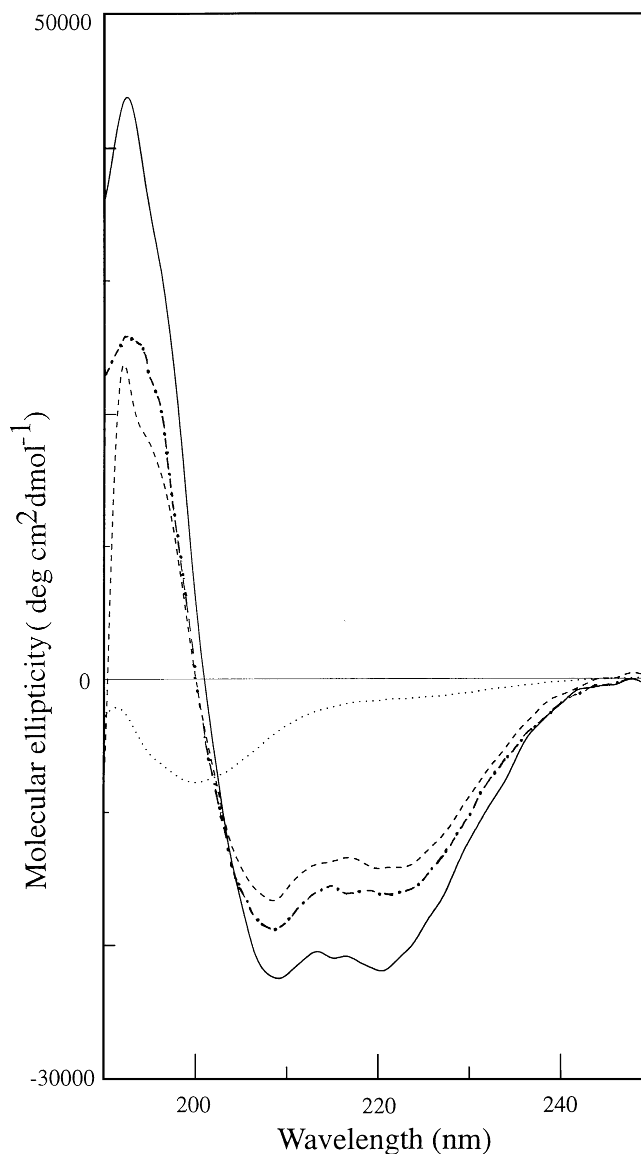


Figure 2 Far-UV spectra of some of the recombinant polypeptides in the present study

The spectra were obtained at 20 °C, in Tris buffer (5 mM), pH 8.5, containing EDTA (0.2 mM) and β-mercaptoethanol (0.2 mM). Dotted line, α1–50; continuous line, α1–154; broken line, β16–17C; and dash-and-dot line, β16–17.

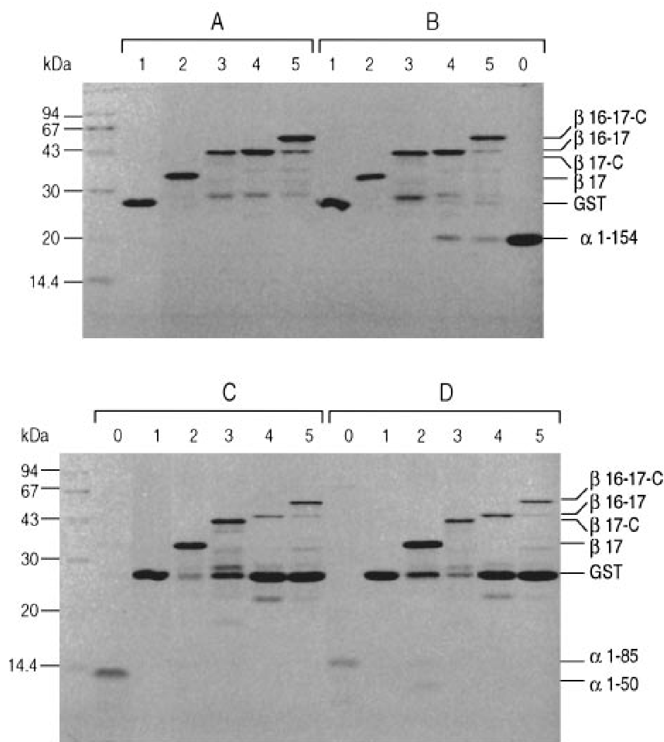


Figure 3 SDS/PAGE analysis of α-and β-spectrin recombinant peptides involved in the spectrin self-association site

GST and β-spectrin GST-fusion peptides (10–15 μM) were incubated overnight at 0–4 °C in the absence of α recombinant peptides (**A**), or in the presence of each of the following spectrin α peptides (25–30 μM): α1–154 (**B**); α1–50 (**C**); and α1–85 (**D**). Unbound α peptides were eliminated by adding glutathione–agarose beads. The washed pellets were analysed by SDS/PAGE on a 14% acrylamide gel. Lanes 0 of (**B**), (**C**) and (**D**) correspond to isolated spectrin α peptides. Lane 1, GST; lane 2, β17 fusion peptide; lane 3, β17C fusion peptide; lane 4, β16–17 fusion peptide; lane 5, β17–16C fusion peptide.

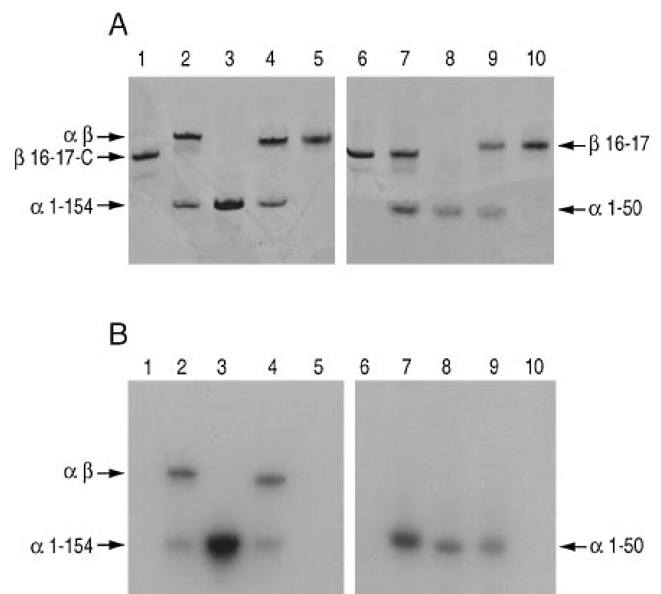


Figure 4 Analysis of spectrin peptides involved in the tetramerization site by non-denaturing electrophoresis

β16–17 and β16–17C peptides (approx. 10 μM) were incubated with ^{125}I-labelled α1–154 and α1–50 peptides (10 μM and 15 μM, respectively) for 4 h at 0–4 °C and analysed by rapid non-denaturing gel-electrophoresis (15 min) at 4 °C using a Phast system (12.5% acrylamide gel). For this purpose, 6 μl of each sample was loaded on the gel. (**A**) Coomassie Blue-stained gels. (**B**) Autoradiograms: lanes 1 and 6, β16–17C peptide (15 μM); lane 2, mixture of β16–17C and α1–154 at 10 μM; lane 3, α1–154 at 30 μM; lane 4, mixture of β16–17 and α1–154 at 10 μM; lanes 5 and 10, β16–17 at 15 μM; lane 7, mixture of β16–17C and α1–50 at 10 and 30 μM, respectively; lane 8, α1–50 at 15 μM; lane 9, mixture of β16–17 and α1–50 at 10 and 15 μM, respectively. The presence of α–β peptide complexes is indicated by arrows.

structure content and stability of the molecules in the free state. For this aim, CD spectroscopy is generally used as a convenient method to evaluate the secondary structure of polypeptides. Figure 2 shows the far-UV CD spectra of some of the protein fragments used in the present study. The α1–154 peptide gives a CD spectrum with all the characteristics of a highly helical polypeptide: two negative bands at 222 nm and 208 nm and a positive one at 192 nm [23]. A simple evaluation, using the molar ellipticity at 222 nm, gives a helical content of about 68%, a value that is somewhat lower than what could be predicted from the comparison with the crystallographic three-dimensional structure of the repetitive segment of spectrin (80%) [7]. Similarly, the protein fragments derived from the sequence of the β chain (β16–17 and β16–17C) have a high content of α-helix secondary structure. In contrast, the smaller polypeptide α1–50 gives a small ellipticity and spectral features suggesting a random-coil structure. In the presence of 2,2,2-trifluoroethanol (30% v/v), a co-solvent which decreases the solvent polarity, the CD spectrum indicates the stabilization of some elements (about 15%) of helical structure (results not shown).

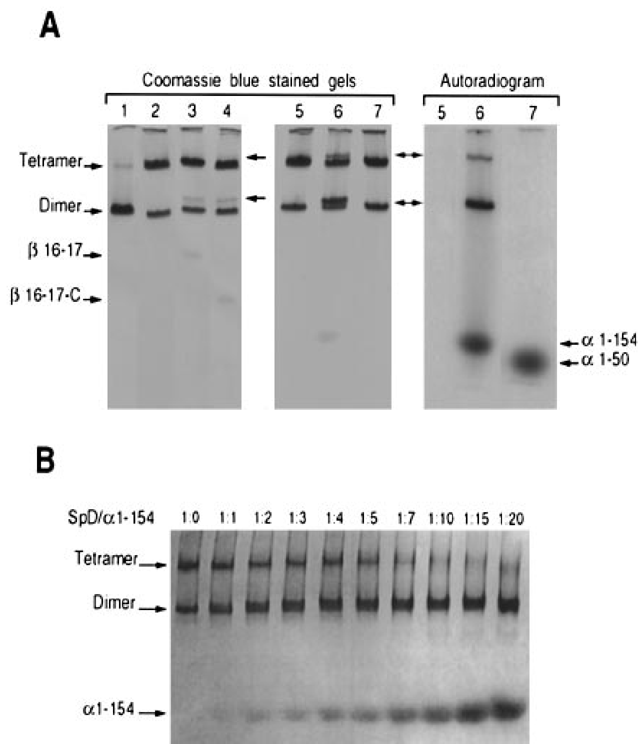
Determination of the peptides involved in the tetramerization site

It was previously shown [24] that polypeptides from the N-terminus of the α chain can readily associate with β-spectrin monomers at low temperature (4 °C) in contrast with the self-association of native SpD, which is infinitesimally slow under these conditions. The different recombinant peptides were therefore tested for their ability to associate with their respective partners at 0 °C in isotonic solution.

In a first approach, each β-spectrin peptide as a GST-fusion protein (10–15 μM) was incubated with each of the different purified α-spectrin peptides, devoid of GST (25–30 μM), and pelleted after attachment to gluthathione–Sepharose beads (Figure 3). A significant proportion of α1–154 peptide was found in the pellet with the β16–17 and β16–17C fusion peptides (Figure 3B, lanes 4 and 5, respectively) whereas no detectable amount of α1–154 peptide was pelleted with the β17 and β17C fusion proteins (Figure 3B, lanes 2 and 3, respectively). No traces of α1–50 and α1–85 peptides were observed in the pellets after incubation with any of the four β-spectrin-fusion peptides (Figures 3C and 3D). GST immobilized on beads did not bind any of the α-spectrin peptides (lanes 1 of Figures 3B, 3C and 3D).

Direct interactions between α- and β-spectrin peptides, devoid of GST, were analysed by rapid non-denaturing gel electrophoresis. In this alternative approach, binding assays were performed between the four isolated β-spectrin peptides and two α1–50 and α1–154 ^{125}I-labelled α-spectrin peptides. As shown in Figure 4, the α1–50 peptide (lane 8), the α1–154 peptide (lane 3) and the β16–17 peptide (lanes 5 and 10) migrated in a short band, and the β16–17C peptide (lanes 1 and 6) migrated in two bands. Only a fraction of the β17 and β17C peptides entered into the gel and migrated in several diffuse bands (results not shown).

Compared with the electrophoretic pattern of each isolated



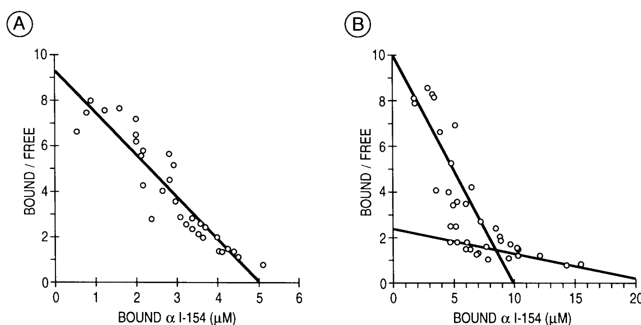


Figure 5 Interaction of α and β peptides with intact SpDs analysed by non-denaturing gel electrophoresis

(**A**) Spectrin dimers (2 μM; lane 1) were incubated for 4 h at 30 °C, either alone (lanes 2 and 5), or in the presence of recombinant peptides: β16–17 (2 μM; lane 3); β16–17C (2 μM; lane 4); ^{125}I-labelled α1–154 (4 μM; lane 6); and ^{125}I-labelled α1–50 (4 μM; lane 7). The α1–154, β16–17 and β16–17C peptides bound extensively to SpDs and to a lesser extent to spectrin tetramers, with the appearance of two new species (indicated by arrows). (**B**) SpDs (2 μM) were incubated with increasing amounts of α1–154 peptide (molar ratio from 1:1 to 1:20).

Figure 6 Scatchard representation of the α–β peptide interactions

β16–17C peptide (5 μM; **A**) and β16–17 peptide (10 μM; **B**) were incubated with increasing amounts of ^{125}I-labelled α1–154 peptide. Free and bound species were separated and analysed by non-denaturing gel electrophoresis. The measured K_d was 0.9 μM for the interaction between β16–17C and α1–154 peptides. Scatchard analysis of the binding between α1–154 and β16–17 revealed two slopes (K_d approx. 1 and 7.5 μM, respectively).

species, an additional band was only observed in the mixture of the α1–154 and β16–17C peptides, with a concomitant decrease in free species (Figure 4A, lane 2). This new band was radioactive (Figure 4B, lane 2) and was recognized by antibodies directed against either α spectrin or β spectrin (results not shown), demonstrating that it corresponds to an α(1–154)–β(16–17C) complex. After electrophoretic separation of the mixture containing the α1–154 and β16–17 peptides (Figure 4A, lane 4), the free α1–154 peptide decreased with no appearance of an additional band. Autoradiograms revealed the presence of an additional labelled band co-migrating with the β16–17 peptide (Figure 4B, lane 4), showing that association of the two peptides had indeed occurred. We confirmed that the α(1–154)–β(16–17) complex has the same electrophoretic mobility as the free β16–17 peptide by immunoblots (results not shown). We were unable to demonstrate an interaction between the α1–154 peptide and either the β17 or β17C peptides, because both of these were unstable. Similar results were obtained when β species were used in the form of fusion proteins instead of isolated peptides.

Incubation of the α1–50 peptide with either the β16–17C or β16–17 peptides led to no detectable association, even after 48 h (Figure 4, lanes 7–9).

Association of recombinant peptides with dimers

The head-to-head interactions of SpDs that lead to formation of a series of oligomers, tetramer, hexamer and so on, are based on the involvement of every α and β chain of the dimers. Therefore, the tetramer formation results from two α–β interactions. The isolated peptides α1–50, α1–154, β16–17C and β16–17 were tested for their ability to bind to native SpD and to compete against tetramer formation after incubation for 4 h at 30 °C (Figure 5). When the concentrations of the peptides α1–154, β16–17C and β16–17 were in the same range as the SpD concentration, they bound to SpD, and to a lesser extent to tetramers, with the appearance of two new species (Figure 5A). These new species were radiolabelled when labelled recombinant peptides were used in the experiment (Figure 5A). When the peptide concentration was increased and present in molar excess, the formation of tetramers was decreased with a concomitant increase in the new species migrating behind the SpD (Figure 5B). The species, which migrate just behind the dimers, correspond to the binding of one molecule of recombinant peptide to its respective partner in the SpD molecule: either an α peptide with the C-terminal end of a β chain or a β peptide with the N-terminal end of α chain. In these complexes, SpD–peptide, one binding site of SpD is involved in an interaction with the recombinant peptide, the other one remains free and can interact with one native SpD molecule to form tetramers. Therefore, the new species migrating behind the tetramers corresponded to the interaction between the SpD–peptide complex and native SpDs. The higher the recombinant peptide concentration, the greater the SpD–peptide formation and the lower the concentration of free SpD. As the SpD–peptide complex cannot interact with another SpD–peptide complex, and the free SpD concentration is decreased, the number of tetramers that form decreases.

The α1–50 peptide did not show any detectable interaction with native SpDs or tetramers.

Determination of the association constants

The kinetics of the interaction between α1–154 and either β16–17C or β16–17 peptides was analysed. ^{125}I-Labelled α1–154 peptide at 10 μM was incubated with equimolar amounts of either β16–17C or β16–17 peptides for different times, and the species present in the mixture were analysed by non-denaturing gel electrophoresis. The interaction was fast, since between 65 and 85 % of the complex had formed after a 5 min incubation and equilibrium was reached after 3–4 h. No detectable redistribution between the free and associated species occurred during the short time of electrophoresis (15 min).

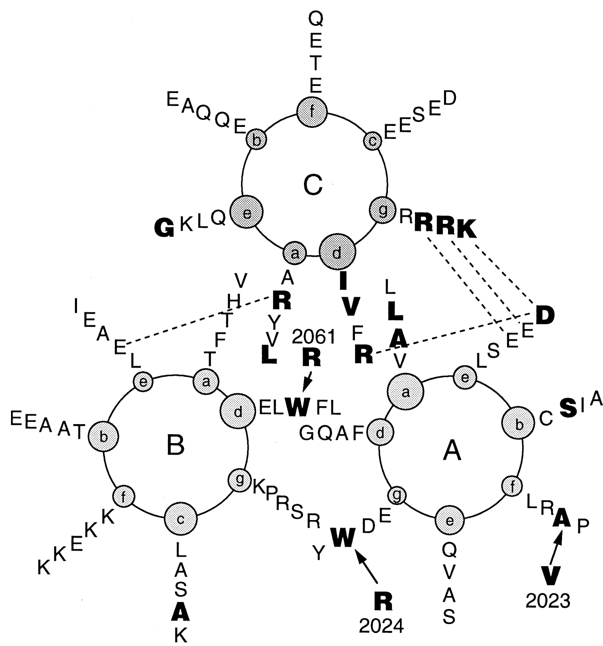


Figure 7 Axial helical projection of the SpD self-association site, based on crystallographic data of a *Drosophila* spectrin repeat [7]

Wheels A and B correspond to A and B helices of the incomplete β17 repeat, and wheel C corresponds to the first C helix of spectrin α chain. The heptad positions (a–g) are shown by circles. Residues that are mutated in HE are shown in bold. The three β^{HE} mutations reproduced by directed mutagenesis are indicated by arrows: A2023V, W2024R and W2061R.

Association constants for binding of α1–154 (0.5–25 μM) to β16–17C or β16–17 peptides have been determined from the equilibrium of two peptide preparations. β16–17C or β16–17 peptides (at 5 or 10 μM) were incubated for 20 h with increasing amounts of ^{125}I-labelled α1–154 peptide. All determinations were in duplicate. The α1–154 peptide interacted with the β16–17C peptide with a K_d value ($\pm$S.D.) of 0.9 ± 0.09 μM ($n = 4$) (Figure 6A). Concerning the interaction between the α1–154 peptide and the β16–17 peptide, missing the non-homologous C-terminal end of the β chain, a Scatchard plot of the results suggested two binding components, one corresponding to a K_d value of 1.09 ± 0.16 μM ($n = 3$), the other to a K_d value of 7.45 ± 1.8 μM ($n = 3$).

Analysis of three β^{HE} mutations on the head-to-head α–β interaction

Three natural β^{HE} mutations discovered in different HE kindreds (A2023V, W2024R and W2061R) were reproduced using directed mutagenesis in the β16–17C peptide. These mutations are located within either the last A helix or the last B helix (Figure 7). The mutated peptides were tested for their ability as free peptides (15 μM) to interact with the α1–154 peptide (up to 40 μM). Saturation curves (Figure 8A) revealed that the A2023V mutant peptide was able to bind to the α1–154 peptide, in a range quite similar to the wild-type, since the K_d value, as determined by the Scatchard plot (Figure 8B) using the Prism software® programme (Graph Pad®, San Diego, U.S.A.) was 8 μM. In contrast, the W2024R mutant peptide showed no ability to interact with the α1–154 peptide, even in a 1:3 molar ratio, and the W2061R mutant peptide had an intermediate binding ability. The K_d values estimated from the Scatchard plot (Figures 8C and 8D) were higher than 100 μM (the K_d was 240 μM and 130 μM for the W2024R and W2061R mutant peptides, respectively).

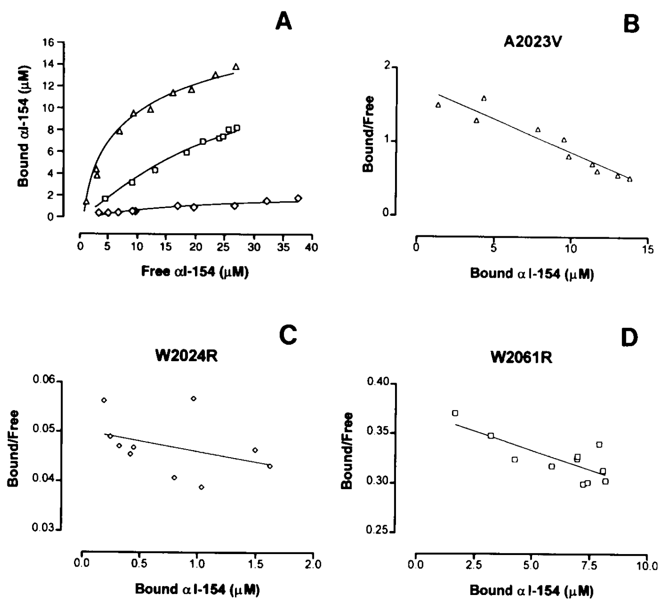


Figure 8 Interaction of β^{HE} mutant peptides with α1–154 peptide

(**A**) Saturation curves. β^{HE} mutant peptides (15 μM) [(△) A2023V, (◇) W2024R and (□) W2061R] were incubated with increasing amounts of ^{125}I-labelled α1–154 peptide. (**B**), (**C**) and (**D**) Scatchard representations of the α1–154 peptide interaction with the β^{HE} mutant peptides.

DISCUSSION

Self-association of SpDs to tetramers is a crucial process for erythrocyte-membrane stability, as clearly demonstrated in HE states in which this function is defective. It is clear that the tetramerization process involves the last two A and B helices of the C-terminal end of the β-spectrin chain and the first C helix at the N-terminal end of α-spectrin chain. In this report, we have demonstrated that these helices alone are insufficient for the interaction. The peptide α1–50, containing the first C helix of the α chain did not manifest any ability to interact with complementary β partners in the form of either recombinant peptides, at 0 °C, or SpDs, at 30 °C. As previously demonstrated [10,12], deletion of the first 45 residues of the α chain abolished binding of the α chain to the β chain. Thus, the first 50 residues, although required for binding, are apparently insufficient for binding to a β partner. The lack of binding could be related to the lack of folding, as indicated by CD studies. The complete helix C–A has no more binding capacity than the first part of the helix. Stable folding and strong interactions only occurred when the C helix was followed by a complete triple-helical repeat (α1–154 peptide). This α1–154 peptide was able to bind to native spectrin and to compete for the self-association site, and therefore contains a functional dimer-binding site. Similar conclusions can be drawn for the β-spectrin end: the last two A and B helices of the β-chain end (β17 and β17C peptides) are unstable and insufficient to establish interactions with the α-spectrin partners; binding only occurred when a complete triple-helical repeat was added (peptides β16–17 and β16–17C). Thus, one adjacent repeat is necessary to stabilize the conformation of both N- and C- terminal

structures directly involved in the interaction site, and is sufficient to generate a binding affinity similar to that of the native molecule (K_d, 1 μM) [24]. In agreement with previous work from Kennedy et al. [15], we found that the non-homologous end of the β chain, i.e. the random domain III, is not required for the interaction with the N-terminal end of the α chain. The β16–17 peptide binds strongly to the α1–154 peptide, as well as to SpDs or tetramers, but the two limbs observed in the Scatchard plot for the β16–17 peptide could suggest the presence of two populations, one with a fully active conformation, like the equivalent peptide with domain III, the other with a lower affinity. Domain III could stabilize the structure of either the last two A and B helices or the triple-helical structure occurring during the interaction.

In the self-association site as defined, several point mutations leading to HE have been located in the first C helix of the α chain, as well as in the last two A and B helices of the β chain. Clinical heterogeneity of HE, as reflected by the variable extent of haemolysis, appears to be related to the severity of the spectrin self-association impairment [21]. Concerning α-spectrin gene mutations, the extent of the spectrin self-association defect depends on two parameters: (i) the location of the mutation in relation to the self-association site; and (ii) the proportion of mutated HE spectrin α chain recruited to the membrane which, in turn, depends on the presence *in trans* (or *cis*) of low-expression α alleles, such as the α^{LELY} allele [16]. Conversely, in HE related to β-spectrin mutations, no low-expressed β-spectrin allele has been described so far and differences in severity as observed within the same α-spectrin HE kindred were not found in β-spectrin HE kindreds. Thus, the clinical heterogeneity of β-spectrin HE stemming from point mutations raises the question of whether a clear relationship exists between the self-association defect and the nature and position of the mutated residue. To address this question, three natural β-spectrin mutations found in different HE kindreds, were reproduced by directed mutagenesis in the self-association site. The A2023V mutation (spectrin^{Paris}) [25] was described in two related patients with fully asymptomatic HE, associated with a minor self-association defect (roughly 13% of SpD in the membrane compared with a normal value of 4%). The W2024R mutation (spectrin^{Linguerre}) [25] was found in two related patients with mild HE, associated with 30% of SpD in the membrane. The W2061R mutation (spectrin^{Cotonou}) [26] was described in a patient with mild HE with 24% of SpD in the membrane. Our studies *in vitro* of the interaction between α- and β-mutated recombinant peptides reproduced well the self-association defects observed in the HE patients. The replacement of either Trp-2061 or Trp-2024 affected considerably the α–β association, giving a K_d of 100 μM (normal value, 1 μM). In contrast, the mutation A2023V affected the interaction to a lesser extent (K_d approx. 8 μM).

In an attempt to understand better the relation between the nature and location of the mutated residues and impairment of the self-association process, Figure 7 shows the self-association site based on the crystallographic data for the *Drosophila* spectrin α14 repeat [7]. Each of the three helices exhibits the heptad repeat pattern found in extended coiled-coil α-helical structures when the positions are conventionally labelled a–g; the residues at positions a and d are generally hydrophobic and lie inside the three-helix bundle. Electrostatic interactions also appear to stabilize the structure, and external interchain salt bridges are often formed by charged residues at positions e and g. All the mutated residues described so far in HE are indicated in bold in Figure 7. The amino acids that are particularly affected are in positions a, d, e and g. These correspond to regions seen to be involved in either hydrophobic or electrostatic interactions. The results suggest that these positions could be crucial for the interaction between the three helices and, consequently, for the tetramer formation. The two mutations studied *in vitro* in this report and that strongly affect the binding are located in the presumed interaction site. The first, the W2061R mutation, affects a residue located within the hydrophobic core. The second, which seems to abolish the interaction, replaces the invariant Trp-2024 with arginine (W2024R). This tryptophan is highly conserved throughout the repeats of erythroid and non-erythroid spectrins, as well as in other spectrin family members (such as dystrophin and α-actinin). Our results indicate the crucial nature of this invariant tryptophan and confirm its contribution to formation of the triple-helical structure, as has been suggested [7,27]. The third mutation, A2023V, is conservative and modifies a residue located outside any presumed region of interaction. Its modest effect on self association could be related either to the kind of the mutation (conservative) or to its location. We have observed another similar conservative mutation, V31A, in an asymptomatic HE (Garbarz, M. and Dhermy, D. unpublished work), also presenting a modest defect on the tetramer formation (7.5% of SpD in the membrane for a control value of 2.5%). Although this mutation is located in the C helix of the spectrin α chain, at a d position in the hydrophobic core, the SpD self-association defect is moderate, suggesting that in this case the kind of mutation determines the reduced deleterious effect.

Taken together, our results showed the molecular basis of a direct correlation between the severity of both the self-association defect and the clinical expression in HE caused by β^{HE} point mutations, and the location and nature of the β-spectrin mutation.

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