The Exon 46-Encoded Sequence Is Essential for Stability of Human Erythroid α-Spectrin and Heterodimer Formation

By Rick Wilmotte, Sandra L. Harper, Jeanine A. Ursitti, Joëlle Maréchal, Jean Delaunay, and David W. Speicher

Human erythroid α-spectrin alleles responsible for hereditary elliptocytosis (α^{HE} alleles) undergo increased incorporation into red blood cell membranes when the polymorphism α^{LELY} (LELY: *L*ow *E*xpression *LY*on) occurs in trans. The α^{LELY} polymorphism is characterized by a mutation in exon 40 at codon 1857 (CTA → GTA, Leu → Val) and the partial (50%) skipping of exon 46, which encodes residues 2177-2182 (Wilmotte et al, *J Clin Invest* 91:2091, 1993). Both of these peptide sequence alterations are located within the region of the α-chain involved in initiating heterodimer assembly, and either or both mutations could potentially contribute to decreased incorporation of α-chains from the α^{LELY} allele in heterozygotes into red blood cell membranes. These possibilities were evaluated by testing the protease resistance and in vitro binding properties of normal and mutant recombinant 4-motif α subunit peptides containing the dimer initiation region. The two forms of α spectrin produced by alternative mRNA splicing of the α^{LELY} allele were represented by α18-21^{1857}, a peptide with the codon 1857 mutation and retaining the exon 46 encoded sequence, and α18-21$^{1857\text{-}\Delta 46}$, a peptide carrying both the 1857 codon mutation and the exon 46 deletion. The properties of these two recombinant peptides were compared with α18-21, a peptide with the normal sequence at codon 1857 and retaining the exon 46 encoded sequence. The codon 1857 mutation does not adversely affect dimer formation, but it is responsible for the increased trypsin cleavage between the αIV and αV domains that was the characteristic feature initially used to identify the α^{LELY} (Sp$\alpha^{V/41}$) polymorphism (Alloisio et al, *J Clin Invest* 87:2169, 1991). Deletion of the six amino acids encoded by exon 46 perturbs folding of the α21 motif, because this region of the α18-21$^{1857\text{-}\Delta 46}$ peptide is rapidly degraded and this recombinant peptide is unusually prone to self-aggregation. Exon 46 deletion reduces, but does not eliminate, dimerization. Comparison of mild trypsin proteolytic products from an α^{LELY} homozygote and the two α^{LELY} recombinant peptides strongly suggests that little, if any, of the 50% of the α chains from the α^{LELY} allele that contain the exon 46 deletion are incorporated into the mature erythroid membrane. Based on the in vitro analysis of recombinant α^{LELY} peptides, the inability of detectable amounts of exon 46^{-} α chains to assemble into the mature membrane skeleton in vivo is probably due to a combination of decreased dimer binding affinity and increased proteolytic degradation of these mutant chains.

SPECTRIN IS THE major protein of the red blood cell (RBC) membrane skeleton. Its physiologic form is a fibrillar $\alpha_2\beta_2$ tetramer in which two $\alpha\beta$ dimers self-associate head-to-head. The human erythroid spectrin α-chain gene encodes 2429 amino acids,[1] maps to chromosome 1q22-q23,[2] and contains 52 exons.[3] The erythroid spectrin β-chain gene encodes 2137 amino acids,[4] maps to chromosome 14q23-q24-2,[5] and contains 36 exons,[6] although only 32 exons are expressed in the RBC. Spectrin $\alpha\beta$ dimers are arranged such that the α- and β-chains associate laterally along the long axis of this flexible rod-like molecule in an antiparallel fashion.[7] Both chains are composed predominantly of many tandem homologous segments (spectrin-type motifs) containing about 106 amino acids (α1 to α21 and β1 to β17).[1,4,8] The phasing or conformational start and stop points of these many tandem homologous motifs is displaced by about 26 amino acids with respect to initial sequence alignments,[9] which were based on partial peptide sequence data.[8] This conformationally defined motif phasing has been confirmed by determination of the crystal structure of the 14th segment or motif of *Drosophila* α-spectrin.[10]

Early studies of spectrin heterodimer association used mild trypsin digestion to dissect dimers into a reproducible pattern of intermediate-sized peptides that were used to evaluate peptides that were laterally associated.[11-13] A subsequent study used purified spectrin monomers and peptides produced by mild tryptic digestion of monomers to study dimer assembly.[14] This study showed that heterodimer formation was initiated exclusively at two complementary sites, which were located near the α-chain C-terminus and near the β-chain N-terminus. Initial association of these dimerization sites was rapidly followed by subsequent lateral pairing of additional α and β motifs along the length of the molecule analogous to closing of a zipper. The smallest mild tryptic peptides that were identified as capable of lateral dimeric assembly corresponded approximately to the homologous motifs α19-21 and β1-4. In contrast, Viel and Branton[15] concluded that the β1-4 region alone was insufficient for dimer assembly and that at least part of the N-terminal actin binding domain of the β-chain was required for initiating high-affinity dimer complexes. Because their study used in vitro-translated peptides, the conformational integrity of the recombinant peptides was not evaluated. Others have shown that some recombinant β-spectrin peptides containing part of the actin binding domain did not fold into native structures or were unstable.[16] The minimum β-spectrin peptide required

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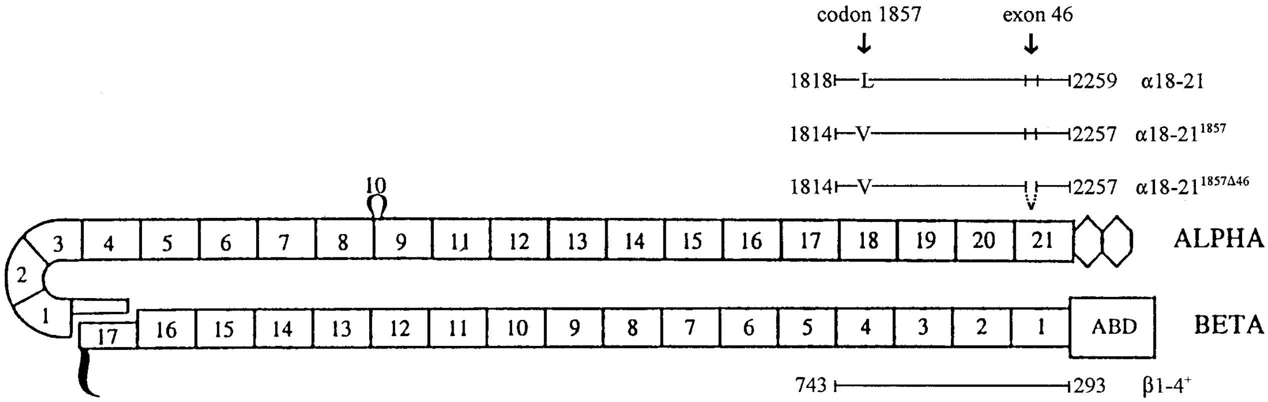

Fig 1. Location of spectrin dimer initiation site peptides used for in vitro binding studies. The arrangement of structural motifs in an antiparallel dimer and the relationship of the recombinant peptides used in this study are shown. The β subunit contains an actin binding domain (ABD), 17 homologous segments or motifs (numbered rectangles), and a small nonhomologous phosphorylated C-terminal domain. The α subunit contains 20 homologous motifs (1 through 9 and 11 through 21), an SH-3 motif (motif 10) located in a loop between the B and C helix of motif 9, and a C-terminal domain consisting of two EF-hand type motifs (diamonds). The locations of the recombinant peptides used in this study are shown by horizontal lines. The relationships of the two gene products from the α^{LELY} allele to a normal α18-21 recombinant peptide are shown. The $\alpha 18\text{-}21^{1857}$ peptide contains the Leu → Val mutation at codon 1857 and contains the normally expressed 6 residues encoded by exon 46. The $\alpha 18\text{-}21^{1857\text{-}\Delta 46}$ peptide represents the second gene product of the α^{LELY} allele, ie, it has both the Leu → Val substitution at codon 1857 and it lacks the exon 46 sequence.

for high-affinity dimer assembly was recently demonstrated using a series of recombinant peptides that were carefully evaluated for proper polypeptide chain folding.[17] These analyses clearly showed that the actin binding domain was not essential for high-affinity dimer assembly, because the first two homologous β motifs bound to α-monomers with a kd of about 230 nmol/L and the first four homologous β motifs had a kd of about 10 nmol/L.

Deleterious α-alleles causing hereditary elliptocytosis (α^{HE} alleles) result in clinical conditions that vary from mild to severe. The clinical severity of a heterozygous elliptocytosis mutation is increased with the occurrence, in trans, of a common low expression polymorphism initially termed the $\alpha^{V/41}$ allele[18] and later renamed as the α^{LELY} allele.[19] The α^{LELY} polymorphism is characterized by three different nucleotide mutations: (1) a point mutation in exon 40 at codon 1857 (CTA → GTA; Leu → Val); (2) a mutation in intron 45 (−12, C → T); and (3) a mutation in intron 46 (−12, G → A). The latter mutation is sometimes also encountered in non-α^{LELY} alleles. In addition, the α^{LELY} allele is associated with partial posttranscriptional skipping (50%) of exon 46, which encodes 6 amino acid residues (residues 2177-2182). The molecular basis of this alternative splicing has not yet been defined. As a result of the 50% skipping of exon 46, two different α chain products are derived from the α^{LELY} allele in approximately equal amounts: α chains with the Val_{1857} mutation and the normal exon 46 sequence and α chains with the Val_{1857} mutation but missing the exon 46 sequence. Both α^{LELY} coding region mutations are located near or within the dimer initiation region; the Leu → Val mutation at residue 1857 is in the α18 motif, and the 6 residues encoded by exon 46 are in the α21 motif.

In this study, we evaluated properties of the two forms of the α chains produced by the α^{LELY} allele in comparison with normal α chains by using 4-motif recombinant peptides of the dimer initiation region. These results show that the conservative Leu → Val mutation at residue 1857 causes the increased trypsin cleavage at residue 1920 between the αIV and αV domains that was originally used to detect and monitor the occurrence of the α^{LELY} allele.[18] However, this mutation does not appear to otherwise affect polypeptide chain folding, stability of the monomer in solution, or dimer assembly. In contrast, deletion of the 6 residues in α21 encoded by exon 46 substantially decreases dimer binding affinity, increases susceptibility of the α21 motif to proteolysis, and increases the propensity of these α recombinant peptides to irreversibly self-aggregate. Hence, the 6 residues encoded by α-subunit exon 46 appear to be essential for maintaining α-chain stability and for proper dimer assembly. Little or no incorporation of the 50% of the α chains from the α^{LELY} allele that lack the exon 46 sequence (and contain the residue 1857 mutation) occurs in mature erythroid membranes, whereas the 50% of the α chains that have only the residue 1857 mutation appear to be incorporated into the membrane skeleton at a rate similar to normal α chains in individuals that are either heterozygotes or homozygotes for the α^{LELY} allele.

MATERIALS AND METHODS

Extraction of spectrin and purification of spectrin β-monomers. The procedure used for the extraction of spectrin from freshly drawn human donor blood has been previously described.[14]

Construction of plasmids encoding glutathione S-transferase (GST) fusion proteins. The following nomenclature will be used: α18-21, a normal α-peptide (residues 1818-2259); $\alpha 18\text{-}21^{1857}$, a peptide carrying the residue 1857 Leu → Val mutation; $\alpha 18\text{-}21^{1857\text{-}\Delta 46}$, a peptide carrying both the residue 1857 Leu → Val mutation and missing the six amino acids encoded by exon 46; β-monomer, normal β-chain; and $\beta 1\text{-}4^{+}$, a normal recombinant peptide (residues 293-743) containing the first four homologous motifs with an 8-residue N-terminal extension of the first motif relative to the common homologous motif phasing. This N-terminal extension, reflected by the ''+'' in the peptide designation, is critical for high-affinity dimerization with the α-chain, as previously described.[17] The relationships of these recombinant peptides to the overall motif structure of a spectrin dimer are shown in Fig 1. As discussed above, the α18-21^{1857} and α18-$21^{1857\text{-}\Delta 46}$ recombinant peptides represent the dimer initiation region of the two types of α chains produced from the α^{LELY} allele by alternative splicing of exon 46.

The α18-21^{1857} and α18-$21^{1857\text{-}\Delta 46}$ (residues 1814-2257) were obtained by reverse transcription-polymerase chain reaction (RT-PCR) from the mRNA of a $\alpha^{LELY}/\alpha^{LELY}$ homozygote (in all cases, residue numbering includes the exon 46-encoded amino acids). The primers used for RT-PCR were 5′ primer (GCAGATCTTGGAAGAATC-CCTAGAATAC) and 3′ primer (GCAGATCTCATTGCTCCA-GGTTGTGTTGCA). Both primers introduced a *Bgl* II restriction site flanking the desired coding region and the 3′ primer introduced a stop codon after codon 2257. These peptides were cloned into a pGEX-3X vector (Pharmacia, Uppsala, Sweden) using the *Bam*HI cloning site, because *Bam*HI and *Bgl* II produce compatible cohesive ends. The β1-4^+ peptide and the normal α18-21 plasmids, which were constructed using the pGEX-2T vector (Pharmacia), were previously described.[17] The integrity of all recombinant vectors was verified by DNA sequencing.

Purification of fusion peptides. Expression and purification of fusion peptides were performed as described,[20] with several modifications. An overnight culture was diluted (1/20) into 600 mL of LB medium containing 50 μg/mL ampicillin, grown at 30°C until the optical density was between 0.5 to 0.7 at 550 nm, and induced with 1 mmol/L (final concentration) of isopropyl-β-thiogalactopyranoside for an additional 3 to 4 hours. Bacteria were collected by low-speed centrifugation and the packed cells were stored at −80°C. The cell pellet was resuspended using 15 mL of lysis buffer (50 mmol/L Tris, 50 mmol/L NaCl, 5 mmol/L EDTA, 1 mmol/L diisopropyl fluorophosphate (DFP), 0.15 mmol/L phenylmethyl sulfonyl fluoride [PMSF], 1 μg/mL leupeptin, 1 μg/mL pepstatin, and 1% Triton X-100, pH 8.0) at 0°C and sonicated. After centrifugation of the lysate (48,000*g*), the supernatant was removed and the fusion peptides were isolated by affinity chromatography on an immobilized reduced glutathione column and eluted in G buffer (50 mmol/L Tris, 10 mmol/L reduced glutathione, pH 8.0). The purification of fusion proteins was monitored using Laemmli sodium dodecyl sulfate (SDS) gels[21] and by high-performance liquid chromatography (HPLC) gel filtration on two analytical (7.8 × 300 mm) TSK columns (G3000SW_{XL} + G2000SW_{XL} TosoHaas) in series at 0.8 mL/min in phosphate-buffered saline (PBS; 10 mmol/L sodium phosphate, 130 mmol/L NaCl, 1 mmol/L EDTA, 0.15 mmol/L PMSF, and 0.05% sodium azide, pH 7.4). The fusion protein GSTβ1-4^+ was further purified by concentration with a 30 K centriprep concentrator followed by repurification using HPLC gel filtration on two preparative (21.5 × 600 mm) TSK columns (G3000SW + G2000SW) in series (TosoHaas) in PBS.

Protease cleavage of purified fusion peptides. The purified fusion peptides were cleaved in G buffer using factor Xa at an enzyme-to-substrate ratio of 1:100 (α18-21^{1857} and α18-$21^{1857\text{-}\Delta}$) at 25°C for 4 hours or using thrombin (α18-21 and β1-4^+ peptides) at 37°C using conditions as previously described.[17] The proteases were inactivated by addition of PMSF (300 μmol/L final concentration). Protease cleavage of fusion proteins was monitored using SDS gels.[21]

Preparative purification of cleaved peptides. After factor Xa or thrombin cleavage, peptides were dialyzed into PBS and purified by rechromatography on glutathione columns to remove the GST moiety and uncleaved fusion proteins. The unbound peak containing the cleaved spectrin recombinant peptide was concentrated using a Centriprep concentrator (Amicon, Beverly, MA). Recombinant peptides were further purified by HPLC gel filtration on two preparative (21.5 × 600 mm) TSK columns (G3000SW + G2000SW) in series (TosoHaas) in PBS to remove improperly folded peptides, aggregates, proteolytic products, and residual GST. When necessary, pooled fractions containing recombinant peptide were concentrated using a Centriprep-30 Concentrator.

Purification of ^{35}S α18-21. Purification of ^{35}S α18-21 was performed essentially as described above with the modifications described below. An overnight culture was diluted 1/10 into 600 mL 1/5 LB medium containing 50 μg/mL ampicillin and grown until the optical density was between 0.5 to 0.7 at 550 nm. The cultures were then induced with 1 mmol/L (final concentration) of isopropyl-β- thiogalactopyranoside, and 1.5 mCi Pro-mix™ L-[^{35}S] in vivo cell labeling mix (Amersham) was added to the culture at the time of induction.

Tryptic digestion of recombinant peptides. Protease resistance of recombinant peptides was evaluated by treatment with trypsin at an enzyme-to-substrate ratio of 1:100 (wt/wt) at 0°C in PBS, pH 7.4. At time points of 0, 15, 30, 60, and 90 minutes, 4 μg of each recombinant protein was removed from the reaction for analysis on a Tris-Tricine gel, which was stained with Coomassie Brilliant Blue. Dilute crude spectrin (200 μg) was digested at an enzyme-to-substrate ratio of 1:20 in 20 mmol/L Tris/0.02% Azide/1 mmol/L 2-mercaptoethanol, pH 7.8, for 90 minutes at 0°C. The tryptic digestion of the recombinant peptide samples as well as the dilute crude spectrin samples were terminated by addition of 1 mmol/L DFP (final concentration). Samples were analyzed by two-dimensional (2D) gels as previously described.[22]

Analytical HPLC gel filtration binding assay. β-Spectrin or the β1-4^+ peptide was mixed with the purified recombinant α dimerization site peptides and incubated at 0°C for different times ranging from 5 minutes to 18 hours. Under most conditions, equilibrium was reached within 5 to 15 minutes; hence, a 25-minute incubation time was routinely used for most binding assays. Protein complexes were separated and quantitated on two analytical (7.8 × 300 mm) TSK-gel columns (G3000SW_{XL} + G2000SW_{XL}) at 4°C with a flow rate of 0.8 mL/min. Eluted proteins were detected by absorbance at 280 nm and were quantified on a data acquisition system (PE Nelson Analytical, Perkin Elmer, Norwalk, CT). Extinction coefficients at 280 nm were calculated from the amino acid sequence composition,[23] and these calculated values were in close agreement with values determined by quantitative amino acid analysis. HPLC peak height and peak area response factors for each protein were determined by replicate injections of known quantities for each component. Molecular weights used for calculating molarity were as follows: β-monomer, 246,000; GSTβ1-4^+, 79,113; β1-4^+, 52,964; α18-21, 51,938; α18-21^{1857}, 51,329; and α18-$21^{1857\text{-}\Delta 46}$, 50,662.

Competitive binding assays using GSTβ1-4^+. In multiple parallel experiments, 250 pmol of ^{35}S α18-21 and 250 pmol GSTβ1-4^+ were mixed with 0, 125, 250, 500, and 1,000 pmol of each competitor (α18-21, α18-21^{1857}, or α18-$21^{1857\text{-}\Delta 46}$) in a final volume of 400 μL in PBS/0.1% bovine serum albumin. Dimer complexes were allowed to reach equilibrium by incubation on ice for 30 minutes. Samples were then added to 100 μL of glutathione Sepharose 4B in a Millipore 0.22-μm filtration unit and incubated at 4°C with agitation for 1 hour. Samples were centrifuged and the filtrate was counted as the unbound fraction. The sedimented complexes were removed by 2 washes with 400 μL 0.2% SDS for 5 minutes at room temperature and centrifuged and the filtrate was counted as the bound fraction.

N-terminal sequence analysis. After separation by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), peptides were transferred onto high retention polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA) as previously described.[24] Membranes were stained with amido black, and the bands of interest were excised and sequenced on a Hewlett Packard G1005A sequencer (Palo Alto, CA) as previously described.[25]

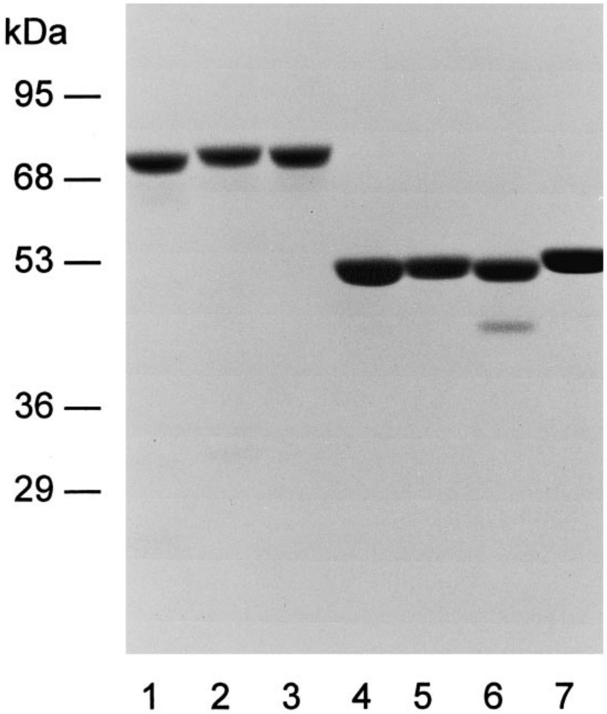

Fig 2. Purified α and β dimerization site peptides. A 10% Laemmli SDS gel stained with Coomassie Brilliant Blue is shown. The GST fusion proteins (2 μg/lane) are as follows: lanes 1 through 3, α18-21, α18-21^{1857}, and α18-21$^{1857\text{-}\Delta46}$, respectively. Recombinant peptides after protease cleavage, rechromatography on glutathione-Sepharose, and preparative HPLC gel filtration are as follows: lanes 4 through 7, α18-21, α18-21^{1857}, α18-21$^{1857\text{-}\Delta46}$, and β1-4^{+}.

Mass spectrometry. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry was performed on a PerSeptive Biosystems Vestec Mass Spectrometer using Voyager software (PerSeptive Biosystems Inc, Framingham, MA). Proteins were dialyzed into 10 mmol/L ammonium bicarbonate, pH 8.0, 1 μL of sample was mixed with 1 μL of matrix solution (saturated solution of α-cyano-4-hydroxycinnamic acid for samples <20 kD and sinapinic acid for samples >20 kD in 0.1% trifluoroacetic acid (TFA), 33% acetonitrile), and the sample-matrix mixture was transferred to the sample target, dried, and analyzed. Expected masses were calculated from known sequences using the GPMAW program (Lighthouse Data, Odense, Denmark).

RESULTS

Purification and characterization of soluble recombinant peptides. The effects of the α^{LELY} mutations on spectrin heterodimer assembly were evaluated by systematically comparing properties of a normal 4-motif recombinant peptide, α18-21, with two 4-motif recombinant peptides representing the two types of α chains produced from the α^{LELY} allele, α18-21^{1857} and α18-21$^{1857\text{-}\Delta46}$ (see Fig 1 and the Materials and Methods). As previously shown,[17] the normal α18-21 peptide is capable of initiating dimer assembly and can bind to either β monomers or a complementary 4-motif β peptide, β1-4^{+}, with very high affinity (kd ~10 nmol/L).

During the course of the present study, it was observed that all four recombinant peptides used here could be obtained in the soluble fraction by growing the bacterial cultures at 30°C instead of 37°C. This approach avoided denaturation and possible incorrect refolding of the recombinant peptides. Yields of the purified fusion proteins ranged from 10 to 50 mg/L when cultures were grown at 30°C, and the final yields after cleaving and removing the GST moiety ranged from 2 to 10 mg/L of original bacterial culture. The integrity of all purified recombinant peptides was confirmed by N-terminal sequencing and mass spectrometry.

The purified fusion proteins and cleaved, repurified recombinant spectrin peptides are shown after SDS-PAGE in Fig 2. All recombinant peptides were highly homogeneous, although α18-21$^{1857\text{-}\Delta46}$ preparations consistently showed a minor lower molecular weight band that represented about 5% of the total protein in the sample (Fig 2, lane 6). This minor band was transferred to a PVDF membrane and analyzed by N-terminal sequence analysis that showed the expected N-terminal sequence of the α18-21$^{1857\text{-}\Delta46}$ recombinant (GILEESLEYLQFMQN). MALDI mass spectrometry analysis showed a mass of 45,206 ± 45 Daltons for this fragment, indicating that the peptide terminated at Arg_{2210} (calculated mass of 45,224 Daltons). This proteolytic cleav-

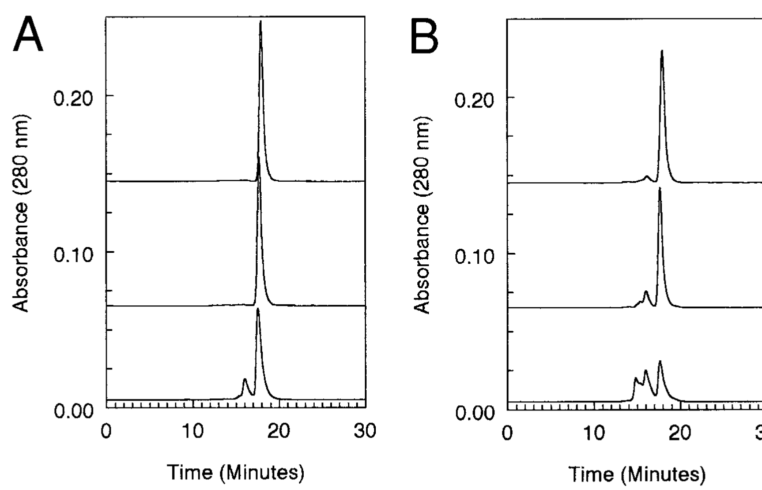

Fig 3. Analytical HPLC gel filtration of spectrin recombinant peptides. Chromatograms are shown at the same absorbance scale with baseline offsets for clarity. Fifty micrograms of each peptide was injected (A) immediately after purification and (B) after storage on ice for 10 days. Chromatograms from top to bottom are α18-21, α18-21^{1857}, and α18-21$^{1857\text{-}\Delta46}$.

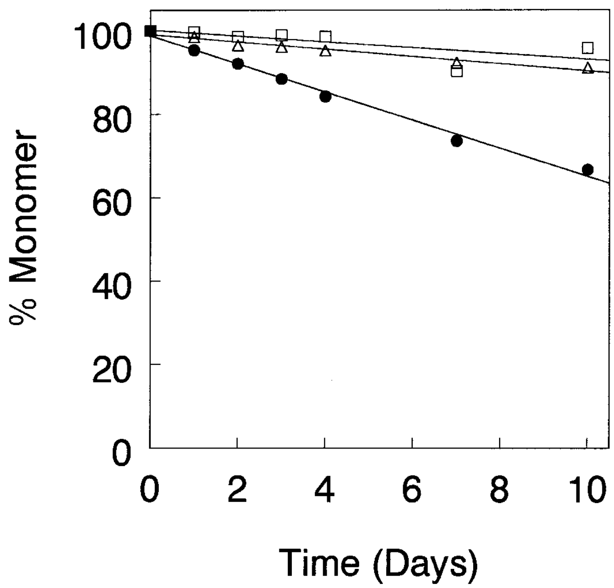

Fig 4. Time-dependent conversion of α dimerization site recombinant peptide monomers into self-aggregates. Recombinant peptide samples were concentrated with a 30K centriprep concentrator to a final concentration of 0.5 mg/mL and 50 μg of protein was immediately analyzed by HPLC gel filtration. The area of each monomer peak on day 0 was set equal to 100%. In parallel experiments, equal amounts of each α spectrin recombinant peptide was injected at intervals over the next 10 days and the amount of monomer in each sample was calculated relative to the amount of monomer observed for that protein at day 0. (□) α18-21; (△) $\alpha18\text{-}21^{1857}$; (●) $\alpha18\text{-}21^{1857\text{-}\Delta46}$.

age site is located near the position of the deleted 6 residues from exon 46. Production of this fragment is apparently caused by a conformational change induced by the exon 46 deletion, because the otherwise identical $\alpha18\text{-}21^{1857}$ peptide did not show detectable cleavage at this site when purified in parallel using identical conditions.

During initial attempts to purify the α^{LELY} recombinant peptides, it was noted that the $\alpha18\text{-}21^{1857\text{-}\Delta46}$ recombinant peptide self-aggregated to varying degrees under conditions in which the α18-21 and $\alpha18\text{-}21^{1857}$ peptides remained almost completely monomeric. In subsequent experiments, potential self-aggregation was minimized by keeping the concentrations of all three recombinants less than 1 mg/mL and by minimizing the length of time that the peptides were stored at 0°C before use. When the three recombinant peptides were purified in parallel under identical conditions as described in the Materials and Methods, HPLC gel filtration analysis performed immediately after purification showed that the α18-21 and $\alpha18\text{-}21^{1857}$ peptides were monomeric, with no detectable higher molecular weight aggregates, whereas the $\alpha18\text{-}21^{1857\text{-}\Delta46}$ peptide was 70% to 80% monomeric, with the remainder of the peptide eluting as a higher molecular weight self-aggregated complex (Fig 3A). Self-aggregation of purified $\alpha18\text{-}21^{1857\text{-}\Delta46}$ monomers continued to occur in a time and concentration dependent manner. When all three peptides were stored at 0°C for 10 days at 0.5 mg/mL, more than 50% of the $\alpha18\text{-}21^{1857\text{-}\Delta46}$ peptide had self-aggregated (Fig 3B). By comparison, α18-21 and the $\alpha18\text{-}21^{1857}$ were still greater than 90% monomeric. The $\alpha18\text{-}21^{1857\text{-}\Delta46}$ self-aggregates could not be significantly dissociated to monomers by dilution, treatment with high ionic strength buffers, or treatment with 5 mol/L urea followed by dialysis to remove the urea. Further time-dependent self-aggregation of the three recombinants after initial isolation (Fig 3A) was systematically analyzed by injecting 100 μL of each recombinant peptide (0.5 mg/mL) onto the HPLC gel filtration columns over a 10-day period. As shown in Fig 4, the α18-21 and the $\alpha18\text{-}21^{1857}$ monomeric peptides very slowly formed small amounts of large complexes under these conditions, whereas the monomeric fraction of the $\alpha18\text{-}21^{1857\text{-}\Delta46}$ sample continued to be converted into aggregates. Therefore, all subsequent experiments were performed within a few days after protein purification, and protein amounts used in binding experiments were based on monomer concentration for all three recombinants as determined by the area of the monomer peak in HPLC gel filtration analyses run on the same day as the binding experiment.

Tryptic digestion of recombinant peptides. A mild trypsin digestion time course was used to evaluate the protease resistance of the normal α18-21 and two $\alpha18\text{-}21^{LELY}$ peptides (Fig 5). Under these conditions, the α18-21 peptide was partially cleaved to produce a band that migrated on SDS gels as an apparent 41-kD peptide. N-terminal sequence analysis of this peptide produced the sequence LQLEDDYAFQ, which is identical to the N-terminal sequence of the 41-kD αV domain produced by mild trypsin digestion of spectrin dimers.[8] The partial trypsin cleavage of the α18-21 from the intact 52-kD recombinant peptide to a 41-kD peptide at the normal αIV-αV site closely resembles the proteolytic susceptibility of this site in intact normal spectrin, because mild trypsin digestion of normal spectrin incompletely cleaves the αIV-V site with moderate yields of the αIV and αV domain peptides. When the $\alpha18\text{-}21^{1857}$ peptide is digested with trypsin (Fig 5, lanes 7 through 10), it is very rapidly and completely cleaved at the αIV-V site analogous to the more

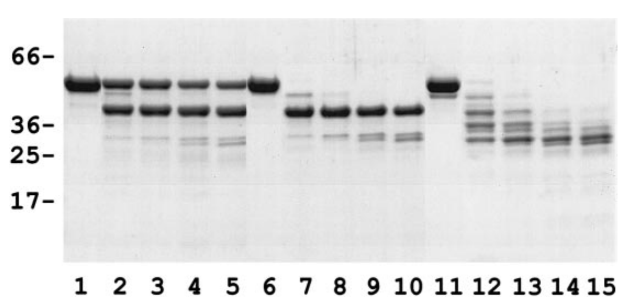

Fig 5. Trypsin digestion time course of α18-21, $\alpha18\text{-}21^{1857}$, and $\alpha18\text{-}21^{1857\text{-}\Delta46}$. Recombinant α spectrin samples were digested with trypsin at an enzyme-to-substrate ratio of 1:100 at 0°C in PBS buffer. Protein samples (4 μg/lane) were analyzed on a 12% Tris-Tricine gel and stained with Coomassie Brilliant Blue. Lanes 1 through 5, α18-21 after 0, 15, 30, 60, and 90 minutes of treatment with trypsin, respectively; lanes 6 through 10, $\alpha18\text{-}21^{1857}$ after 0, 15, 30, 60, and 90 minutes of treatment with trypsin, respectively; lanes 11 through 15, $\alpha18\text{-}21^{1857\text{-}\Delta46}$ after 0, 15, 30, 60, and 90 minutes of treatment with trypsin, respectively.

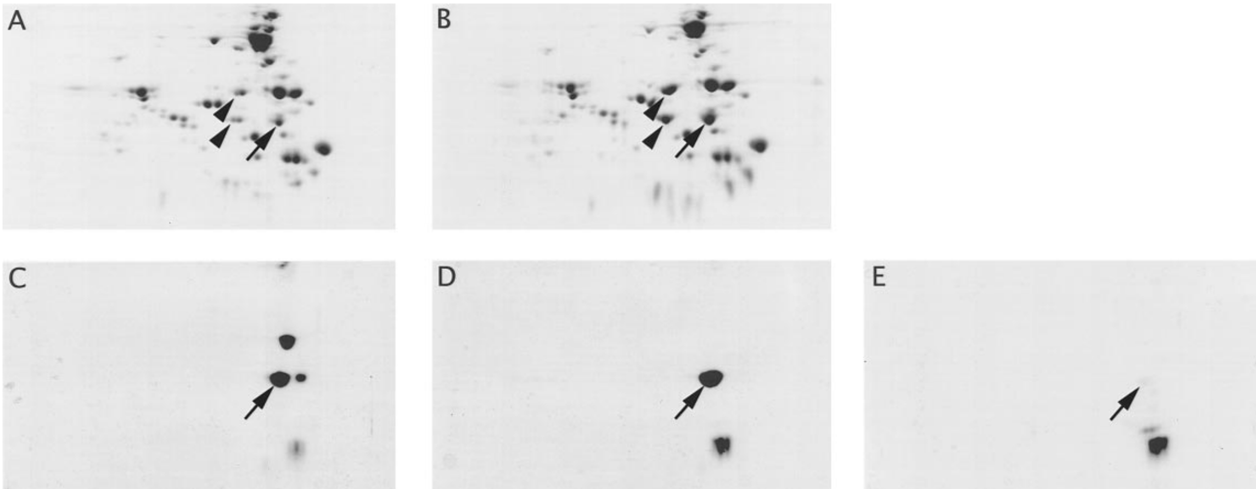

Fig 6. 2D gels of tryptic peptides from spectrin dimers and α dimerization site recombinant peptides. Isofocusing was in the horizontal direction (basic side on the left) followed by separation on a 12% SDS gel that was stained with Coomassie Brilliant Blue. (A) Two hundred micrograms of spectrin digested with trypsin from a normal donor; (B) 200 μg of spectrin digested with trypsin from a donor homozygous for the α^{LELY} mutation; (C through E) 20 μg of each recombinant spectrin peptide digested with trypsin. (C) α18-21; (D) α18-21^{1857}; (E) α18-$21^{1857\text{-}\Delta46}$. Arrows indicate the position of the normal αV 41-kD tryptic domain in all panels. The arrowheads indicate the major αIV domain peptides in (A) and (B).

efficient cleavage of intact spectrin from the α^{LELY} allele that was originally used to identify this polymorphism.[18] In contrast, the α18-$21^{1857\text{-}\Delta46}$ recombinant peptide was highly unstable and was quickly degraded into smaller peptides. Based on mapping of several proteolytic fragments using 2D gels and N-terminal sequence analysis, it appears that these fragments were due to both the increased proteolysis at the αIV-V site caused by the Val_{1857} mutation and extensive proteolysis at the C-terminal region of the peptide, primarily in the α21 motif.

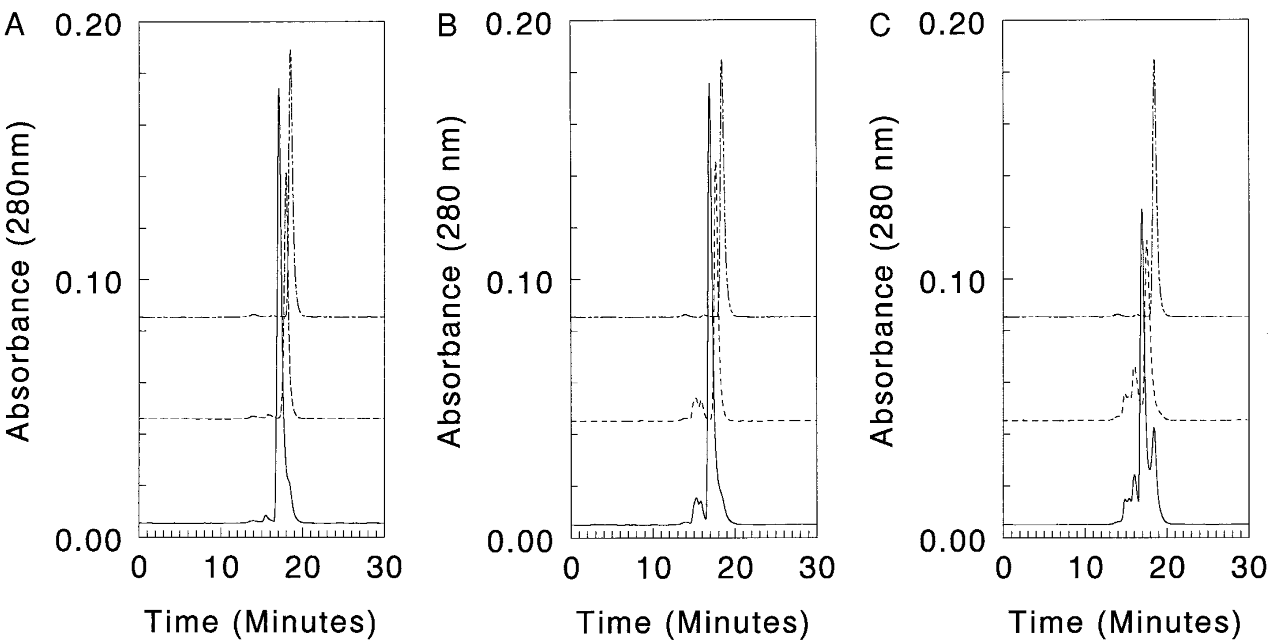

Fig 7. Dimerization of α and β peptides. Chromatograms are shown at the same scale with baseline offsets for clarity. (A) (– -- –) 1,000 pmol β1-4^+ recombinant peptide; (---) 1,000 pmol α 18-21 recombinant peptide; (—) 1,000 pmol each of β1-4^+ and α18-21 incubated on ice 25 minutes before gel filtration. (B) (– -- –) 1,000 pmol β1-4^+ recombinant peptide; (---) 1,000 pmol α18-21^{1857} peptide; (—) 1,000 pmoles each of β1-4^+ and α18-21^{1857} incubated on ice 25 minutes before gel filtration. (C) (– -- –) 1,000 pmol β1-4^+ recombinant peptide; (---) 1,000 pmol of a 18-$21^{1857\text{-}\Delta46}$; (—) 1,000 pmol each of β1-4^+ and a 18-$21^{1857\text{-}\Delta46}$ incubated on ice 25 minutes before gel filtration. All quantities are based on the amount of monomer species present in the sample at the time of the experiment.

The relationships between tryptic fragments of intact spectrin and the recombinant peptides are also shown in Fig 6 using 2D gels. The position of the 41-kD αV domain peptide is shown by an arrow in all panels. The superposition of the tryptic 41-kD peptides produced from α18-21 and α18-21^{1857} with the positions of the tryptic 41-kD peptides from digestions of normal spectrin and spectrin from an α^{LELY} donor were verified by mixing digested recombinant peptide and spectrin samples before 2D gel analysis (data not shown).

Heterodimer assembly of monomeric α^{LELY} recombinant peptides. Dimer binding assays of the three α dimerization site peptides with the β1-4$^+$ peptide were performed in parallel to evaluate their ability to form heterodimeric complexes. Representative results using equimolar amounts of α and β peptides are shown in Fig 7. As expected based on previous studies,[17] the normal α18-21 and β1-4$^+$ recombinant peptides formed a high-affinity complex with nearly all of the individual peptides forming an earlier eluting heterodimer complex (Fig 7A). The α18-21^{1857} peptide exhibited similar strong binding affinity for the β1-4$^+$ peptide (Fig 7B). In contrast, when equimolar amounts (based on monomer concentration) of α18-21$^{1857\text{-}\Delta46}$ were mixed with β1-4$^+$ as shown in Fig 7C, a substantial amount of the β1-4$^+$ peptide was not incorporated into the dimer complex peak (compare peak height of top chromatogram with the corresponding peak in bottom chromatogram). In this experiment, the unbound monomeric α18-21$^{1857\text{-}\Delta46}$ peptide elutes between the complex and the unbound β1-4$^+$ peptide and is not resolved. There was no detectable change in the peak heights or areas of the early eluting self-aggregate peptides and no β1-4$^+$ peptide was detected by SDS gel analysis of these peaks, indicating that the α18-21$^{1857\text{-}\Delta46}$ aggregates could not participate in dimerization.

Competitive binding assays using GSTβ1-4$^+$ fusion protein as the complementary binding partner. The ability of the three recombinant peptides (α18-21, α18-21^{1857}, and α18-21$^{1857\text{-}\Delta46}$) to form heterodimer complexes was further explored by evaluating their capability to compete with ^{35}S-α18-21 for binding to GSTβ1-4$^+$ (Fig 8). The α18-21^{1857} recombinant was as effective a competitor as unlabeled α18-21. In contrast, the α18-21$^{1857\text{-}\Delta46}$ peptide was a less effective competitor than the other two recombinant peptides in this assay.

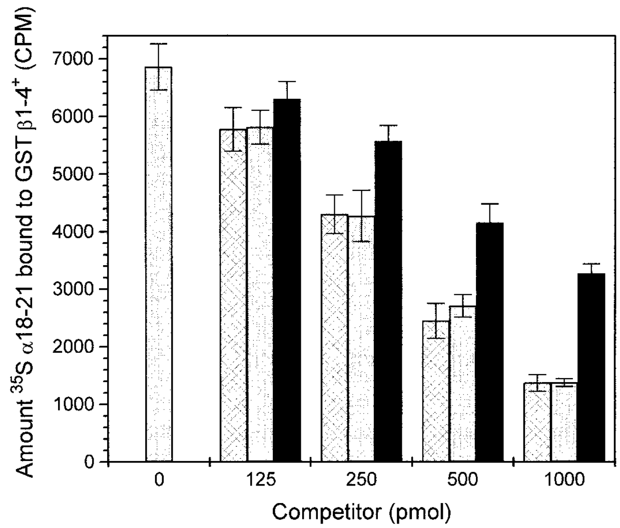

Fig 8. Competitive binding of α18-21, α18-21^{1857}, or α18-21$^{1857\text{-}\Delta46}$ to GSTβ1-4$^+$. In each assay, 250 pmol of ^{35}S α18-21 and 250 pmol of GSTβ1-4$^+$ were mixed with 0, 125, 250, 500, and 1,000 pmol of each competitor (α18-21, α18-21^{1857}, or α18-21$^{1857\text{-}\Delta46}$) in a final volume of 400 μL in PBS/0.1% bovine serum albumin for 30 minutes on ice. Bound complexes were sedimented with glutathione Sepharose 4B in a Millipore 0.22-μm filtration unit and the filtrate was counted as the unbound fraction. The resin was washed twice and bound complexes were eluted with two aliqouts of 400 μL 0.2% SDS. Background corrected values for the amount of ^{35}S α18-21 from three different experiments were averaged (error bars show standard deviation). (⊠) α18-21; (□) α18-21^{1857}; (■) α18-21$^{1857\text{-}\Delta46}$.

DISCUSSION

In a previous study,[19] we hypothesized that the low incorporation of the α^{LELY} allele-derived α chains into dimers in vivo was related to the partial skipping of exon 46 in 50% of the transcripts. These α-chains, lacking the six amino acids encoded by exon 46 and located in helix A of the α21 motif, were thought to be unable to undergo the dimer initiation process and therefore would be degraded. In this model, the loss of half the α chains from one allele in a heterozygote or even half the α chains from both alleles in α^{LELY} homozygotes would be expected to be a neutral polymorphism under normal circumstances, because an excess of α chains is usually synthesized.[26-28] However, this polymorphism influences the clinical expression of α^{HE} alleles occurring in trans. Because of the reduced ability to form dimers, α chains from the α^{LELY} allele are underrepresented in dimers and subsequently in the mature RBC membrane. Consequently, an α^{HE} mutation on the same allele as the α^{LELY} polymorphism would be underrepresented on the mature membrane,[29] whereas a α^{HE} mutation on the opposite allele from the α^{LELY} polymorphism would be overrepresented on the mature membrane skeleton.[18]

In this study, we evaluated the effects of the two structural mutations associated with the α^{LELY} polymorphism on dimer assembly using recombinant peptides. The conservative Leu → Val mutation at residue 1857 as represented by the α18-21^{1857} recombinant peptide does not appreciably affect dimer assembly. This implies that the 50% of the α chains derived from the α^{LELY} allele, which contain this mutation and also contain the exon 46 encoded residues, should assemble normally into dimers and therefore onto the cell membrane. These observations are consistent with the observation that α^{LELY} homozygotes, in which 100% of the α chains produced contain the residue 1857 mutation, have normal membrane stability. In addition, mild trypsin treatment of the normal α18-21 and the two α^{LELY} recombinant α dimerization site peptides shows that the residue 1857 mutation is responsible for the observed increased protease sensitivity of α chains from the α^{LELY} allele at the αIV-V junction.

The recombinant peptide α18-21$^{1857\text{-}\Delta46}$, which has both the residue 1857 substitution and lacks the exon 46 encoded 6 residues in the α21 motif, represents the other half of the α chains produced from the α^{LELY} allele. This peptide exhibited three features that distinguish it from the normal α recombinant or the other product of the α^{LELY} allele (α18-21^{1857}), namely decreased dimer binding affinity, increased sensitivity to proteolysis, and an increased propensity for forming self-aggregates. Any of these three properties of the recombinant peptide in vitro could be expected to interfere with the in vivo assembly of full-length α chains lacking the 6 residue exon 46 encoded sequence. Regardless of the relative contributions of these three potentially important properties, it is apparent that α chains lacking this 6 residue sequence are not incorporated into mature RBC membrane skeletons in any appreciable amount. The absence of appreciable amounts of exon 46^{-} α chains in RBCs is supported by several lines of evidence from analysis of mild tryptic peptide patterns (Fig 6). First, inspection of the mild trypsin digestion of spectrin isolated from an α^{LELY} homozygote (Fig 6B) shows a proportional increase in the αIV domain peptides (arrowheads) and the 41-kD αV domain peptide (arrow). Because the 41-kD peptide is not protease resistant when the exon 46 encoded sequence is deleted (Figs 5 and 6), the presence of an appreciable amount of exon 46^{-} α chains on the membrane of an α^{LELY} homozygote would be expected to result in a decreased yield of the αV 41-kD peptide relative to the αIV domain peptide rather than the observed proportional increase. In addition, when the α18-$21^{1857\text{-}\Delta 46}$ recombinant peptide was digested with trypsin, a series of unique intermediate-sized peptides were observed that had the expected 41-kD peptide N-terminal sequence, indicating that they were produced by proteolysis within the α21 motif (Fig 5). However, these unique exon 46^{-} related fragments could not be detected on spectrin digests from an α^{LELY} homozygote, even when gels were overloaded to emphasize minor components. Although the possible presence of a small amount, perhaps up to 10%, of exon 46^{-} α chains might not be detected by these methods, these results do support the conclusion that the 50% of the α chains from the α^{LELY} allele that lack the exon 46 sequence are not appreciably incorporated into RBC membranes.

It is not surprising that a 6 residue deletion in the α21 motif prevents incorporation of α chains with this mutation into stable heterodimers on the mature RBC membrane. As shown previously,[14,17] the α21 motif is part of the minimum region required for initiating spectrin dimerization and mutations in this region might reasonably be expected to affect efficiency of dimerization. In addition, the strong conservation in length of most spectrin motif units indicates that the lengths of motifs impart important structural characteristics to the spectrin molecule. Any mutation that affects the length of a motif would therefore be expected to disrupt polypeptide chain folding. A number of reported low expression spectrin variants with small deletions, which are also associated with elliptocytosis, support this conclusion (for review, see Lux and Palek[30]). Some examples include spectrin Oran ($\alpha^{II/21}$), which is missing amino acids 822 to 862 (helix B of the α8 motif), and the $\alpha^{I/36}$ spectrin Sfax variant, which has a nine amino acid deletion in helix C of the α4 motif (amino acids 363-371).

It is interesting to compare the exon 46 encoded deletion of 6 residues in α21 with pathogenic mutations of spectrin that usually disrupt tetramer assembly. Many of the pathogenic tetramer binding site mutations are located in either the α0 or β17 partial motifs, which form the tetramerization binding site, and most of these mutations are relatively conservative single amino acid mutations.[22] In comparison, a 6 residue deletion in the middle of helix A, as occurs with the 50% of the α chains from the α^{LELY} allele that lack the exon 46 sequence, would be expected to more severely affect polypeptide chain folding and any associated function. Hence, it is not surprising that this mutation perturbs both dimerization and polypeptide stability as reflected by resistance to proteolysis and self-aggregation. The lack of appreciable incorporation of exon 46^{-} α chains into mature RBC membranes is probably due to both decreased dimerization affinity and decreased polypeptide stability, because the reduced dimer binding affinity of the α18-$21^{1857\text{-}\Delta 46}$ peptide by itself does not appear to be sufficient to prevent detectable incorporation of some exon 46^{-} chains into dimers.

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