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

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

Human erythroid α-spectrin alleles responsible for hereditary elliptocytosis (αHE alleles) undergo increased incorporation into red blood cell membranes when the polymorphism αLELY (LELY: Low Expression L(Yon)) occurs in trans. The αLELY 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 (Wilmutte 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 αLELY allele 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 αLELY allele were represented by α18-211857α, a peptide with the codon 1857 mutation and retaining the exon 46 encoded sequence, and α18-211857β-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 αLELY (SpαIV/IV) 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 α18-211857-αLELY 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 αLELY homozygote and the two αLELY recombinant peptides strongly suggests that little, if any, of the 50% of the α chains from the αLELY allele that contain the exon 46 deletion are incorporated into the mature erythroid membrane. Based on the in vitro analysis of recombinant αLELY 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.

© 1997 by The American Society of Hematology.

SPECTRIN IS THE major protein of the red blood cell (RBC) membrane skeleton. Its physiologic form is a fibrillar αβ tetramer in which two αβ 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 1q23-q24-2,5 and contains 36 exons,6 although only 32 exons are expressed in the RBC. Spectrin αβ 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.9 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.1,4 The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. section 1734 solely to indicate this fact. © 1997 by The American Society of Hematology. 0006-4971/97/9010-0021$3.00/0

From the Laboratoire de Génétique Moléculaire Humaine, CNRS URA 1171, Institut Pasteur de Lyon, Lyon, France; and The Wistar Institute, Philadelphia, PA.

Submitted June 24, 1996; accepted July 9, 1997.

Supported by the “Centre National de la Recherche Scientifique” (URA 1171 and PICS 221, a joint program with the “Ministère des Affaires Etrangères,” Paris, France and the Ministry of Education [Monbusho], Tokyo, Japan), the “Université Claude-Bernard Lyon 1” (CRE 930405), the “Conseil de la Région Rhône-Alpes,” the “Fondation pour la Recherche Médicale,” the “Association de Recherche contre le Cancer,” the “Institut Pasteur de Lyon,” and the “Programme Hospitalier de Recherche Clinique (1995-1997).” Also supported by National Institutes of Health Grants NO. HL63794 (to D.W.S.) and HD08840 (to J.A.U.) and by partial support from National Cancer Institute Cancer Core Grant NO. CA10815.

Address reprint requests to David W. Speicher, PhD, The Wistar Institute, 3601 Spruce St, Philadelphia, PA 19104.
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 α21 recombinant peptide are shown. The α21-1857 peptide contains the Leu → Val mutation at codon 1857 and contains the normally expressed 6 residues encoded by exon 46. The α21-1857-46 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^{HIE} 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 α^{4741} allele18 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 Val1857 mutation and the normal exon 46 sequence and α chains with the Val1857 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); α18-21^{1857}, a peptide carrying the residue 1857 Leu → Val mutation; α18-21-1857-46 a peptide carrying both the residue 1857 Leu → Val mutation and the six amino acids encoded by exon 46; β1-4+, 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.15 The relationships of these recombinant peptides to the overall motif structure of a spectrin dimer are shown in Fig 1. As discussed above, the a18-211857 and a18-211857-246 recombinant peptides represent the dimer initiation region of the two types of α chains produced from the a18-21 allele by alternative splicing of exon 46.

The a18-211857 and a18-211857-246 (residues 1814-2257) were obtained by reverse transcription-polymerase chain reaction (RT-PCR) from the mRNA of a a18-21 plasmids, which were constructed using the pGEX-2T vector (Pharmacia). BamHI cloning site, because BamHI and Bgl II produce compatible cohesive ends. The β1-4′ peptide and the normal a18-21 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,22 with several modifications. An overnight culture was diluted (1/20) to 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) gels21 and by high-performance liquid chromatography (HPLC) gel filtration on two analytical (7.8 × 300 mm) TSK columns (G3000SWXL + G2000SWXL, 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 centricon preparator 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 (a18-211857 and a18-211857-246) at 25°C for 4 hours or using thrombin (a18-21 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 Centricon 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 Centricon-30 Concentrator.

Purification of 35S a18-21. Purification of 35S a18-21 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-35S 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, and 60 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/100 mmol/L 2-mercaptopethanol, 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 (2-D) 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 (G3000SWXL + G2000SWXL) at 4°C with a flow rate of 0.8 mL/min. Eluted proteins were detected by absorbance at 280 nm and were quantitated 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; a18-21, 51,938; a18-211857, 51,329; and a18-211857-246, 60,662.

Competitive binding assays using GSTβ1-4′. In multiple parallel experiments, 250 pmol of 35S a18-21 and 250 pmol GSTβ1-4′ were mixed with 0, 125, 250, 500, and 1,000 pmol of each competitor (a18-21, a18-211857, or a18-211857-246) 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
SPECTRIN aLELY POLYMORPHISM

RESULTS

Purification and characterization of soluble recombinant peptides. The effects of the aLELY mutations on spectrin heterodimer assembly were evaluated by systematically comparing properties of a normal 4-motif recombinant peptide, a18-21, with two 4-motif recombinant peptides representing the two types of a chains produced from the aLELY allele, a18-211857 and a18-211857-D46 (see Fig 1 and the Materials and Methods). As previously shown,37 the normal a18-21 peptide is capable of initiating dimer assembly and can bind to either b monomers or a complementary 4-motif b peptide, b1-4', 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 a18-211857-D46 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 a18-211857-D46 recombinant (GILEESELYLQFMQN). MALDI mass spectrometry analysis showed a mass of 45,206 ± 45 Daltons for this fragment, indicating that the peptide terminated at Arg220 (calculated mass of 45,224 Daltons). This proteolytic cleav-

---

Fig 2. Purified a and b 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, a18-21, a18-211857, and a18-211857-D46, respectively. Recombinant peptides after protease cleavage, rechromatography on glutathione-Sepharose, and preparative HPLC gel filtration are as follows: lanes 4 through 7, a18-21, a18-211857, a18-211857-D46, and b1-4'.

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 a-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).

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 a18-21, a18-211857, and a18-211857-D46.
partially cleaved to produce a band that migrated on SDS analysis performed immediately after purification showed described in the Materials and Methods, HPLC gel filtration AID Blood 0039 / 5h41$$$761 09-25-97 12:18:04 blda WBS: Blood 0.5 mg/mL, more than 50% of the a when all three peptides were stored at 0°C, more than 90% of the monomer resistance of the normal spectrin recombinant peptide was injected at an enzyme-to-substrate ratio of 1:100 at 0°C for 10 days at 4°C. Normal 52-kD recombinant peptide, to a 41-kD peptide at the D 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 analogous to the more 18-21 1857 peptides were still greater than 90% monomeric. The α18-21 1857Δ46 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 α18-21 1857 monomeric peptides very slowly formed small amounts of large complexes under these conditions, whereas the monomeric fraction of the α18-21 1857Δ46 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 α18-21LELY 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. 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-αV domain peptides. When the α18-21 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 α18-21LELY recombinant peptides. It was noted that the α18-21 1857 1857-Δ46 recombinant peptide self-aggregated to varying degrees under conditions in which the α18-21 and α18-21 1857-Δ46 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 α18-21 1857 peptides were monomeric, with no detectable higher molecular weight aggregates, whereas the α18-21 1857Δ46 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 α18-21 1857-Δ46 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 α18-21 1857-Δ46 peptide had self-aggregated (Fig 3B). By comparison, α18-21 and the α18-21 1857 were still greater than 90% monomeric. The α18-21 1857Δ46 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 α18-21 1857 monomeric peptides very slowly formed small amounts of large complexes under these conditions, whereas the monomeric fraction of the α18-21 1857Δ46 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.

![Figure 4](image-url) 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; (△) α18-21 1857; (●) α18-21 1857Δ46.

![Figure 5](image-url) Trypsin digestion time course of α18-21, α18-21 1857, and α18-21 1857Δ46. 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, α18-21 1857 after 0, 15, 30, 60, and 90 minutes of treatment with trypsin, respectively; lanes 11 through 15, α18-21 1857Δ46 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<sup>LELY</sup> mutation; (C through E) 20 μg of each recombinant spectrin peptide digested with trypsin. (C) α18-21; (D) α18-21<sup>1857</sup>-D<sup>46</sup>; (E) α18-21<sup>1857</sup>-D<sup>46</sup>. 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<sup>LELY</sup> allele that was originally used to identify this polymorphism. In contrast, the α18-21<sup>1857</sup>-D<sup>46</sup> 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<sub>1857</sub> 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<sup>1</sup> recombinant peptide; (-----) 1,000 pmol α 18-21 recombinant peptide; (-----) 1,000 pmol each of β1-4<sup>1</sup> and α18-21 incubated on ice 25 minutes before gel filtration. (B) (-----) 1,000 pmol β1-4<sup>1</sup> recombinant peptide; (-----) 1,000 pmol α18-21<sup>1857</sup> peptide; (-----) 1,000 pmol each of β1-4<sup>1</sup> and α18-21 incubated on ice 25 minutes before gel filtration. (C) (-----) 1,000 pmol each of β1-4<sup>1</sup> and α18-21<sup>1857</sup>-D<sup>46</sup>; (-----) 1,000 pmol each of β1-4<sup>1</sup> and a 18-21<sup>1857</sup>-D<sup>46</sup> 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 α18-21β1857 with the positions of the tryptic 41-kD peptides from digests of normal spectrin and spectrin from an αLELY donor were verified by mixing digested recombinant peptide and spectrin samples before 2D gel analysis (data not shown).

Heterodimer assembly of monomeric αLELY 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 α18-21β1857 peptide exhibited similar strong binding affinity for the β1-4+ peptide (Fig 7B). In contrast, when equimolar amounts (based on monomer concentration) of α18-21β1857β46 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 α18-21β1857β46 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 α18-21β1857β46 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, α18-21β1857, and α18-21β1857β46) to form heterodimer complexes was further explored by evaluating their capability to compete with [35S]α18-21 for binding to GSTβ1-4+ (Fig 8). The α18-21β1857 recombinant was as effective a competitor as unlabeled α18-21. In contrast, the α18-21β1857β46 peptide was a less effective competitor than the other two recombinant peptides in this assay.

DISCUSSION

In a previous study,19 we hypothesized that the low incorporation of the αLELY 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 αLELY 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 α18-21 alleles occurring in trans. Because of the reduced ability to form dimers, α chains from the αLELY allele are underrepresented in dimers and subsequently in the mature RBC membrane. Consequently, an αHE mutation on the opposite allele from the αLELY polymorphism would be overrepresented on the mature membrane, whereas a αHE mutation on the opposite allele from the αLELY polymorphism would be underrepresented on the mature membrane skeleton.29

In this study, we evaluated the effects of the two structural mutations associated with the αLELY polymorphism on dimer assembly using recombinant peptides. The conservative Leu → Val mutation at residue 1857 as represented by the α18-21β1857 recombinant peptide does not appreciably affect dimer assembly. This implies that the 50% of the α chains derived from the αLELY 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 αLELY 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 αLELY recombinant α dimerization site peptides shows that the residue 1857 mutation is responsible for the observed increased protease sensitivity of α chains from the αLELY allele at the αIV-V junction.

The recombinant peptide α18-21β1857β46, 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 αLELY allele. This peptide

---

Fig 8. Competitive binding of α18-21, α18-21β1857, or α18-21β1857β46 to GSTβ1-4+. In each assay, 250 pmol of [35S]α18-21 and 250 pmol of GSTβ1-4+ were mixed with 0, 125, 250, 500, and 1,000 pmol of each competitor (α18-21, α18-21β1857, or α18-21β1857β46) 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 aliquots of 400 μL 0.2% SDS. Background corrected values for the amount of [35S]α18-21 from three different experiments were averaged (error bars show standard deviation). (□) α18-21; (■) α18-21β1857; (■) α18-21β1857β46.
exhibited three features that distinguish it from the normal \( \alpha \) recombinant or the other product of the \( \alpha^{LELY} \) allele (\( \alpha^{18-21^{655}} \)), 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 \( \alpha \) chains lacking the 6 residue exon 46 encoded sequence. Regardless of the relative contributions of these three potentially important properties, it is apparent that \( \alpha \) 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 \( \alpha \) chains in RBCs is supported by several lines of evidence from analysis of mild trypsin peptide patterns (Fig 6). First, inspection of the mild trypsin digestion of spectrin isolated from an \( \alpha^{LELY} \) homozygote (Fig 6B) shows a proportional increase in the \( \alpha IV \) domain peptides (arrowheads) and the 41-kD \( \alpha V \) 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 \( \alpha \) chains on the membrane of an \( \alpha^{LELY} \) homozygote would be expected to result in a decreased yield of the \( \alpha V \) 41-kD peptide relative to the \( \alpha IV \) domain peptide rather than the observed proportional increase. In addition, when the \( \alpha^{18-21^{655-246}} \) 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 \( \alpha 21 \) motif (Fig 5). However, these unique exon 46 related fragments could not be detected on spectrin digests from an \( \alpha^{LELY} \) 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 \( \alpha \) chains might not be detected by these methods, these results do support the conclusion that the 50\% of the \( \alpha \) chains from the \( \alpha^{LELY} \) 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 \( \alpha 21 \) motif prevents incorporation of \( \alpha \) chains with this mutation into stable heterodimers on the mature RBC membrane. As shown previously, 14,17 the \( \alpha 21 \) 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 (\( \alpha^{221} \)), which is missing amino acids 822 to 862 (helix B of the \( \alpha 8 \) motif), and the \( \alpha^{256} \) spectrin Sfax variant, which has a nine amino acid deletion in helix C of the \( \alpha 4 \) motif (amino acids 363-371).

It is interesting to compare the exon 46 encoded deletion of 6 residues in \( \alpha 21 \) with pathogenic mutations of spectrin that usually disrupt tetramer assembly. Many of the pathogenic tetramer binding site mutations are located in either the \( \alpha 0 \) or \( \beta 17 \) 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 \( \alpha \) chains from the \( \alpha^{LELY} \) 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 \( \alpha \) 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 \( \alpha^{18-21^{655-246}} \) peptide by itself does not appear to be sufficient to prevent detectable incorporation of some exon 46 \( \alpha \) chains into dimers.

ACKNOWLEDGMENT

The authors thank Peter Hembach, Tara DeSilva, and Curtis Lawrence for excellent technical assistance, David Reim for performing the sequence analyses, Kaye Speicher for mass analyses, and N. Connon for preparing the manuscript.

REFERENCES

8. Speicher DW, Marchesi VT: Erythrocye spectrin is comprised of many homologous triple helical segments. Nature 311:177, 1984
12. Sears DE, Marchesi VT, Morrow JS: A calmodulin and \( \alpha \)-
subunit binding domain in human erythrocyte spectrin. Biochim Biophys Acta 870:432, 1986
The Exon 46-Encoded Sequence Is Essential for Stability of Human Erythroid α-Spectrin and Heterodimer Formation

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