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MOLECULAR EPITOPES OF THE ANKYRIN-SPECTRIN INTERACTION

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Abstract

Isoforms of ankyrin and its binding partner spectrin are responsible for a number of interactions in a variety of human cells. Conflicting evidence, however, had identified two different, nonoverlapping human erythroid ankyrin subdomains—Zu5 and 272—as the minimum binding region for β -spectrin. Complementary studies on the ankyrin binding domain of spectrin have been somewhat more conclusive, yet have not presented binding in terms of well-phased, integral numbers of spectrin repeats. Thus, the objective of this study was to clearly define and characterize the minimal ankyrin-spectrin binding epitopes. CD wavelength spectra of the aforementioned ankyrin subdomains show that these fragments are 30–60% unstructured. In contrast, human erythroid β -spectrin repeats 13, 14, 15, and 16 (prepared in all combinations of two adjacent repeats) demonstrated proper folding and stability as determined by CD and tryptophan wavelength and heat denaturation scans. Native PAGE gel shifts as well as affinity pull-down assays implicated Zu5 and β -spectrin repeats 14–15 as the minimum binding epitopes. These results were confirmed by analytical ultracentrifugation to sedimentation equilibrium by which a 1:1 complex was obtained if and only if Zu5 was mixed with β -spectrin constructs containing repeats 14 and 15 in tandem. Surface plasmon resonance yielded a K_D of 15.2 nM for binding of β -spectrin fragments to the ankyrin subdomain Zu5, accounting for all of the binding observed between the intact molecules. Collectively, these results show the 14th and 15th β -spectrin repeats comprise the minimal, phased region of β -spectrin which binds ankyrin at the Zu5 subdomain with high affinity.

Stress on the human red cell membrane, particularly during passage of the red cell through capillaries, is relieved by transmission to the underlying skeletal protein network (1). As a result, inter-protein connections between the membrane and the skeletal network, termed “vertical” (2)—as opposed to inter-protein connections within the skeletal network, termed “horizontal” (2)—are crucial to cell deformability and integrity. The vertical connections

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SUPPORTING INFORMATION AVAILABLE

Supplemental Experimental Methods

Supplemental Results

Supplemental Discussion

Supplemental Figure S1: Prediction of Intrinsic Disorder in Zu5 and 272.

Supplemental Figure S2: HSQC Spectrum of ¹⁵N-labeled Zu5 Reveals a Surprisingly Well- Folded Protein.

Supplemental Figure S3: Nickel Pull-down Assay with His-tagged Zu5, but not His-tagged 272, Indicates Binding to HE β 1415.

Supplemental Figure S4: HE β 1314, 1415 and 1516 are stably folded on Heat Denaturation Monitored by CD.

Supplemental Figure S5: Gel Shift Assay with Zu5 Implicates HE β 1415 as Minimal Binding Repeats.

Supplemental Figure S6: Nickel Pull-down Assay with His-tagged Zu5 Confirms HE β 1415 as Minimally Necessary for Binding.

Supplemental Figure S7: Analytical Ultracentrifugation to Sedimentation Equilibrium Indicates Zu5 Stably Binds to HE β 1415

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consist of two different “bridges” of proteins—one (3) from the integral membrane protein glycophorin C to a protein complex (Band 4.1, spectrin, and actin) (4) in the skeletal network and a second from the anion exchange transporter Band 3 in the membrane via the adaptor protein ankyrin directly to β -spectrin in the skeletal network (5). Lack of effect on red cell mechanical properties upon severing the former glycophorin-containing bridge (6), however, speaks to a greater importance of the ankyrin-containing bridge, which is demonstrable in red cell ghosts by single particle tracking and with optical tweezers (7).

Most isoforms of ankyrin are comprised of an 89 kDa N-terminal Band 3 binding domain, a central 62 kDa Spectrin binding domain and a 55 kDa C-terminal regulatory domain (5) (Fig. 1). Much is known about the associations of this ubiquitous protein’s multiple isoforms (8, 9), the static and kinetic properties of the ankyrin-Band 3 interaction (10), the consequences of ankyrin mutations for cardiac (11, 12) and other functions (12, 13). Characterization of its molecular structure, however, has been confined largely to its Band 3 binding domain of so-called ankyrin repeats. An X-ray crystal structure of a fragment of repeats (14) suggests origins of its spring-like behavior, which were later demonstrated by atomic force microscopy (15). The mechanisms of binding of this ankyrin domain to the C-terminal tail of Band 3 have also been elucidated through crystal structure determination (16) and recombinant DNA/ligand testing (17).

In comparison with progress on the Band 3 binding domain, structural information for the Spectrin Binding Domain (SpBD) and the regulatory domain of ankyrin is meager. Given its critical role as part of the bridge directly tethering the skeletal network to the cell membrane (18), SpBD is particularly crucial during reversible deformation of red cells. In order to address the molecular properties of this force-transmitting site, questions as to the identity of the molecular epitope(s) of ankyrin interacting with spectrin needed to be resolved. A spectrin binding epitope within SpBD—originally identified by Wallin et al. (19)—was first reported to be “272” (20), according to immunoblot binding of SpBD fragments to biotinylated spectrin and to competition between fusion proteins and ankyrin for radiolabeled spectrin on inside-out red cell vesicles. More recently, a non-overlapping SpBD peptide sequence containing a Zu5 domain plus 55 residues C-terminal to it—herein referred to as “Zu5”—was reported to be a β 2-spectrin binding epitope from results of yeast 2-hybrid assays (21). According to Zhang *et al.* (22), Zu5 is a “functionally unknown domain found in ZO-1 and UNC5H receptors”.

This first portion of this study was begun to identify the molecular epitope(s) of ankyrin involved in binding spectrin (20, 21). The results presented here show that the Zu5 subdomain binds specifically to fragments containing β -spectrin repeats 14 and 15 according to a native polyacrylamide gel shift assay, affinity pull-downs, as well as analytical ultracentrifugation to sedimentation equilibrium. In contrast, 272 did not evidence binding by any of these methods. To complement the information gathered regarding the minimal spectrin-binding regions of ankyrin, similar studies were performed with the ankyrin-binding domain of spectrin. While there has been less controversy over the regions of spectrin implicated in binding, structural studies of other spectrin repeats have revealed the necessity for proper “phasing” of cloned spectrin repeats.

The 37 triple-helical repeats of the repeating unit domain of the α - and β -spectrin heterodimer are often represented in diagrams by identical symbols (Fig. 2). This implication of identity has been heuristically useful for measuring (23) and modeling (24) mechanical properties of the red cell skeletal network, although recent protein unfolding studies suggest heterogeneity among repeats with respect to their stabilities of folding (25, 26) and melting temperatures (27). More obvious functional correlates of this heterogeneity are the specific affinities of certain spectrin repeats reviewed by Djinoovic-Carugo *et al.* (28),

which indicate that the low (20–30%) amino acid sequence identity among repeats (29) has evolved to afford functional specialization within the repeating unit domain of spectrin. This reasoning also applies, of course, to the other prominent members of the spectrin family, α -actinin and dystrophin (30). It remains a mystery, however, as to what dictates this structural versatility of the signature spectrin repeating unit motif (31) in spite of the availability of X-ray crystal structures of two (25, 32, 33) and three (34) repeat fragments of spectrin and α -actinin. An exception is the structure of four repeat fragments of α -actinin (35) with a lengthwise twist which enables interaction of α -actinin with actin at each end.

The specialization of interest here is the affinity of certain spectrin fragments for the adaptor protein ankyrin. A minimal version of the ankyrin binding subdomain had originally been identified as β -spectrin repeat 15 by cloning and testing of incrementally smaller regions of the C-terminal end of β -spectrin for binding to inside-out red cell vesicles (36). However, it became apparent subsequently that this “repeat 15” was actually the C-terminal third of repeat 14 plus the whole of repeat 15, encompassing the start and stop sites of canonical spectrin repeats defined by surveying the CD spectra and protease sensitivities of differently phased fragments (37). Nevertheless, the identity of this region as the ankyrin binding site is supported by its harboring 1) a sequence in the 15th repeat, which is well-conserved among β -spectrins I, II and III with ankyrin binding activity, and 2) a 43 residue region of low homology with other repeats (38). Four cloned permutations of repeat 15 were recently reported to yield somewhat different results, depending on whether ankyrin binding activity was assayed by interaction with intact ankyrin, interaction with a PE/PC monolayer, binding to PE/PC liposomes or inhibition of spectrin binding to PE/PC liposomes (39). Perhaps because of slight differences in phasing of start and stop sites, ankyrin binding activity was obtained with a fragment missing the C-terminal half of β -spectrin repeat 15 (39), which had been previously found to be necessary (36).

In order to determine which repeats are minimally necessary for binding to the spectrin binding subdomain of ankyrin, three 2-repeat fragments of β -spectrin—HE β 1314, HE β 1415, HE β 1516—and one 3-repeat fragment, HE β 1315, were cloned. With concern for physiological relevance of the results in the present study, these four β -spectrin fragments were designed to conform to the start and stop sites of a canonical, 106 amino acid spectrin repeat (37), although 6 residues were added to the N- and C-termini of HE β 1415 and 2 residues to the N- and C-termini of HE β 1315 to minimize “fraying.” Such small additions have been shown to enhance the stabilities of folding of dystrophin (40) as well as spectrin (41) repeats. Furthermore, in addition to CD and tryptophan fluorescence spectra, free energies of heat-induced unfolding, i.e. ΔG 's, were determined for evaluation of the stabilities of folding of the candidate peptides. Finally, these phased and well-folded fragments were tested for binding to tagged and untagged versions of the Zu5 + neighboring 55 residue subdomain of the Spectrin Binding Domain of ankyrin (SpBD), previously reported to exhibit spectrin binding activity *in vivo* (20, 21, 42). Among the tested fragments, only β -spectrin fragments containing repeats 14 and 15 were able to bind the subdomain of ankyrin “Zu5”, in agreement with the earlier finding that ankyrin binding activity requires both the C-terminal third of repeat 14 and the whole of repeat 15 (36). Binding measurements indicated a K_D of ~15 nM, suggesting these minimal binding regions are responsible for nearly all of the binding observed between the intact molecules (43). Taken together these results provide for the first time a promising system for determining the molecular basis of this affinity which is critical to the reversible deformation of red cells.

EXPERIMENTAL PROCEDURES

Preparation of non-tagged ankyrin constructs

cDNA coding for both ankyrin peptides, Zu5 (residues 911 – 1068) (21) and 272 (residues 1102–1193) (20), was human erythroid ankyrin cDNA Ank15 kindly given by Dr. Patrick Gallagher (44, 45). Zu5 and 272 were cloned into pET-3c and pET-30b, respectively, by amplifying the target sequences via PCR, gel purifying the PCR products, and ligating them into the target vector. *Escherichia coli* DH5 α cells were transformed with the recombinant plasmids by electroporation and plated on LB agar with the appropriate antibiotics. After overnight incubation at 37 °C, single colonies were cultured for plasmid amplification followed by DNA miniprep purification (Promega Wizard kit). To ensure fidelity of cloning and to verify construct identity, each was sequenced by the Northwestern University Sequencing Facility.

Preparation of His-tagged ankyrin constructs

His-tagged versions of Zu5 and 272 were created via ligation-independent cloning into pICANTE (a pMCSG7 (46) derived vector created for these studies) and pCDF-2 Ek/LIC (Novagen), respectively, using standard methods.

Preparation of spectrin constructs

cDNA coding for spectrin constructs was human erythroid β -spectrin (HE β) cDNA (47), and chicken brain α -spectrin (CB α) cDNA (48), kindly provided by Dr. Bernard Forget and the late Dr. Matti Saraste, respectively. The new target constructs—HE β 1314 (residues 1585–1798), HE β 1415 (residues 1685–1910), HE β 1516 (residues 1799–2010), and HE β 1315 (residues 1583–1906)—were PCR amplified for cloning into the ligation-independent vector pMCSG7 (46). Sizes of the PCR products were verified by agarose gel electrophoresis and the amplified DNA products purified (Qiagen Gel Extraction Kit). Exonuclease treatment for incorporation into the vector was performed according to Stols *et al.* (46). The recombinant plasmids were transformed into *Escherichia coli* NovaBlue competent cells (Novagen) by heat shock and plated on LB agar with 100 μ g/mL ampicillin. Plasmid isolation and sequence were performed as described above. Cloning and preparation of HE β 89 in pET-23d and CB α 1617 in pET-3d were completed as previously (25, 26, 33).

Protein expression of Zu5

E. coli Rosetta2(DE3) competent cells (Novagen) were transformed by heat shock and plated on LB agar containing 100 μ g/mL ampicillin and 34 μ g/mL chloramphenicol. After overnight incubation at 37 °C, single colonies were inoculated into 50 mL LB with antibiotics and grown for ~16 h at 37 °C with shaking. The starter cultures were then transferred to 1 L LB cultures and incubated at 37 °C with shaking until the OD₆₀₀ was 0.5. The cultures were briefly cooled and protein expression was induced with 0.5 mM IPTG. Cultures were then incubated overnight at 16 °C with shaking. Cells were harvested by centrifugation at 6,000 rpm and the cell pellet stored at –80 °C.

Protein expression of 272

Expression of 272 was carried out as described for Zu5 except that 50 μ g/mL kanamycin was used in each step rather than ampicillin, cultures of 272 were maintained at a constant 37 °C, and protein expression was induced for 3–4 h at this temperature followed by harvesting as above.

Expression of spectrin constructs

Recombinant plasmids bearing cDNA for β -spectrin repeats were transformed into *E. coli* BL21(DE3) by heat shock and grown on LB agar plates containing the appropriate antibiotic. After overnight incubation at 37 °C, single colonies were inoculated into 50 mL LB media with antibiotic and grown at 37 °C with shaking at 300 rpm. After ~16 h, this starter culture was centrifuged at $6000 \times g$ for 15 min at 4 °C, the supernatant removed, and the cell pellet resuspended in 10 mL fresh LB. The resuspended culture was then transferred to 1 L of LB with antibiotic (initial OD₆₀₀ of ~0.1) and incubated at 37 °C with shaking at 300 rpm. After ~2.5 h (OD₆₀₀ of 0.5–0.6), protein expression was induced with 1 mM IPTG for three hours, after which the cells were harvested at $5,000 \times g$ for 30 min at 4 °C. The resulting cell pellet was frozen and stored at –80 °C.

Protein purification of non-tagged Zu5 and 272

Frozen cell pellets from 1 L of culture were resuspended in 20 mL PED (20 mM sodium phosphate, pH 8.0, 1 mM EDTA, 1 mM DTT) with 1 mL protease inhibitor cocktail containing: AEBSF, EDTA, Bestatin, Pepstatin A and E-64 (Sigma P8465). After five cycles of sonication (one min on, one min off) at 4 °C, the cell lysate was centrifuged at $35,000 \times g$ at 4 °C for 1 h. The supernatant was loaded onto a carboxymethyl sepharose (Sigma) column pre-equilibrated with PED, then washed with 20 mL PEDP (PED buffer with 0.1 mM PMSF). A 0 – 0.5 M NaCl gradient in PEDP was used to elute the bound species. Fractions containing the target protein were then loaded on an S (Sulfonyl) methacrylate (Bio-Rad) column and eluted with a 0–1.0 M NaCl gradient in PEDP. When necessary, the target protein was further chromatographed on a P-10 (Bio-Rad) size exclusion column. Zu5 and 272 were concentrated by centrifugation in Amicon Ultra centrifugal filter devices. Purity of the proteins was monitored by SDS-PAGE on 4–20% polyacrylamide gradient gels (Pierce) or on 15% polyacrylamide gels and protein concentration determined by $A_{\lambda=280}$ extinction coefficients calculated from UV absorbing amino acids in the sequences.

Expression and purification of His-tagged ankyrin constructs

Protein expression of the His-tagged ankyrin constructs and cell lysis were performed as described for their non-tagged counterparts. After centrifugation, the resulting supernatant was first purified by affinity chromatography on Ni-CAM resin (Sigma). Subsequent S (Sulfonyl) methacrylate (Bio-Rad) column and P-10 (Bio-Rad) size exclusion column purification were also performed as described above. When necessary, Zu5 and 272 were concentrated with Amicon Ultra centrifugal filter devices. Purity of the proteins was monitored by SDS-PAGE on 4–20% polyacrylamide gradient gels (Pierce).

Purification of spectrin constructs

After thawing, cells were resuspended in 20 mL 50 mM sodium phosphate, pH 8.0, 10 mM imidazole and lysed by five cycles of sonication (one min on, one min off). Cell debris was then removed by centrifugation at $35,000 \times g$ for 30 min. For His-tagged constructs, the resulting supernatant was purified by affinity chromatography on Ni-CAM resin (Sigma). Subsequently, the His-tag was removed with TEV protease (1:20 m/m ratio construct:protease) at 4 °C for ~20 h while simultaneously dialyzed into 50 mM sodium phosphate, pH 8.0. TEV protease and any remaining uncleaved protein were removed with Ni-CAM resin. Untagged constructs were purified as described previously (26, 33). Ion exchange chromatography on Q-Sepharose (Sigma) and size exclusion chromatography on S-100 (Bio-Rad) were then carried out for all target proteins in TEDP-based buffers (20 mM Tris, pH 8.0, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF). Fractions were tested by SDS-PAGE; those which contained protein of the highest purity and in highest yield were pooled

and further purified. The resulting purified proteins were desalted into 10 mM HEPES, pH 7.5, on a 10DG desalting column (Bio-Rad), concentrated to ~200 μ M, and stored at 4 °C. Typical yields ranged from 5–50 mg of purified protein per liter of culture. Protein concentration was determined from absorbance at 280 nm and calculated extinction coefficients.

Circular dichroism

CD measurements were obtained with a Jasco J715 spectropolarimeter equipped with a Peltier device and calibrated with *d*-10-camphorsulfonic acid (Keck Facility, Northwestern University). The data pitch was 1 nm, the scan speed 20 nm/min, the response time 8 s, and the band width 1 nm. Wavelength scans of Zu5 at room temperature and of 272 at 6 °C were performed in buffer containing 10 mM sodium phosphate, pH 8.0, 0.14 M NaF and 1 mM TCEP using a 0.2 mm path length demountable cuvette. Wavelength scans of spectrin fragments at room temperature were carried out at a final protein concentration of 15–20 μ M in 10 mM sodium phosphate, pH 8.0, 0.14 M NaF (0.2 mm path length demountable cuvette). Heat denaturation scans (final protein concentration of 1 μ M in a 1 cm cuvette) were measured at 222 nm from 10 to 85 °C. Samples were heated at a rate of 1 °C/min with stirring. Data for wavelength scans are given after buffer subtraction and presented in units of molar ellipticity ([θ]), deg·cm²·dmol⁻¹.

Tryptophan fluorescence

Purified spectrin fragments were diluted to 4–6 μ M in 10 mM sodium phosphate, pH 8.0, 0.14 M NaCl. Samples were excited at 295 nm and emission scans from 300 to 380 nm were collected with a Hitachi F-4500 Fluorescence Spectrometer. Data was collected in a 0.5 cm square quartz cuvette. Slit widths of 5 nm were used throughout.

Thermodynamic analysis of thermal unfolding

Stabilities of folding based on CD-monitored thermal unfolding were determined as previously (34, 41). Buffer-subtracted spectra were fit with SigmaPlot 8.0 to equations describing 2-state (41) or 3-state (34) transitions to yield transition temperatures (T_M 's) and slopes of the transition (ΔT 's). Free energies of unfolding, ΔG 's, at 25 °C were obtained from equation 1.

$$\Delta G = 4RT_M (T_M - T) / \Delta T \quad (1)$$

where R = gas constant 1.987 cal·K⁻¹·mol⁻¹ and T = 298 K.

Gel shift assay

The indicated proteins were mixed in a 1:1 molar ratio, except where noted for stoichiometry determination, and incubated on ice for 15 min before loading of the gel. Ten percent polyacrylamide gels (with 5% stacking gel when indicated) at pH 7.4 (49) were pre-run for 30 min at 30 V before loading. Samples were mixed with 6× loading buffer (40% sucrose), and run at 30 V for roughly 4 h at 4 °C (measured gel running temperature was 4.6 °C). Protein was visualized by Coomassie Blue staining.

Analytical ultracentrifugation to sedimentation equilibrium

Sedimentation equilibrium experiments with Zu5 and 272 were carried out in a Beckman XL-A analytical ultracentrifuge at 20 °C and 6 °C, respectively, in 10 mM sodium phosphate, pH 8.0, 0.1 M NaCl, 1 mM EDTA. One hundred thirty microliters of sample from 0.25 mg/mL to 0.52 mg/mL were centrifuged at 20,000 rpm for 24 h and scans taken at 18 and 24 h to establish attainment of equilibrium. Where indicated, additional scans at

35,000 and 48,000 rpm were taken sequentially; samples were allowed 12 h to reach equilibrium before scanning at each of these higher speeds. Concentration distributions were measured at 280 nm and determined on SedPhat (version 4.4b) (<http://www.analyticalultracentrifugation.com/sedphat/sedphat.htm>) by fitting the data to equations describing single ideal, two ideal, non-ideal, or association models. Correspondence between the data and the calculated fit, randomness of residuals, and global reduced χ^2 values close to one were used to assess the quality of the fit.

Mass spectrometry

Purified proteins were diluted in water to 0.25–2.5 μM and applied to a Phenomenex Jupiter Proteo Column (1.0 mm $\times$ 150 mm, 4 μm particle size) at 50 $\mu\text{L}/\text{minute}$. In total, ~ 1 pmol (4 μL) of material was injected. A mobile phase gradient from 0.1% formic acid in water to 0.08% formic acid in acetonitrile was used to chromatograph each sample over 6 minutes, which was then taken for in-line electrospray ionization mass spectrometry (ESI-MS). Mass spectra were obtained on an Agilent 6510 Accurate-Mass Q-TOF LC/MS with an accelerating voltage of 4100 V. Deconvolution was performed with the included software, Agilent MassHunter Qualitative Analysis version B.01.02. Masses of all proteins were within 1.0 Da of those predicted.

Surface plasmon resonance

Binding affinities and kinetics of biotinylated spectrin fragments to Zu5 were measured with a Biacore 3000 equipped with a streptavidin biosensor. Prior to immobilization, three purified spectrin fragments (HE β 1315, HE β 1415, and CB α 1617) were individually biotinylated. Each of these constructs contained a single cysteine near the C-terminus which allowed for directed biotinylation with 1-Biotinamido-4-[4'-(maleimidomethyl)cyclohexanecarboxamido]butane (Pierce, manufacturer's protocol followed). The biotinylated spectrins (ligands) were diluted in HBS-P (10 mM HEPES, pH 7.4, 0.15 M NaCl, 0.005% (v/v) surfactant P20, Biacore) to a final concentration of 1 nM and applied to individual flow cells of a streptavidin-modified biosensor (sensor chip SA, GE Healthcare) at a rate of 20 $\mu\text{L}/\text{min}$ at 25 $^{\circ}\text{C}$. Between 100 and 150 response units (R.U.) were immobilized for each biotinylated ligand. Immobilization was judged to be stable with no noticeable decay in response. Purified Zu5 (analyte) in HBS-P was diluted serially to the concentrations indicated and maintained at 10 $^{\circ}\text{C}$ until the time of injection. Injections were performed in parallel with Zu5 being applied over all flow cells simultaneously at a flow rate of 20 $\mu\text{L}/\text{min}$ and a sensor temperature of 25 $^{\circ}\text{C}$. Injections were performed in triplicate using HBS-P as the system buffer; association and dissociation phases were 60 seconds each. Zu5 completely dissociated after ~ 180 s; nevertheless, a 300 s post-dissociation phase wash was included in the run parameters to ensure complete removal of Zu5 from previous injections. Data were analyzed using the included BIAevaluation software (version 4.1). Sensorgrams for each set of injections were processed globally (and simultaneously for k_a and k_d) after reference cell and buffer subtraction. Fits were assessed by correspondence between the data and the calculated fit, randomness of residuals, and nearness of global reduced χ^2 values to 1.0. Values presented for the k_a , k_d , and K_D (calculated by k_a/k_d) represent the average $\pm$ average standard deviation for the three replicates for each ligand.

RESULTS

Isolation of ankyrin subdomains Zu5 and 272

Two neighboring but non-overlapping subdomains of the Spectrin Binding Domain (SpBD) of human erythroid ankyrin (Fig. 1) were previously reported to be minimally necessary to interact with the ankyrin binding site on human erythroid β -spectrin: i) "Zu5," a 17,588 dalton peptide of 158 residues (21) and ii) "272," a 10,187 dalton peptide of 92 residues

(20). These two different molecular epitopes putatively involved in spectrin binding had been cloned and tested largely as proteolysed peptide fragments in immunoblot assays with biotinylated spectrin or as fusion proteins competing with ankyrin on inside-out red cell vesicles for radiolabeled spectrin (20) or in yeast two-hybrid assays (21). With the goal of eventual structure determination of the spectrin-ankyrin binding site crucial to red cell skeletal integrity (50), it became necessary for us to clone, isolate, and study these candidate epitopes free of unrelated domains which could potentially influence their conformations in a non-physiological manner. 272 and Zu5 were cloned, expressed, and purified separately to greater than 99% purity (**EXPERIMENTAL PROCEDURES**) as shown by SDS-PAGE in Fig. 3. The DNA sequence of both proteins was found to be correct by sequencing; masses of these proteins were verified to within 1.0 Da by mass spectrometry. Properties which hindered the recovery of these proteins were the tendencies of Zu5 to become insoluble during expression and to precipitate over time, once purified, and of 272 to aggregate at temperatures greater than 4 °C.

Greater than 30% of 272 and Zu5 are unstructured by modeling of their CD data

One reason for the above and other difficulties encountered in isolating and purifying Zu5 and 272 became apparent on measurement of CD spectra of Zu5 (Fig. 4A) and 272 (Fig. 4B). Solutions of each protein in 10 mM sodium phosphate, pH 8.0, 0.15 M NaF, with 1 mM TCEP were scanned from 250 to 185 nm and yielded a mean residue ellipticity at 222 nm of 3,923 deg·cm²·dmol⁻¹ for Zu5 and 3,267 deg·cm²·dmol⁻¹ for 272. The spectra were then analyzed with standard algorithms in conjunction with appropriate protein reference sets on the website Dichroweb (51, 52). The algorithm giving by far the best fit to the spectra of both Zu5 and 272 was CONTINLL (53, 54), based on CONTIN, devised by Provencher and Glockner (55) and modified by van Stokkum *et al.* (56), in combination with reference sets 6 and 3, respectively. These conditions gave an NRMSD of ~0.05 and a virtually superimposable fit of the calculated spectra to the measured spectra of Zu5 (Fig. 4A) and of 272 (Fig. 4B), whereas the next best combination of algorithm and reference set gave an NRMSD of 0.17 and a poor fit of the calculated spectra to the measured spectra (not shown). The average percentages of predicted secondary structure given in each figure are shown in Table 1.

Zu5 forms a complex with HEβ1315 or with HEβ1415 but not with HEβ89 or CBα1617 on native polyacrylamide gels

To corroborate the specific interaction of Zu5 or 272 with HEβ1315 or HEβ1415, but not with CBα1617, each protein was subjected to native 10% polyacrylamide gel electrophoresis Fig. 5A.

Unfortunately, 272 (pI 9.5) did not enter the gel at pH 7.4 (Fig. 5A, rightmost lane) nor was an observable shift in spectrin fragment migration noted when spectrin fragments were mixed with 272 and run at pH 7.4 (not shown). Several attempts were made to demonstrate 272-containing complexes via gel shift by varying protein ratios, adjusting the pH of the gel and running buffer (pH ranged from ~4.0 to ~8.5 in 0.5 pH unit increments), and electrophoresing in the (–) cathode direction. None of these variations demonstrated formation of 272-containing complexes making this assay definitive only for characterizing Zu5 binding to HEβ1315 and to HEβ1415.

In contrast with 272, however, Zu5 was shown to form specific complexes. Fig. 5B shows the gel lanes marked according to the proteins added. Zu5 alone is in the extreme right lane. The first four lanes on the left show that either HEβ1315 or HEβ1415 forms a complex with Zu5; the next four lanes show that neither HEβ89 nor CBα1617 forms a complex with Zu5. In the lanes with both Zu5 and HEβ1315 or HEβ1415, a small amount of excess HEβ1315

and HE β 1415 are seen below the complexes (i.e. migrating more quickly). These conclusions were also validated using Nickel affinity pull-down (methods and results can be found in the accompanying supplement).

Zu5 forms a complex with HE β 1315 and with HE β 1415 but not with CB α 1617 at sedimentation equilibrium

To examine the affinities of Zu5 and 272 for the ankyrin binding site on spectrin in solution, sedimentation equilibrium experiments were performed. Plots of the 280 nm absorbance versus centrifugal radius for a representative of each sample type are shown in Fig. 6, accompanied by fits and residuals.

For Zu5-containing mixtures, the single ideal species model gave the best fits with global reduced χ^2 values close to 1.0 and randomly distributed residuals. Zu5 alone (Fig. 6, top left) behaved as a monomer in the absence of spectrin fragments at pH 8.0 at 6 °C. Furthermore, a molecular mass within 10% that of a 1:1 complex was found for Zu5 with the 3-repeat fragment, HE β 1315 (Fig. 6, top center), and for Zu5 with the 2-repeat fragment, HE β 1415 (Fig. 6, bottom center). In contrast, Zu5 with the 2-repeat fragment, chicken brain α -spectrin 16 and 17 (CB α 1617), yielded a molecular mass of 25.1 kDa (only 60% of a complex) (Fig. 6, bottom left). Presumably, the similar mass of Zu5 alone was obscured by the fit of this sample.

In comparison, 272 (Fig. 6, top right) existed in a monomer-aggregate equilibrium under the same conditions. Interestingly, this aggregation was found quite reproducibly in the 40–80 kDa range at speeds <35,000 rpm but at those >35,000 rpm (not shown) this larger species could be “removed”—presumably by pelleting the aggregated 272. Thus, data for 272 alone fit the association model at speeds <35,000 rpm but the single ideal species model at those >35,000 rpm. In the presence of spectrin fragments, data for 272 fit the two ideal species model which indicated 272 was not binding to spectrin fragments. Data for HE β 1315 with 272 is shown in the lower right panel of Fig. 6.

Finally, in view of their proximity within the spectrin binding domain, Zu5 and 272 were also tested by ultracentrifugation for simultaneous binding to HE β 1415. This experiment yielded a molecular mass which is 79% of a ternary complex but 99% of a complex of Zu5 + HE β 1415 (not shown).

Defining the minimum number of β -spectrin repeats interacting with the minimum spectrin binding subdomain of ankyrin

Progress towards identifying the ankyrin binding region of β -spectrin began with the observation that repeat 15 alone constitutes the evidenced binding to ankyrin on inside-out-red cell vesicles (36). Only upon later structural determination of spectrin repeats did it become apparent that this “repeat 15” (Kennedy *et al.*, 1991) actually comprised the C-terminal third of repeat 14 and the whole of repeat 15, according to the phasing of start and stop sites of canonical spectrin repeats (37). In order to test well-folded spectrin fragments for binding to the ankyrin subdomain Zu5, re-cloning of different combinations of canonically phased, two and three repeat fragments in the region of the 14th and 15th repeating units of human erythroid β -spectrin was undertaken. Fig. 2 indicates the locations of the 13th, 14th, 15th and 16th β -spectrin repeats of interest (filled ellipses) among other repeats (open ellipses) within two α - and β -spectrin dimers forming a tetramer.

In view of the greater stability of spectrin (41) and spectrin-related dystrophin (40) fragments with up to seven extra residues at the N- and C-termini, the following β -spectrin fragments were cloned, expressed and purified, as described in **EXPERIMENTAL PROCEDURES**:

- HE β 1314 composed of the 13th and 14th repeats in tandem,
- HE β 1415 composed of the 14th and 15th repeats in tandem plus six extra N- and C-terminal residues,
- HE β 1516 composed of the 15th and 16th repeats in tandem,
- HE β 1315 composed of the 13th, 14th and 15th repeats in tandem plus two extra N- and C-terminal residues.

The fragments were purified to >99% in high yield as shown by SDS-PAGE (Fig. 7). Two-repeat fragments, HE β 89 (25) and CB α 1617 (33), previously characterized by X-ray crystallography are also shown in Fig. 7 for comparison.

CD and fluorescence spectra indicate β -spectrin candidates for ankyrin binding are folded

To demonstrate that all four β -spectrin fragments are well-folded, CD (Fig. 8A) and tryptophan fluorescence (Fig. 8B) wavelength spectra were measured. Fig. 8A shows that all four fragments exhibit the signature α -helical CD profile expected of triple-helical repeats of spectrin (31) with troughs at 208 and 222 nm. Their mean residue ellipticities in deg.cm².dmol⁻¹ and percent α -helical structure, according to a 100% value of 36,000 deg.cm².dmol⁻¹ (57), varied slightly: 31,973 (88.8% α -helical) for HE β 1315; 30,188 (83.9% α -helical) for HE β 1415; 35,211 (97.8% α -helical) for HE β 1314; and 32,894 (91.4% α -helical) for HE β 1516. Spectra were also analyzed with a standard secondary structure analysis algorithm on Dichroweb, CDDSTR, with reference protein set 3 giving the lowest NRMSDs. All β -spectrin peptides were found to be predominantly α -helical at room temperature: 68% regular α -helix and 28% distorted α -helix in HE β 1315, 67% regular α -helix and 28% distorted α -helix in HE β 1415, 67% regular α -helix and 26% distorted α -helix in HE β 1314, 68% α -helix and 25% distorted α -helix in HE β 1516, which are nearly the same as the 67% regular α -helix and 28% distorted α -helix in HE β 89 and only slightly lower than the 74% regular α -helix and 23% distorted α -helix in CB α 1617. The NRMSD of each fit was < 0.01 and the calculated spectra indistinguishable from experimental spectra (not shown). Fig. 8B shows that excitation of tryptophan at 295 nm in all four samples yielded emission spectra with blue-shifted peaks (335 nm for HE β 1315, 336 nm for HE β 1415, 338 nm for HE β 1314 and 338 nm for HE β 1516), as expected of tryptophans shielded within tertiary structure from the bulk phase (58). Finally, each spectrin fragment was found to fold stably as measured by CD-monitored heat-induced unfolding (supplemental information included).

β -spectrin repeats 14 and 15 are both necessary and sufficient to form a 1:1 complex with Zu5 as demonstrated by native polyacrylamide gel electrophoresis

In order to ascertain the stoichiometry of binding and to ensure that the nonbinding of HE β 1314 and HE β 1516 could not be ascribed to their very weak binding to Zu5, the effect of varying the molar ratios of all β -spectrin peptides to Zu5 was examined using native gels (Fig. 9). In Fig. 9A nearly all of the HE β 1315 forms a complex with Zu5 at a 1:1 molar ratio. The slight excess of HE β 1315 may indicate a more weakly binding subspecies. At a molar ratio of 1:2, the most slowly migrating free Zu5 appears near the top of the gel and continues to increase in amount as the molar ratio is reduced to 0:1. Likewise, in Fig. 9B, nearly all of the HE β 1415 forms a complex with Zu5 at a molar ratio of 1:1, although a very faint excess of HE β 1415 is detectable, perhaps again representing a more weakly binding subspecies. In contrast with Fig. 9A and 9B, Fig. 9D of HE β 1516 with Zu5 reveals no complex located between these two proteins at any molar ratio. Fig. 9C of HE β 1314 with Zu5 also reveals no complex located between these two proteins at any molar ratio. A 1:1 complex of these spectrin fragments with Zu5 was also found by analytical ultracentrifugation to sedimentation equilibrium (supplemental information). A 1:1 complex of these spectrin

fragments with Zu5 was also found by analytical ultracentrifugation to sedimentation equilibrium (supplemental information).

Affinity and kinetic analysis of ABD to Zu5 by surface plasmon resonance

Finally, mutual affinities of the minimal binding domains of these proteins were quantified by surface plasmon resonance (Fig. 10). Biotinylated spectrin fragments HE β 1315 (Fig. 10A), HE β 1415 (Fig. 10B), and CB α 1617 were immobilized on separate flow cells of a streptavidin-modified biosensor; Zu5 at various concentrations was then applied over all flow cells simultaneously. For HE β 1315, the average values for the association/dissociation rates and dissociation constant were: $k_a = 2.82 \times 10^6 \pm 4.44 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$; $k_d = 4.23 \times 10^{-2} \pm 6.58 \times 10^{-4} \text{ s}^{-1}$; and $K_D = 15.0 \text{ nM}$. Similarly, for HE β 1415: $k_a = 3.61 \times 10^6 \pm 5.70 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$; $k_d = 5.45 \times 10^{-2} \pm 7.46 \times 10^{-4} \text{ s}^{-1}$; and $K_D = 15.2 \text{ nM}$. CB α 1617 did not evidence any binding to Zu5 even at higher concentrations—the control shown in Fig. 10A corresponds to a Zu5 injection at 1 μM over the CB α 1617 flow cell. In all cases a simple 1:1 binding model with a drifting baseline was used to fit the data. Global reduced χ^2 values ranged from 0.93 to 1.25 indicating good agreement between the modeled fit and the experimental data.

DISCUSSION

Cloned ankyrin subdomain Zu5, but not 272, forms a complex with ankyrin binding repeats of human erythroid β -spectrin

17.6 kDa Zu5 (21) but not 10.9 kDa 272 (20) of the spectrin binding domain of ankyrin (Fig. 1) has been shown for the first time to specifically form a complex in a defined system with repeats 14 + 15 and repeats 13 + 14 + 15 of human erythroid β -spectrin by three methods: native PAGE of complexes of spectrin fragments with Zu5 (Fig. 5B), Nickel-based pull-down assays of His-tagged Zu5 (but not His-tagged 272) bound to β -spectrin fragments (Supplemental Fig. S3), and analytical ultracentrifugation to sedimentation equilibrium of complexes of β -spectrin repeats 14 + 15 with Zu5, but not with 272 (Fig. 6). Although technical difficulties prevented a straightforward assessment of the inability of 272 to bind to β -spectrin fragments by native PAGE, the results presented here confirm the findings by yeast two-hybrid assays in intact cells (21, 42) that Zu5 with its C-terminal neighboring 55 residues (see Experimental Procedures) is the minimal β -spectrin binding subdomain of ankyrin. In contrast, the results presented here do not confirm the findings by immunoblot assay of red cell ghost membrane preparations (20) that 272 is a β -spectrin binding subdomain of ankyrin. The purity of ankyrin fragments (Fig. 3) and spectrin peptides (Fig. 7) cloned for this study makes it unlikely that *in vivo* versus *in vitro* discrepancies can be ascribed to protein contaminants. Previous findings which utilized undefined sources of binding partners (containing intact spectrin as well as other membrane and non-membrane proteins) could account for the different outcomes in earlier versus the present work. Perhaps for this reason, it is not uncommon that other interactions of proteins originally detected in undefined systems cannot be confirmed in defined systems (59).

Inability to demonstrate binding of 272 to HE β 1315 by native PAGE

As described in the **RESULTS** and above, it was not possible to show in a straightforward manner by native PAGE alone that 272 does not form a complex with the ankyrin binding epitope of β -spectrin because either 272 or the spectrin fragments disappeared from the gel during electrophoresis at all pH's tested (e.g. 272 is not evidenced on the gel in Fig. 5A). Apparently the high pI of 272 (9.5) and generally low pI's of spectrin fragments (ranging from 4–6) resulted in their migration to opposite electrodes so that retention of one peptide species on the gel precluded that of the other. When spectrin fragments were visible on the gel, however, their bands appeared to be of the same intensity and at the same R_f regardless

of the presence or absence of 272 in the starting samples. This suggests that none of the spectrin fragments had been lost to a complex with 272. Furthermore, that 272 was not visible when spectrin fragments could be seen in the gel (Fig. 5A) rules out precipitation of 272 as a reason for non-binding. It is also unlikely that the absence of 272-spectrin fragment complexes was due to electric field-induced dissociation during native PAGE, since complexes between β -spectrin fragments and Zu5 (with a nearly equally high pI of 8.5) remained stable.

Self-aggregation of 272 is not responsible for its nonbinding to spectrin fragments

The possibility that aggregation of the cloned 272 fragment demonstrated by analytical ultracentrifugation (Fig. 6, upper right panel) prevented its binding to spectrin fragments seems unlikely. The K_A of 272 self-aggregation (roughly estimated to be 230 M^{-1} based on analytical ultracentrifugation) is too low to have reduced the 272 monomer level below that required for at least some 272 binding to spectrin fragments. Binding constants of the affinity of spectrin for the spectrin binding domain of ankyrin-R lacking 70 of its N-terminal residues have been reported in the 10 to 200 nM range (60). Had 272 bound to β -spectrin fragments, analytical ultracentrifugation to sedimentation equilibrium should have enabled detection of this strength of binding as in the case of cloned β -spectrin repeats 1 + 2 binding to α -spectrin repeats 19 + 20 with K_D 's from 20–600 nM (61). Finally, although His-tagged Zu5 on Ni-NTA agarose could selectively pull-down β -spectrin repeats 14 + 15 (Supplemental Fig. S3A), His-tagged 272 was clearly unable to do so (Supplemental Fig. S3B). Here again, an objection might be made that the His-tag on 272 changed its conformation so as to disallow its binding to the β -spectrin fragments, but since His-tagged Zu5 could bind to the β -spectrin fragments, the suggestion that such a conformational change occurred is unduly speculative.

It should also be noted that repeated attempts were made to demonstrate the interactions between Zu5 or 272 and the β -spectrin repeats 14 + 15 both by CD difference spectra and by changes in the sum of the free energies of unfolding of each peptide alone versus that of a mixture of both fragments by procedures reviewed by Greenfield (62). Neither strategy was successful (not shown). Attempts to demonstrate binding of 272 to spectrin fragments by these methods were particularly hampered by the tendency of 272 to precipitate in spite of measurements being performed close to 4 °C.

Both Zu5 and 272 and their parent spectrin binding domain of ankyrin contain regions which lack regular secondary structure

A third important new finding in this study is the likely occurrence of substantial intrinsically unstructured regions in Zu5 and 272, according to their CD spectra (Fig. 4) and from modeling of their sequences (Supplemental Fig. S1) with disordered sequence predictors disEMBL (63), GlobPlot (64), PONDR (65, 66), and DISOPRED2 (67). As noted in the **RESULTS**, the predicted presence of some of the intrinsically unstructured sequences of 272 and Zu5 in their parent spectrin binding domain attests to the context independence of this predicted disorder. For this reason it is unlikely that these unstructured regions resulted from the sequence design of the cloned fragments.

Having identified Zu5 as the primary β -spectrin binding domain, we initiated basic NMR studies to clarify the structural nature of Zu5. Surprisingly, HSQC data collected with ^{15}N -labeled Zu5 indicated an unexpectedly well-folded protein (Supplemental Fig. S2). In the context of the aforementioned CD data and disorder predictions, it seems likely that Zu5 contains little secondary structure yet still adopts a folded conformation. With this finding, carefully differentiating between “unstructured” (folded, yet lacking secondary structure elements) and “disordered” (not adopting a fold) was key to the interpretation of these data.

Structural elucidation of the Zu5 subdomain should certainly prove interesting both for its implications in protein folding as well as β -spectrin binding.

Additionally, the content of intrinsically unstructured and/or disordered sequences could have accounted for the limited solubility of Zu5 and 272, although the expression of these protein fragments in bacterial systems unable to glycosylate, fatty acid acylate, or otherwise post-translationally modify may also have contributed to limited solubility. In addition to putative post translational modifications noted by the website ExPASy, fatty acid acylation of ankyrin has been reported (68).

Repeats 14 + 15 of β -spectrin are minimally necessary and sufficient to form complexes with the minimal spectrin binding ankyrin

Among fragments of all combinations of phased, well-folded, human erythroid β -spectrin repeats previously implicated in ankyrin binding (36, 39)—i.e. repeats 13 + 14, repeats 14 + 15, repeats 15 + 16, and repeats 13 + 14 + 15—only fragments containing both repeats 14 and 15 were observed to bind to the Zu5 epitope for β -spectrin. Native PAGE (Figs. 5 and 9), Nickel affinity pull-down assays (Supplemental Figs. S3 and S6), and analytical ultracentrifugation to sedimentation equilibrium (Fig. 6 and Supplemental Fig. 7) results, when taken together, show conclusively that tandem repeats 14 + 15 are necessary and sufficient to bind to the β -spectrin binding site on ankyrin.

CD and tryptophan fluorescence spectra and ΔG 's of heat-induced unfolding indicate β -spectrin fragments are similarly well- and stably folded

The CD wavelength spectra of all four β -spectrin fragments in this study exhibit the signature α -helical trace expected for triple-helical bundles (Fig. 8). The β -spectrin fragments gave the lowest NRMSD with the CD modeling program CDDSTR (54) with reference protein set 6 on Dichroweb (52) yielding $67.5\% \pm 0.3$ regular α -helix and $26.8\% \pm 1.5$ distorted helix. The calculated spectra were indistinguishable from those measured (not shown). The discrepancy between the modeled sum of helical conformations equaling 94% and the helical regions in X-ray crystal structures of repeats equaling 87% (25, 33, 34) may reflect the assignment of some loop regions as distorted helix in the latter.

The differences in the tryptophan fluorescence intensities of the fragments (Fig. 8B) appear to represent differences in the locations of the tryptophans within a heptad repeating pattern serving to identify the residues participating in inter-helical hydrophobic interactions (69). Thus, HE β 16 in HE β 1516 with the lowest fluorescence intensity possesses a tryptophan in an “e” or solvent exposed position, whereas HE β 13 in HE β 1314 with an intermediate intensity possesses a tryptophan in a “b” or more solvent-protected position. All other tryptophans are in “a” or “d” positions, protected from the solvent and participating in helix-stabilizing, hydrophobic interactions. As expected, these intensities parallel the wavelengths of maximum emission; the less quenched, and therefore more intense, spectra being correlated with the lowest wavelengths of maximum emission: 335.6 nm for HE β 1315, 336.4 nm for HE β 1415, 338.8 nm for HE β 1314 and 338.8 nm for HE β 1516. Most importantly, ΔG 's of heat-induced unfolding monitored by CD were nearly the same for all β -spectrin peptides, as shown in heat denaturation curves in Supplemental Fig. S4. Hence, nonbinding to Zu5 of spectrin fragments without repeats 14 or 15 (i.e. HE β 1516 or HE β 1314) cannot be attributed to relative instability of folding of those fragments.

1:1 Stoichiometry of binding of β -spectrin repeats to ankyrin subdomain Zu5 by native polyacrylamide gel electrophoresis

Not only did native polyacrylamide gel electrophoresis demonstrate the binding of β -spectrin repeats 14 + 15 and 13 + 14 + 15 to Zu5 (Fig. 5), this method was also a convenient

way of demonstrating near 1:1 stoichiometry (Fig. 9A, 9B). In addition, binding did not occur between Zu5 and the “incomplete” ankyrin binding domain fragments HE β 1314 and HE β 1516, even at a spectrin:Zu5 of 10:1 (Fig. 9C and 9D, respectively). Thus, HE β 1314 and HE β 1516 appeared to be entirely inactive in binding to Zu5, not merely weakly binding. However, although no freely migrating complex with HE β 1314 is observed in Fig. 9C, careful inspection of Supplemental Fig. S5 reveals that at 1:1 HE β 1314 is almost imperceptibly retarded in its mobility whereas Zu5 has migrated slightly faster. This result is highly reproducible and easily discerned when a 5% stacking gel is used. These slight differences in the extent of migration during electrophoresis could signify conformational changes occurring upon interaction of Zu5 with HE β 14, which are necessary but not sufficient for stable complex formation.

Kinetic and affinity measurements of β -spectrin fragments to Zu5

SPR binding measurements of Zu5 to biotinylated spectrins validated the results of many of the aforementioned studies while also providing quantitative kinetic and affinity measurements. Zu5 binding was again found to be specific to repeats 14 + 15 with 1:1 stoichiometry, as there was no binding to CB α 1617 (Fig. 10A, black circles). Interestingly, comparisons of Zu5 binding to HE β 1315 (Fig. 10A) versus HE β 1415 (Fig. 10B) exhibited very slight differences in binding kinetics with no appreciable change in K_D (15.2 versus 15.0 nM, respectively). This was somewhat surprising as additional spectrin repeats are known to stabilize spectrin fragments (41) yet did not have an impact on SpBD binding affinity. Additionally, the reasonably strong binding of these fragments was slightly tighter than expected based on studies of intact spectrin and ankyrin (~25 nM (43)), suggesting that these minimal regions may be responsible for nearly all of the binding affinity. Further characterization of this interaction by ITC is in progress in order to determine the enthalpic and entropic contributions of binding in a similar way to those reported for the spectrin heterodimer assembly site (70).

In conclusion, these binding epitopes of ankyrin and β -spectrin continue to present a host of tantalizing questions—most recently with respect to dysfunction in lateral membrane biogenesis and abnormal distributions of membrane proteins in various cell types due to mutations of polar to alanine residues within the spectrin binding domain without an apparent effect on ankyrin folding (42). The possibility that those mutated residues occupy sites of ankyrin- β -spectrin interaction calls for testing in the type of defined system amenable to structural analysis which has been presented here. A second major question is whether the regions of SpBD which lack secondary structure might eventually undergo folding on interacting with ankyrin binding epitopes or other function-determining sites of β -spectrin. A number of such intrinsically unstructured regions do fold on meeting their binding partners (71), whereas others persist and function in an unstructured state (72). After many years of intense study of protein folding, a new era is literally and figuratively unfolding so that alternative paradigms may provide greater understanding of the relationship between the structure and function of proteins with intrinsically unstructured regions, such as the β -spectrin binding domain of ankyrin.

In view of the finding by Winograd *et al.* (1991) that cloning of well-folded spectrin repeats required their being “phased” or consisting of integral, non-fractional numbers of repeats, the binding activity of “unphased” repeats might be considered to be of dubious physiological significance. Nevertheless, the results presented here on “phased” repeats agree well with those on “unphased” repeats by Kennedy *et al.* (1991) and by Hryniewicz-Jankowska *et al.* (2004), insofar as the former identified the C-terminal third of β -spectrin repeat 14 and the whole of repeat 15 and the latter found a fragment from repeat 13 to the middle of repeat 15 to be the spectrin binding site on ankyrin. Upon closer inspection, however, the minimal “phased” region of maximal binding consisted of only β -spectrin

repeats 14 + 15 with no kinetic or thermodynamic advantage evidenced by inclusion of β -spectrin repeat 13.

Now that a defined system of well-folded peptides is available for structural study of the interactions between the minimal β -spectrin binding domain of ankyrin and the minimal ankyrin binding domain of β -spectrin, we hope to characterize the β -spectrin/ankyrin binding interface comprising the C-terminal third of β -spectrin repeat 14 and the N-terminal half of β -spectrin repeat 15 at the molecular level. Interest in this interface has been heightened by the recent finding of the force-induced accessibility of the single cysteine in repeat 15 of β -spectrin to IAEDANS labeling in intact red cells (73).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations used in this manuscript

AEBSF	4-(2-Aminoethyl) benzenesulfonyl Fluoride Hydrochloride
CBα	Chicken Brain α -spectrin
CD	Circular Dichroism
DTT	Dithiothreitol
EDTA	Ethylenediaminetetracetic acid
ESI	Electrospray Ionization
HEβ	Human Erythroid β -spectrin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid
HSQC	Heteronuclear Single Quantum Coherence
IAEDANS	5-[[[(2-iodoacetyl)amino]ethyl]amino]-naphthalene-1-sulphonic acid
IPTG	Isopropyl β -D-1-thiogalactopyranoside
ITC	Isothermal Titration Calorimetry
LB	Luria-Bertani Medium
NRMSD	Normalized Root Mean Square Difference
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PED(P)	20 mM sodium phosphate, pH 8.0, 1 mM EDTA, 1 mM DTT, (0.1 mM PMSF)
PE/PC	Phosphatidylethanolamine/Phosphatidylcholine
PMSF	Phenylmethylsulphonyl Fluoride
Q-TOF	Quadrupole Time-of-Flight
RU	Response Units
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SpBD	Spectrin Binding Domain
SPR	Surface Plasmon Resonance

TCEP	Tris(2-carboxy-ethyl)phosphine Hydrochloride
TEDP	20 mM Tris, pH 8.0, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF
TEV	Tobacco Etch Virus
UV	Ultraviolet

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Human Erythrocyte Ankyrin

Fig. 1. Diagram of Human Erythrocyte Ankyrin

The three domains of ankyrin (N-terminal membrane domain, spectrin binding domain, and C-terminal regulatory domain) are diagrammed to scale. The hatched region within the 55 kDa Regulatory Domain indicates the location of a ~10 kDa Death Domain. Below, magnification of the spectrin binding domain (SpBD) indicates the positions and sizes of the putative minimal binding regions, Zu5 and 272. Corresponding amino acid residue numbers for the SpBD are provided.

Fig. 2. Diagram of the Human Erythrocyte Spectrin Tetramer

The human erythrocyte spectrin tetramer consists of two α and two β chains, arranged as shown. Each spectrin repeat of the tetramer is depicted as an ellipse; repeats of interest in studying spectrin-ankyrin binding (13, 14, 15, and 16) are shaded and numbered. Divided ellipses represent the dimerization sites formed from the N-terminus of α -spectrin and the C-terminus of β -spectrin. Triangles indicate the SH3 domain of α -spectrin.

Fig. 3. Purity and Apparent Molecular Masses of SpBD Constructs

A Coomassie Brilliant Blue-stained 4–20% SDS-PAGE gel of purified Zu5 and 272 flanked by molecular mass markers is shown.

Fig. 4. Conformational Analysis by Circular Dichroism (CD)

A, CD wavelength scan for Zu5 (diamonds) and reconstructed fit (line). B, CD wavelength scan for 272 (diamonds) and reconstructed fit (line). Fitting was performed with CONTINLL and reference set 3 on DICHROWEB. NRMSD values were 0.054 and 0.050 for Zu5 and 272, respectively.

Fig. 5. Gel Shift Assays with Zu5 Demonstrate Binding to HEβ1415

A, Native 10% PAGE of all purified constructs show that the spectrin repeats, with relatively low pI's of about 4–6 migrated more quickly than Zu5 with a pI of 8.5. 272, with a pI of 9.5, failed to enter the gel when run toward the (+) anode. *B*, Native 10% PAGE of HEβ1315, HEβ1415, HEβ89, and CBα1617 in the presence or absence of Zu5 are shown. A noticeable shift is observed for both HEβ1315 (lane 2) and HEβ1415 (lane 4) but not for HEβ89 or CBα1617. Zu5 alone is shown in the far right lane for comparison. Separate regions of the same gel are divided by a dashed line.

Fig. 6. Analytical Ultracentrifugation of Sedimentation Equilibrium Indicates Zu5, but not 272, Stably Binds HE β 1315 and HE β 1415

Analytical ultracentrifugation results for ankyrin constructs in the presence and absence of spectrin constructs are shown with fits and residuals. Zu5 (top left panel) exists as a monomer in solution and forms a stable complex with HE β 1315 (top center panel) and HE β 1415 (bottom center panel). To confirm the stability of this complex, data was analyzed at two speeds; the data presented show the global fit for each mixture. In contrast, Zu5 did not form a complex with CB α 1617 (bottom left panel) demonstrating the specificity of complex formation. 272 (top right panel) existed in a monomer-aggregate equilibrium which reproducibly gave molecular masses of ~ 10 and ~ 60 kDa when measured at 6°C . The 60 kDa aggregate could be “cleared” from the sample by spinning at high speed (data not shown). Finally, 272 did not evidence complex formation with HE β 1315 (bottom right

panel). All data were collected at 6 °C. Filled circles indicate data collected at 20,000 rpm, open circles 35,000 rpm, and shaded triangles 48,000 rpm.

Fig. 7. Purity and Apparent Molecular Masses of Spectrin Constructs

A Coomassie Brilliant Blue-stained 4–20% SDS-PAGE gel of the spectrin constructs flanked by molecular mass markers is shown. All fragments were obtained in high yield (5–50 mg per liter culture) with >99% purity as determined by SDS-PAGE.

Fig. 8. Circular Dichroism (CD) and Tryptophan Fluorescence Wavelength Scans
 A, HEβ1315 (red circles), HEβ1415 (blue squares), HEβ1314 (green triangles) and HEβ1516 (purple diamonds) yield typical α -helical CD spectra with troughs at 222 and 208 nm. The plotted molar ellipticities for these constructs were expected and obtained except for HEβ1315 as it consists of 3 repeats rather than 2. B, Tryptophan emission spectra obtained by exciting at 295 nm to exhibit blue-shifted wavelengths of maximum emission ranging from 335 to 338 nm. Intensities reflect the positions of the tryptophans in a repeating heptad pattern described in **RESULTS**. Symbols are as in A.

Fig. 9. Gel Shift Assays with Varying Stoichiometry of Spectrin Fragments to Zu5 Show 1:1 Binding

Spectrin fragments and Zu5 mixed in ratios ranging from 1:0 to 0:1 to determine binding stoichiometries. In the case of HEβ1314 and HEβ1516 this assay was performed to rule out the possibility that their binding to Zu5 is weak and could be shifted towards complex formation by adjusting the molar ratios. The two uppermost gels indicate that the spectrin:Zu5 binding ratio is between 1:1 and 1:1.5. When mixed with increasing relative amounts of HEβ1314 but not HEβ1516, Zu5 migration modestly increases; this effect is more prominent in Fig. 5 due to the 5% stacking gel.

Fig. 10. Surface Plasmon Resonance Determination of Binding Affinity and Kinetics

Representative sensorgrams and fits are shown for the binding of Zu5 to HEβ1315 (A) and HEβ1415 (B) at various concentrations. Data from each concentration series were fit globally and simultaneously for k_a and k_d . The sensorgrams presented reflect the reference and buffer subtracted curves. Zu5 was injected for 60 s beginning at $t = 0$; an additional 60 s was allowed for dissociation. For HEβ1315 (A), the average values of the three trials were: $k_a = 2.82 \times 10^6 \pm 4.44 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$; $k_d = 4.23 \times 10^{-2} \pm 6.58 \times 10^{-4} \text{ s}^{-1}$; and $K_D = 15.0 \text{ nM}$. For HEβ1415 (B): $k_a = 3.61 \times 10^6 \pm 5.70 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$; $k_d = 5.45 \times 10^{-2} \pm 7.46 \times 10^{-4} \text{ s}^{-1}$; and $K_D = 15.2 \text{ nM}$. CBα1617 provided a negative control to demonstrate specific binding, as a $1 \mu\text{M}$ injection of Zu5 applied over the CBα1617 flow cell resulted in no

binding (A, black circles). Sensorgrams and fits among the triplicate samples were nearly identical and demonstrated good correspondence between the fit and experimental data with global reduced χ^2 close to 1.

Table 1
Secondary Structure Analysis of Ankyrin Fragments Suggests Intrinsically Unstructured Regions

Secondary structure modeling analysis of CD data by DICHROWEB for Zu5 and 272 are shown. Dichroweb discriminates between “regular” and “distorted” forms of α -helix and β -strand, which are assigned according to the secondary structure prediction program DSSP (74) but were pooled for presentation here, since the two forms are sufficiently similar for present purposes.

Secondary Structure	Zu5 using reference set 3 NRMSD = 0.054	272 using reference set 3 NRMSD = 0.051	272 using reference set 6 NRMSD = 0.051
α -helix	7.8%	19.2%	7.4%
β -strand	39.5%	16.1%	22.1%
turns	19.8%	26.2%	10.0%
unstructured	32.9%	38.7%	60.5%