

Ankyrin Binds to the 15th Repetitive Unit of Erythroid and Nonerythroid β -Spectrin

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Abstract. Ankyrin mediates the attachment of spectrin to transmembrane integral proteins in both erythroid and nonerythroid cells by binding to the β -subunit of spectrin. Previous studies using enzymatic digestion, 2-nitro-5-thiocyanobenzoic acid cleavage, and rotary shadowing techniques have placed the spectrin-ankyrin binding site in the COOH-terminal third of β -spectrin, but the precise site is not known. We have used a glutathione S-transferase prokaryotic expression system to prepare recombinant erythroid and nonerythroid β -spectrin from cDNA encoding approximately the carboxy-terminal half of these proteins. Recombinant spectrin competed on an equimolar basis with ^{125}I -labeled native spectrin for binding to erythrocyte membrane vesicles (IOVs), and also bound ankyrin in vitro as measured by sedimentation velocity experiments. Although full length β -spectrin could inhibit all spectrin binding to IOVs, recombinant β -spectrin encompassing the complete ankyrin binding domain but lacking the amino-terminal half of the molecule failed to inhibit about 25% of the binding capacity of the IOVs, suggesting that the ankyrin-independent spectrin membrane binding site must lie

in the amino-terminal half of β -spectrin. A nested set of shortened recombinants was generated by nuclease digestion of β -spectrin cDNAs from ankyrin binding constructs. These defined the ankyrin binding domain as encompassing the 15th repeat unit in both erythroid and nonerythroid β -spectrin, amino acid residues 1,768-1,898 in erythroid β -spectrin. The ankyrin binding repeat unit is atypical in that it lacks the conserved tryptophan at position 45 (1,811) within the repeat and contains a nonhomologous 43 residue segment in the terminal third of the repeat. It also appears that the first 30 residues of this repeat, which are highly conserved between the erythroid and nonerythroid β -spectrins, are critical for ankyrin binding activity. We hypothesize that ankyrin binds directly to the nonhomologous segment in the 15th repeat unit of both erythroid and nonerythroid β -spectrin, but that this sequence must be presented in the context of a properly folded spectrin "repeat unit" structure. Future studies will identify which residues within the repeat unit are essential for activity, and which residues determine the specificity of various spectrins for different forms of ankyrin.

SPECTRIN, the major component of the erythrocyte membrane cytoskeleton, is a heterodimer composed of two subunits (α , β) of 280,000 and 246,000 D, respectively (Coleman et al., 1989; Sahr et al., 1990; Winkelmann et al., 1990). Each subunit is composed predominantly of multiple 106 amino acid residue repeats, α -spectrin containing 22 such repeats and β -spectrin 17 repeats. Each subunit also contains regions of nonhomologous sequence, and the average repeat-to-repeat sequence identity is $\sim 30\%$. The primary linkage of spectrin to the membrane in erythrocytes is mediated by the binding of β -spectrin with ankyrin. Inherited defects in the linkage of spectrin to ankyrin causes reduced erythrocyte stability and hemolytic disease in both mice (White et al., 1990) and humans (Lux et al., 1990; Lambert et al., 1990). The recognition that analogues of both spectrin

and ankyrin are present in probably all cell types, and that ankyrin can link a wide variety of integral membrane proteins to the cytoskeleton including Na,K-ATPase (Nelson and Veshnock, 1987; Morrow et al., 1989), the voltage gated sodium channel (Srinivasan et al., 1988), and others (Bourguignon et al., 1986; Kalomiris and Bourguignon, 1988), has heightened the importance of understanding the way spectrin and ankyrin interact.

The location and nature of the ankyrin binding site in spectrin are not well defined. Previous studies using enzymatic digestion and 2-nitro-5-thiocyanobenzoic acid (NTCB) cleavage identified a 50-kD fragment arising from the COOH-terminal third of β -spectrin which could inhibit the binding of spectrin to stripped erythrocyte membranes (Morrow et al., 1980). Rotary-shadowed electron micrographs of spec-

Portions of this work have been previously presented in abstract form (Kennedy, S. P., S. L. Warren, B. G. Forget, J. C. Winkelmann, and J. S. Morrow. 1990. *J. Cell Biol.* 111[No. 5, Pt. 2]:46a[Abstr.]).

1. *Abbreviations used in this paper:* IOV, spectrin and actin free erythrocyte inside-out vesicles; NTCB, 2-nitro-5-thiocyanobenzoic acid.

trin-ankyrin complexes were consistent with this assignment (Tyler et al., 1980). In addition, ankyrin was found to bind with higher affinity to spectrin tetramers and oligomers compared with dimeric spectrin, suggesting that the ankyrin binding site might be sensitive to conformational changes emanating from the spectrin self-association site (Cianci et al., 1988).

In the present study, as a first step to elucidating the molecular mechanisms by which spectrin-ankyrin binding may be controlled, we have used deletional analysis of recombinant β -spectrin to define precisely the structural determinants of the ankyrin binding domain. We find that ankyrin binding requires nearly the complete 15th repetitive unit of β -spectrin (residues 1,768–1,898), and that it is highly sensitive to deletions that appear to modify the conformation of this repeat. We also provide evidence that a similar ankyrin binding site exists in recombinant human nonerythroid β -spectrin (β -fodrin), and that an ankyrin independent membrane binding site exists in the amino-terminal half of β -spectrin. Collectively, these results provide a molecular framework for understanding the complexity of the spectrin-ankyrin interaction and its apparent regulation by allosteric and posttranslational mechanisms.

Materials and Methods

Construction of Full-length and Truncated β 28-Spectrin Expression Plasmids

The methods used to construct expression plasmids have been described by Maniatis and colleagues (1982). All enzymes and oligonucleotides were purchased from New England Biolabs. The pGEX vectors were the generous gift of Dr. James M. Anderson, Yale University, New Haven, CT, and may presently be purchased from Pharmacia LKB Biotechnology, Inc. (Piscataway, NJ). All other reagents were of analytical grade and purchased from Sigma Chemical Co., St. Louis, MO.

Plasmid pBR322 β 28 (Winkelmann et al., 1988), which contains ~40% of the coding sequence through the COOH terminus of human erythrocyte β -spectrin, was digested with NcoI and blunt ended by treatment with Klenow fragment of DNA polymerase I and deoxynucleoside triphosphates (dNTPs) as shown in Fig. 1. A BamHI linker (d(CGGATCCG)) was ligated onto the blunt end with T4 DNA ligase and the resultant plasmid was sequentially digested with BamHI and EcoRI. A 2.494-kb β 28 restriction fragment was purified from an agarose gel and ligated into complementary sites in pGEX-3X. Truncated forms of β 28 (see Fig. 2) were constructed by digesting pGEX-3X β 28 with the appropriate restriction endonuclease, treating with klenow (for XhoI, NdeI) or T4 DNA polymerase I (for SphI) and dNTPs, and inserting a nonsense codon linker, d(CTAGCTAGCTAG), containing a unique NheI site. Construction of the ScaI plasmid required isolation of the desired restriction fragment from a partial ScaI digest.

Sequential carboxy-terminal truncations (Fig. 5 a) were constructed by digestion of β 28XhoI with the NheI followed by time-dependent digestion with nuclease Bal 31 at 30°C. Reactions were terminated by extraction with phenol:chloroform (1:1) saturated with 10 mM Tris, 1 mM EDTA, pH 8 (TE buffer). Digested plasmids were blunt ended with Klenow, and NheI nonsense codon linkers were ligated onto this site. The resultant constructs were digested with NheI and BamHI, and truncated restriction fragments were purified and ligated onto β 28XhoI that had sequence between the BamHI and NheI sites removed.

Sequential amino-terminal truncations (Fig. 5 b) were constructed by digesting β 28Xho I-5C-ScaI with BamHI followed by digestion with nuclease Bal 31 and treatment with Klenow. A BamHI linker, d(CGCGGATCCGCG), was ligated into this site, truncated BamHI-EcoRI restriction fragments purified, and ligated into complementary sites in pGEX-IN, -2T, and -3X vectors.

β 28Sca I-4N-SphI and β 28Sca I-10N-NdeI (Fig. 6) were constructed by digesting β 28XhoI-5C-ScaI-4N and β 28XhoI-5C-ScaI-10N with SphI and NdeI, respectively. Both constructs were blunt ended and nonsense codon linkers were ligated into these sites. β 28XhoI-5C-SphI (Fig. 6) was con-

structed by removal of sequence between the BamHI and SphI sites of β 28XhoI-5C, treatment with T4 DNA polymerase, and ligation of the blunt-ended construct. Some β 28-constructs in pGEX-3X were subcloned into pGEX-2T by digestion with BamHI, followed by Klenow treatment, EcoRI digestion, and ligation of the BamHI (Blunt)-EcoRI restriction fragment into pGEX-2T. The β -fodrin expression plasmid was constructed by subcloning a 1.589-kb β -fodrin EcoRI restriction fragment, originally isolated from a human endothelial cell cDNA library, into pGEX-3X. The complete characterization of this clone will be reported separately (Chang, J.-G., and B. G. Forget, manuscript in preparation). Its identity as a bonafide β -fodrin clone has been verified by a comparison of the predicted amino acid sequence of overlapping genomic and cDNA clones with direct protein sequence obtained from purified human brain β -spectrin (Green, L. A. D., A. S. Harris, D. W. Speicher, and J. S. Morrow, unpublished data).

Expression and Purification of Recombinant β 28-Spectrin Fusion Proteins

Recombinant proteins were expressed and purified from *Escherichia coli* as described (Smith and Johnson, 1988). Overnight cultures of *E. coli* (JM101 or W3110) transformed with pGEX- β 28 constructs were diluted 1:10 with Luria broth medium and grown for 1 h at 37°C. Isopropylthiogalactopyranoside (1 mM) was added and after 3–4 h of growth, bacteria were pelleted and resuspended in 1:50 culture volume of a sonication buffer containing 50 mM Tris, 50 mM NaCl, 1 mM EDTA, and 0.15 mM PMSF, pH 8.0 at 4°C. Induction was maximal at 3–4 h and fusion proteins comprised 20–80% of total bacterial protein, as determined by densitometry of Coomassie blue-stained SDS gels (not shown). All subsequent steps were performed at 0–4°C. Bacteria were treated with 1 mg/ml lysozyme for 30 min and then 10 μ g/ml leupeptin, 1 mM PMSF, 1 mM DTT, 1 mM EDTA, 1 mM benzamidine, 10 μ g/ml aprotinin, and 1 μ g/ml pepstatin A were added. The suspension was frozen on dry ice, thawed at 30°C, and incubated with Triton X-100 (1%), deoxyribonuclease I (0.1 mg/ml), and MgCl₂ (10 mM) for 30 min. Diisopropylfluorophosphate (0.5 mM) was added and the suspension was sonicated with a sonifier (70 W; Branson Sonic Power, Danbury, CT) for 30 s. The lysate was centrifuged at 48,000 g for 20 min and the supernatant applied to a 2–4 ml reduced glutathione agarose column. The column was washed with 25 ml of phosphate buffer containing 130 mM NaCl, 7.2 mM Na₂HPO₄·7H₂O, 2.8 mM Na₂H₂PO₄, 1 mM DTT, 0.1 mM PMSF, 1 mM EDTA, pH 7.5 (PBSI), and 0.5% Triton X-100, followed by 100 ml of PBSI and 25 ml sonication buffer. Bound fusion protein was eluted with sonication buffer containing 5 mM reduced glutathione. For removal of the glutathione transferase tail, proteins bound to the glutathione column were eluted by the addition of 250 U of human plasma thrombin in 50 mM Tris, 150 mM NaCl, 2.5 mM CaCl₂, pH 8.0 for 1 h at 37°C. Samples were concentrated by centrifugation in centricon microconcentrators (Amicon Corp., Danvers, MA). Recombinant proteins that remained insoluble after lysing the bacteria were solubilized in freshly prepared 8 M urea, 50 mM Tris, 1 mM DTT, 1 mM EDTA, pH 8.0 at room temperature for 1 h. The urea extract was diluted 1:10 in sonication buffer and applied to the glutathione agarose column as above.

Erythrocyte Inside-Out Vesicle (IOV) Binding Studies

Spectrin and actin-free erythrocyte vesicles (IOVs), resealed ghosts, and ¹²⁵I-labeled spectrin tetramer were prepared as before (Morrow et al., 1980; Howe et al., 1985). Spectrin tetramer was iodinated using immobilized lactoperoxidase and glucose oxidase (Bio-Rad Enzymobeads, Richmond, CA). Fusion proteins were tested for ankyrin binding inhibitory activity by incubation with 40 μ g IOV protein at 25°C for 30 min, followed by incubation with radiolabeled spectrin for 2 h. Unbound spectrin was separated from vesicles by sedimentation through a 5% sucrose cushion and both the bound and unbound fractions were quantitated by γ counting (1282 Compugamma CS gamma counter; LKB Instruments, Gaithersburg, MD).

Sedimentation Velocity Studies

Human erythrocyte ankyrin was prepared by high salt extraction of IOVs followed by DEAE-cellulose chromatography (Tyler et al., 1980). Recombinant spectrin proteins (15–25 μ g), iodinated with Na [¹²⁵I] in the presence of Enzymobeads, were incubated with 100 μ g of ankyrin in isoKCl buffer (130 mM KCl, 20 mM NaCl, 10 mM Tris, 1 mM EDTA, 0.1 mM PMSF, 5 μ g/ml leupeptin, 0.5 mM β -mercaptoethanol) for 5 h at 0–4°C. Samples were centrifuged at 200,000 g for 20 h at 4°C in a 5–20% sucrose gradient in isoKCl. Approximately 0.35-ml fractions were collected and assayed for radioactivity by γ counting. Protein content was assayed by OD₂₈₀ and

SDS-PAGE. Gradients were calibrated with equine heart cytochrome C (1.83S), bovine serum albumin (4.31S), rabbit skeletal muscle aldolase (7.35S), and bovine liver catalase (11.3S).

Other Procedures

SDS-PAGE and protein transfer to nitrocellulose (Western blots) were performed as described (Towbin et al., 1979; Laemmli, 1970). SDS-PAGE gels were stained with Coomassie brilliant blue. Antibodies were prepared in New Zealand white rabbits using the isolated β -spectrin subunit or the COOH-terminal phosphorylated NTCB fragment eluted from SDS-PAGE gels (Mische, 1988). Western blots were developed by incubation with 125 I-labeled staphylococcal protein A (Lux et al., 1990) followed by autoradiography. Protein determinations were by the method of Lowry et al. (1951), with bovine serum albumin used as a standard. DNA sequencing was performed using a Sequenase kit Version 2.0 (United States Biochemical Corp., Cleveland, OH).

Results

Expression of Recombinant β -Spectrin Proteins in *E. coli*

A 2.679-kb β -spectrin cDNA, β 28 (amino acid residues 1,333–2,137, numbering according to Winkelmann et al., 1990) was used for these studies since the encoded sequence extended beyond the boundaries of the 50-kD NTCB fragment previously shown to contain some or all of the ankyrin binding site (Morrow et al., 1980). A β 28 NcoI-EcoRI restriction fragment encoding from the center of β -spectrin repeat 11(1397) to the carboxy terminus(2137) was subcloned into a glutathione S-transferase fusion protein expression vector as shown schematically in Fig. 1. The β 28(1397–2137) construct was truncated by insertion of translation termination codons at restriction endonuclease sites thought to be close to or distant from the ankyrin binding site (Fig. 2). These constructs allowed a determination of the general boundaries of the ankyrin binding site.

Induction of bacteria containing pGEX-3X β 28(1397-2137) constructs with isopropyl-thiogalactopyranoside resulted in the appearance of fusion protein bands not present in uninduced bacteria and at the predicted molecular weights (data not shown). These fusion proteins were purified directly from bacterial lysates on a glutathione affinity column and analyzed by SDS-PAGE (Fig. 2). Yields of purified recombinant proteins were highly variable, from 1–10 mg/liter of culture, depending largely on the size and solubility of each protein. Truncation at the NdeI(1865) site caused a dramatic decrease in the solubility of this fusion protein, however, it remained soluble after treatment with urea followed by slow dialysis into, or dilution with isotonic buffer (isoKCl). Fragments of spectrin have previously been shown to recover ankyrin binding activity after treatment with denaturants (Morrow et al., 1980).

To demonstrate that the β 28 fusion proteins were expressed in the correct reading frame and represented bonafide β -spectrin, they were transferred to nitrocellulose and immunoblotted with anti- β -spectrin antibodies (Fig. 2). These studies also clearly indicated that most of the lower molecular mass bands which appeared to copurify on the glutathione column with the recombinant spectrin peptides did not react with the anti-spectrin antibodies. All fusion proteins were recognized at their predicted relative molecular masses by an antibody specific for β -spectrin. Moreover, as expected, only native spectrin and the full-length β 28 recombinant protein

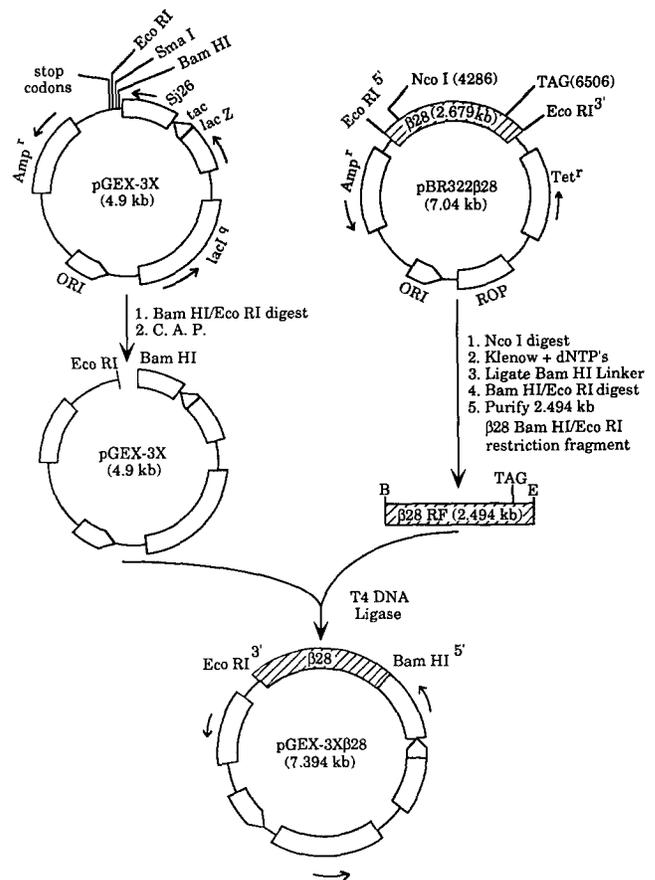


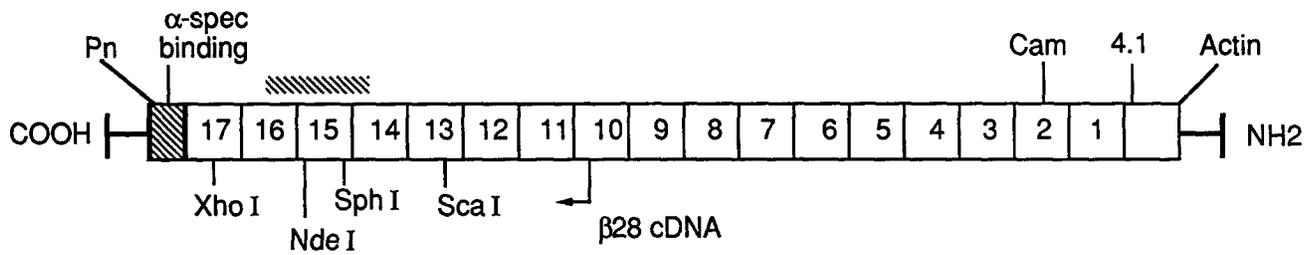
Figure 1. Construction of the β 28-spectrin prokaryotic expression vector. The pGEX-3X β 28 vector was constructed by cloning the 2.494-kb β -28-spectrin cDNA restriction fragment at the EcoRI and the BamHI cloning sites. The pGEX vectors are available in three different reading frames, pGEX-IN, -2T, and -3X. PGEX-2T and -3X vectors contain thrombin and factor Xa protease sites in the multiple cloning site respectively, which facilitates removal of the glutathione S-transferase(Sj26) portion of the fusion protein.

were recognized by antibody (β -1), which was specific for the extreme carboxy terminus of β -spectrin. Of technical interest in these studies was the small amount of product at 115,000 *M*, which appeared when the ScaI(1397-1589) construct was expressed in JM101 bacteria. This product also reacted with the β -1 antibody, and in separate studies was found, unlike the truncated ScaI(1397-1589) construct product (50,000 *M*), to be competent for ankyrin binding. The genesis of this minor read-through product was traced to nonsense suppression of the stop codon inserted into the ScaI(1589) site by the JM101 bacteria, a problem that was readily eliminated by expressing the same construct in W3110 bacteria, a strain that is nonsense suppressor defective (*sup*⁻) (Bachmann, 1983). All subsequent experiments were carried out in the W3110 strain of bacteria to avoid this problem.

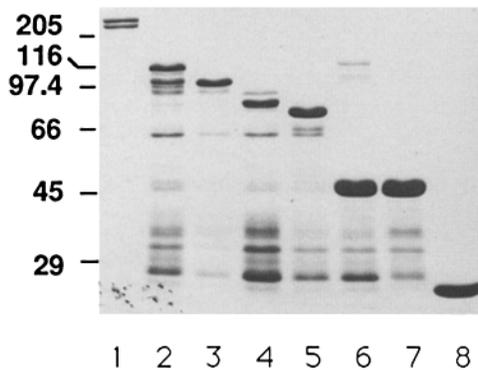
The β 28(1397-2137) and β 28-XhoI(1397-2015) Fusion Proteins Competitively Inhibit Ankyrin-dependent Spectrin-Membrane Binding

β 28 fusion proteins were examined for ankyrin binding activity by their ability to compete with radiolabeled spectrin

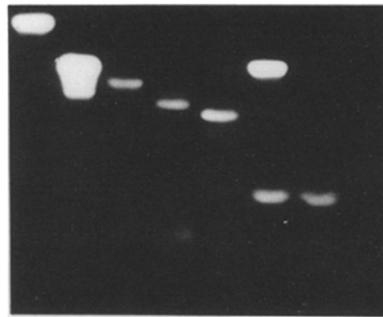
a



b



c



d

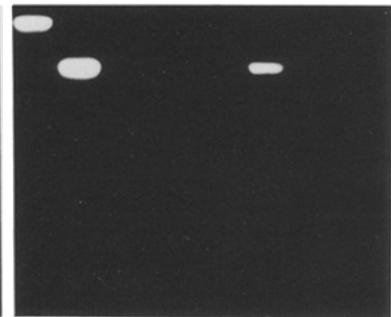


Figure 2. Recombinant β 28-spectrin proteins react with β -spectrin antibodies. (a) The repetitive unit structure of erythrocyte β -spectrin is shown with the approximate binding sites for α -spectrin (Morrow et al., 1980), calmodulin (Anderson and Morrow, 1987), protein 4.1 (Becker et al., 1990), and actin (Tyler et al. 1980). The approximate region of ankyrin binding as deduced from earlier studies is also depicted by the shaded bar (Morrow et al., 1980). The restriction endonuclease sites used to truncate the β 28 cDNA are illustrated. (b) β -Spectrin fusion proteins were expressed and purified by glutathione affinity chromatography from bacteria (*E. coli* W3110 unless otherwise noted), electrophoresis on 10% SDS gels and stained with Coomassie blue. Lane 1, purified erythrocyte spectrin; lane 2, rec- β 28 spectrin; lane 3, rec- β 28XhoI; lane 4, rec- β 28NdeI spectrin; lane 5, rec- β 28SphI spectrin; lane 6, rec- β 28ScaI spectrin (expressed in JM101); lane 7, rec- β 28ScaI spectrin; lane 8, pGEX vector (glutathione S-transferase) alone. The approximate size (kilodaltons) of standards and the major translation products are as indicated. (c) Autoradiogram of Western blot of gel shown in b, stained with polyclonal rabbit antibodies to β -spectrin. (d) Autoradiogram of Western blot of gel shown in b, stained with a polyclonal rabbit antibody specific for a COOH-terminal phosphorylated peptide generated by NTCB cleavage of β -spectrin (β -1). Note that all β 28-fusion proteins reacted with antibodies specific for the entire β -spectrin, and that only native spectrin and the full-length rec- β 28 protein reacted with an antibody specific for the extreme carboxy-terminus of β -spectrin. The immunoreactivity observed at \sim 115 kD in lane 6 (rec- β 28ScaI) is "read through" product due to expression in *E. coli* (JM101), which can suppress the termination codon inserted at the ScaI site in this construct. No read-through product appears when this construct is expressed in W3110 *E. coli* (lane 7).

for binding to erythrocyte IOVs, an interaction generally attributed to ankyrin (Bennett and Stenbuck, 1979) (Fig. 3). Unlabeled spectrin (2.4 μ M) inhibited over 95% of the binding of 125 I-spectrin to IOVs. In the same assay, the β 28(1397-2137) and β 28-XhoI(1397-2015) fusion proteins inhibited \sim 75 percent of maximal spectrin binding, and the β 28-NdeI(1397-1865), β 28-SphI(1397-1790), and β 28-ScaI(1397-1589) fusion proteins were without inhibitory activity (only the result for β 28-NdeI(1397-1865) is shown). Identical results were obtained with fusion protein concentrations up to 50 μ M and with the glutathione S-transferase protein removed (data not shown), indicating that the failure of the β 28(1397-2137) and β 28-XhoI(1397-2015) constructs to fully inhibit spectrin binding was not due simply to a reduced affinity for ankyrin (also see below). These findings, in con-

junction with other data discussed below, suggest that an ankyrin-independent membrane binding site(s) must reside in the amino-terminal half of β -spectrin.

Although the β 28(1397-2137) and β 28-XhoI(1397-2015) fusion proteins could not achieve a net level of inhibition equivalent to that of native spectrin, at low concentrations these peptides were just as effective as native spectrin, as shown in Fig. 4. Spectrin, β 28(1397-2137), and β 28-XhoI(1397-2015) all demonstrated competitive inhibition of 125 I-spectrin binding to IOVs, with nearly identical inhibition constants (K_i) of 1–3 μ M. Conversely, β 28-NdeI(1397-1865), β 28-SphI(1397-1790), and β 28-ScaI(1397-1589) fusion proteins were inactive. This placed the carboxy-terminal boundary of the ankyrin binding site between the XhoI(2015) and NdeI(1865) sites in the β 28 cDNA sequence (Fig. 2).

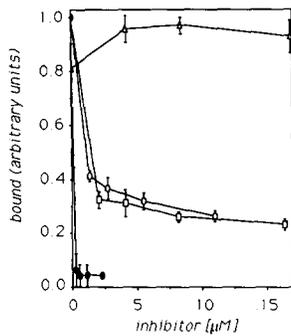


Figure 3. Recombinant β 28-spectrin competes effectively for 75% of the binding sites on erythrocyte IOVs. Recombinant β 28-fusion proteins were tested for their ability to inhibit the binding of radiolabeled spectrin to IOV's. Unlabeled native spectrin (●) inhibited nearly all of the binding of 125 I-labeled spectrin to IOVs. Rec- β 28 spectrin (○) and rec- β 28-XhoI spectrin (□) maximally inhibited ~65-75% of spectrin binding, even at concentrations of the recombinant peptides up to 50 μ M (data not shown). The rec- β 28-NdeI spectrin (Δ) did not inhibit spectrin binding. Each point is the mean of three determinations; errors represent the SEM.

Ankyrin Binding Requires the Full 15th Repeat Unit of β -Spectrin

To more precisely define the carboxy-terminal sequence of the ankyrin binding site, the pGEX-3X β 28-XhoI(1397-2015) plasmid was sequentially nuclease digested from the XhoI(2015) site to the inactivating NdeI(1865) site. The truncated fusion proteins were purified and examined for inhibitory activity in the IOV binding assay (Fig. 5 a). The truncated plasmids were also sequenced to define the new 3' terminus created by the digestion. Activity was not affected by 3' deletions until the boundaries of the 15th repeat unit were reached, whereupon activity was abruptly abolished (β 28-NdeI(1397-1865)). This result placed the carboxy-terminal boundary of a functionally active inhibitory peptide at amino acid 1,898 in the β -spectrin sequence. Truncations from the amino terminus of the smallest active fusion protein, β 28-XhoI-5C-(1397-1898), down to the amino-terminal ScaI(1589) site did not affect ankyrin binding activity (Figs. 2 and 5 b). However, truncation to the SphI(1790) site abolished activity (not shown), placing the amino-terminal boundary of the ankyrin binding site between these two restriction sites. Therefore, β 28-XhoI-5C-ScaI(1589-1898) was sequentially digested from the 5' terminus to the SphI(1790) site, and the resulting deletion mutants were expressed in each of three reading frames. Their fusion proteins were purified, and those making bonafide spectrin peptides were examined for inhibitory activity. As shown in Fig. 5 b, progressive deletion of amino-terminal sequence resulted in a gradual loss of inhibitory activity, which rapidly became variable and then fell to null values as the truncations approached the boundaries of the 15th repeat unit. These results suggested that the fusion protein β 28-XhoI-5C-ScaI-7N(1712-1898) represented the minimal sequence necessary for ankyrin binding. Alternatively, it was possible that the glutathione S-transferase fusion protein was interfering with ankyrin binding in the amino-terminal deletion recombinants. Therefore, further studies were carried out after removal of the fusion protein tail.

As shown in Fig. 6, removal of the glutathione S-transferase tail from β 28-XhoI-5C-ScaI-10N(ScaI-10N(1768-1898)) with thrombin, resulted in the return of measurable IOV spectrin binding inhibitory activity to this otherwise inactive protein. To ensure that the ScaI-10N(1768-1898) protein represented the minimal sequence required for ankyrin binding, it was

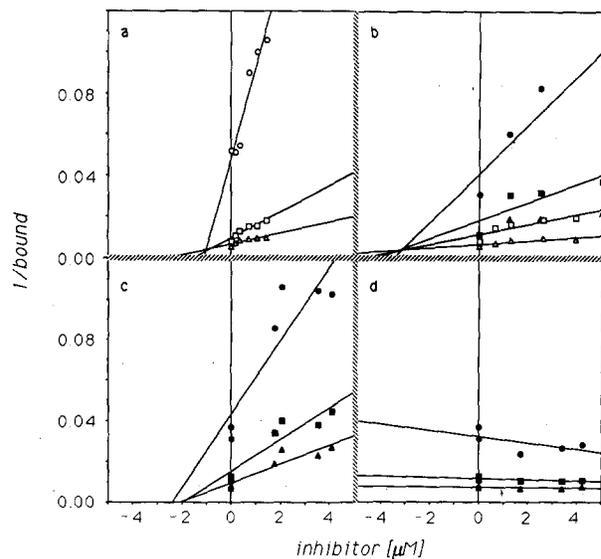


Figure 4. Recombinant β -spectrin proteins inhibit radiolabeled spectrin binding to IOVs with affinities equal to that of intact spectrin. Radiolabeled spectrin (○, □, Δ, ●, ■, ▲; 1, 11, 22, 1.2, 3.5, 6.5 μ g, respectively) was incubated with IOVs in the absence or presence of (a) spectrin, (b) rec- β 28 spectrin, (c) rec- β 28-XhoI spectrin, or (d) rec- β 28-NdeI spectrin. The approximate K_i s for all active proteins (including native spectrin) ranged from 1-3 μ M. The rec- β 28-NdeI spectrin was inactive.

further truncated from the amino (5C-SphI(1790)) or carboxy (10N-NdeI(1865)) termini and the glutathione S-transferase tails removed (Fig. 6). Both of these truncations abolished the inhibitory activity of the ScaI-10N(1768-1898) protein, indicating that this entire sequence was essential to fully inhibit spectrin-ankyrin binding. To ensure that the inhibitory activity that was being measured in the IOV assays was unique to the 15th repeat unit, a recombinant protein of equivalent size to ScaI-10N(1768-1898) but encoding repeat unit 14 was prepared. As shown in Fig. 6, this repeat 14 protein, β 28-ScaI-4N-SphI(1660-1789), which included sequence in common with the amino end of the ScaI-10N protein(1768-1898), was inactive.

Since spectrin has been shown to also bind to IOVs by ankyrin-independent mechanisms (Howe et al., 1985; Steiner et al., 1988), it was necessary to demonstrate that the recombinant proteins which were competent for inhibitory activity in IOV binding assays were indeed able to bind ankyrin. Therefore, active (β 28(1397-2137), ScaI-10N(1768-1898)) and inactive (4N-SphI(1660-1789), 10N-NdeI(1768-1865), 5C-SphI(1790-1898)) recombinant proteins were 125 I-labeled and assayed for direct binding to purified ankyrin by velocity centrifugation in sucrose gradients (Fig. 7). As expected, only the β 28(1397-2137) and ScaI-10N(1768-1898) proteins bound and comigrated with ankyrin, confirming the conclusions of the IOV-spectrin competition assays.

A Recombinant Nonerythroid β -Spectrin Peptide Encompassing the 15th Repeat Also Binds Ankyrin

Nonerythroid spectrin, or fodrin (240,000-235,000 M_r) can

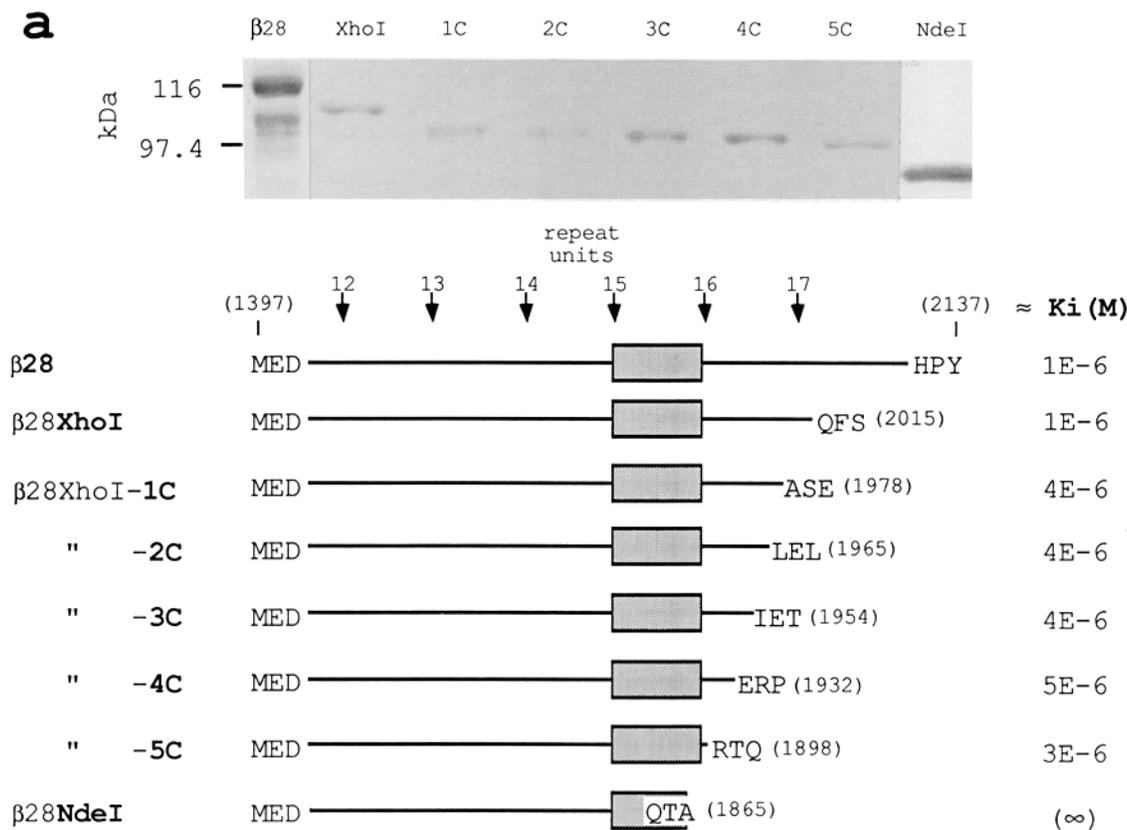


Figure 5. The carboxy- and amino-terminal boundaries of the β -spectrin ankyrin binding domain include repeat 15. Purified recombinant proteins were electrophoresed on 10 (NH₂-terminal) or 7% (COOH-terminal) SDS gels, and assayed for ankyrin binding activity as described in Fig. 3. Approximate K_i s were determined from Dixon plots. Amino acid positions within the β -spectrin sequence are indicated in parentheses. (a) The pGEX-3X $\beta 28$ -Xho I construct was sequentially digested from the XhoI site to the NdeI site with Bal 31 nuclease. The purified peptides are shown on SDS gels at the top, the location of the truncation, as determined by sequencing, is depicted at the bottom. (b) Removal of sequence between the 5' terminus and ScaI site of $\beta 28$ -XhoI-5C did not affect activity, therefore the 5C-ScaI construct was nuclease digested from the ScaI site to the SphI site. The purified recombinant peptides and the location of the truncations is shown as in a. ∞ , no inhibition up to 50 μ M of inhibitory peptide. * Although the 5C-ScaI-10N fusion protein was inactive, removal of the glutathione S-transferase tail with thrombin restored activity, as described below (Fig. 6).

also bind erythrocyte ankyrin, albeit with lower affinity than the erythrocyte protein (Harris et al., 1986). To determine if the ankyrin binding site in nonerythroid spectrin resided in a similar region of the β -subunit, a β -fodrin cDNA (1.589 kb) encompassing repeat 15 was expressed and purified as a glutathione S-transferase fusion protein (90,000 D) (Fig. 8). This β -fodrin recombinant, which represents approximately the COOH-terminal quarter of β -fodrin, was recognized by an antibody to intact fodrin (not shown) and was able to bind ankyrin as indicated by its comigration with purified erythrocyte ankyrin during velocity sedimentation (Fig. 8). Thus, ankyrin binds to similar regions of both human erythrocyte β -spectrin and human β -fodrin. As shown below (Fig. 9), the 15th repeat of these two proteins share many similar features, which distinguish them from other spectrin repeats.

Discussion

The data presented here indicate that ankyrin binds to the 15th repetitive unit of human erythrocyte and nonerythroid

β -spectrin, and that the binding site requires a complete 15th unit and some portions of the 16th unit. This conclusion is supported by the following observations: (a) an erythroid β -spectrin recombinant peptide which begins at the second residue in the 15th repeat and extends 25 residues into the 16th repeat is fully active in vitro for ankyrin binding; (b) recombinant peptides with NH₂-terminal deletions 24 residues into the repeat are inactive; (c) recombinant peptides with COOH-terminal deletions 8 residues into the 15th repeat are inactive; (d) a nonhomologous 43-residue sequence that is unique to the 15th repeat of erythroid spectrin is highly conserved in the 15th repeat of β -fodrin; and (e) recombinant β -fodrin peptides encompassing the 15th unit bind ankyrin in vitro. These results are summarized in Fig. 9.

In addition, it has been demonstrated that recombinant β -spectrin peptides encompassing repeat 11 through the carboxy terminus inhibit only the ankyrin-dependent binding of spectrin to erythrocyte membranes (IOVs), but not the ankyrin-independent binding. Since previous studies have found that all spectrin-membrane binding to IOVs can be inhibited by the intact β -spectrin subunit (Morrow et al.,

b

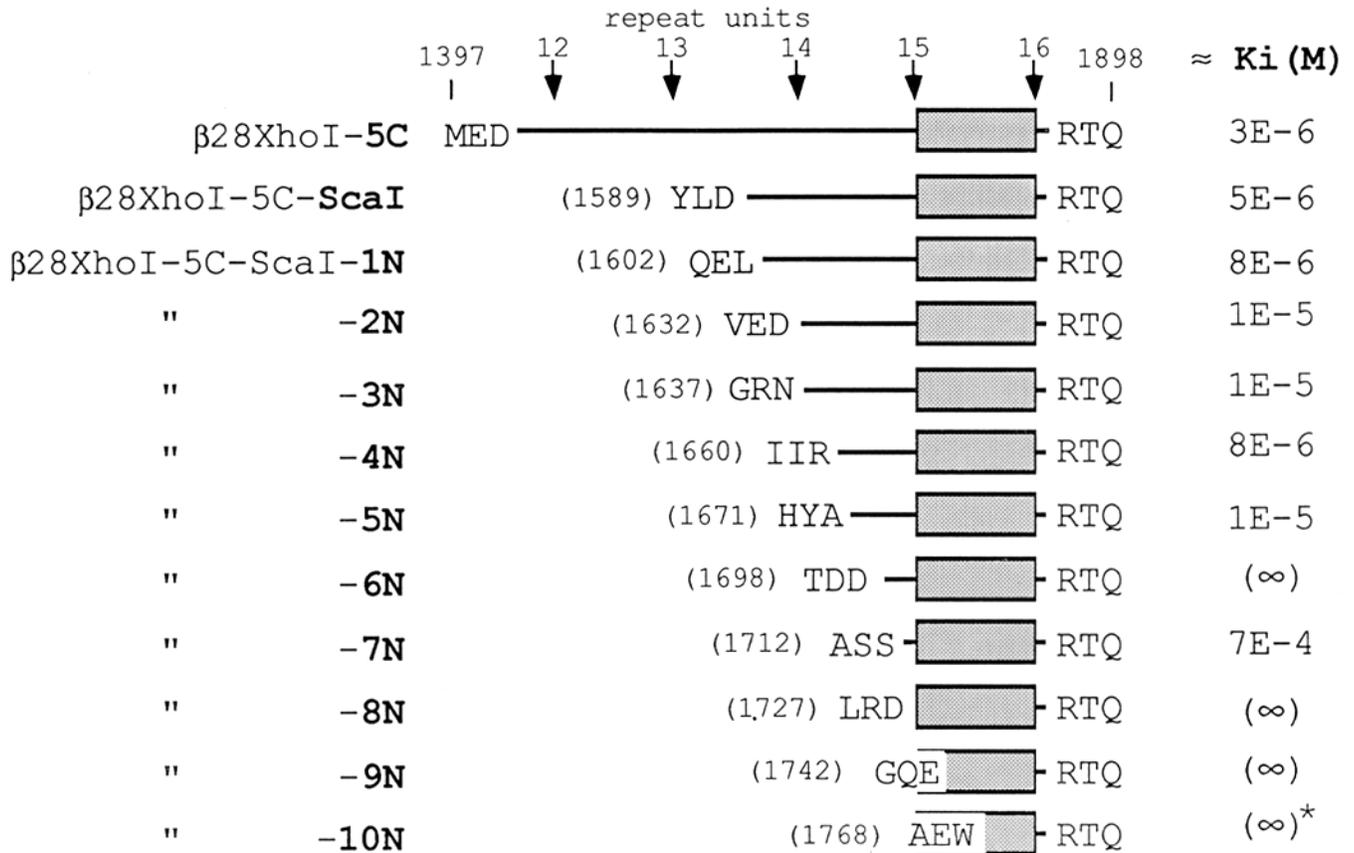
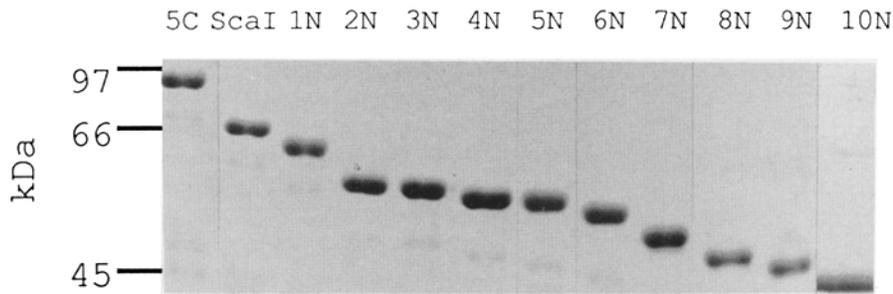


Figure 5.

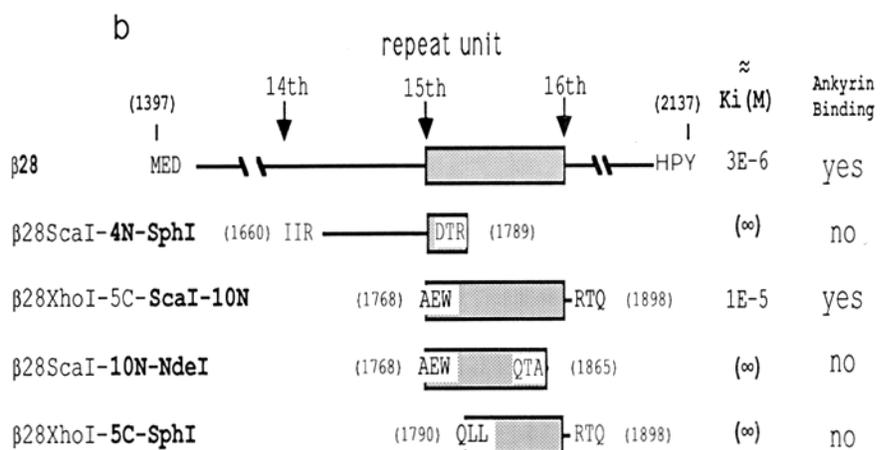
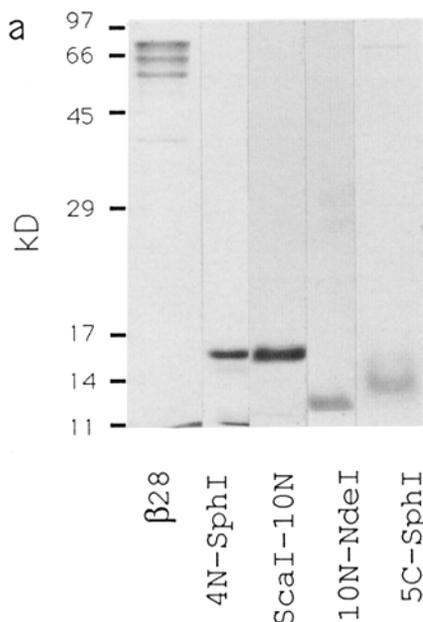


Figure 6. Only recombinant β -spectrin proteins containing the complete 15th repeat unit have ankyrin binding activity. (a) Coomassie blue-stained 15% SDS gel of β -spectrin recombinant proteins with glutathione S-transferase fusion protein tails removed. (b) β -28XhoI-5C-ScaI-10N was further truncated from its amino and carboxy termini, and β 28ScaI-4N-SphI, which contained the 14th repeat unit of β -spectrin, were prepared and assayed for both IOV binding inhibitory activity and for direct ankyrin binding activity (Fig. 7).

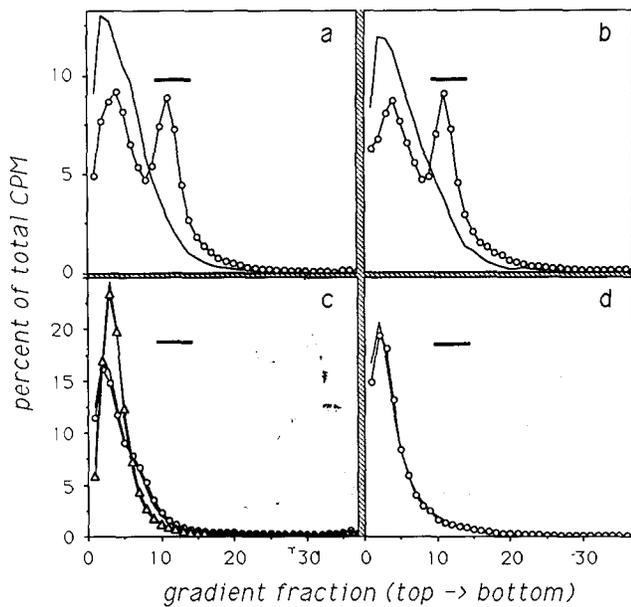


Figure 7. Recombinant $\beta 28$ XhoI-5C-ScaI-10N spectrin binds directly to ankyrin. Recombinant proteins with glutathione S-transferase tails removed were labeled with 125 I, incubated with (O, Δ) or without (lines only) purified ankyrin and centrifuged on a 5–20% sucrose gradient. The solid black bar indicates the migration position of ankyrin at 6.3S, as determined by OD₂₈₀ and SDS-PAGE analysis. Counts in each fraction were normalized relative to total counts in the gradient. (a) rec- $\beta 28$ spectrin; (b) rec- $\beta 28$ XhoI-5C-ScaI-10N spectrin; (c) rec- $\beta 28$ ScaI-10N-NdeI spectrin (O) and rec- $\beta 28$ XhoI-5C-SphI spectrin (Δ); (d) rec- $\beta 28$ ScaI-4N-SphI spectrin.

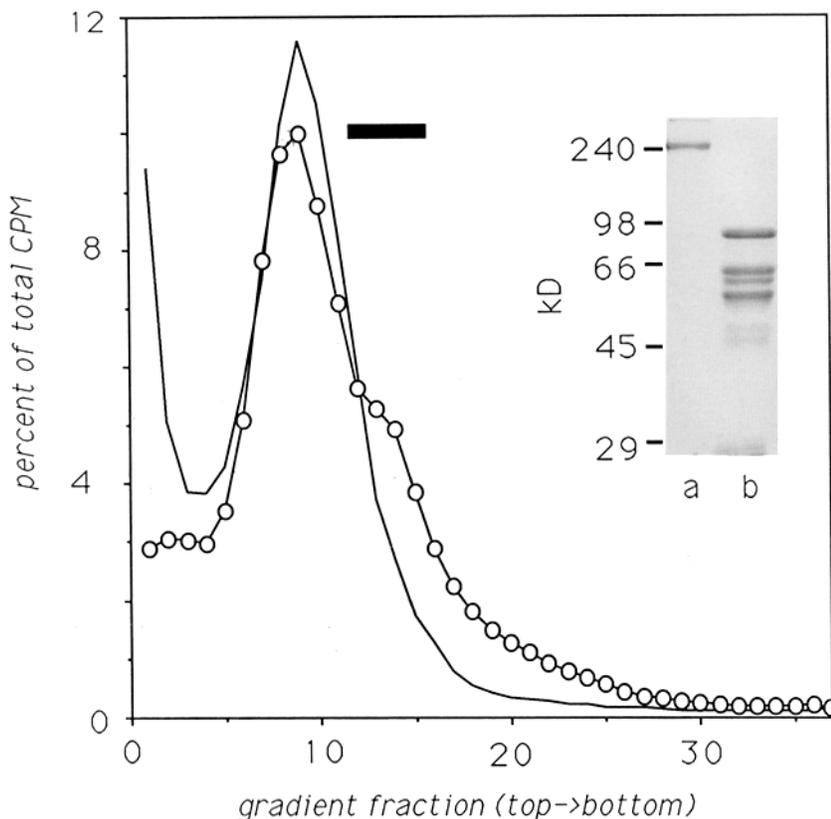
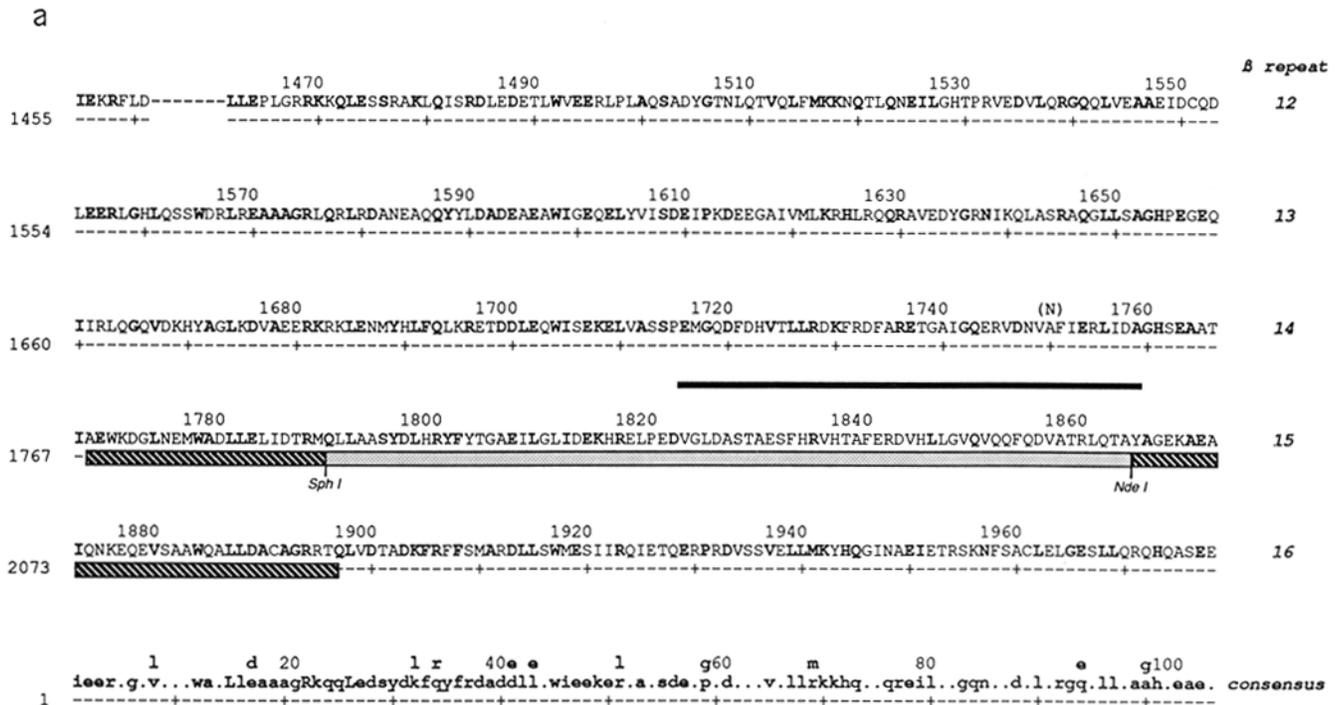


Figure 8. A recombinant human β -fodrin fusion protein binds ankyrin. A β -fodrin cDNA (10 D) encompassing the 15th repeat unit was expressed as a fusion protein and assayed for direct ankyrin binding as described in Fig. 7. The shift in migration of the rec- β -fodrin 10-D protein after incubation with ankyrin (O) compared to its migration without ankyrin (line) was reproduced in three separate experiments. Intact native fodrin also bound ankyrin in this assay (data not shown). (Inset) Purified human brain fodrin (lane a) and recombinant β -fodrin (10 D) (lane b, 90 kD) were analyzed by SDS-PAGE on a 10% gel.

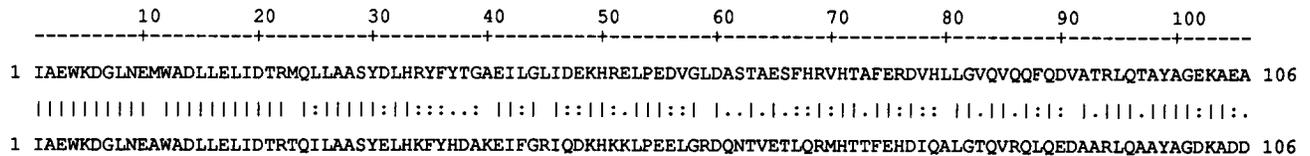
1980), our present results imply that the ankyrin-independent membrane binding site(s) must exist in the NH₂-terminal half of β -spectrin (repeats 1–10). Since this is also the location of the protein 4.1 binding site (Becker et al., 1990), further deletional analysis studies will be required to distinguish the relative contributions of the 4.1 site from other ankyrin independent membrane binding sites in erythroid and nonerythroid β -spectrin.

The large size of the minimum ankyrin binding domain, 15,300 D, implies that both linear and conformational determinants contribute to the ankyrin binding function of spectrin. Comparison of 15th repeat sequence with that of other repeats that do not bind ankyrin (Fig. 9 a) revealed several unique features, which may contribute to its ability to bind ankyrin. The most striking difference in the 15th repeat is the absence of the strongly conserved tryptophan at repeat position 45 (residue 1,811) (Winkelmann et al., 1990). A second difference is the presence of a highly nonhomologous 43-residue sequence in the latter half of this repeat, residues 1,823–1,865. This sequence is only 7% identical to a consensus sequence derived from repeats 12–16, compared to an average identity of 39% for repeats 12, 13, 14, and 16. Both of these distinguishing features are also preserved in the 15th repeat of β -fodrin (Fig. 9 b), where the nonhomologous 43-residue sequence is 51% identical to that in the erythrocyte protein but dissimilar to other β -fodrin repeats. The differences in amino acid sequence in these two nonhomologous segments probably accounts for the different affinities of erythrocyte and brain spectrin for ankyrin (Harris et al., 1986).



b

repeat 15



repeat 16

	110	120	130		Similarity	Identity
107	IQNKEQEVSAAWQALLDACAGRRRTQ	131		β -spectrin		
	.:: ..				81.1%	60.3%
107	IQKRENEVLEAWKSLLDACESRRVR	131		β -fodrin		

Figure 9 (a) The 15th repeat ankyrin binding domain is atypical and contains a nonhomologous segment. Erythroid β -spectrin repeat units 12-16 were aligned (Winkelman et al., 1990), and compared to their consensus sequence. Consensus residues are depicted in bold letters. The boxed area represents the extent of the ankyrin binding rec- β 28-ScaI-10N protein; hatched sections represent inactivating truncations at the SphI and NdeI sites. The solid bar indicates a 43-amino acid nonhomologous segment. (b) The 15th repeat of both erythroid (top) and nonerythroid β -spectrin (bottom) share many similarities. Note the absence of trp 45 in both proteins, and the nonhomologous segment in the latter third of the repeat. Presumably, the amino acid differences between β -fodrin and β -erythrocyte spectrin shown here account for the \sim 100-fold lower affinity of the brain protein for erythrocyte ankyrin (compared to erythrocyte β -spectrin; see Harris et al., 1986).

A third unusual feature of the 15th repeat of β -spectrin emerges from a comparison of the spectrin and fodrin sequence (Fig. 9 b). The first 30 residues of the 15th repeat are unusually well conserved between β -fodrin and β -spectrin, being 90% identical compared to 33, 30, 53, and 60% identity for the 12th, 13th, 14th, and 16th repeats, respectively (Forget, B. G., unpublished data). This observation, together with the sensitivity of ankyrin binding to deletions in this region, indicate that this portion of the 15th unit also

must be particularly important for ankyrin binding activity. However, since recombinant peptides encompassing the 14th unit of β -spectrin and the initial 22 residues of the 15th repeat do not bind ankyrin (Fig. 6), it appears that this highly conserved sequence alone is insufficient for activity.

Collectively, these observations suggest that while the 43-residue nonhomologous segment in the terminal third of the 15th spectrin repeat unit is probably most critical for ankyrin binding activity, other conformational determinants provided

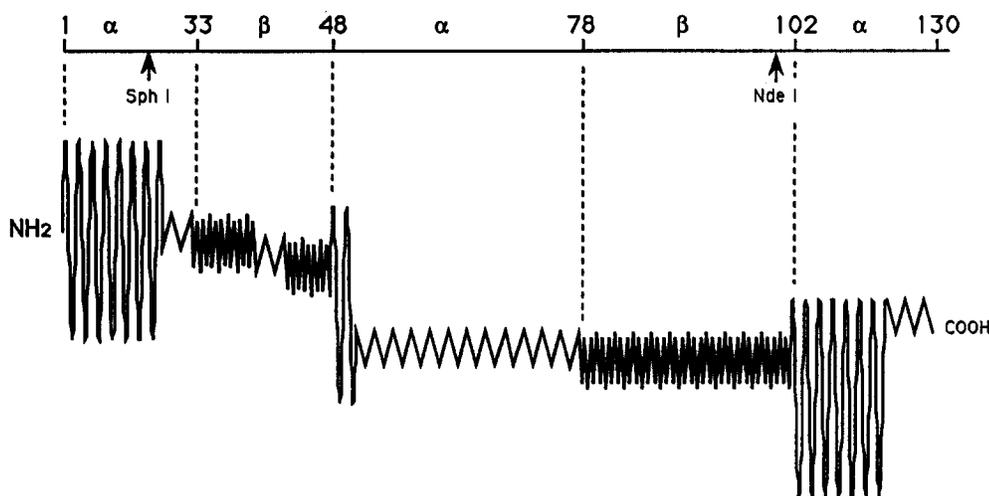


Figure 10. Predicted secondary structure (Chou-Fasman) of the β -spectrin ankyrin binding domain. The Chou-Fasman algorithm (Wisconsin Genetics Computer Group, Nucleic Acids Research 12(1); 387-395) was used to analyze the 130-amino acid ankyrin binding domain (residues 1,768-1,898; Winkelmann et al., 1990). Predicted α -helical (α) and β -sheet (β) conformations as well as inactivating truncations (SphI, NdeI) are shown. Note the strong α -helical structure predicted for the conserved first 30 residues of the repeat, and for the COOH-terminal portions of the ankyrin binding peptide (which extends into the 16th repeat).

by flanking sequences are also required. The most important of these flanking sequences appears to be the initial 30 residues in the repeat. Algorithms predicting secondary structure (Chou and Fasman, 1978) suggest that in the nonhomologous segment, α -helix is largely replaced by β -sheet or other nonhelical structure (Fig. 10), while the initial 30 residues of the repeat are predicted to be strongly α -helical. The sequences exiting the repeat are also predicted to be largely α -helical. Truncations which abolish ankyrin binding activity in the ScaI-10N(1768-1898) peptide (SphI(1790) and NdeI(1865)) are thus predicted to remove α -helical conformation. We interpret these observations to mean that ankyrin binding activity requires the nonhomologous 43-residue segment, which must be presented in the context of a proper "spectrin repeat" tertiary folded structure with α -helices entering and exiting the repeat unit.

The complexity of the ankyrin binding site and its apparent sensitivity to conformational constraints suggests that small deletions or post-translational modifications may significantly alter spectrin's ankyrin binding activity. These changes probably need not occur at sites directly involved with ankyrin binding, since they must only impact the secondary or tertiary structure of the 15th repeat unit. We therefore anticipate that inherited mutations affecting spectrin-ankyrin binding will prove to be relatively common, but perhaps more subtle in their effects than those affecting dimer-dimer self-association (Knowles et al., 1983). At the same time, the sensitivity of ankyrin binding to several factors within the repeat unit suggests a structural basis for the modulation of ankyrin binding by the oligomeric state of spectrin (Cianci et al., 1988) or by the phosphorylation state of ankyrin (Lu et al., 1985). Further studies using site-directed mutagenesis may be required to identify which residues within the repeat unit are most essential for activity, and which residues contribute to the specificity of various spectrins for different forms of ankyrin.

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