

Identification of functional domains of human erythrocyte spectrin

(peptide maps/cytoskeleton/membrane binding/peptide association/protein structure)

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ABSTRACT Isolated human erythrocyte spectrin is a dimer of two unique polypeptide chains. The dimer ($\alpha\beta$) undergoes reversible salt- and temperature-dependent association to form ($\alpha\beta$)₂ tetramers. Spectrin also binds with high affinity to a protein receptor on the cytoplasmic surface of erythrocyte membrane vesicles. By cleavage of spectrin at its cysteine residues with 2-nitro-5-thiocyanobenzoic acid, a 50,000-dalton peptide fragment has been isolated which inhibits the binding of spectrin to erythrocyte membrane vesicles. This peptide arises from a terminal region of the β chain. An 80,000-dalton peptide generated by restricted trypsin digestion binds preferentially to dimeric spectrin. This peptide arises from a terminal portion of the α chain. Multiple peptides involved in noncovalent associations between the chains have also been identified. These associations indicate that the two subunits of spectrin are aligned parallel to one another and that the tetramer formation site and the high-affinity membrane binding site are in close proximity to one another.

The composition and organization of the erythrocyte cytoskeleton has become the object of intense investigative effort (1-16). A major component of the cytoskeleton and the predominant protein associated with the cytoplasmic surface of the erythrocyte membrane is spectrin (6, 7). It is likely that the cytoskeleton controls the viscoelastic properties of the erythrocyte and the lateral distribution of intrinsic membrane proteins.

To understand these phenomena in molecular terms, one must identify both the participating proteins and the factors that regulate their assembly into the macromolecular arrays characteristic of the cytoskeleton. Toward this end, a number of specific protein-protein interactions have been identified. Band 2.1 is a high-affinity binding site for spectrin present on the cytoplasmic surface of the erythrocyte membrane (2, 10). Another putative spectrin-binding protein is band 4.1 (3, 4). Actin (band 5) also may bind spectrin, possibly as short filaments of F-actin (11, 12); some investigators believe that band 4.1 is necessary for this interaction (3). Spectrin by itself demonstrates at least two specific protein-protein interactions. The functional unit of spectrin appears to be an $\alpha\beta$ dimer that exhibits a reversible salt- and temperature-dependent association to form a tetramer ($\alpha\beta$)₂ (13).

In a preceding paper (1), we demonstrated that the two chains of spectrin are unique polypeptides. Proteolytically resistant peptide domains ranging in molecular weight from 80,000 to 28,000 were identified and aligned by peptide mapping. In the present paper, we demonstrate that specific functional domains also exist in spectrin; the tetramer-forming site and the high-affinity membrane-binding site are each contained in specific peptide subsets of the intact molecule. In addition, we have identified multiple chain-chain ($\alpha\beta$) asso-

ciation sites, allowing the alignment of the two spectrin polypeptide chains with respect to each other.

MATERIALS AND METHODS

Extraction and Purification of Spectrin. Unless otherwise noted, all procedures were performed at 0-4°C. Hemoglobin-free erythrocyte membranes were prepared as described (1, 5). Spectrin was extracted by incubating the membranes at 37°C for 30 min in 5-10 vol of extraction buffer [0.3 mM sodium phosphate/0.1 mM EDTA/0.2 mM diisopropyl fluorophosphate (iPr₂P-F)]. After centrifugation at 300,000 × g for 1 hr, the supernatant contained primarily spectrin and actin. This spectrin/actin mixture was concentrated by ultrafiltration and purified by gel filtration on a 2.5 × 90 cm column of Sepharose CL-4B (Pharmacia) in isotonic KCl buffer [10 mM Tris/20 mM NaCl/130 mM KCl/0.5 mM 2-mercaptoethanol/0.03 mM phenylmethylsulfonyl fluoride (PhMeSO₂F)] (18). The NaDodSO₄ step (1) was omitted to preserve native inter-chain associations. This made no difference in the limited tryptic digestion pattern.

Tryptic Digestion of Spectrin and Purification of 80,000-Dalton Peptide. Samples of purified spectrin were digested at 0°C in 10 mM Tris/20 mM NaCl/130 mM KCl, pH 8.0. Trypsin (TPCK-treated, Worthington) was added at an enzyme-to-substrate ratio of 1:20. Digestion times varied from 45 min to 2 hr. For some experiments, lower salt concentrations were used, usually 40 mM NaCl. Digestion was terminated by inactivating trypsin with 1 mM iPr₂P-F (final concentration) and pancreatic trypsin inhibitor (Worthington) at 1 mg/mg of trypsin.

Alternatively, spectrin was digested *in situ* by treatment of intact erythrocyte ghosts (2 mg/ml) in 5 mM phosphate, pH 8/20 mM NaCl/20 μg of trypsin per ml. Other conditions were as given above. After inhibition of trypsin, the ghosts were removed by centrifugation at 30,000 × g for 1 hr.

The 80,000-dalton peptide was a prominent soluble component of either digest and was purified by gel filtration at room temperature in 0.1% NaDodSO₄/40 mM sodium phosphate/1 mM EDTA/0.03 mM PhMeSO₂F/0.1 mM dithiothreitol, pH 7.5, on a 5 × 90 cm Ultrogel Aca 34 (LKB, Sweden) column. After gel filtration, the pooled fractions containing 80,000-dalton peptide were concentrated by ultrafiltration on an Amicon XM-50 membrane, and the NaDodSO₄ was removed by exhaustive dialysis against isotonic KCl buffer. Alternatively, 80,000-dalton peptide was purified by gel filtration at 4°C in isotonic KCl buffer on a 2.5 × 90 cm Ultrogel Aca 34 column without detergent.

Abbreviations: iPr₂P-F, diisopropyl fluorophosphate; IOV, inside-out vesicle; NTCB, 2-nitro-5-thiocyanobenzoic acid; PhMeSO₂F, phenylmethylsulfonyl fluoride; Gdn-HCl, guanidinium hydrochloride.

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Preparation of 50,000-Dalton Peptide. Purified spectrin was cleaved at its cysteine residues as described (18). Spectrin was dissolved at 6 mg/ml in 200 mM Tris-HCl, pH 8/7.5 M guanidinium HCl (Gdn-HCl)/1 mM EDTA. Cleavage was initiated by incubating the solution for 1 hr at room temperature after the addition of 2-nitro-5-thiocyanobenzoic acid (NTCB) to 2 μ M. Subsequently, 1 M Tris was added to raise the pH to 9.0, and the solution was incubated at 37°C overnight. The reaction was terminated by the addition of 2-mercaptoethanol to 50 mM, and the Gdn-HCl was removed by dialysis. NTCB was synthesized as described (19). The 50,000-dalton peptide was purified from the crude reaction mixture by preparative electrophoresis in the presence of NaDodSO₄ using a UNIPHOR system (LKB Instruments).

Inside-Out Vesicle (IOV) Binding Assay. Purified spectrin was labeled with ¹²⁵I and IOVs were prepared from fresh ghosts as described (5). Peptides were tested for inhibitory activity by preincubation of the peptide with 40 μ g of IOVs at room temperature for 30 min, followed by the addition of 15 μ g of radiolabeled spectrin. This mixture was incubated at 37°C for 30 min and then centrifuged at 40,000 \times g for 10 min; the extent of spectrin binding was measured by using a Beckman Biogamma counter.

Polyacrylamide Gel Electrophoresis. Electrophoresis under nondenaturing conditions was performed in detergent-free 6 \times 100 mm polyacrylamide gels at 4°C. These gels generally contained between 4–8% acrylamide in 40 mM Tris/20 mM sodium acetate/2 mM EDTA, pH 7.4. This buffer system is similar to that of Fairbanks *et al.* (8). The bisacrylamide content was 4% of total acrylamide. Samples were made 5% in sucrose immediately before electrophoresis to facilitate loading; electrophoresis was carried out in the same Tris/sodium acetate buffer used for preparation of the gels.

Nondenaturing gradient slab gels were prepared 3 mm thick ranging from 3.5% to 25% acrylamide by using the above buffer and acrylamide preparations. Two-dimensional polyacrylamide gels were prepared, using the nondenaturing 6 \times 100 mm gels instead of isoelectric focused gels, by the method of O'Farrell (20). The second dimension was generally a 10% acrylamide gel containing 0.1% NaDodSO₄ as described by Laemmli (21). Proteins were visualized by staining with Coomassie blue R-250 (Bio-Rad).

Two-Dimensional Peptide Maps. Tryptic and chymotryptic maps were prepared from Coomassie blue-stained peptide bands by the method of Elder *et al.* (22), with modifications as described (1).

RESULTS

Binding of Spectrin to IOVs. Spectrin bound selectively to the cytoplasmic surface of erythrocyte membrane IOVs in a saturable and inhibitable manner, as reported (5, 23). When NaDodSO₄-purified α or β chains (7) were examined for their ability to inhibit the binding selectively, the inhibitory activity was found exclusively on the β chain (Fig. 1A), confirming a previous report that only the β chain is able to bind IOVs (5). Limited trypsin digests of spectrin did not contain inhibitory peptides smaller than 100,000 daltons (data not shown), but cleavage of spectrin at its cysteine residues with NTCB (18) yielded a mixture of smaller peptides with reduced but still significant inhibitory activity. The smallest active fragment in this mixture was a 50,000-dalton peptide (Fig. 1B Inset).

This peptide was able to inhibit the binding of ¹²⁵I-labeled spectrin to IOVs (Fig. 1B). When the vesicles incubated with the 50,000-dalton NTCB peptide were washed in 0.1 mM

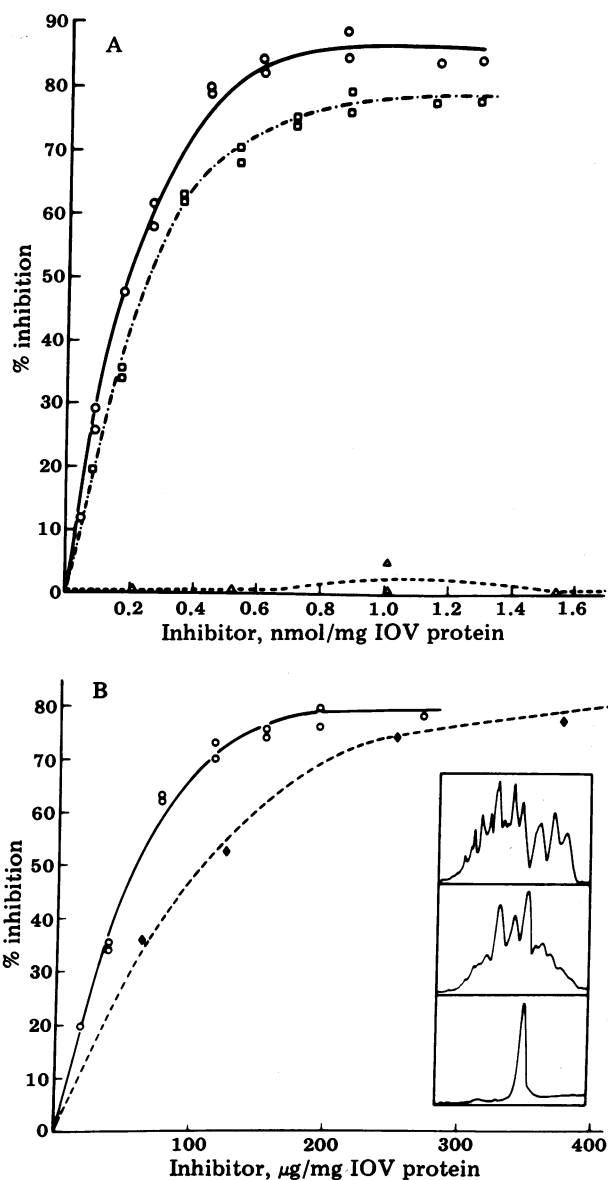


FIG. 1. Inhibition of binding of ¹²⁵I-labeled spectrin to IOVs. The indicated amount of peptide was incubated with 40 μ g of IOVs for 30 min at room temperature; 15 μ g of ¹²⁵I-labeled spectrin was added and the mixture was incubated for an additional 30 min at 37°C in a 400- μ l Microfuge tube. The IOVs and bound spectrin were sedimented by centrifugation at 40,000 \times g for 10 min, and the bound spectrin was quantitated by gamma counting. The percent inhibition is expressed as extent of spectrin bound with inhibiting peptide compared to controls without competing peptide. In all cases, non-specific binding to resealed ghosts was deducted from the amount bound to the vesicles, as described (5, 23). (A) Inhibition of binding by spectrin (O) or NaDodSO₄-purified α (Δ) or β (□) chains. (B) Inhibition by spectrin (O) or 50,000-dalton peptide (◆). (Inset) Sequential densitometric scans of Coomassie blue-stained polyacrylamide gel electrophoretic analysis of NTCB-cleaved peptides with inhibitory activity during different stages of purification. The top tracing represents the NTCB cleavage peptides of spectrin. The middle tracing represents a fraction with inhibitory IOV binding activity. The bottom tracing represents the purified 50,000-dalton NTCB peptide.

EDTA at pH 8.0 before addition of the ¹²⁵I-labeled spectrin, no inhibition of spectrin binding occurred.

To identify the origin of this peptide, its peptide map was compared with maps obtained from tryptic peptides after re-

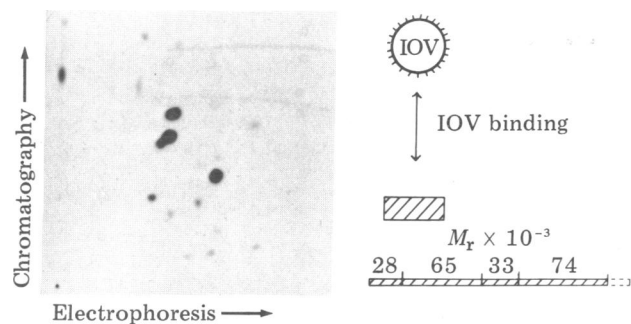


FIG. 2. Chymotryptic peptide map of 50,000-dalton NTCB peptide. The scheme at the right indicates the relationship of this map relative to the maps of reference tryptic peptides and the alignment of these peptides in the primary sequence of the β -chain (see ref. 1). The tryptic map of this peptide (not shown) was consistent with this alignment.

stricted proteolysis of isolated α and β chains (1). In Fig. 2 the chymotryptic map of this peptide is shown, along with the position it corresponds to in the linear arrangement of tryptic peptides. The map of the NTCB peptide shared unique features with the maps of the β -chain 28,000- and 65,000-dalton tryptic peptides (see ref. 1). We conclude that this peptide, which is strongly inhibitory of spectrin binding to IOVs, arises from the β -subunit and overlaps two of the peptides generated by restricted tryptic digestion of spectrin.

Tetramer Formation Involves an 80,000-Dalton α -Chain Peptide. A prominent soluble product of restricted trypsin digestion of either purified spectrin or intact ghosts was the 80,000-dalton peptide. This peptide was obtained at >95% purity by gel filtration either with or without detergent. The presence of NaDodSO₄ during the purification did not affect any of the measured properties of the peptide. The purest preparations were obtained by digestion of intact hemoglobin-free ghosts, followed by chromatography in 0.1% NaDodSO₄ (Fig. 3).

Polyacrylamide gel electrophoresis in the absence of denaturants provided a rapid and accurate means of assessing oligomer populations of spectrin and of measuring 80,000-dalton peptide binding (Fig. 4). When incubated in the presence of spectrin, the 80,000-dalton peptide bound preferentially to the dimer species, yielding a new species with an apparent molecular weight of 560,000. No binding to the tetramer was detected at the concentrations of 80,000-dalton peptide and spectrin used (approximately 0.5 mg/ml). The amount of tet-

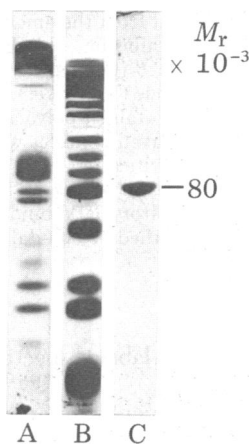


FIG. 3. Coomassie blue-stained NaDodSO₄/polyacrylamide gels. (A) Erythrocyte ghosts; (B) supernatant after tryptic digestion of ghosts; (C) purified 80,000-dalton peptide after recycling gel filtration chromatography.

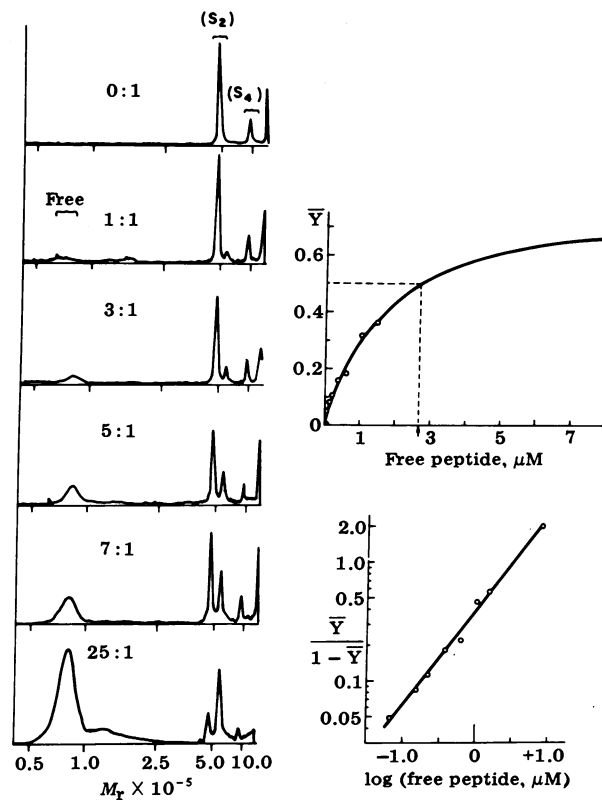


FIG. 4. Binding of 80,000-dalton peptide to dimer spectrin. (Left) Densitometric scans of Coomassie blue-stained 4% nondenaturing 6×100 mm tube gels containing native spectrin and increasing amounts of 80,000-dalton peptide (ratio of peptide to spectrin is shown). Samples were electrophoresed for 1000 V-hr at 4°C. S₂ and S₄ represent dimer and tetrameric spectrin, respectively. The new species which appeared at an apparent molecular weight near 560,000 represents 80,000-dalton peptide bound to dimer. Similar binding to tetramer was not detectable; note reduction in the amount of tetramer and dimer as the amount of the new species increased. (Right) Quantitation of binding of 80,000-dalton peptide on Coomassie blue-stained gels. A simple relationship exists between the association constants for 80,000-dalton peptide binding and tetramer formation. The ratio of these constants for all experiments so far determined approaches unity (1.3 ± 0.9 ; error limits represent 2 SD). Hill coefficient $n = 0.8$. K_a for peptide binding to dimer at 30°C in isotonic KCl at pH 7.6 was $4 \times 10^5 \text{ mol}^{-1}$ (arrow indicates $1/K_{80}$).

ramer formed at 30°C after incubation for 6 hr was decreased when the 80,000-dalton peptide was present.

The kinetics of binding of the 80,000-dalton peptide to dimer spectrin at 30°C paralleled those for tetramer formation (data not shown), with equilibrium being achieved after approximately 1 hr; at 0°C, several days were required. The association constant for 80,000-dalton peptide binding to dimer at 30°C in isotonic KCl at pH 7.6 was estimated to be $4 \times 10^5 \text{ moles}^{-1}$, based on the concentration of 80,000-dalton peptide yielding half saturation. The Hill coefficient for the same series of measurements was 0.8.

The overall amino acid composition of the 80,000-dalton fragment is similar to that of spectrin (Table 1). Peptide mapping confirmed its origin as a terminus of the spectrin α chain.

α and β Chains Associate Noncovalently at Multiple Sites. Nondenaturing polyacrylamide gel electrophoresis was used to examine associations of peptides after limited trypsin digestion. The components of each association were identified by electrophoresis in a second dimension using NaDodSO₄. Fig. 5 Top shows such an experiment. Native spectrin (a mixture

Table 1. Amino acid composition of 80,000-dalton peptide

Amino acid	Composition, mol %	
	80,000-dalton peptide	Spectrin
Asp	11.2	10.5
Thr	4.3	4.2
Ser	5.8	5.8
Glu	21.0	19.6
Pro	1.4	2.3
Gly	4.0	4.7
Ala	8.4	8.8
Val	5.9	5.2
Met	1.3	1.8
Ile	3.6	4.1
Leu	12.4	12.4
Tyr	2.6	2.1
Phe	2.9	3.2
His	3.5	2.9
Lys	7.6	6.7
Arg	6.0	6.0

Duplicate hydrolysis in 6 M HCl/2% phenol at 110°C *in vacuo* for 24, 63, and 90 hr. The amino acids were quantitated on a Durrum D-500 amino acid analyzer. The reported values of Thr and Ser represent extrapolations to time zero. After 90 hr of hydrolysis, 15% of the Thr and 30% of the Ser were destroyed.

of dimer and tetramer forms) was digested for 1 hr at 0°C with trypsin (enzyme/substrate ratio, 1:20) in isotonic KCl buffer. After inhibition with iPr₂P-F, 100 µg of the digest was electrophoresed in an 8% nondetergent gel at 4°C. After separation in the first-dimension, a 3-mm longitudinal slice of the gel was electrophoresed in the second dimension in a 10% polyacrylamide NaDodSO₄ slab gel. Multiple noncovalently associated complexes were identified; their component peptides were revealed by NaDodSO₄ electrophoresis in the second dimension.

It is possible that two peptides of different molecular weight might coelectrophorese in the absence of detergent, due to compensatory differences in charge. To avoid misinterpretation of such coincidental "associations," two additional experiments were conducted. Urea (4 M) was incorporated into the first-dimension gel. Under these conditions, few noncovalent associations remain, but the net charge of each peptide is largely retained. Alternatively, a 3.5–25% nondetergent gradient acrylamide gel was used in the second dimension. In such a gel, the peptides (and peptide complexes) migrate until trapped in the increasing acrylamide gradient on the basis of their effective Stokes radii. Thus, the extent of migration reflects the true size of a complex, and unassociated peptides would be separated on the basis of size, regardless of their charge differences. The groups of associated peptides discussed below have withstood these tests and reflect specific, noncovalently associated, spectrin peptides.

One complex involved peptides of 80,000 and 65,000 daltons; or 80,000, 52,000, and 28,000 daltons, as illustrated in Fig. 5 *Top*. By mapping, the 80,000-dalton component was identified as the terminal α chain peptide. The 65,000- and 28,000-dalton components were identified as β chain peptides (1). The 52,000-dalton peptide was a slightly shortened version of the 65,000-dalton peptide. These associations are depicted in Fig. 5 *Middle*.

Another peptide complex involved peptides of 104,000, 92,000, and 50,000 daltons. This complex was demonstrated by using a 5% acrylamide nondenaturing gel in the first dimension; it is not clearly evident in Fig. 5 *Top*. Tryptic and

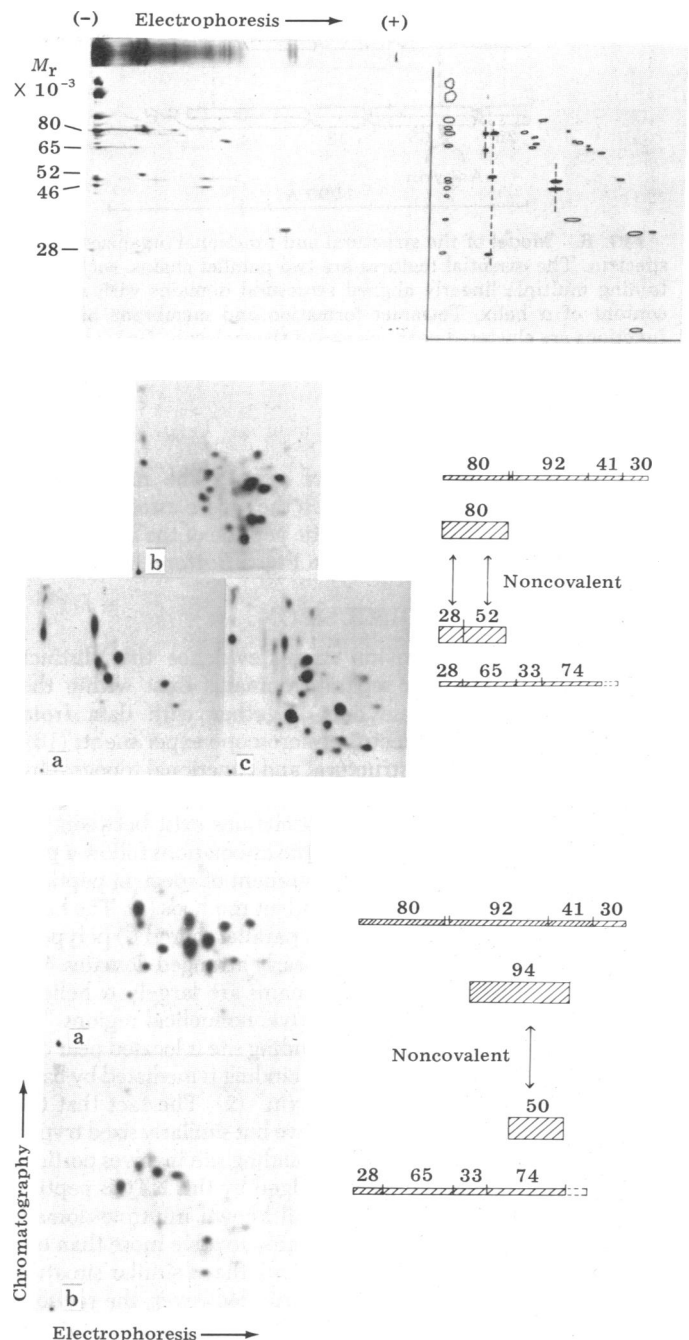


FIG. 5. Identification of noncovalent associations between α and β chains. (*Top*) Two-dimensional polyacrylamide gel electrophoresis of native spectrin digested by trypsin (enzyme/substrate, 1:20) at 0°C in isotonic KCl buffer. The first-dimension nondenaturing gel was 8% in acrylamide. Noncovalent associations were identified by their alignment in the second (NaDodSO₄) dimension. Multiple gels of varying acrylamide concentration were required in the first dimension to demonstrate all associations (data not shown). (*Middle and Bottom*) Chymotryptic maps of two sets of associated peptides. Comparison with the maps of reference tryptic peptides allowed the alignment depicted on the right (see ref. 1). The tryptic maps of these peptides confirmed their placement. (*Middle*) a, 28,000-dalton, β chain; b, 80,000-dalton, α chain; c, 52,000-dalton β chain. (*Bottom*) a, 94,000-dalton; α chain; b, 50,000-dalton, β chain.

chymotryptic peptide maps indicate a precursor-product relationship for the 104,000- and 92,000-dalton species and demonstrate their origin to be the α chain, spanning the ref-

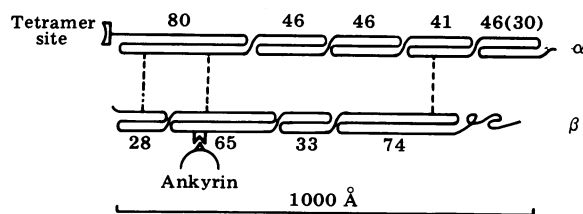


FIG. 6. Model of the structural and functional organization of spectrin. The essential features are two parallel chains, each containing multiple linearly aligned structural domains with a high content of α helix. Tetramer formation and membrane binding functions are clustered near one end of the molecule. One (or both) end of the β -chain is especially sensitive to proteolytic attack by trypsin.

erence peptides 46a and 41c of ref. 1. The map of the 50,000-dalton peptide, with which they were associated, was a subset of the 74,000-dalton tryptic peptide of the β chain (1). These associations are depicted in Fig. 5 *Bottom*.

DISCUSSION

The present results provide strong evidence that distinct functional but spatially separate domains exist within the spectrin molecule. These data, together with data from chemical (14, 15) and electron microscope experiments (16), suggest a model for the structural and functional topography of the spectrin molecule. The essential features of this model are shown in Fig. 6. Multiple associations exist between the spectrin chains, but in every case the associations follow a pattern consistent with the linear alignment of spectrin peptides previously determined by independent methods (1). The basic structural motif appears to be two parallel (α and β) polypeptide chains folded into multiple linearly arranged domains. We hypothesize that the spectrin domains are largely α helical, separated by proteolytically sensitive, nonhelical regions.

The high-affinity membrane binding site is located near one end of the β chain. The membrane binding is mediated by band 2.1, more recently termed "ankyrin" (2). The fact that the 50,000-dalton NTCB peptide is active but similarly sized tryptic peptides are not suggests that the binding site involves portions of the two structural domains bridged by this NTCB peptide (28,000 and 65,000 daltons). In all known multiple-domain proteins, active sites almost invariably involve more than one structural domain (24). It seems likely that a similar situation exists for ankyrin binding to spectrin. However, the reduced molar specific activity of the 50,000-dalton peptide (Fig. 1B) suggests that the active site is not perfectly preserved in this peptide. Adjacent to the ankyrin binding region, but located on the α chain, is a domain important in tetramer formation. This 80,000-dalton peptide binds preferentially to dimer, competes with tetramer, and has an association constant indistinguishable from that for tetramer formation. Thus, the ankyrin and tetramer binding sites are closely related spatially. This result agrees with the morphological studies by Tyler *et al.* (4), which have directly visualized 2.1 protein bound to spectrin tetramers.

Spectrin is a true "multifunctional protein," as defined by Kirschner and Bisswanger (25). The domains reported here probably do not represent the smallest independently refoldable

structural units within the molecule. Compared with other proteins with well-documented domain structure, the peptide domains represented here are 2 to 4 times larger (24, 25). It is not clear whether the functional domains defined here represent the minimum size of the functional units. Other experiments (data not reported) indicate that proteolytic removal of even a 6000-dalton peptide from the 80,000-dalton peptide results in loss of dimer binding activity. Similarly, Anderson (26) has reported evidence for a series of 8000- to 10,000-dalton domains at the phosphorylated end of the β chain. The implication is that each of the specific functions of spectrin involves multiple structural domains, complicating their identification and purification.

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