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Membrane Skeletons and Cytoskeletal–Membrane Associations 75–93

Mechanisms of Cytoskeletal Regulation: Functional and Antigenic Diversity in Human Erythrocyte and Brain Beta Spectrin

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A study of human erythrocyte and brain spectrin with particular emphasis on the beta subunits revealed a structural homology but functional dissimilarity between these two molecules. Six monoclonal antibodies raised to human erythrocyte beta spectrin identify three of the four proteolytically defined domains of erythrocyte beta spectrin. Five of these monoclonal antibodies cross-react with human brain spectrin. None of a previously identified set of alpha erythrocyte spectrin monoclonal antibodies [Yurchenco et al: J Biol Chem 257:9102, 1982] reacted with brain spectrin. A domain map generated by limited tryptic digestion shows that brain spectrin is composed of proteolytically resistant domains analogous to erythrocyte spectrin, but the brain protein is more basic. The binding of brain spectrin to erythrocyte ankyrin, both in solution and on erythrocyte IOVs, yielded an association constant approximately 100 times weaker than for erythrocyte spectrin. The binding of azido-calmodulin under native conditions was specific for the erythrocyte beta subunit but was not calcium dependent. In contrast, azido-calmodulin bound only to the alpha subunit of brain spectrin in a calcium-dependent manner. The similarity of structure but modified functional characteristics of the brain and erythrocyte beta spectrins suggest that these proteins serve different cellular roles.

Abbreviations used: BSA, bovine serum albumin; CHAPS (3[(3-chloramidopropyl)-dimethylammonio)] 1-propane sulfonate; DEAE, diethyl aminoethyl; DFP, diisopropylfluro-phosphate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(beta-amino ethyl ether)) N,N,N′N′-tetraacetic acid; ELISA, enzyme-linked immunosorbant assay; HEPES, N-2-hydroxethylpiperazine-N′-2 ethanesulfonic acid; IEF, isoelectric focusing; IOV, human erythrocyte inside out vesicle; MOPS, 3-(N-morpholino) propanesulfonic acid; NTCB, 2-nitro-5-thiocyanobenzoic acid; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; RSG, resealed human erythrocyte ghost; SDS, sodium dodecyl sulfate; TPCK, N-p-tosyl-l-phenyl-alanine chlormethyl ketone.

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Proteins of the spectrin family enjoy widespread tissue distribution [for review, see 1,2, and references therein]. Mammalian erythrocyte spectrin primarily stabilizes the overlying lipid bilayer and probably also organizes the distribution of the integral membrane proteins [for review, see 3]. In the avian intestinal brush border, an enterocyte-specific form of spectrin [4] stabilizes the microvilli by joining the basal segments of adjacent microfilamentous rootlet bundles [5]. This spectrin appears to have little or no membrane binding role [5,6]. Less clear is the function of the most widespread class of spectrins, such as those purified from neural tissue [7–14]. Spectrins of this class, also called fodrin [8] or calspectin [9], comprise two subunits, a 240-kDa alpha spectrin and a 235-kDa subunit variably called beta or gamma spectrin [10]. All avian spectrins appear to share a common 240-kDa subunit; functional differences in this species derive from diversity of the subunit joined to alpha spectrin. At least four different alpha binding avian spectrins have been identified. Their molecular weights range from 220 kDa to 260 kDa; their names from beta to beta′, gamma, and simply TW260.

Mammalian tissues may display even greater spectrin diversity. While an enterocyte-specific spectrin has not been identified, multiple skeletal muscle isoforms have been [15], and at least two different alpha spectrins exist [11]. Since many of the characteristic functional properties of erythrocyte spectrin are features of the beta subunit, including phosphorylation [3], ankyrin binding [17], and probably 4.1 binding [18], an understanding of structural and functional diversity in the beta spectrins should allow insight into the various roles of this important class of proteins.

Toward this end, human erythrocyte and brain spectrin have been compared with respect to their amino acid composition, degree of cross-reactivity with a battery of monoclonal antibodies, proteolytic resistant domain structure, and their ankyrin- and calmodulin-binding properties, focusing primarily on the beta subunit. The results reveal a remarkable degree of overall structural and antigenic similarity between these two tissue specific beta spectrins, despite significant differences in their ability to be phosphorylated and to bind erythrocyte ankyrin and calmodulin.

EXPERIMENTAL PROCEDURES

Preparation of Spectrin

Human erythrocyte spectrin was prepared from fresh erythrocyte ghosts by extraction of 0.1 mM EDTA at pH 9.0 followed by gel filtration chromatography on Sepharose CL-4B (Pharmacia) [19]. Isolated subunits of alpha or beta erythrocyte spectrin were prepared by calmodulin affinity chromatography in 6 M urea [20]. Human brain spectrin was prepared from cadaver brains removed at autopsy 2–6 hr postmortem [11]. Briefly, aliquots of frozen brain were thawed and homogenized in 10 volumes of buffer A (200 mM sucrose, 10 mM HEPES, 5 mM EGTA, 5 mM $MgCl_2$, 1 mM DTT, 0.5 mM DFP, 0.09 mM PMSF, 1 μg/ml bestatin [Sigma], 1 μg/ml antipain [Sigma], 2 μg/ml leupeptin [Sigma], pH 7.40) at 0°C. After removal of nuclear debris and soluble material, the resulting membranes were washed two times in 10 mM HEPES, 5 mM EDTA, 1 mM DTT, 0.1 mM DFP, 0.09 mM PMSF, pH 7.40, at 0–4°C, and were subsequently extracted at pH 9.0 in 0.6 M KCl, 2.5 mM HEPES, 2.5 mM EDTA, 2 mM DTT, 0.5 mM DFP, and 1 μg/ml each of antipain, bestatin, and leupeptin at 37°C for 45 min following established procedures [11]. Brain spectrin was purified by gel filtration and ion exchange chromatography [21].

Preparation of Monoclonal Antibodies

BALB/c, NZB, and AJ mice were immunized with human erythrocyte spectrin emulsified in complete Freund's adjuvant as previously described [22]. Either native spectrin or a spectrin digest prepared by limited trypsin treatment [23] was used as the immunogen. Spleen cells from hyperimmune mice were fused with the nonsecreting myeloma SP2/0 Ag14 [22,24]. Supernatants from surviving fusions were screened for spectrin reactivity by ELISA. Master colonies producing spectrin-reactive antibodies were secondarily screened for reactivity with either alpha or beta human erythroid spectrin. The beta reactive colonies were next screened by immunoblotting of erythroid spectrin digested with either trypsin (Worthington) [23] or NTCB [25]. On the basis of their reactivity with specific beta spectrin domains, master colonies were selected for cloning by the method of limiting dilution. Cells were plated at an average density of 1/2 cell per well, and inspected to ensure selection of only those with one microscopic colony per well. Larger quantities of antibody, when needed, were purified from the ascites fluid of BALB/c mice that had been injected with approximately 10^7 log phase hybridoma cells per mouse [22]. Purification was by ammonium sulfate precipitation (40%) of the clotted ascitic fluid, followed in most cases by chromatography employing DEAE cellulose [26].

Assay of Antibody Reactivity

Measurement of antibody reactivity with either brain or erythrocyte spectrin was by ELISA, employing 1–2 μg of protein per microtiter well and an avidin-biotin-horseradish peroxidase and biotinylated horse antimouse IgG detection system (Vector Laboratories) [27,28]. Immunoblots of spectrin or spectrin digests were prepared from SDS polyacrylamide gels after electrophoresis [29] employing 125-I staph protein A and rabbit antimouse IgG for detection, and the monoclonal or polyclonal antibodies at dilutions of 1:100 [22].

Enzymatic and Chemical Digestion of Spectrin

Restricted enzymatic digests of either brain or erythrocyte spectrin were prepared by incubating purified spectrin with TPCK-treated trypsin (Worthington) at 0°C for various times at an enzyme:substrate ratio of 1:20 [23,25]. The digestion was terminated by the addition of 0.5 mM DFP followed in 30 min by a two-fold molar excess of pancreatic trypsin inhibitor. Cleavage at cysteine residues was accomplished by reaction with NTCB as previously described [25].

Erythrocyte Inside-Out Vesicle (IOV)-Binding Studies

The spectrin- and actin-free erythrocyte vesicles remaining after spectrin extraction were harvested for binding studies as described [30]. Resealed ghosts (RSG's) used for nonbinding control membranes were prepared from freshly made ghosts [31]. The binding of spectrins to human erythrocyte IOV's and RSG's was assayed at 0°C in 75 mM NaCl, 20 mM sodium phosphate, pH 7.4, with 1% bovine serum albumin in the final assay mixture. 125-I-labeled spectrin (either brain or erythrocyte) was prepared by lactoperoxidase and glucose oxidase iodination (Bio-Rad Enzymo-

beads, 125-I from Amersham-Searle) and used at a specific activity of approximately 20–50,000 cpm/μg, as detected by gamma counting. Forty to fifty micrograms of IOV or RSG protein was used per assay, in a total volume of 150 μL. The incubation mixture was incubated for 1 hr; unbound spectrin was separated from the vesicles by sedimentation of 16,000g for 30 min through a 150-μL cushion of 10% sucrose in incubation buffer [30]. Inhibition studies were performed in 50 mM Tris-HCl, 130 mM KCl, 20 mM NaCl, 0.1 mM EDTA, 0.1% BSA, pH 7.40, by allowing the membranes to incubate with the unlabeled inhibitor protein for 30 min at 0°C before the addition of 125-I-labeled spectrin.

Ankyrin Binding Studies

Human erythrocyte ankyrin was prepared by high salt extraction of IOV's followed by DEAE cellulose chromatography [32]. Spectrin (20–40 μg) was incubated with increasing concentrations of 125-I-labeled ankyrin (prepared by the method of Boulton and Hunter [33], Amersham-Searle) in 130 mM KCl, 20 mM NaCl, 25 mM Tris-HCl, 0.1 mM EDTA, 0.5 mM DTT, pH 7.40, for 60 min at 0°C. Binding to spectrin was measured by nondenaturing PAGE [34] at 4°C. The amount bound to spectrin vs the amount free was determined by autoradiography of the gels after electrophoresis and staining and by gamma counting of the bands representing free and bound material.

Photoaffinity Calmodulin Labeling of Spectrin

Calmodulin was prepared from bovine brain by standard methods [11]. Its activity after all modifications was assayed by its ability to stimulate phosphodiesterase [35]. 125-I-labeled material was prepared by lactoperoxidase-catalyzed coupling employing immobilized enzymes as described above. Azido-calmodulin, the photoreactive analog, was prepared by reaction with methyl-4-azidobenzimidate (Pierce Chemical Co.) in 10 mM MOPS, 0.2 mM $CaCl_2$, 1.0 mM 2-mercaptoethanol, pH 7.1 [36]. Photocoupling was activated by irradiation with long-wavelength ultraviolet light (Minerlite) for 5 min at 0°C. Immediately after photocoupling, possible reactive intermediates were quenched by 2 mM DTT, 2 mM ascorbic acid, and 10 mM ethanolamine at pH 7.1.

Polyacrylamide Gel Electrophoresis and Other Procedures

SDS-PAGE and two-dimensional IEF/SDS-PAGE were performed by the methods of Laemmli [37] and O'Farrell [38], respectively. Nondenaturing PAGE was performed by the method of Fairbanks, as modified by Morrow [19,34,49]. Proteins were visualized by Coomassie blue staining. Protein determinations were by the method of Lowry [39], and by amino acid analysis on a Durram D-500 analyzer after 20 hr hydrolysis at 110°C with 6 N HCl/2% phenol in vacuo [19].

RESULTS

Human Erythrocyte and Brain Spectrins Have Nearly Identical Amino Acid Composition

A comparison of the amino acid composition of two human spectrins (heterodimers) is shown in Table I. The two proteins are nearly indistinguishable on this basis, although the glx and leu content of the brain protein is slightly reduced, and the ser is

TABLE I. Human Erythrocyte and Brain Spectrin Have Similar Amino Acid Compositions*

Amino acid	Erythrocyte	Brain
Asx	10.4	10.8
Thr	4.3	4.4
Ser	5.9	7.1
Glx	20.3	19.6
Pro	2.4	2.4
Gly	4.4	4.4
Ala	9.0	9.0
Val	4.3	4.4
Met	1.8	2.3
Ile	3.3	3.4
Leu	12.1	10.3
Tyr	2.0	2.0
Phe	3.1	3.1
His	2.9	2.8
Lys	7.3	7.5
Arg	6.2	6.3

*Composition: mole %. Duplicate hydrolysis in 6 M HCl/2% phenol at 110°C in vacuo for 20 h. The amino acids were quantitated on a Durram D-500 amino acid analyzer.

slightly increased. The degree of similarity reported here is greater than that originally noted in a previous cross-species comparison [21].

Monoclonal Antibodies Identify Specific Beta Erythrocyte Spectrin Domains

Forty-three stable master colonies reactive with native spectrin by ELISA were identified. Of these, 19 were positive for the beta subunit (Table II). Nearly all beta-reactive colonies were obtained from BALB/c mice immunized with digested spectrin. Conversely, AJ strain mice injected with the same antigen produced only alpha spectrin antibodies. Of the 19 beta erythroid spectrin-reactive colonies, nine would react by one-dimensional immunoblotting with either limited trypsin or NTCB digests. Five of these were cloned, and their beta spectrin domain reactivity was identified.

The proteolytic resistant domains of erythrocyte beta spectrin are shown in Figure 1. The linear sequence of the peptides representing the largest unique fragment of each domain (T28, T65, T33, T74) was initially established on the basis of shared spots on cellulose peptide maps with overlapping peptides generated by NTCB or milder enzymatic cleavage [23,25]. Many of these alignments have more recently been confirmed by partial sequence analysis [40]. The domain reactivity of the monoclonals is shown in Figure 2, depicted in Figure 1, and listed in Table II. No monoclonals were reactive with the Beta-III domain, although 125-I peptide mapping of the NTCB fragments reactive with monoclonal 7-C4(VIIIC7) indicated that this antibody reacted with peptides that bridged the Beta-II and Beta-III domains (Fig. 3). The reaction of this monoclonal with only the largest Beta-II tryptic fragment (Fig. 2) supported this observation and allowed localization of its binding site to a well defined region near the amino terminus of the Beta-II domain (Fig. 1).

Both Beta-I-reactive monoclonals 7-F11(VIIF7) and 7-F2(IVF8) reacted with the 28- and 17-kDa tryptic peptides (Fig. 2) and the 25-kDa NTCB-phosphorylated

TABLE II. Erythroid Beta Spectrin Master Clones and Monoclonal Antibodies

Master colony	Immunizing antigen	Mouse strain	Mono-clone	Immunoblot reactive	Domain specificity	Reactive fragments	Brain reactive
6(IVC9)	Native	NZB	C2	Yes	B-IV	T74,T28	(+++)
7(IVD2)	Digest	BALB/c	*	No	ND	—	ND
7(IVD3)	Digest	BALB/c	*	No	ND	—	ND
7(IVD5)	Digest	BALB/c	*	No	ND	—	ND
7(IVE7)	Digest	BALB/c	G10	No	ND	—	ND
7(IVF8)	Digest	BALB/c	F2	Yes	B-I	T28,T17 N25,N21	(+/−)
7(VG5)	Digest	BALB/c	*	No	ND	—	ND
7(VIE5)	Digest	BALB/c	*	Yes	(A)	—	ND
7(VIIC10)	Digest	BALB/c	*	No	ND	—	ND
7(VIID4)	Digest	BALB/c	*	Yes	(B)	—	ND
7(VIID5)	Digest	BALB/c	*	Yes	(C)	—	(—)
7(VIIE3)	Digest	BALB/c	*	No	ND	—	ND
7(VIIF4)	Digest	BALB/c	*	No	ND	—	ND
7(VIIF7)	Digest	BALB/c	F11	Yes	B-I	T28,T17, N25	(+)
7(VIIIB6)	Digest	BALB/c	*	Weak	(D)	—	ND
7(VIIIC7)	Digest	BALB/c	C4	Yes	B-II	T65,N52, N28	(+++)
7(VIID6)	Digest	BALB/c	*	No	ND	—	ND
T(XF4)	Digest	BALB/c	*	No	ND	—	ND
6(VD4)	Native	NZB	C5	Yes	B-IV	T74,T68, T28	(+++)

*, not cloned, (−) not reactive, (+/−) weakly reactive, (+) moderately reactive, (+++) strongly reactive. (A), reactive fragment on one-dimensional SDS gel: T17; (B), reactive fragment on one-dimensional SDS gel: T46; (C), reactive fragment on one-dimensional SDS gel: T65, T46, N88, N76; (D), not reactive.

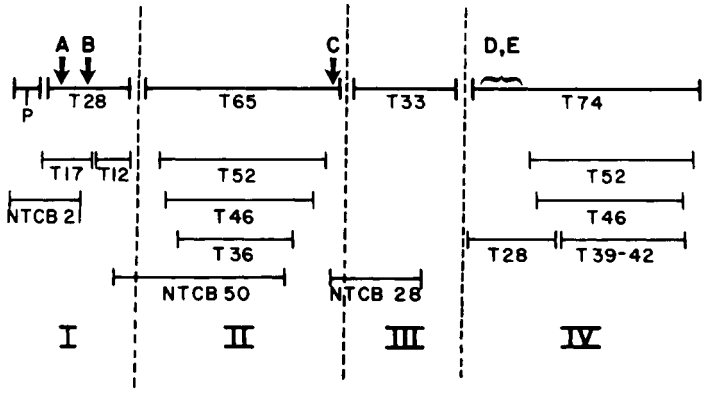

Fig. 1. Human erythrocyte beta spectrin is composed of four unique proteolytically defined domains. Peptides generated by limited tryptic or NTCB digestion are labeled T and NTCB, respectively, whereas the molecular weights are expressed in kilodaltons. The binding sites of five monoclonal antibodies are depicted: A) 7-F2(IVF8), B) 7-F11(VIIF7), C) 7-C4(VIIIC7), D) 6-C2(IVC9), E) 6-C5(VD4).

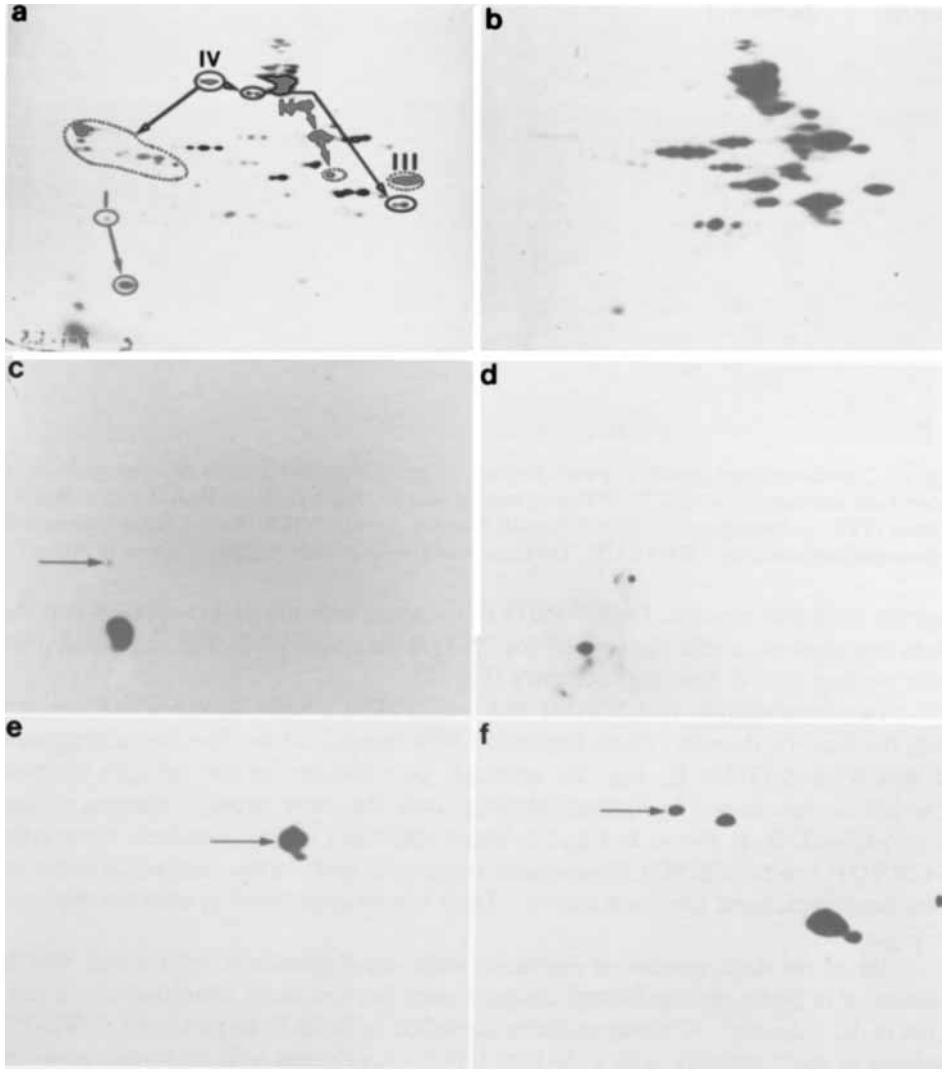

Fig. 2. Monoclonal antibodies to the beta subunit of erythrocyte spectrin react with specific peptides within each domain. Spectrin tryptic peptides were separated by two-dimensional IEF/SDS-PAGE, transferred electrophoretically onto nitrocellulose sheets, and reacted with either polyclonal antibodies (b) or monoclonal antibodies (c–f) as described under "Experimental Procedures." The immunoreactive peptides were subsequently incubated with 125-I-labeled S. aureus protein A and detected by autoradiography. The identity of each of the monoclonal reactive peptides was established by superimposition on Coomassie blue-stained spots (a) and polyclonal autoradiogram spots (b). a) Coomassie blue-stained two-dimensional IEF/SDS-PAGE of spectrin after limited tryptic digestion. The largest peptide in each of the beta domains are labeled. The solid lines depict those peptides that were reactive with the monoclonal antibodies, while the dotted lines encompass those peptides identified as derived from the beta chain [23,25], but that were not reactive with these monoclonal antibodies. b) Immunoblot of spectrin digest reacted with polyclonal antierythrocyte spectrin antisera. All of the beta domains were reactive with this antisera. c) Peptide specificity of Beta-I monoclonal, 7-F2(IVF8). Arrow indicates the position of the parent domain peptide, T28. d) Peptide specificity of Beta-I monoclonal, 7-F11(VIIF7). The reactivity with the domain peptide T28 was minimal. * indicates an artifact generated during immunoblotting procedure. e) Peptide specificity of Beta-II monoclonal, 7-C4(VIIIC7). Arrow indicates the position of the parent domain peptide, T65. f) Peptide specificity of Beta-IV monoclonal, 6-C5(VD4). Arrow indicates the position of the parent domain peptide, T74. The peptide specificity of the Beta-IV monoclonal, 6-C2(IVC9), was indistinguishable from 6-C5(VD4) (data not shown).

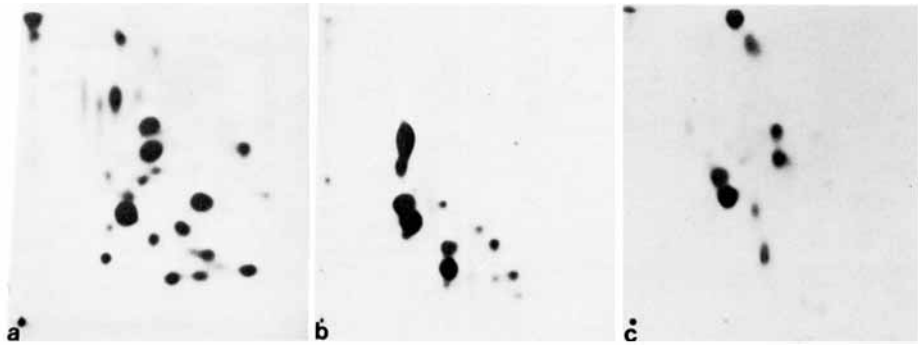

Fig. 3. Two-dimensional peptide maps of 125-I-labeled spectrin peptides identify the binding of site of monoclonal antibody 7-C4(VIIIC7). a) Peptide map of Beta-II domain (T65). b) Peptide map of beta-III domain (T33). c) Peptide map of Beta-II-Beta-III bridging peptide (NTCB 28) that is immunoreactive with monoclonal antibody 7-C4(VIIIC7). The linear relationship of these peptides is shown in Figure 1.

peptide from this domain. Only 7-F2(IVF8) reacted with the 21-kDa NTCB peptide (data not shown), a subfragment of the 25-kDa fragment [23]. The relationship of their binding sites is therefore as shown (Fig. 1).

Two monoclonals (6-C5(VD4) and 6-C2(IVC9)), were identified that reacted with the Beta-IV domain. These antibodies both recognized the 28-kDa subfragment of this domain (Table II, Fig. 2), although they differed in the strength of their reaction as determined by immunoblotting (only the most strongly reacting of the two, 6-C5(VD4), is shown in Fig. 2). These antibodies are not identical, since only 6-C5(VD4) reacts with NTCB-generated fragments, and the two antibodies differ in their isoelectric point (data not shown). Their approximate binding sites are depicted in Figure 1.

Based on their pattern of reactivity with one-dimensional tryptic and NTCB digests, it is likely that additional unique clones beyond those identified above also exist in the collection of master colonies identified in Table II. In particular, 7(VIID5) appears to react strongly with a 76-kDa NTCB peptide and with no tryptic peptides smaller than 46 kDa. This combination of reactivity is consistent with a binding site between the Beta-I and Beta-II domains, the site of ankyrin binding [19].

The Beta-II Monoclonal Antibody Inhibits Spectrin Binding to IOV's

Since erythrocyte ankyrin binds at a site adjacent to the Beta-I and Beta-II domains, it was of interest to test if antibodies directed toward this region interfered with binding. As shown in Figure 4, 7-C4(VIIIC7) weakly inhibited the binding of 125-I-labeled spectrin to IOV's, an interaction mediated primarily by ankyrin [17].

Erythrocyte Beta Spectrin Monoclonal Antibodies Selectively React With Brain Spectrin

Despite the similar composition of brain and erythrocyte spectrin, only about 1% of epitopes in the two proteins were strongly cross-reactive, as measured in an earlier study [11]. The degree of cross-reactivity of the monoclonal antibodies was, therefore, of interest. While none of a previously identified set of alpha erythroid spectrin monoclonal antibodies [22] reacted strongly with the brain protein (data not

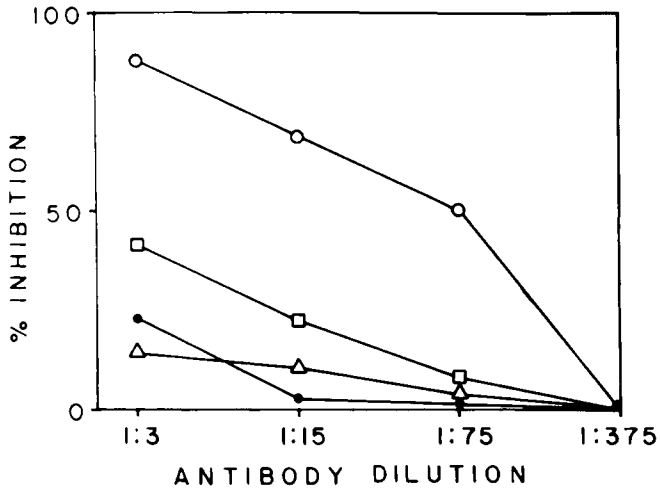

Fig. 4. Antibodies reactive with erythrocyte spectrin at or near the ankyrin binding site inhibit the binding of 125-I-labeled spectrin to IOV's. (125-I-labeled spectrin and monoclonal antibodies were incubated in 80 mM NaCl, 10 mM sodium phosphate, 0.1 mM EDTA, and 0.5% BSA for 60 minutes at 30°C, after which time IOVs (50 μg protein) were added and the incubation was continued for 30 min at 37°C. The free and bound 125-I-labeled spectrin was separated and quantitated as described in "Experimental Procedures." (○) polyclonal rabbit anti-human erythrocyte spectrin, (□) monoclonal antibody 7-C4(VIIIC7), which reacts with the Beta-II domain, (△) monoclonal antibody 6-C7(IID2), reactive against the Alpha-I (T80 domain [22]), (●) nonimmune rabbit serum.

shown), this was not the case for the monoclonals reported here. As shown in Figure 5 and summarized in Table II, only the monoclonals directed toward the Beta-I domain failed to react strongly with intact human brain spectrin by ELISA. In addition, a master colony was identified that failed to react with the brain protein, despite good affinity for erythrocyte spectrin. While this colony has not yet been cloned, preliminary evidence based on its pattern of reactivity (Table II) with one-dimensional NTCB and tryptic spectrin digests suggest a binding site between domains I and II.

Erythrocyte and Brain Spectrin Have Similar Sized Protease-Resistant Domains

A typical two-dimensional IEF/SDS-PAGE pattern of peptides obtained from limited trypsin digestion of erythrocyte spectrin after 1 hour at 0°C is shown in Figure 6A. Brain spectrin digested under identical conditions generates a series of similar sized fragments, although the overall 2-D pattern is different, which is due in part to the more basic isoelectric point of the brain protein. Brain spectrin also exhibits greater proteolytic resistance than does the erythrocyte protein (data not shown), a feature noted by others [11,12]. Cellulose 125-I mapping studies of the protease-resistant fragments of the brain protein indicate a remarkably similar domain structure between the two proteins (data not shown), consistent with their similar composition.

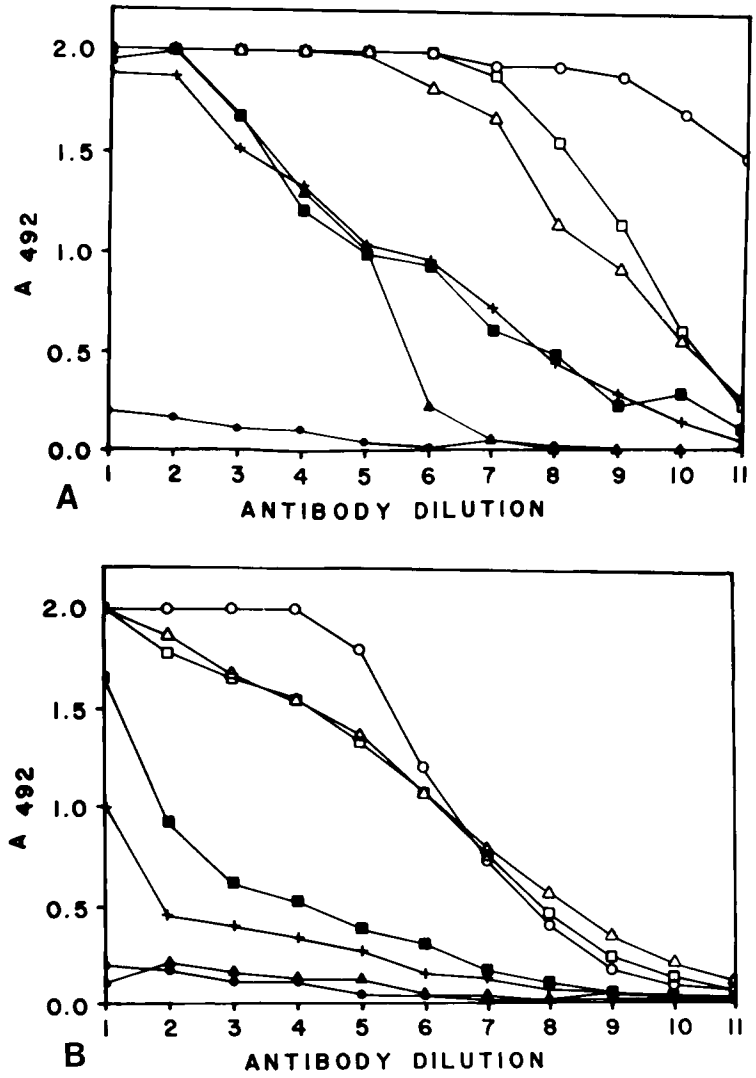

Fig. 5. Monoclonal antibodies raised to human erythrocyte beta spectrin do not uniformly react with human brain spectrin. A) ELISA of erythrocyte beta spectrin monoclonal antibodies demonstrates the strength of the reaction when spectrin is the substrate. The antibody dilution ranged from 1:3 (1) to 1:177147 (11). (○) 6-C5(VD4); (□) 7-C4(VIIIC7); (△) 6-C2(IVC9); (●) hybridoma medium, (■) 7-F11(VIIF7); (▲) 7-(VIID5); (+) 7-F2(IVF8). B) ELISA of the same monoclonal antibodies demonstrates the differential reactivity when brain spectrin is the substrate.

Human Brain Spectrin Binds Human Erythrocyte Ankyrin Weakly

One important property of erythrocyte spectrin is its ability to bind erythrocyte ankyrin [17]. While brain spectrin from other species has been shown to have this ability [7,13,41], uncertainty persists as to the strength of this interaction. The low cross-reactivity of beta-spectrin monoclonals directed to sites near the ankyrin binding region stimulated a reevaluation of the ability of these two proteins to bind IOV's and purified erythrocyte ankyrin in vitro. As measured by direct IOV binding assay (Fig.

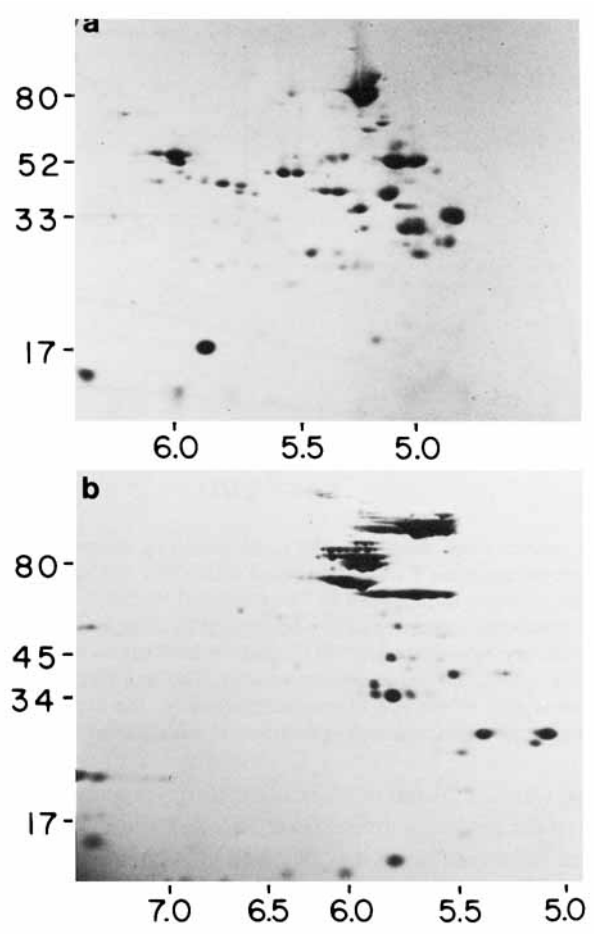

Fig. 6. Limited tryptic digestion of human erythrocyte and brain spectrin yield different "domain maps" for each of the proteins. a) 2-dimensional IEF/SDS-PAGE map of human erythrocyte spectrin digested at approximately 1 mg/ml in 20 mM Tris-HCl, pH 8.00, 0.1 mM EDTA, and 0.1 mM 2-mercaptoethanol. The pH profile of the IEF dimension is indicated at the bottom of the gel. b) IEF/PAGE domain map of human brain spectrin digested under the same conditions as described above. The ampholine content of the IEF gel was altered to accommodate the more basic nature of the peptides, and the nonionic detergent was changed from Triton X-100 to CHAPS to facilitate solubilization of the peptides. The pH profile of the IEF gel is depicted at the bottom. The enhanced resistance to protease digestion of the brain protein is evident by the larger peptides present in its domain map.

7), brain spectrin binds IOV membranes poorly when compared to the erythrocyte protein. This binding could not be saturated at the levels of free protein achieved (0.6 μM), although there was specificity for the cytoplasmic side of the membrane (Fig. 7, inset). Alternatively, the ability of brain spectrin to compete with the binding of erythrocyte spectrin was measured (Fig. 8). In this assay, weak inhibition was detected, with an apparent K_i of $3 \times 10^5\ M^{-1}$.

The direct binding of purified erythrocyte ankyrin with brain spectrin was measured by the comigration of 125-I-labeled erythrocyte ankyrin with spectrin on PAGE under native conditions (Fig. 9). While erythrocyte ankyrin binds strongly to

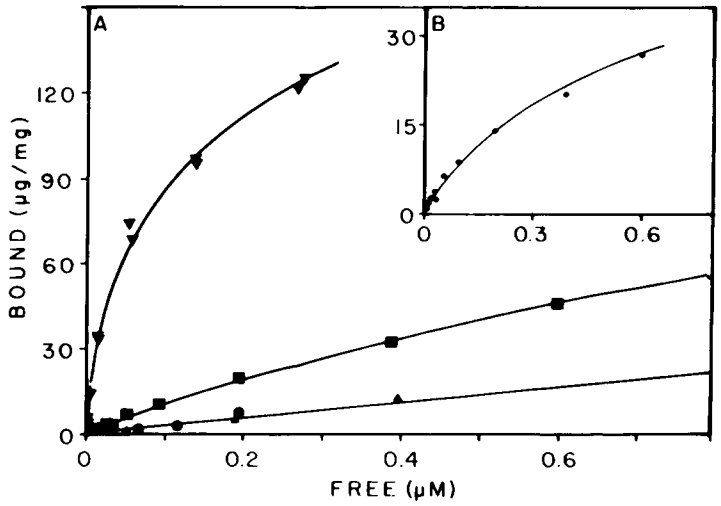

Fig. 7. Human brain spectrin binds weakly to IOV's. A) Increasing amounts of human brain spectrin (■) and human erythrocyte spectrin (▼) were incubated with IOV's (53 μg) for 1 hr, 0°C at pH 7.4. The bound spectrin was separated as described in "Experimental Procedures." Binding to the external side of the erythrocyte membrane (nonspecific) was determined by using RSG's, as shown in the bottom curve. Both brain (▲) and erythrocyte spectrin (●) bound the RSG's in an identical fashion. B) Human brain spectrin binding is specific for the inside-out vesicles. The data from A are reexpressed as the specifically bound material after subtraction of nonspecific binding. The hyperbolic nature of the curve suggests a saturable binding isotherm, although saturation was not achieved experimentally.

erythrocyte spectrin (lanes 2,3),the binding of erythrocyte ankyrin to brain spectrin is barely discernable in the autoradiograms (lane 10). Association constants on the order of $1 \times 10^5\ M^{-1}$ are needed to be clearly detected in such assays. Thus it appears that brain spectrin has an association constant for erythrocyte ankyrin of approximately this order of magnitude, a value similar to the inhibition constant for IOV binding.

Azido-Calmodulin Labels Different Subunits in Brain and Erythrocyte Spectrin

Although the role of calmodulin in modulating spectrin function remains an enigma, the nonerythroid alpha spectrins bind calmodulin strongly in a calcium-dependent manner. Conversely, human erythrocyte spectrin binds calmodulin only weakly under native conditions [56]; in 6 M urea, a specific binding site within the beta-IV domain has been identified [20]. A photoactivatable derivative of calmodulin has allowed a comparison of the sites of calmodulin binding to erythroid and brain spectrins under native conditions (Fig. 10). The alpha subunit of brain spectrin is strongly labeled by this derivative in a calcium-dependent manner, as evidenced by the incorporation of label into the 240-kDa subunit and its 150-kDa proteolytic fragment. In the presence of 10 mM EGTA, the extent of labeling is strongly reduced. Based on the apparent size of the labeled protein (260 KDa, Fig. 10), alpha brain spectrin binds a single molecule of calmodulin. No higher molecular weight labeled species are observed.

Only the beta subunit of human erythrocyte spectrin reacts significantly with the photoactivated calmodulin (Fig. 10) under native conditions. Unlike the case for

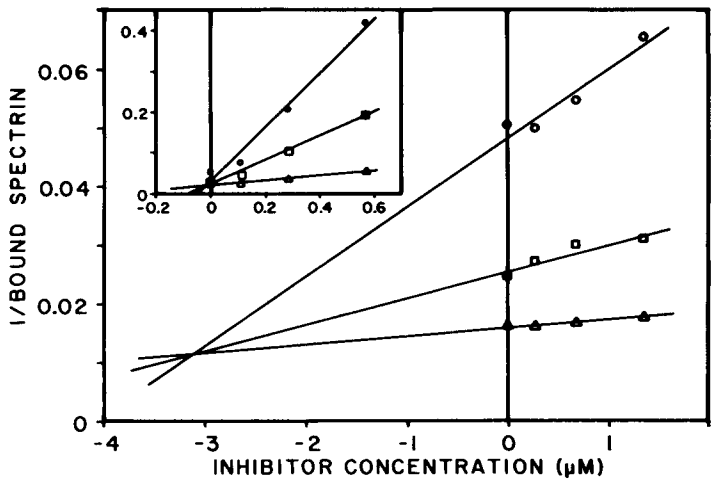

Fig. 8. Human brain spectrin weakly inhibits erythrocyte spectrin rebinding to IOV's. Human brain spectrin was incubated with IOV's for 30 min, followed by the addition of 125-I-labeled human erythrocyte spectrin. Bound and free protein were separated and quantitated as described in "Experimental Procedures." (○) 6 μg/ml 125-I-labeled erythrocyte spectrin, (□) 12 μg/ml 125-I-labeled erythrocyte spectrin, (△) 21 μg/ml 125-I-labeled erythrocyte spectrin. The data are plotted according to Dixon [74], which yields a K_i of 3×10^5 (1/M). Inset: Unlabeled erythrocyte spectrin strongly inhibits the binding of 125-I-labeled erythrocyte spectrin to IOV's ($K_i = 5 \times 10^7$ (1/M)).

brain spectrin, this binding is calcium independent. The molecular weight of the labeled complex (235 kDa) is again consistent with a binding stoichiometry of one calmodulin per erythrocyte beta spectrin. In ghosts, this same subunit of erythrocyte spectrin is photolabeled by azidocalmodulin [77]. Additional proteins are also selectively labeled as observed by others [69]. The binding of beta erythroid spectrin to azidocalmodulin is not due to random collisional labeling, since neither alpha spectrin nor added bovine serum albumin react under these conditions. The binding is also specifically inhibited by unlabeled calmodulin or by phenothiazine, but not by serum albumin or other proteins [77, data not shown].

DISCUSSION

Nineteen stable master colonies producing antibodies to beta erythrocyte spectrin have been isolated. Nine of these reacted with erythrocyte spectrin peptides after immunoblotting. Of these, five were cloned, and their sites of reaction were determined. Each of the monoclonal antibodies was specific for a given beta erythrocyte spectrin domain, extending our own [22] and others' [12] earlier observations of unique epitopes in each of the spectrin domains.

The strong cross-reactivity of several of these antibodies with spectrin purified from human brain tissue suggests that these two proteins may be much more closely related than previously recognized. Detailed studies on the antigenic variation of the influenza virus hemagglutinin protein indicate that marked reductions in monoclonal antibody binding are caused by even a single conservative amino acid substitution, such as arg to lys on exposed loops [42]. This high degree of discrimination is

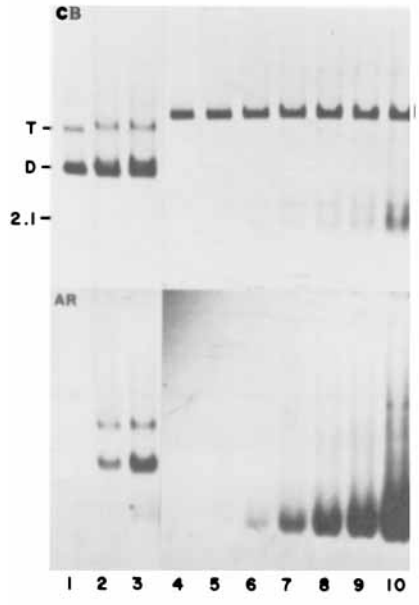

Fig. 9. Human erythrocyte ankyrin binds strongly to erythrocyte spectrin, but weakly to human brain spectrin. Nondenaturing PAGE of erythrocyte spectrin, 17 μg (lanes 1–3), and brain spectrin, 17 μg (lanes 4–10), incubated in the absence (lanes 1,4) or the presence (lanes 2,3,5–10) of 125-I-labeled erythrocyte ankyrin (30,000 cpm/μg). Both the dimeric and tetrameric forms of the erythrocyte protein bound ankyrin as seen by the retarded mobility of those species in the Coomassie blue stained gel (top) and the presence of two bands in the autoradiogram (bottom). The binding of 125-I-labeled ankyrin to brain spectrin was only discernable at the highest concentration of ankyrin (lane 10), and this was detectable only in the autoradiogram (bottom). Lane 2, 4 μg ankyrin; lane 3, 8 μg ankyrin; lane 5, 2 μg ankyrin; lane 6, 4 μg ankyrin; lane 7, 8 μg ankyrin; lane 8, 12 μg ankyrin; lane 9, 15 μg ankyrin; lane 10, 30 μg ankyrin. The brain spectrin is a tetramer, but its mobility is slower than erythrocyte tetramer owing to its increased molecular weight and greater isoelectric point (Fig. 6). T, tetramer; D, dimer; 2.1, ankyrin.

consistent with the unique domain reactivity of all erythrocyte spectrin monoclonal antibodies that we have examined, since the average degree of identity between 106 amino acid repeats within erythrocyte spectrin is approximately 25% [43]. The finding that three of the five beta spectrin monoclonal antibodies cross-react strongly with brain spectrin suggests a very high degree of homology between these proteins, at least for certain segments. This conclusion is supported by the near identity of their amino acid compositions and by limited sequence information indicating that certain 20 residue regions of human brain spectrin are 77% identical to the erythroid protein [44]. Cross-reactivity has also been noted in 13 of 24 monoclonal antibodies directed against the porcine spectrins [12].

The distribution of cross-reactive sites within spectrin is also of interest. Previous studies that used polyclonal antisera demonstrated that the highest degree of antigenic similarity between brain and erythrocyte spectrin occurred in the most central domains [11,41]. With the possible exception of areas responsible for actin binding, antigenic overlap between brain and red cell spectrin specifically appeared to exclude regions of functional specialization. The results with the monoclonal

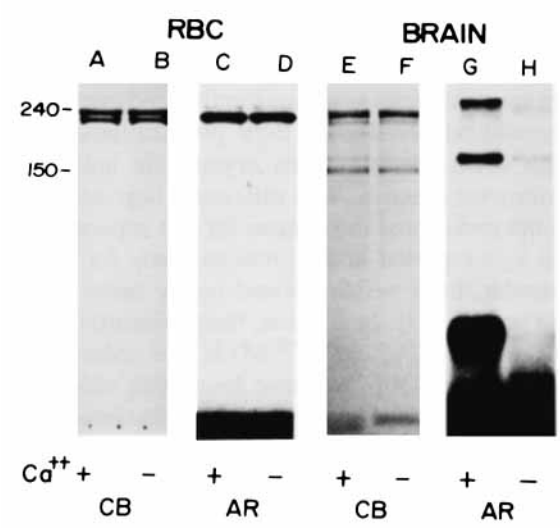

Fig. 10. Human erythrocyte and brain spectrin bind azido-calmodulin under native conditions, but on different subunits, when analyzed by SDS-PAGE. The strength and location of the azido-calmodulin binding to erythrocyte spectrin is calcium independent (lanes A–D). The molecular weight of the labeled chain (237 kDa) is consistent with binding of one calmodulin to one beta subunit. Brain spectrin binds azido-calmodulin strongly in the presence of calcium (lanes E, G), but only weakly in the absence of calcium (lanes F, H). The binding appears to be specific for the alpha subunit, as evidenced by the molecular weight of the labeled parent chain (260 kDa) and the labeling of the 150-kDa proteolytic fragment of the alpha subunit [11,41]. CB, Coomassie blue stained gel; AR, autoradiogram. (+) 1 mM Ca^{++}, (−) 10 mM EGTA.

antibodies extend these observations. Those antibodies raised to erythroid beta spectrin that are strongly cross-reactive with brain spectrin bind to the central portions of erythrocyte beta spectrin domains II and IV. Antibodies directed toward the site responsible for oligomer formation (Beta-I) or erythrocyte ankyrin binding (between Beta-I and Beta-II, tentatively identified as 7[VIID5]), react more weakly with brain spectrin. Since antibodies most often react with exposed and conformationally mobile areas of a protein [45–48], areas most likely to be associated with the various specialized functions of spectrin, the reduced cross-reactivity of antibodies directed to regions encompassing the functional sites is perhaps not surprising. A similar correlation between monoclonal antibody cross-reactivity and a preference for internal or functionally preserved (such as actin binding) regions of the molecule is also apparent in the data reported for porcine spectrin [12]. In that study, four of nine antibodies raised to brain spectrin cross-reacted with both the brain and erythrocyte proteins. All of these reacted with the central or actin binding regions of the molecule.

A marked predilection of the cross-reactive monoclonal antibodies for the beta subunit is also apparent. None of nine previously identified alpha erythroid spectrin monoclonal antibodies [22] react strongly with the brain protein [Green, Harris, and Morrow, unpublished observations]. In the study of pig brain spectrin by Glenney et al [12], three of four beta brain spectrin antibodies but only one of five alpha spectrin antibodies reacted with the erythroid analog. Thus, based on antigenic criteria the erythroid and brain beta spectrins appear to be more similar than erythroid and brain alpha spectrin, despite clear functional differences between beta subunits.

The spectrins differ significantly in their functional specialization. Human erythrocyte spectrin undergoes a facile self-association involving the Alpha-I and Beta-I domains leading to high molecular weight oligomers much larger than tetramer [34,49]. Human brain spectrin exists as an apparently nondynamic and stable tetramer under comparable conditions [17,50,51]. Both proteins bind erythrocyte ankyrin [7,21,41,52], although brain spectrin binds erythrocyte ankyrin 100 times more weakly than does erythrocyte spectrin. This difference is greater than that previously reported [13]. We do not understand the reasons for this apparent discrepency, except to note that while the k_d's reported in the previous study for brain and erythrocyte spectrin were very similar, their vesicles bound nearly seven times more erythroid spectrin than the brain protein [13]. In addition, their measured k_d value for erythroid spectrin binding (230 μg/ml = 0.5×10^{-6} M) is one order of magnitude weaker than accepted literature values [30]. We have found that chloramine-T iodinations may lead to oxidative protein damage and cross-linking that interferes with the binding assays, and suggest that such damage may be a potential complicating factor in the earlier study. Conversely, brain spectrin has been reported to bind the brain analog of ankyrin with high affinity [64,76].

Another difference between brain and erythrocyte spectrin is their ability to bind calmodulin. Several studies have reported the binding of calmodulin to mammalian nonerythroid alpha spectrin [1,9,11,13,14,16,51,53–55], including human brain spectrin [11,41]. Human erythrocyte spectrin binds calmodulin much more weakly under native conditions [56], although this binding is enhanced in 6 M urea [20,57]. Under these denaturing conditions, calmodulin binds at a specific site in the Beta-IV domain [20]. The studies reported here employing a photoactivatable calmodulin derivative confirm the binding of calmodulin in a calcium-dependent manner to alpha brain spectrin, and demonstrate for the first time a specific binding of calmodulin under native conditions to the beta subunit of erythrocyte spectrin. While the role of calmodulin spectrin binding in the erythrocyte is uncertain and may be vestigial [16], these results do indicate that the two spectrins at least differ in the regions of their alpha and beta subunits responsible for the calmodulin interactions.

Finally, it is worthwhile to consider the possible implications of the structural and functional differences identified in vitro on the probable role of the spectrins in vivo. Human erythrocyte spectrin presumably assembles at the membrane surface because of the high affinity interaction between beta spectrin and membrane-bound ankyrin [49]. This compartmentalization at the membrane surface by high-affinity membrane receptors favor subsequent interactions involving other membrane-bound molecules, lending cooperativity to the assembly of the membrane skeleton (a process we originally termed "affinity modulated assembly" [34,49]). Other factors including allosteric regulation [58] and differential rates of synthesis and degradation of skeletal components are also involved [59–62]. In the erythrocyte, the propensity of spectrin to oligomerize and simultaneously bind actin may help to establish an extensive supportive structure required for membrane stability, erythrocyte spectrin's role being primarily structural [3].

Brain spectrin does not appear to undergo the concerted self-polymerization to high molecular weight oligomers characteristic of erythrocyte spectrin [11,49]. It also does not bind erythrocyte ankyrin strongly, although strong binding to a brain analog of ankyrin has been reported [64,76]. These large differences in affinity of the two spectrins for two types of ankyrin thus provide the quantitative basis for the asym-

metric distribution of spectrin isoforms elegantly demonstrated in the developing chicken nervous system [10,63]. Brain spectrin also possesses a bonafide calcium-dependent calmodulin binding site on the alpha subunit, suggesting regulation of at least some function for this protein. Since brain spectrin can undergo patching and capping without disruption of the plasma membrane [65–67,75], it seems unlikely that the brain isoform is required for global membrane support even though linked to surface receptors. This suggestion is supported by microinjection studies [68], which clearly indicate that spectrin can be aggregated within the cell without apparent injury to the plasma membrane. Taken together, these findings suggest that brain spectrin may not act as a structural unit in a supportive membrane skeleton. Instead, we speculate that it might serve as a regulatable (eg, by Ca^{++}/calmodulin) and specific linker of some surface receptor molecules to the filamentous cytoskeleton. Other components in this linkage might presumably be receptor-specific mediating proteins such as brain ankyrin [76]. It is clear that mechanisms exist in cells for interpreting receptor organization induced by external events [70]. It is probably by such mechanisms that cells respond to polyvalent ligands [71,72] and the extracellular matrix [73]. Thus, we hypothesize that by linking receptor molecules undergoing external organization to the cytoskeleton, nonerythroid spectrins such as fodrin form a crucial and regulatable link in the translation of this organizational event into an intracellular "signal." In this sense, some spectrins may serve a transductional role.

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